INTERNATIONAL WEBINAR

ONE HEALTH OVER BORDERS

Tatiana Emanuelli
Maria Rosa Chitolina
Alessandra Marcon Gasperini
Darci Raquel Fonseca
(organizers)

Capes PrInt/UFSM

PPGART editora

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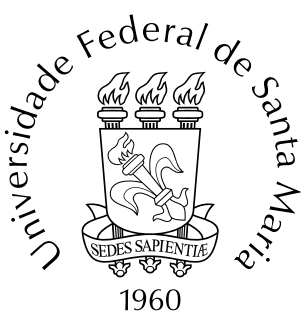

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I61 International webinar [recurso eletrônico] : one health over borders / Tatiana Emanuelli ... [et al.] (organizers). – 1. ed. – Santa Maria, RS : Ed. PPGART, 2022.
1 e-book : il.

ISBN 978-65-88403-56-3

1. One health 2. Human health 3. Animal health 4. Environment 5. Bioactive compounds 6. Food safety I. Emanuelli, Tatiana

CDU 614(063)
502.3(063)

Ficha catalográfica elaborada por Lizandra Veleda Arabidian - CRB-10/1492
Biblioteca Central - UFSM

International Webinar One Health Over Borders
ISBN: 978-65-88403-56-3
Organização do e-book: Alessandra Marcon Gasperini, Tatiana Emanuelli, Maria Rosa Chitolina e Darci Raquel Fonseca
Revisão: Tatiana Emanuelli, Alessandra Marcon Gasperini, Aniélen Dutra da Silva, Maria Rosa Chitolina e Raul Dotto Rosa
Projeto Gráfico: Cristina Landerdahl, Hosana Celeste
Identidade Visual: Alice Veleda Wendt
Diagramação: Evandro Bertol e Cristina Landerdahl

Todos os direitos desta edição estão reservados à Editora PPGART.
Av. Roraima 1000. Centro de Artes e Letras, sala 1324.
Bairro Camobi | Santa Maria / RS
(55) 3220-9484 | (55) 3220-8427
editorappgart@ufsm.br e *seceditorappgart@gmail.com*
http://coral.ufsm.br/editorappgart/

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PREFACE

Tatiana Emanuelli
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Maria Rosa Chitolina
Chair of the Organizing Committee

Alessandra Marcon Gasperini
Chair of the e-Book Committee

It is a great pleasure to present the e-book "**One Health over Borders – Capes/PrInt-UFSM**" as a result of the **International Webinar One Health over Borders – Capes/PrInt-UFSM** that was held by the Federal University of Santa Maria/Brazil, on November 23 to 25, 2021. The Webinar was organized within the CAPES-PrInt Internationalization Program from the Federal University of Santa Maria. Since 2018, the CAPES/PrInt Program supports the cooperation of researchers from UFSM with foreign partners under two network transdisciplinary projects within the **One Health theme:** ***Animal health and welfare*** and ***Pharmacological and nutritional strategies for health promotion***.

The One Health Concept highlights the inseparable relationship between all living species and the environment that are interconnected in a symbiotic way. Since its creation, that occurred less than 20 years ago, the One Health Concept has been recognized and promoted by the United Nations and other relevant international scientific and socioeconomic players. Moreover, the emergence of the coronavirus 2019 pandemic showed the urgency of this transdisciplinary approach. However, infectious diseases are not the only threat to the human and animal health. Anthropogenic activity may pose many other risks to the maintenance of life on Earth. Thus, the development of medicines and interventions to fight non-communicable diseases and the production of safe, healthy and affordable food while assuring animal welfare and ecofriendly practices are current challenges.

The e-book is organized as a collection of 14 chapters from lectures of the Webinar. The **International Webinar "One Health over Borders – Capes/PrInt-UFSM"** merged the disciplinary frontiers by joining distinguished speakers in round-table transdisciplinary sessions to discuss integrative solutions for current animal-environment-human challenges. The Webinar was organized under three major themes ***"Shared environment", "Shared medicines and interventions" and "Safe food and food systems"***, which were discussed in the following sessions:

Viral diagnosis and vaccines: from animal to human health

Environmental pollutants: a hidden hazard for human health

Pain control: a complex challenge for animal and human welfare

Cancer research: perspectives of new compounds and non-animal approaches

Drug repurposing applicable to COVID-19

From fish welfare assurance to safe food products

Clean technologies for food processing and extraction of bioactive compounds

Food safety from farm-to-fork

During the webinar we had the opportunity of showing you relevant data obtained in the CAPES/PrInt network transdisciplinary projects and discussing how they contribute to the One Health approach. We also had the opening of the Art Exhibition "Grass Flower" from professor Raquel Fonseca (UFSM) that calls our attention to the beauty of undervalued plant species from our local biodiversity. By articulating researchers from different areas of knowledge, such as animal health and welfare, human health, food safety, education in science and visual arts, the Webinar promoted holistic discussions to drive the knowledge progress within and among these different areas and make an effective contribution to public health.

We had more than 360 registrations for the webinar, which joined participants from institutions of 13 different states of Brazil and also from Nigeria, Chile, Guatemala, Mexico, United States, Canada, Portugal, Spain, Italy, and the United Kingdon.

CARDIOVASCULAR AND DIABETES RISKS; THE INFLUENCE OF HERBS/DIETS IN THE PATHOGENESIS OF ERECTILE DYSFUNCTION

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ABSTRACT

Sexual dysfunction (SD) is a sexual behavior and sensation problem that manifests as an abnormality or lack of sexual psychology and physiological response. It is a catch-all word for a variety of symptoms, including erectile dysfunction (ED), failure of sexual intercourse, and libido/desire loss. According to studies, 52 percent of males between the ages of 40 and 70 have some form of SD. Furthermore, a range of biological and psychological variables contribute to the development of these disorders. Around 15% of couples in the world suffer from sexual discord, with male factors accounting for 40 to 50% of these cases. Because of the sensitivity of the male reproductive system, it is easily influenced by a variety of risk factors, including chronic diseases, environmental pollutants, drug toxicity, and an unhealthy lifestyle, to name a few. Significant progress has been made in recent years toward understanding the various varieties of male SD and putative pathogenic causes. There are only a few treatments in reproductive medicine that are being addressed against SD. The goal of this review is to sum up recent findings of SD pathogenic factors in clinical and animal studies, to elaborate the underlying mechanisms of these diseases from *in vivo* and *in vitro* studies, to analyze risk factors, and to describe the management strategies traditionally recommended for male sexual dysfunction. The findings of the review reveal systematic approaches to effectively preventing certain disorders.

KEYWORDS

Sexual dysfunction, risk factors, animal studies, clinical studies.

Introduction

Sexual dysfunction in males is caused by medical illnesses such as hypertension, diabetes mellitus, and cardiovascular disease (CVD), as well as psychological problems like depression and anxiety. CVD and hypertension cause the arteries to constrict and stiffen, reducing blood flow to the corporal bodies, which is necessary for achieving an erection. Because diabetes can impair both the blood vessels and the nerves that supply the penis, it is a major cause of sexual dysfunction. Erectile dysfunction (ED) generally is linked to a number of risk factors for cardiovascular disease, including age, cigarette smoking, hypertension, dyslipidemia, and diabetes mellitus (DM) (FELDMAN et al., 1994, p 151; BILLUPS AND FRIEDRICH, 2000, p 147; SCHACHTER, 2000, p 9). Patients receiving treatment for diabetes, heart disease, or hypertension had a 4-fold higher risk of getting ED than age-matched controls without these illnesses, according to the Massachusetts Male Aging Study (MMAS) (FELDMAN et al., 1994, p 151). This was verified in an 8-year follow-up of a sample of the MMAS population, where the prevalence of mild or total ED in patients with CV risk factors was 31% compared to 20% in the age-matched, disease-free control group (GREENSTEIN et al., 1997, p 123; FELDMAN et al., 2000, p 328). ED, on the other hand, is substantially connected to the occurrence of later CV episodes (THOMPSON et al., 2005, p 2996). Atherosclerotic disease affecting not only the coronary but also the penile vasculature, resulting in insufficient blood flow, and endothelial dysfunction in combination with hyperlipidemia (WEI et al., 1994, p 140; SCHACHTER, 2000, p 9), hyperglycemia (Seftel et al., 1997, p 1016; De Angelis et al., 2001, p 1155), or hypertension (TADDEI et al., 1998, p 41), impairing the fundamental relationship could be vascular endothelial dysfunction. It occurs frequently in people with CV disease and/or diabetes mellitus before the formation of atherosclerotic plaques (KIRBY et al., 2000, p 614; DE ANGELIS et al., 2001, p 1155). Men with DM had a 5- to 6-fold higher risk of any degree of ED than the general population (FELDMAN et al., 1994, p 151), and this risk is enhanced when other CV risk factors are present. Because ED and CVD have many of the same risk factors, it's no surprise that men with CVD have a higher rate of ED. MONTORSI et al. (2003, p 360) discovered that ED was present in nearly half of

individuals with acute chest pain and angiography-confirmed coronary artery disease (CAD). In this investigation, ED was found to be the cause of CVD in over 70% of cases. Similarly, many men with ED have been discovered to have pre-existing cardiovascular disease (CVD). VLACHOPOULOS et al. (2005, p 996) looked at the prevalence of asymptomatic CVD in 50 males with ED in a study. These researchers discovered that 19% of males with ED had asymptomatic CVD. Mulhall and colleagues discovered that 20% of males with ED and vascular insufficiency on the penile duplex had asymptomatic CVD (MULHALL et al., 2009, p 820).

These findings imply that individuals with ED and CV risk factors should be assessed for silent CVD and undergo a comprehensive CV evaluation. Men with new onset ED and no signs or symptoms of CVD are at increased risk for experiencing a CV event in the subsequent 3–5 years (HODGES et al., 2007, p 2019). Cardiovascular diseases (CVD) continue to be the largest cause of death and morbidity in Western countries, and they represent a major impediment to long-term human growth (ROTH et al., 2017, p 1). According to ROTH et al. there were 422.7 million prevalent instances of CVD and 17.92 million CVD deaths worldwide in 2015. Ischemic heart disease (IHD) is the main cause of death and disability worldwide (ROTH et al., 2017, p 1). For a long time, a gender difference in CVD risk was observed, with males having a higher risk than females (ALWAN, 2011). Between 1990 and 2015, all high-income and several middle-income nations reported significant reductions in age-standardized CVD death rates (ROTH et al., 2017, p 1). As a result, identifying high-risk people is a critical step in developing an effective primary preventive program. Several population-specific CV risk engines have been created for this purpose (ROSENBLIT, 2019, p 61). They are fundamentally based on multivariate risk prediction equations derived from large prospective cohort studies (ROSENBLIT, 2019, p 61). Although these instruments have shown to be useful in clinical practice, due to some drawbacks, such as lower reliability when used to populations with different features from those for which they were developed (ROSENBLIT, 2019, p 61). As a result, the Framingham risk engine, which was designed in the United States, was found to overestimate CV risk in European populations (MARRUGAT et al., 2003, p 634). Furthermore, another confusing issue in CV risk categorization is the

concept of "residual risk." According to data from the general population, the majority of CV events occur in people who are classified as "lower-risk" by traditional measures (FISHER et al., 2009, p 866; FISHER et al., 2012, p 2785; ZANNAD et al., 2012, p 163; RASTRELLI et al., 2012, p 3227; RASTRELLI et al., 2014, p 173). As a result, identifying additional parameters that contribute to this "residual CV risk" will be a difficult task in the near future.

ED is a multifaceted disease that affects more than 100 million people worldwide, with an estimate of more than 300 million affected people by 2025. ED is not only a complication of CVD, but it is also frequently a precursor to future CVD, according to a significant body of research (GUO et al., 2010, p 2805; DONG et al., 2011, p 1378; YAMADA et al., 2012, p 43673; VLACHOPOULOS et al. 2013, p 99; FAN et al., 2018, p 1681; ZHAO et al., 2019, 1005). Therefore, ED patients should be viewed as paradoxically fortunate, because the onset of symptoms gives a valuable temporal window [2-5 years] for earlier modification of related risk factors and, perhaps, improved outcomes (CORONA et al., 2008, p 193). Diabetes mellitus (DM) is a global public health issue and the prevalence of diabetes is thought to be linked to the aging process, as is the prevalence of reduced glucose tolerance as people get older (MELMED et al., 2017, p 1358). Type II diabetes is a chronic disease caused mostly by two polygenic abnormalities (KALIN et al., 2017, p 267). Insulin resistance and insulin secretion defects in the beta cell are two of these (HOGAN AND HULL, 2017, p 952). Insulin resistance is linked to sarcopenia and is hypothesized to have a role in the physiopathology of hypertension, hyperlipidemia, and obesity (SRIKANTHAN et al., 2010, p 10805).

Sarcopenia is described as a loss of muscle strength or function that is accompanied by a reduction in muscle mass. Sarcopenia can be caused by vascular, neurological, and hormonal factors, as well as low calorie intake and cytokines (MORLEY, 2008, p 452). Obesity, diabetes, metabolic syndrome and Sarcopenia have all been linked to androgenic hormones that regulate sexual functioning (KAMEL et al., 2002, p 865; LUNENFELD et al., 2015, p 5). Depending on the age, kind, and duration of diabetes, the prevalence of diabetic ED ranges from 30 to 90 percent (MCCULLOCH et al., 1980, p 279). Although several factors have been linked to the physiopathology of ED in diabetic men, hypogonadism, diabetic neuropathy, and

endothelial dysfunction have been identified as the most important (CELLEK et al., 2013, p 1). Multiple processes are implicated in the pathophysiology of sarcopenia and ED, which are becoming more common, especially among middle-aged and advanced-age diabetic men, impairing physical function, sex life, and overall quality of life. Due to the treatment process, incurred treatment expenses, comorbidities, and rising prevalence in recent years, sarcopenia and ED have become a global public health problem.

The impact of dietary supplementation with plant/food items has recently gotten increased attention. Because of their long history of use, herbal medications can be used to treat ED; also, natural remedies are becoming increasingly popular as a means of preserving good health around the world. Further research on the efficacy and safety of herbal medicine for the treatment of ED is needed to better understand the significance and relevance of traditional medicinal knowledge in health care. The development of natural supplements from medicinal plants is gaining traction due to their high effectiveness, low cost, and lack/less of adverse effects (EKOR, 2013, p 177). As a result, medicinal herbs having aphrodisiac properties must be investigated further. I will highlight certain herbal/food products that have been shown to have considerable pharmacological activity in this review. Alkaloids, flavonoids, tannins, xanthones, triterpenes, and quinones are some of the phytoconstituents that influence reproductive function. Herbal plants are commonly used to treat sexual dysfunction due to their vascular protection or oxygen radical scavenging properties through a variety of processes (SHI et al., 2017, p 562). In a two-year Mediterranean diet experiment, a diet rich in whole grains, fruit, vegetables, nuts, legumes, and olive oil was linked to improved erectile performance in people with metabolic syndrome (ESPOSITO et al., 2006, p 405). This intervention significantly improved endothelial function and reduced carbonyl-reactive protein concentrations, highlighting the similar underlying mechanisms that link diet, inflammation, vascular function, and ED. The review evaluates the association between CV and diabetic risks in the pathogenesis of ED as well as the influence of diet and herbs.

Treatment Options

Lifestyle adjustments, which are regarded as first-line therapy in many medical diseases, can have a beneficial influence in ED management, and men should be encouraged to make the required changes for the benefit of their sexual function as well as their general health. Despite the benefits of behavior modification, men who present with ED want the doctor to assist them with fast solutions.

Phosphodiesterase-5 (PDE-5) inhibitors, intracavernosal injectable therapy, vacuum constriction devices (VCDs), intraurethral prostaglandin suppositories, and surgical insertion of a penile prosthesis are all alternatives for treatment beyond behavior retraining (RANDRUP et al., 2015, p 166). PDE-5 inhibitors, the principal second-line medication, have been the cornerstone of ED treatment since the release of sildenafil citrate (Viagra) in 1998, with the creation of a slew of others since then, with more on the way. For the vast majority of men, these drugs improve erectile function by increasing blood flow in the corpora cavernosa. These drugs are usually taken on demand and should be taken around an hour before sexual intercourse. Tadalafil (Cialis) has a longer acting time and is available in a daily preparation, which could eliminate the requirement for 'on-demand' medication. The daily dosing of tadalafil, 2.5–5 mg/day, has also been approved by the FDA for treatment of symptoms of Benign prostatic hyperplasia (BPH) (WRISHKO et al., 2009, p 2039). PDE-5 inhibitors are not recommended for men who are using nitrates, although they are otherwise quite safe and effective. When PDE-5 inhibitors are used with nitrates, they can cause severe hypotension and systemic vasodilation. Because many patients with ED are elderly and share risk factors with patients with coronary artery disease (CAD), medication combinations like these are frequently explored or seen in clinical practice (SEFTEL et al., 2004, p 2341). However, various negative effects have been documented as a result of using these synthetic medications, including blurred vision, bleeding, and other issues. This has opened the door to natural, safer, and less expensive alternative therapies. Plant/food products have been used in the management/treatment of ED in numerous forms, some of which are highlighted here.

Huoxue Tongluo Qiwei Decoction

Leech, centipede, red peony root, *Achyranthis radix*, *Angelica sinensis*, cowherb seed, *Radix curcumae*, green tangerine peel, *Radix bupleuri*, *Tribulus terrestris* L, *Epimedium wushanense*, and *Morinda officinalis* are the major ingredients of Huoxue Tongluo Qiwei Decoction. Huoxue Tongluo Qiwei Decoction is a traditional herbal preparation that can help patients with erectile dysfunction (ED) symptoms and has a good therapeutic impact on diabetic erectile dysfunction (DIED). Huoxue Tongluo Qiwei Decoction's major function is to accelerate blood circulation and dredge collaterals, remove blood stasis, and thereby bringing comfort/relief (WENXIONG et al., 2019, p 954]. XIAO et al. [2021, p 114674] designed animal tests to investigate the mechanism of Huoxue Tongluo Qiwei Decoction in the therapy of DIED. They discovered that the decoction inhibited the production of protein and mRNA of PKC signaling pathway related molecules in DIED rats and improved erectile function by curing vascular endothelial injury, increasing antioxidant capacity, and preventing platelet activation.

Moringa oleifera

Before being stressed for seven days, Wistar male rats were given oral dosages of *M. oleifera* leaf extract (10, 50, and 250 mg/kg) for 45 minutes. The levels of PDE-5 and testosterone were measured using suitable kits, and mating activity was assessed using video. The plant extract reduces PDE-5 action, increasing blood testosterone levels and mating efficiency. PRABSATTROO et al. (2015, p 179) found that this extract increased the number of testicular Leydig cells and spermatogenesis. Another study found that giving male albino rats 100, 200, or 500 mg/kg of *M. oleifera* aqueous seed extract each day for 21 days boosted their libido and copulation (ZADE et al., 2013, p 129).

Recently, OYELEYE et al. (2021, p 13323) discovered treatments of diabetic rats with acarbose (ACA) and co-fed with 2% and 4% of Moringa leaf and seed inclusive diets for 14 days, could be of great benefit toward the management of ED caused by diabetes, as evident by the expression of

some ED-related biomarkers [acetylcholinesterase (AChE), monoamine oxidase (MAO), angiotensin-I converting enzyme (ACE), adenosine deaminase (ADA) and arginase activities, and thiobarbituric acid reactive species (TBARS) level] in the penile tissue of diabetes male rats, compared to that of ACA treated alone. Thus, a large-scale clinical investigation to manage diabetes complications, such as the possible benefit of Moringa leaf and seed inclusive diets co-administered with ACA for the holistic therapy of diabetes-induced erectile dysfunction, seems acceptable (ED). Clinical trials on the safety and efficacy of *M. oleifera*, however, are not yet available.

Hunteria umbellata

In South-western Nigeria, *Hunteria umbellata* (HU) is known as Abeere. Many components of HU (leaf, seed, root, and bark) have been reported effective for the treatment of various human disorders in Nigerian traditional medicine, including pregnancy-related diseases, inflammation, and the plant is recognized to have anthelmintic properties (GILL, 1992, p 134; FALODUN et al., 2006, p 256). OBOH et al. (2017, p 2016) investigated the biochemical indices involved in the diabetic risk of ED, as well as diabetes and impotence in traditional medicine. The effects of the extracts (leaf, seed, root, and bark) on activities of several enzymes relevant to erectile dysfunction (arginase and phosphodiesterase-5) and type-2 diabetes (α-amylase and α-glucosidase) were investigated in order to determine their erectogenic and antidiabetic characteristics. Several antioxidant tests (DPPH, OH) were used to evaluate the antioxidant capabilities of these extracts. HPLC was also used to evaluate and quantify the phenolic components. Gallic acid, chlorogenic acid, caffeic acid, ellagic acid, and quercetin are among the bioactive phytochemicals found in HU, with caffeic and quercetin taking the lead of all the polyphenols. In the article recently published by OLABIYI et al. (2021, p 13864), the positive effect of quercetin was confirmed on some of these biochemical parameters involved in ED using hypertension model in rats.

African walnut (*Tetracarpidium conophorum Müll.* Arg.)

Tetracarpidium conophorum Müll. Arg. is a tropical Euphorbiaceae climbing shrub that reaches 10–20 feet tall and is called in *Southern* Nigeria as ukpa (igbo), Western Nigeria as *awusa* or asala (Yoruba), and the littoral and western Cameroon as kaso or ngak. It contains bioactive components such as plant sterols, vitamin C, and omega-3 fatty acids (KANU et al., 2015, p 215). Walnut eating has been demonstrated to lower serum cholesterol levels and to prevent lipid peroxidation from starting and progressing (AKOMOLAFE et al., 2016, p 15). Walnut leaves and peel have also been proven to have an anti-hyperglycemic impact in diabetic rats. Our study also discovered that walnut hydroalcoholic extract has a modulatory effect on ectonucleotidases (OLABIYI et al., 2018, p 140). In Nigeria's South Western region, walnut has long been utilized as a male sexual stimulant. In a recent study, OLABIYI et al. (2019, p 251) investigated the effects of dietary supplementation of *T. conophorum* on critical biochemical variables that determine erectile function and copulatory behavior in a hypertensive rat model. ADEMILUYI et al. (2019, p 12815) also investigated the influence of the seed shell on biological activity. They looked at how African walnut extract interacts with phosphodiesterase-5 (PDE-5), arginase, angiotensin-converting enzymes (ACE), and acetylcholinesterase (AChE) in isolated penile tissue, and Fe^{2+}-induced malonaldehyde (MDA) generation. The extracts suppressed these relevant enzymes as well as MDA generation, with Walnut cooked with shell having the greatest effect. This was supported by higher levels of phenolic acids and flavonoids in African walnuts cooked with the shell compared to those cooked without shell. They came to the conclusion that one of the possible mechanisms of action of African walnut in the management/treatment of ED could be enzyme inhibition and antioxidative potential. Cooking walnut seed with the shell appears to be a significant element, as it may prevent phytochemicals from seeping out, which could be responsible for these biological impacts.

Tiger nut (*Cyperus esculentus* L.)

Cyperus esculentus L. belongs to the Cyperaceae family and it is known in the Middle East as Tiger nut (English) and "Hab Al-zulom" (Arabic), which means "men's seed" (Allouh et al., 2015, p 1). In Ayurvedic medicine, the herb has been used as an aphrodisiac to improve ED and sexual activity (ALLOUH et al., 2015 p 1; OLABIYI et al., 2017, p 152). In a study by ALLOUH et al. (2015, p 1) the aphrodisiac function of *C. esculentus* in adult male rats was confirmed. In comparison to controls, oral ingestion of 1–2 g/kg of raw *C. esculentus* powder every day for 30 days boosted testosterone levels in rats and increased intromission frequency in moderately active rats. Vitamin C, vitamin E, quercetin, and zinc components were also found in the *C. esculentus* methanolic extract after LC–MS phytochemical screening. It's considered that Vitamin C, vitamin E, quercetin, and zinc components were responsible for the rats' high serum hormone levels (ALLOUH et al., 2015, p 1).

In another work by OLABIYI et al. (2017, p 152) adult male rats were fed a diet enriched with processed and raw *C. esculentus* (both 10 and 20 percent) for 14 days and after the corpus cavernosum was removed and cultured. A 20% raw plant diet lowered arginase, AChE, and adenosine deaminase (ADA) activity while increased NO generation, suggesting that a 20% raw plant diet could improve erectile sexual response. In the cerebral cortex (CC) of hypertensive rats, we discovered that *C. esculentus* improved behavioral responses, modulated purinergic receptor densities, ectonucleotidases (E-NTPDase), and adenosine deaminase (ADA) activities, increased NO levels, and prevented oxidative stress related to erectile function (OLABIYI et al., 2021, p 13862). The findings demonstrate that the nut can be used as a feed for both animals and humans in the treatment of ED. It should be noted that the nut had no toxicological effects. Furthermore, there have been no clinical trials on the safety and efficacy of *C. esculentus* in the ED so far.

Conclusion

It can be seen from the above findings, the modulatory effects of natural compounds on the sexual behavioral activities, antioxidant status as well as relevant biochemical

parameters responsible for erectile function. These effects on arginase activity, nitric oxide synthase, insulin resistance, glucose oxidation-induced superoxide production, the renin-angiotensin system, and acetylcholinesterase are all pathophysiologic processes implicated in the treatment of ED. The unfavorable effects of synthetic PDE inhibitors, alprostadil, penile prosthesis, and hormonal replacement treatment piqued researchers' interest, prompting them to look into new medicinal plant species and natural active substances with less/no adverse effect to help lessen and treat ED. However, only a few clinical trials have looked into the safety and efficacy of medicinal plants/plant foods in the management/treatment of ED.

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PLANT ESSENTIAL OILS AND PHYTOCHEMICALS AGAINST AEROMONOSIS IN TROPICAL FISH

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ABSTRACT

Bacteria of the genus *Aeromonas* are very common pathogens in aquaculture, causing hemorrhagic septicemia, cutaneous ulcerations and enteritis in aquatic organisms. Antimicrobial, immunostimulant and antioxidant properties of plant essential oils (EOs) and their isolated compounds have been extensively studied. This chapter reviewed recent data about the preventive and curative effects of plant EOs and phytochemicals in the feed or via bath on survival of tropical fish with aeromonosis. The vast majority of studies used silver catfish (*Rhamdia quelen*) and Nile tilapia (*Oreochromis niloticus*) as experimental models, and used strains of *Aeromonas hydrophila*. Therefore, studies involving other species of tropical fish and other Aeromonas species are scarce. Most studies with a preventive function administered the substances orally through the feed. Almost all studies with curative function administered the substances via bath. The preventive efficacy of the substances derived from plants was generally satisfactory, with percentage of survival increase compared to untreated control ranging from 11.11% to 400% after infection. Similarly, the curative efficacy of the substances derived from plants in treating infected animals was promising, with percentage of survival increase compared to untreated control ranging from 22.22% to 200.03%. These studies demonstrate very promising results, presenting alternative treatments to conventional antimicrobials with high efficacy and environmentally sustainable. In addition, the use of natural substances as antimicrobials contributes to the reduction of conventional antimicrobial residues in the aquatic environment and, consequently, in fish meat, slowing down the process of bacterial resistance and improving the safety of fish-based foods for human consumption.

KEYWORDS

Bath; Diet supplementation; Prevention; Survival rates; Treatment.

The genus *Aeromonas* corresponds to Gram-negative rods, oxidase-positive and facultatively anaerobic, that grow readily on basic laboratory media (JANDA & ABBOTT, 2010). These bacteria are very common pathogens in aquaculture, causing hemorrhagic septicemia, cutaneous ulcerations and enteritis in aquatic organisms (BEAZ-HIDALGO & FIGUERAS, 2013). In Brazil, economic losses related to parasitic and infectious diseases in fish are estimated to be approximately US$84 million per year, mainly due to increased mortality rate, decreased growth rate and drug expenses for prophylaxis and treatment of these animals (TAVARES-DIAS & MARTINS, 2017). *Aeromonas* have the potential to be a foodborne pathogen to humans. As a psychrotroph, *Aeromonas* grows in foods during refrigeration. The main disease caused in humans is gastroenteritis. Its pathogenicity depends of the expression of some virulence factors, such as exotoxins, cytotoxins, endotoxins, siderophores, invasins, adhesins, S-layers and flagella (DASKALOV, 2006). The presence of multi-resistant strains increases the concern (BLASCO et al., 2008).

Plants have been used in traditional medicine since ancient times and pharmacological properties are attributed to their secondary metabolites, mainly phenols. There are several benefits of using these compounds, mainly involving antimicrobial, antioxidant, nutritional and pharmaceutical activities (LAI & ROY, 2004). Essential oils (EOs) are volatile natural mixtures extracted from different plant parts (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots), and are composed of terpenoid with broad pharmacological activities. They are usually obtained by hydro or steam distillation or by cold pressing (in the case of citrus peel oils). Antimicrobial properties of EOs and their isolated compounds have been extensively reported (SEOW et al., 2014; CUNHA et al., 2018a). There are several studies focusing on the use of EOs and their major compounds in the prevention and/or treatment of fish bacterial diseases, such as aeromonosis. Applications of these substances via bath or diet supplementation have shown promising results (CUNHA et al., 2018a).

Bath administration ensures that all animals come into contact with the same drug concentration, including animals with anorexia. However, it is more difficult to apply and handle in fish farms, compared to the administration of the drug in feed. On the other hand, although diet supplementation is more practical,

debilitated fish may not eat or may eat smaller amounts of feed, which means that they may not come into contact with the active ingredient at therapeutic concentrations. The preventive and curative effects of plant EOs and phytochemicals in the feed or via bath on survival of tropical fish with aeromonosis are shown in Tables 1 and 2, respectively.

There are studies in this area with only three species of tropical fish: silver catfish (*Rhamdia quelen*), Nile tilapia (*Oreochromis niloticus*) and tambaqui (*Colossoma macropomum*). The vast majority of studies used silver catfish and Nile tilapia, which reinforces the need for further studies with other species of tropical fish. In addition, most studies used strains of the species *A. hydrophila*, while only one study used a strain of *A. veronii*. Therefore, studies involving other *Aeromonas* species and tropical fish are scarce. Most studies with a preventive function administered the substances orally through the feed. Only one study with a preventive function administered the substance via bath, which was *Melaleuca alternifolia* EO (SOUZA et al., 2016). Almost all studies with curative function administered the substances via bath. Only one study with curative function administered the substance orally, which was *Nigella sativa* EO (HAL & EL-BARBARY, 2021).

The preventive efficacy of the substances derived from plants was generally satisfactory, with percentage of survival increase compared to untreated control ranging from 11.11% to 400% after infection. A 100% of survival was reported in Nile tilapia infected with *A. hydrophila* and treated orally with curcumin (MAHMOUD et al., 2017) and *Petroselinum crispum* EO (FARAG et al., 2021). Similarly, the curative efficacy of the substances derived from plants in treating infected animals was promising, with percentage of survival increase compared to untreated control ranging from 22.22% to 200.03%. CUNHA et al. (2018b, 2019) reported 100% of survival in silver catfish infected with *A. hydrophila* and treated via bath with *Origanum majorana* EO and the phytochemical terpinen-4-ol.

Several EOs and their isolated compounds have been tested against bacteria of the genus *Aeromonas*. For example, the phytochemical carvacrol and the oregano EO (*Origanum vulgare* carvacrol-rich EO) have been reported for their antibacterial activity against *A. hydrophila* in tambaqui and Nile tilapia, respectively (OLIVEIRA et al., 2020; SILVA et al., 2021). Another example is the antibacterial effect of the

phytochemical terpinen-4-ol and tea tree EO (*M. alternifolia* terpinen-4-ol-rich EO) against *A. hydrophila* in silver catfish (CUNHA et al., 2019; SOUZA et al., 2016). Often the main compound of EO is responsible for its antibacterial effect. However, this is not always true, as in some cases, there may be additive, synergistic or antagonistic actions between different phytochemicals in the same EO. An example of a synergistic action is the combination of the phytochemical *trans*-cinnamaldehyde with the EOs of *Thymus vulgaris* and *Eugenia caryophyllus* against bacteria of the genus *Aeromonas* (KOT et al., 2019). Testing these substances separately and in combination is the best way to explore these actions.

Generally, an *in vitro* antibacterial screening test is performed before testing the EOs and their phytochemicals *in vivo*. In this way, only those compounds with the most promising activities in the *in vitro* test are selected, in order to reduce the number of animals to be used in the *in vivo* test, following ethical principles. For example, BANDEIRA JUNIOR et al. (2019) tested the *in vitro* antibacterial activity of EOs of *Achyrocline satureioides*, *Aniba parviflora*, *Aniba rosaeodora*, *Anthemis nobilis*, *Conobea scoparioides*, *Cupressus sempervirens*, *Illicium verum*, *Lippia origanoides* and *M. alternifolia* against bacteria isolated from fish, and selected only the most promising (*L. origanoides* EO) for the *in vivo* test against aeromonosis in silver catfish. On the other hand, there are several EOs that have been tested *in vitro* against bacteria of the genus *Aeromonas*, have shown good results, but have never been tested *in vivo*, demonstrating that this area still needs to be further explored. Two examples are the EOs of cinnamon (*Cinnamomum cassia*) and lemongrass (*Cymbopogon citratus*), which have shown good results *in vitro* against different species of *Aeromonas* (STARLIPER et al., 2015). However, most EOs that were evaluated to treat bacterial infections using baths showed effect at concentrations below the minimum inhibitory concentration (MIC) observed *in vitro*. An analysis of the MIC and the minimum effective bath concentrations or dietary supplementation doses of EOs found in the literature demonstrated that there is no correlation between them (CUNHA et al., 2018a).

With the aquaculture development and the constant use of antibiotics for prophylaxis and treatment of bacterial diseases, concerns about the emergence of bacterial resistance

and the presence of antibiotic residues in the fish meat arise (CABELLO, 2006; WATTS et al., 2017). Focusing on these problems, research has emerged aiming at the combined use of substances isolated from plants with conventional antimicrobials, in order to reduce the amount of antibiotics used. In this line, it has been reported that the phytochemical linalool has synergistic activity with the conventional antimicrobials florfenicol and oxytetracycline against *A. hydrophila* in an *in vitro* "checkerboard" study (BANDEIRA JUNIOR et al., 2018). In a similar *in vitro* study, SOUZA et al. (2017) reported synergistic effect of the combination of *Aloysia triphylla* and *Lippia alba* EOs with florfenicol against *Aeromonas* sp. DEEPIKA et al. (2019) reported synergistic antibiofilm potential of the combination of rutin with florfenicol against *A. hydrophila*. In the same study, this combination of rutin and florfenicol enhanced therapeutic spectrum against drug resistant *A. hydrophila*. Despite this, there is still a lack of *in vivo* studies to confirm these *in vitro* synergistic effects.

It is known that the effect of EOs and their isolated compounds on the survival of fish infected with *Aeromonas* is not only due to their antibacterial activity. The immunostimulant and antioxidant effects of substances isolated from plants are well recognized for improving the health status of these animals, consequently increasing their survival rate (CITARASU, 2010). On this subject, YILMAZ (2019) reported the immunostimulant effect of caffeic acid on Nile tilapia. A significantly increase in the phagocytic index, potential killing activity, respiratory burst activity, serum myeloperoxidase activity and serum catalase activity was detected after dietary supplementation with caffeic acid. Furthermore, increased expression of genes related with immune system was reported in fish supplemented with caffeic acid. MONTEIRO et al. (2021) reported an increased leukocyte respiratory activity in tambaqui fed diet supplemented with *Lippia sidoides* EO after experimental infection with *A. hydrophila*, indicating immunostimulant action of this EO. Stimulating the fish's immune system is a desirable action in case these animals have an infectious disease.

According to BANDEIRA JUNIOR & BALDISSEROTTO (2020), most studies on oxidative status in fish with aeromonosis pointed to an increase in reactive oxygen species, malondialdehyde and protein carbonylation levels after infection, indicating possible oxidative damage. Therefore, the

use of substances isolated from plants with an antioxidant effect is desirable, as they could prevent changes in the oxidative status caused by the infection. MAHMOUD et al. (2017) reported a significant increase of both catalase activity and reduced glutathione content with a significant decrease in the malondialdehyde level in plasma of healthy Nile tilapia fed diet supplemented with curcumin, indicating an antioxidant action of curcumin. Similarly, ROSA et al. (2019) reported that the dietary addition of rutin in silver catfish diminishes the *A. hydrophila* induced-rise in nitric oxide and superoxide anion levels and reestablishes superoxide dismutase activity in the muscle. In addition, it reduces *A. hydrophila* induced-lipid peroxidation, markedly elevates ascorbic acid and thus reestablishes total antioxidant capacity in the muscle, whose levels were decreased after *A. hydrophila* challenge.

Essential oils and their isolated compounds can also act by inhibiting the expression of bacterial virulence factors, such as the ability to form biofilm and cause hemolysis. In this sense, BANDEIRA JUNIOR et al. (2018) reported that phytochemicals carvacrol, citral, eugenol, linalool and thymol inhibited biofilm formation and hemolysis activity of an *A. hydrophila* strain. Similarly, SUTILI et al. (2015) reported that EOs of *Hesperozygis ringens*, *Ocimum americanum* and *Ocimum gratissimum* inhibited hemolysis activity of another strain of *A. hydrophila*. Another example is the ability of rutin to inhibit the biofilm formation of an *A. hydrophila* strain (DEEPIKA et al., 2019).

These studies demonstrate very promising results, presenting alternative treatments to conventional antimicrobials with high efficacy and environmentally sustainable. In addition, the use of natural substances as antimicrobials contributes to the reduction of conventional antimicrobial residues in the aquatic environment and, consequently, in fish meat, slowing down the process of bacterial resistance and improving the safety of fish-based foods for human consumption.

Acknowledgments

Authors thank the support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, finance code 001) of Brazil.

Table 1 - Preventive effect of plant EOs and phytochemicals in the feed or via bath on survival of tropical fish with aeromonosis

Plant EO (plant part) or phytochemical	Fish species	Bacterial species	Administration	Best dosage or concentration[1]	Survival increase compared to control (%)[2]	Source
Aloysia triphylla (leaves)	*Rhamdia quelen*	*Aeromonas hydrophila*	Orally, incorporating the EO in the feed for 21 d before infection	1.76 g/kg of feed	↑ 75.47%	SANTOS et al. (2017)
Caffeic acid	*Oreochromis niloticus*	*Aeromonas veronii*	Orally, incorporating the phytochemical in the feed for 60 d before infection	5 g/kg of feed	↑ 67.59%	YILMAZ (2019)
Carvacrol	*Colossoma macropomum*	*Aeromonas hydrophila*	Orally, incorporating the phytochemical in the feed for 60 d before infection	10 g/kg of feed	↑ 59.95%	SILVA et al. (2021)
Curcumin	*Oreochromis niloticus*	*Aeromonas hydrophila*	Orally, incorporating the phytochemical in the feed for 84 d before infection	0.05 g/kg of feed	↑ 42.86%[3]	MAHMOUD et al. (2017)
Cymbopogon flexuosus (leaves)	*Oreochromis niloticus*	*Aeromonas hydrophila*	Orally, incorporating the EO in the feed for 45 d before infection	1.77 g/kg of feed	↑ 400%	SOUZA et al. (2020)
Lippia alba (leaves)	*Oreochromis niloticus*	*Aeromonas sp.*	Orally, incorporating the EO in the feed for 45 d before infection	1.8 g/kg of feed	↑ 111.11%	SOUZA et al. (2019)
Lippia sidoides (leaves)	*Colossoma macropomum*	*Aeromonas hydrophila*	Orally, incorporating the EO in the feed for 60 d before infection	0.625 g/kg of feed	↑ 100 %	MONTEIRO et al. (2021)
Melaleuca alternifolia (leaves)	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 1 h for 7 d before infection	44.9 mg/L	↑ 100 %	SOUZA et al. (2016)
Origanum vulgare (leaves)	*Oreochromis niloticus*	*Aeromonas hydrophila*	Orally, incorporating the EO in the feed for 30 d before infection	5 g/kg of feed	↑ 30.02%	OLIVEIRA et al. (2020)
Petroselinum crispum (leaves)	*Oreochromis niloticus*	*Aeromonas hydrophila*	Orally, incorporating the EO in the feed for 60 d before infection	1.27 g/kg of feed	↑ 11.11%[3]	FARAG et al. (2021)
Rutin	*Rhamdia quelen*	*Aeromonas hydrophila*	Orally, incorporating the phytochemical in the feed for 14 d before infection	1.5 g/kg of feed	↑ 12.5%	ROSA et al. (2019)

[1]When necessary, values were transformed from volume to mass, based on the compound density. When not available in the reports, EOs density values of the same plant species were sourced from the internet.

[2]Survival increase calculated by the equation: [(survival treatment X 100)/survival control]–100.

[3]No mortality in the treated group.

Table 2 - Curative effect of plant EOs and phytochemicals in the feed or via bath on survival of tropical fish with aeromonosis

Plant EO (plant part) or phytochemical	Fish species	Bacterial species	Administration	Best dosage or concentration[1]	Survival increase compared to control (%)[2]	Source
Eugenol	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 1 h for 5 d after infection	10 mg/L	↑ 100%	SUTILI et al. (2014)
Hesperozygis ringens (leaves)	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 1 h for 5 d after infection	20 mg/L	↑ 75%	SUTILI et al. (2015a)
Lippia alba (leaves)	*Rhamdia quelen*	*Aeromonas* sp.	Continuous bath after infection	16 mg/L	>80% survival vs. 0% survival in control	SUTILI et al. (2015b)
Lippia origanoides (leaves)	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 1 h for 4 d after infection	4.3 mg/L	58.33% survival vs. 0% survival in control	BANDEIRA JUNIOR et al. (2019)
Nigella sativa (seeds)	*Oreochromis niloticus*	*Aeromonas hydrophila*	Orally, incorporating the EO in the feed for 14 d after infection	66.5 g/kg of feed	↑ 22.22%	HAL & EL-BARBARY (2021)
Ocimum americanum (leaves)	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 1 h for 5 d after infection	20 mg/L	↑ 87.5%	SUTILI et al. (2015a)
Origanum majorana (leaves)	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 1 h for 5 d after infection	18.4 mg/L	↑ 122.22%[3]	CUNHA et al. (2018b)
Terpinen-4-ol	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 30 min for 6 d after infection	5 mg/L	↑ 200.03%[3]	CUNHA et al. (2019)
Thymol	*Rhamdia quelen*	*Aeromonas hydrophila*	Daily baths of 30 min for 6 d after infection	5 mg/L	↑ 166.67%	CUNHA et al. (2019)

[1]When necessary, values were transformed from volume to mass, based on the compound density. When not available in the reports, EOs density values of the same plant species were sourced from the internet.

[2]Survival increase calculated by the equation: [(survival treatment X 100)/survival control]–100.

[3]No mortality in the treated group.

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CHRONIC EXPOSURE TO CHEMICAL RESIDUES IN LOW CONCENTRATIONS: THE INVISIBLE RISKS

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ABSTRACT

Humans and all other living beings are commonly exposed to environmental contamination at low concentrations for a long time. We are showing examples of emerging contaminants present in water bodies, that even at very low concentrations, cause toxic effects. For some chemicals, there is not a threshold for gene expression changes and for cancer. Theoretically, exposures below the threshold would be safe. Silent chronic exposures to chemicals cause an invisible cumulative toxic effect that will manifest in the future. For neurotoxic chemicals, this is an irreversible process. The refinement of toxicological methodologies and bioassays to get more sensitivity can be the key to protect human life and the ecosystems.

KEYWORDS

Threshold; Toxicity; Chronic; Pesticides; Emerging

Introduction

Chronic studies are commonly used to investigate the adverse effects arising from prolonged or repeated exposures to low levels of chemicals over the whole or greater part of a lifetime. These studies are more expensive to perform because they involve the use of many animals for a long-time. The prolonged exposures allow us to understand the toxic effects arising from the long and latent periods (EATON and GILBERT, 2008, p. 98). Results from chronic studies are very important for regulatory agencies to protect the population from residues of food additives, residues of pesticides in food, residues of pharmaceuticals and pesticides in drinking water, which we are all daily exposed through our lives (WRIGHT and WELBOURN, 2002, p. 128).

Chronic long-term assays have been replaced by shorter tests with durations of weeks or few months, based on the sensitivity of a bioassay. The employment of most susceptible stages of the life cycle such as embryos and larvae and using sublethal concentrations have been widely used with different animal species. A variety of endpoints might be used as concentration-response relationship, growth, reproduction, delay in development, weight loss, determination of mechanisms of action, biochemical markers, and behavior alterations (WRIGHT and WELBOURN, 2002, p. 129). Doses/concentrations selections for a chronic test might be previously determined in an acute or sub chronic assay to avoid premature mortality during the test.

For chronic studies in rodents, the determination of values such as Lowest Observable Adverse Effect Level (LOAEL) and Non-Observable Adverse Effect Level (NOAEL) is very important in case of regulation of new chemicals. Normally LD50% is obtained from acute tests. These are reference values used for governmental authorities in the determination of the maximum residue limits of chemicals to establish safety limits for protecting human health (WORTH *et al.*, 2007, p. 2).

Recently, toxicologists have become aware of using rodents in long-term assays, due to ethical responsibilities and consequently, they are replacing them with alternative protocols. The OECD protocol n. 215 (2000, p. 4) is designed to assess the effects of prolonged exposure to chemicals

in fish, among them zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). In this chronic test Effective Concentration ($EC_{20,30,50\%}$), No-Observable Effect Concentration (NOEC), and Lowest Observable Effect Concentration (LOEC) can be obtained and used to calculate the PEC and PNEC respectively (Predictive Effective Concentration and Predictive Non-Effect Concentration) respectively. Besides that, to get PEC/PNEC values the assessment of persistence, bioaccumulation, and toxicity of a chemical using different species, might be obtained to support these calculations (BREITHOLTZ *et al.*, 2006, p. 331).

The chemicals long used as pesticides and pharmaceuticals are continuously released to the environment and soon or later, they end up in water bodies. In this context, risk assessment is used to protect the environment and human health. The PNEC is the measurement of a chemical found in the ecosystem at concentrations below of adverse effects to living beings. For this reason, PNEC values obtained for different species in an ecosystem are used by regulatory authorities in the risk assessment of chemical contaminants in water (SMIT *et al.*, 2005, p. 442).

Figure 1 shows different fish species sensitivity to antidepressant fluoxetine based on LC50% gathered from literature, besides LC50% from our laboratory in zebrafish embryos. The LC50% varied from 0.164 to 2.0 mg/L. For calculations of PEC/PNEC in the risk assessment evaluation, the most sensitive species with LC50% available might be chosen based on the precautionary principle (FARIAS *et al.*, 2019, p. 7).

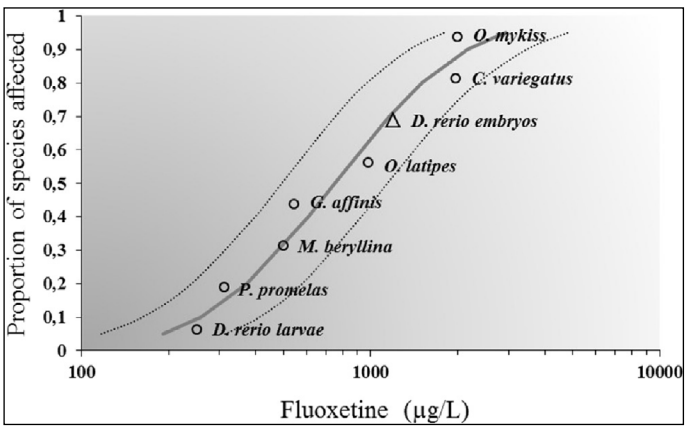

Figure 1. Different species sensitivity of fish exposed to fluoxetine based on LC50%. Triangle represents own data and the circles other fish species. The dotted line denotes upper and lower limits (FARIAS et al., 2019, p. 6).

New chemicals have arisen in water bodies, called emerging contaminants like pharmaceuticals and personal care products. They achieve different aquatic environments at very low concentrations of ng, µg and mg/L. They are biologically active even at very low concentrations. Then, chronic exposures at low concentrations may be of concern to human populations as well as aquatic life. They are continuously released to the environment mainly through wastewater, and they have been found also in drinking water, because up to now there is no technology to remove such chemicals in the drinking water treatment plants (METCALFE *et al.*, 2003, p.268; BENOTTI *et al.*, 2009, p. 599).

Cases of chemicals causing adverse biological effects even at very low exposure levels

We will show below some results of studies carried out in our laboratory and also for other groups reporting adverse side effects of emerging contaminants in water present at very low concentrations. Even at low concentration in water bodies, they have showed adverse biological activities.

Fluoxetine (FLX): a selective serotonin reuptake inhibitor antidepressant widely used in clinics and very often found in environmental samples of urban aquatic ecosystems in concentrations ranging from ng/L to µg/L. Zebrafish embryos were exposed immediately after fertilization to seven different concentrations of FLX (0; 0.01, 0.27, 0.74, 2.02, 5.51, 15.0 mg/L). Acetylcholinesterase activity was also measured after exposure at 6 µg/L. FLX affected the embryo development and inhibited acetylcholinesterase (FARIAS *et al.*, 2019, p. 108836). Zebrafish adults exposed at 0.33 and 2.7 µg/L of FLX for 30 days showed tissue alterations in liver and intestine (Farias *et al.*, 2020, p. 108836). Guppies (*Poecilia reticulata)* chronically exposed to FLX at 0.03 and 0.5 µg/L for 28 days resulted in a decreased survival and growth, and altered behavioral responses (PELLI and CONNUGHTON, 2015, p. 205).

Carbamazepine (Cbz): an anticonvulsant widely prescribed for the treatment of psychomotor epilepsy, bipolar disorder, and trigeminal neuralgia and have been detected in surface

waters and sediments. Zebrafish adults were exposed for 63 days to Cbz (0 - control, 10 µg/L - concentration found in effluents, and 10,000 µg/L. Several biochemical markers were changed a decrease in catalase activity in the liver and gills, lactate dehydrogenase – LDH was increased in the liver which means cell injury, and glutathione S-transferase – GST was increased. Histopathological changes in previtellogenic follicles in ovarian tissues were observed after exposures for 63 days to Cbz. At these concentration levels, no micronuclei and nuclear abnormalities in peripheral erythrocytes were found (SANTOS *et al.*, 2018, p. 230).

Ibuprofen: an analgesic and anti-inflammatory medicine used for the treatment of headaches and general pain (SYCHA *et al.*, 2003, p. 170). Ibuprofen has been identified in untreated sewage at concentration 4 up to 24.6 µg/L. Treated and untreated wastewater enter into aquatic ecosystems carrying pharmaceuticals products (METCALFE *et al.,* 2003, p. 2885; FENT *et al.*, 2006, p. 132). *Oryzias latipes* adults (Fish - medaka) were exposed to ibuprofen for 6 weeks at concentrations of 1.0, 10.0 and 100 µg/L. Reproductive parameters as spawning, fecundity, egg size, and rate of fertilization, were measured. As expected, no acute toxicity was observed. No pathological damage was evident in the gills, livers and kidneys in the fish of exposed groups. However, there was interference with reproductive performance causing a delay in the spawning frequency. The COX (cyclooxygenase enzyme) is directly involved with ibuprofen metabolism. The COX activity was significantly inhibited in the liver of medaka fish (FLIPPIN *et al.*, 2007, p. 76).

2,4-dichlorophenol (2,4-DCP): belong to chloroaromatics group, commonly found biocides, flame retardants, wood treatment compounds, and solvents. The 2,4-DCP is used in the production of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) widely used around the world. According to HOUSE *et al.* (1997, p. 344), the 2,4-D is one the most abundant herbicide found in the aquatic environment. The freshwater fish *Carassius auratus* were exposed at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0 mg/L of 2,4-DCP for 40 days. The antioxidative defense mechanisms in liver tissue exposed to 2,4-DCP was investigated through the measurement of the enzymes SOD,

CAT, Se-GPx, GST, GR, GSH and GSSG. The results show that CAT, SOD and Se-GPx were associated with the oxidative stress caused by 2,4-DCP. The GST was slightly affected. The GSH was not sensitive to 2,4-DCP. The oxidative damage induced by 2,4-DCP on the fish liver demonstrate their toxic effects, even at low concentrations (ZHANG *et al.*, 2004, p. 170).

Glyphosate (GLY): (N-[phosphonomethyl] glycine)-based herbicide has been the most widely used around the world, present in more than 750 different herbicide formulations in agricultural and non-agricultural areas. Commonly, such GLY formulations contain the surfactant POEA – polyoxyethylene amine with specific toxicity, and in water breakdown to aminomethylphosphonic acid (AMPA). In our laboratory, we could demonstrate that POEA and AMPA are more toxic to zebrafish embryos than the active ingredient glyphosate (RODRIGUES *et al.*, 2019, p. 98). POEA and AMPA induced DNA breaks (comet assay) in zebrafish larvae at very low concentrations of 0.4, 0.8, 1.6 and 4.0 mg/L, and 1.7, 5.0, 10.0, 23.0 and 50.0 mg/L respectively, while GLY was more effective at concentrations of 23.0, 50.0 and 100.0 mg/L (RODRIGUES *et al.*, 2019, p. 99). In Florida (USA), manatees are chronically exposed to GLY, being detected in 55.8% of plasma samples with an average concentration of 0.17 ± 0.47 µg/L. At these exposure levels GLY can cause immune dysfunction and inflammatory stress (De MARIA *et al.*, 2021, p. 106493). Larvae of *Apis mellifera* were chronically exposed to 2.5 mg in food. Individuals without any observable signs of toxicity showed gene expression changes, when their transcriptomes were analyzed. About 29% of asymptomatic larvae had transcriptional changes in many genes after the GLY chronic intake (VAZQUEZ *et al.*, 2020, p. 114148).

Auramine: dyes are widely used in several industrial products, including textiles, paper, plastic, leather, food, cosmetics, and household products. In Brazil, dyes have been found in the aquatic environment with concentrations ranging from 0.0118 to 6.81 µg/L (UMBUZEIRO *et al.* 2005, p. 58; CARNEIRO *et al.* 2010, p. 490). In Canada, dyes were detected in water samples, with concentrations ranging from 3.0 to 17.0 µg/L (MAGUIRE 1992, p. 2879). The dye Auramine, referred as Solvent Yellow 34, is used for dyeing leather, jute, cotton, and paper, widely

used due to their versatility in colors and bright (GESSNER and MAYER, 2000, p. 12). Auramine dyes are classified as possibly human carcinogens (Group 2B, IARC - International Agency for Research on Cancer, 2000). AZEVEDO *et al.* (2020, p. 1869) carried out a study to estimate the auramine PNEC using four species of aquatic organisms from three trophic levels, *Raphidocelis subcapitata* (algae), *Daphnia similis* (microcrustacea), *Hydra attenuatta (freshwater cnidarian)* and *Danio rerio (zebrafish).* Tests in *R. subcapitata* were based on biomass growth. The algae population was exposed to different dye concentrations of 20, 60, 200, 600, and 2000 µg/L. Growth inhibition was observed through photocolorimetric test. The observed values of EC_{10} = 46 µg/L and EC50 = 300 µg/L were obtained after 72 h of exposure. The chronic tests in *D. similis*, with exposure of 14 days, found values of EC_{10} =500 µg/L, and EC_{50} = 900 µg/L. The *H. attenuatta* were exposed to auramine dissolved in Hydra medium at concentration of 500, 1000, 2000 and 7000 µg/L for 96 h. Morphological alteration were analyzed in each growth stages. At 1000 µg/L morphological alteration occurred in 60% of organisms. In the highest tested concentration (7000 µg/L) the morphological alteration induced were irreversible. The zebrafish test followed the OECD protocol n. 236 (FET – Fish Embryotoxicity Test). Embryos were exposed at concentrations of 1000, 1900, 3700 7100 and 13600 µg/L for 96 h. Lethality and malformations were evaluated: lack of otolith formation, lack of eye, somite formation, edemas, tail detachment, yolk sac non-absorption, delay in development. The LC_{10} = 1300 µg/L and LC_{50} = 1900 µg/L were calculated for lethality based on lethality of auramine. The development deformities as edema, tail malformation, and delay in yolk absorption were the main alterations induced by auramine in zebrafish embryos. In this study *R. subcapitata* was the most sensitive species, and therefore the EC_{10} value was used for PNEC calculation of 0.92 µg/L.

Where are the toxicological thresholds for knowing the true risks?

In theory, exposures to chemicals at concentrations below of the threshold of toxic concern would be safe. The threshold concept was firstly adopted by regulatory authorities in the U.S. Food

and Drug Administration for regulation of food additives. Later, it was adopted also by the Joint FAO/WHO Expert Committee on Food Additives and the European Food Safety Authority to evaluate flavoring substances (KROES *et al.*, 2005, p. 228).

Threshold values are established experimentally in animal testing, through chronic exposures for the determination of NOAEL. It means that the threshold value would be the maximum exposure level to a chemical that we do not observe any adverse (toxic) effect (figure 2). In reality, many different threshold values can be obtained for a chemical depending on the study design of the chronic test, besides the animal species used. Then, are the exposures below the threshold really safe?

Regarding genotoxic carcinogens, the presence of just only one molecule inside the body is enough to cause DNA interactions showing some carcinogenic risk. For these substances, a safe threshold is not considered. The risk increases according to exposure level (figure 2-A) (NOHMI, 2018, p. 284).

In the regulation of chemicals like food additives, pesticides, and pharmaceuticals, once judged as genotoxic carcinogens it must be banned because risk exists at any level of exposure. Recently, this approach has been contested, because genotoxic carcinogens chemicals can be metabolized and inactivated before reaching DNA, DNA damaged can be repaired, and there are several steps where mutations can be suppressed (NOHMI, 2018, p. 285). In contrast, for non-genotoxic carcinogens, exposures below the threshold can be accepted, because it is assumed that any possible adverse effects are neglected and the risks are acceptable.

Based on NOAEL and threshold, the regulatory authorities determine the acceptable daily intake (ADI) (Figure 2-B). The ADI is the daily intake level of a chemical, which no adverse effects occur in a person for their entire life. Many chemicals are approved by regulatory authorities to be used in society based on intake levels below the ADI. Thus, the determination of genotoxicity and the threshold for adverse toxic effects is critical for the regulation of chemicals (NOHMI, 2018, p. 287).

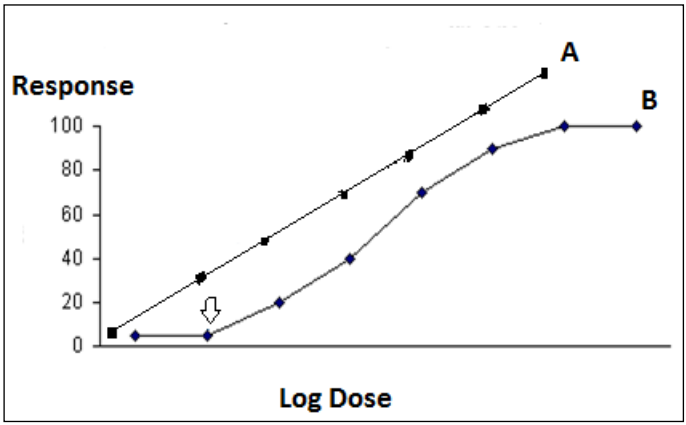

Figure 2: A – dose-response correlation for a chemical that there is not a threshold. **B** – dose-response correlation for a chemical that present a safety threshold. The arrow in B curve means the determined threshold.

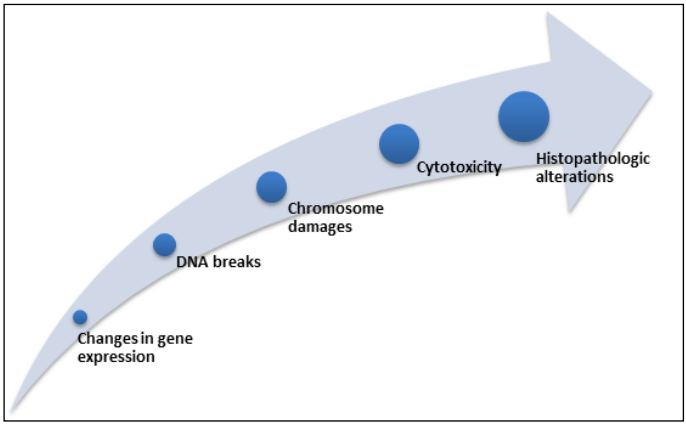

Figure 3: Different levels of disturbances that could be used to define a threshold.

In the NOAEL determination, what is the meaning of a non-observable adverse effect level? The cytotoxicity is an adverse observable effect, but before the cytotoxicity detection many other molecular adverse events have occurred. Before chromosome damages observation, certainly many other cellular adverse effects have occurred, such as DNA breaks. In the case of using DNA breaks to define the threshold for genotoxicity, what really happens inside cells to result in DNA breaks? (Figure 3).

The chronic studies carried out with antidepressant pharmaceuticals in our laboratory at the University of Brasilia, the adverse effects for genotoxicity were observed even at very low exposures. Zebrafish were chronically exposed to carbamazepine for 60 days and no genotoxicity through micronucleus test were found after exposure at 0.60 and

2,812 µg/L. However, using a more sensitive endpoint such as changes in gene expression through microarrays of transcriptome, 994 and 1425 genes were differentially expressed at the same exposure levels respectively (Santos *et al.*, 2018, p. 300; OLIVEIRA, 2021, p. 118). In chronic studies with amitriptyline, exposures occurred at 0.006 and 28.120 µg/L for four weeks. No micronucleus nor DNA breaks occurred, however 123 and 411 genes were differentially expressed respectively (Saxena and Ahuja, 1988, p. 428; Oliveira, 2021, p. 123). In studies with nortriptiline, zebrafish were chronically exposed at 0.10 and 10.0 µg/L for 28 days. At these exposure levels neither micronucleus nor comet assay indicated genotoxicity. However, 66 and 184 genes were differentially expressed respectively (Oliveira, 2021, 128).

In these three cases, no adverse genotoxic effects were found when using the micronucleus test and comet assay. On the other hand, using a more sensitive endpoint as gene expression changes, our observed results were quite different. It means that the NOAEL for the same chemical varies according to the sensitivity of the endpoints used in the chronic study. To protect human health and the environment from dangerous chemicals, regulatory authorities should rely on the most sensitive organisms and the most refined methods for determining the NOAEL and identifying the threshold for decision making.

The threat of the endocrine-disrupting chemicals (EDCs)

They are defined as "an exogenous agent that interferes with production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (KAVLOCK et al., 1996, p. 720). Different classes of chemicals act as endocrine disruptors, such as polychlorinated biphenyls organotins, phthalates (plastics), alkylphenols (surfactants), dioxins, pesticides (organochlorine, organophosphates, carbamates and pyrethroids), etc. They have the ability to interact with hormone receptors causing a wide range of metabolic perturbations. Many of them are stable and persistent in the environment, acting at very low concentrations (CASALS-CASAS and DESVERGNE, 2011, p. 142).

The EDCs are involved with many metabolic diseases, reproductive disturbances and cancer. Traditionally, hormones act by binding to specific cell receptors at very low concentration (levels of pg/L, ng/L and µg/L). They mimic a natural hormone blocking or overexpressing receptors and interfering with normal physiology. Many EDCs are lipophilic and then bioaccumulate and bio magnify in the ecological chains. The genetic effects of EDCs are related with epigenetic changes, involving DNA methylation and histone modifications in a variety of different genes. They do not cause DNA breaks and chromosome aberrations. Alterations in imprinted genes can promote disease states (SKINNER, 2008, p. 4). In the regulatory processes, to protect human life, the threshold for EDCs as residues in food and drinking water should be based on epigenetic changes. In this case, the LOEC would be the first level of the figure 3 (changes in gene expression), however epigenetic changes are not used for threshold determination.

Conclusion

Silent exposures to chemicals in early life involving the developing brain and nervous system can cause permanent injury for the whole life. The mechanisms of slow and invisible toxicities are poorly understood and the challenge is how to make such toxicities visible and intervene to prevent it. One answer would be the refinement of the toxicological tests to get greater sensitivity. There is a temporal gap between adverse effects that we cannot see now, but they will be seen tomorrow. For example, while exposures to chronic low concentrations of pesticides may not produce immediate health effects in a given population, but over time will contribute to an increased number of cancers. Regulatory authorities have the obligation of combatting future harm for their population based on long-term exposures knowledge to dangerous chemicals instead of interests in the developmentalist benefits. The States must invest in curtailing future harm. Finally, studies based on science and technology to protect society inspire analysis of the ways that risk is evaluated and the ambiguity of toxicity, as demonstrated most clearly by the difficulty of establishing legal proof of toxic harm.

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ART ET ENVIRONNEMENT. REPENSER LE RÉEL À L'AUNE DU VIRTUEL

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ABSTRACT

Dans la relation intime que nous entretenons avec l'environnement, dans la mise en relation entre l'image numérique et l'environnement, entre le vivant et l'environnement, nous nous demandons dans quelle mesure la vie artificielle nous amène à repenser la relation entre le réel et le virtuel. Le projet environnemental de 1999 de Natalie Jeremijenko, au croisement de l'art, des sciences et de l'ingénierie, relève de la double définition de la vie artificielle (Langton), imitation et création de vies. Il rend tangible le fonctionnement complexe de notre écosystème et permet de repenser le réel à l'aune du virtuel; notre perception du réel est transformée, voire augmentée à l'aune du virtuel. Il amène à nous interroger sur la limite entre vie naturelle et vie artificielle et révèle ainsi notre responsabilité envers l'environnement (Michaud) et envers le vivant.

KEYWORDS

Création; Vivant; Environnement; Vie Artificielle; Ecosystème.

Les définitions de la «vie artificielle» fluctuent en fonction du point de vue selon lequel nous nous plaçons, celui de l'ingénierie, de la biologie ou de l'informatique. Quoi qu'il en soit, la vie artificielle a été définie en 1987 par Christopher Langton comme «l'étude des systèmes construits de mains d'hommes qui ont des comportements caractéristiques des systèmes naturels vivants.» (LANGTON, 1989, p. 1). L'idée était que la vie n'est pas réduite à la chimie organique, mais qu'elle relevait de processus tels que par exemple l'émergence, l'autoréplication, la morphogénèse, la mutation, la relation à l'environnement et l'apprentissage, et que de ce fait il devait être possible de la créer informatiquement, non pas de la simuler mais de l'engendrer.

Du point de vue de l'artiste, c'est la question de l'incarnation de la vie qui est posée à l'ère des technologies contemporaines, du numérique et des biotechnologies, qui fait émerger la complexité que représente la vie biologique, technologique et sociale.

Questionnant la relation intime avec l'environnement, la mise en relation entre l'image numérique et l'environnement, le vivant et l'environnement, nous nous demandons dans quelle mesure la vie artificielle nous amène à repenser la relation entre le réel et le virtuel et ceci d'autant plus lorsque les installations artistiques combinent les deux, de façon hybride. L'idée qui est aussi soulevée est que l'innovation est ce qui pourrait nous permettre de changer le monde (*innovare*, vient de *novare* qui veut dire changer[1]) si nous l'anticipions.

Nous allons nous appuyer sur un projet environnemental de 1999 de Natalie Jeremijenko qui travaille au croisement de l'art, des sciences et de l'ingénierie, dans des créations qui articulent technologies et environnement. Ce projet est constitué de trois expérimentations et œuvres en soi, ou trois volets, qui nous confrontent directement à la nature tant par les actions artistiques réalisées que par les interrogations et les enjeux afférents : un logiciel de vie artificielle *A-trees,* des arbres biologiques clonés *OneTrees*, et un virus électronique intitulé *Stump.*

1 *TLF*, http://atilf.atilf.fr/dendien/scripts/tlfiv5/advanced.exe?8;s=3375811125;

A-trees (*Artificial Tree*, de *Artificial Life*) est un logiciel qui simule un arbre qui fonctionne comme une plante de bureau; dans les deux sens du terme, car elle est aussi la plante installée sur le bureau de l'ordinateur (*desktop*), une plante virtuelle qui va croître comme une plante réelle. Cet arbre a été programmé pour pousser progressivement grâce au L-system — ou système de Lindermayer — qui, par procédé algorithmique, grâce à une grammaire récursive, permet de modéliser le processus de croissance d'êtres vivants comme les plantes ou les bactéries[2] et ceci par autoréplication (BERSINI, 2005, p. 410)[3]; c'est un système fractal. Issue d'une hybridation entre le L-system et un agent qui traite les interactions avec l'environnement, la modélisation se fait selon des facteurs ontogénétiques d'une part, la programmation étant caractéristique d'un arbre réel, et d'autre part en fonction du taux de dioxyde de carbone de l'environnement ambiant, l'ordinateur étant relié à un détecteur.

La 2^{e} œuvre *OneTrees* est une plantation de cent arbres biologiques clonés, génétiquement identiques, dispersés dans seize lieux différents de Bay Aerea de San Francisco en Californie. Ces arbres avaient tous été initialement exposés, sous la forme de plantules, au Yerba Buena Center for the Arts en 1999 à San Francisco (JEREMIJENKO, *OneTrees*). L'arbre en question est un Paradox Vlach, hybride réalisé en laboratoire, dérivé du Paradox qui est un hybride de première génération du noyer. Le Paradox est en effet un croisement de deux noyers (un noyer noir de Californie et un noyer persan)[4] (PREECE, MCGRANAHAM) nommé ainsi à la fin du XIXe siècle par l'horticulteur américain Luther Burbank, à cause de sa grande vigueur et de sa rapidité de croissance bien supérieures à celles des noyers d'origine. Il ne produisait par ailleurs ni fruit et peu de pollen ce qui en faisait un arbre idéal pour le milieu urbain : il est peu allergène (JEREMIJENKO, *OneTrees)*. Le Vlach Paradox porte ce nom car il a été planté en 1904 dans la propriété d'un certain Vlach, dans la ville de Modesto en Californie, renommée pour la très mauvaise qualité de son air.

2 «L-System - Définition et Explications», https://www.techno-science.net/definition/11374.html

3 C'est «la possibilité pour un système de produire par son fonctionnement propre, une copie conforme à lui-même".

4 Juglans hindsii (noyer noir du nord de la Californie) x J. regia (noyer persan).

Stump (Souche), dernier volet du projet, est un virus informatique qui comptabilise le nombre de feuilles émises par l'imprimante de l'utilisateur. Celle-ci diffuse automatiquement une vue en coupe stylisée d'un arbre lorsqu'elle a consommé l'équivalent en pulpe de la plante (JEREMIJENKO, *OneTrees)*. L'œuvre appelle ainsi à la prise de conscience de notre rapport à la consommation et par là même de notre participation à la destruction des forêts et plus largement de notre écosystème.

L'arbre, testeur de notre relation à l'environnement.

L'œuvre de Natalie Jeremijenko procède d'une réflexion sur les biotechnologies et notamment sur le clonage. Des théoriciens intègrent les biotechnologies dans ce qu'on appelle la «Vie artificielle», c'est le cas par exemple du biologiste et philosophe de la nature danois Claus Emmeche qui a classé la vie artificielle en deux groupes principaux, les versions triviales ou vie artificielle faible basée sur la recherche d'imitation, ou les versions non triviales, ou vie artificielle forte fondée sur la recherche de création de vies (EMMECHE, 1994). Nous retrouvons ces deux versions de vie artificielle dans l'œuvre de Natalie Jeremijenko à travers les créations numériques et le clonage d'arbres biologiques. En effet, *OneTrees* est fondée sur le clonage d'arbres biologiques de la même façon que l'arbre virtuel sur le bureau d'ordinateur (*A-trees)* fonctionne par clonage, par processus algorithmique, et notamment par autoréplication.

Prenant à revers les discours sur le déterminisme génétique, l'artiste révèle l'impact de nos actions sur l'environnement et l'incidence capitale de celui-ci sur notre évolution. En effet, dans la baie de San Francisco qui a pour particularité d'être soumise à différents microclimats, les arbres génétiquement identiques vont se développer de façons différentes, dans leur configuration, leur vitalité et dans les images qu'ils donnent à voir, et ceci en fonction de leur emplacement, des précipitations, des caractéristiques du sol, de l'exposition au soleil, du niveau en CO_2, des polluants inhérents à la vie urbaine (ils sont exposés dans seize lieux différents de la baie)... De la même façon, chaque propriétaire d'une version de *A-trees* a la possibilité de voir croître son arbre virtuel en fonction du taux de CO_2 contenu dans l'air

qui l'entoure (dans les années 2000, la revue londonienne *Mute* diffuse un CD-ROM avec une version de *A-trees* remis dans le contexte du projet).

La croissance des arbres biologiques est aussi le témoin des disparités sociales profondément ancrées dans la baie de San Francisco. En effet, les choix géographiques des habitants qui sont liés aux conditions environnementales (qualité des sols, conditions d'expositions au soleil et aux intempéries, investissement des pouvoirs publics dans l'entretien des espaces publics...) sont malheureusement souvent conditionnés par leurs niveaux socio-culturels. La fracture sociale entre les différents lieux d'implantation est telle que Natalie Jeremijenko parle d'un «contexte social hautement balkanisé» (JEREMIJENKO, *OneTrees*) de la baie de San Francisco.

Les trois volets du projet se font donc écho dans le sens où ils sont tous trois des instruments qui nous rappelle au fonctionnement de notre écosystème ainsi qu'à notre responsabilité : l'arbre de bureau *A-trees* révèle le taux d'oxyde de carbone auquel notre environnement nous soumet; les arbres clonés *de OneTrees* dressent une cartographie des conditions environnementales de la baie de San Francisco ainsi que celle des disparités sociales. Ils sont des marqueurs de notre relation à l'environnement comme le sont les anneaux de croissance des arbres représentés par l'œuvre *Stump*. En effet, les périodes de sécheresse, de pluies abondantes, de feux, d'épidémies d'insectes ou de maladies, de blessures, d'éclaircies, ou de pollution atmosphérique sont autant d'évènements qui laissent leur marque dans leurs anneaux annuels (JEREMIJENKO, *OneTrees*).

En interaction permanente avec son milieu, d'une part virtuel, celui de la simulation, et d'autre part actuel, celui de notre environnement ambiant, *A-Trees* nous renvoie à l'écosystème dans lequel sont intégrés les arbres biologiques de l'œuvre *OneTrees* soumis à son biotope : en effet, le gaz carbonique contenu dans l'atmosphère est partie prenante du réseau et de la dynamique d'échanges de matière, d'énergie, et participe à l'évolution de l'environnement et au développement de la vie. *Stump* fonctionne quant à lui comme agent pathogène (le virus), qui est là pour réguler le déséquilibre de la biosphère, tout au moins ici sous la forme d'une alerte ou d'une atteinte à la conscience.

La simulation, révélatrice de la complexité de notre écosystème.

En juxtaposant la simulation informatique *A-trees* et son équivalent biologique *OneTrees,* le projet fait la démonstration que la simulation ne représente pas autant que ce que fait son équivalent biologique (JEREMIJENKO, *OneTrees*)[5] insiste Natalie Jeremijenko. En effet, même si les artistes donnent à voir les concepts avec lesquels ils jonglent dans la redéfinition constante qu'ils font du réel, et même si «[l]es arts sont un terrain d'essai pour la réalité» (GESSERT, 2003, p. 52), la simulation reste une représentation simplifiée de ce qu'est la vie. Elle permet cependant de donner forme, de rendre tangible des données environnementales qui semblent abstraites ou inaccessibles parce que trop complexes ou spécialisées. Elle rend compte cependant de cette complexité des interactions liées à l'environnement. Même s'il y a une inspiration biomimétique des arbres biologiques, les images qui s'inscrivent à l'écran sont l'interprétation graphique d'une suite d'instructions mathématiques. Les L-systèmes à l'origine de l'œuvre virtuelle donnent à voir des processus morphogénétiques similaires à la morphogénèse végétale, mais ne signifient pas cependant que les génomes végétaux procèdent d'un mécanisme identique. Ils révèlent comment fonctionne le vivant pour mieux le comprendre, mais aussi pour mieux le préserver, pour mieux anticiper; en ce sens la vie artificielle, tel que l'exprime C. G. Langdon n'est pas qu'imitation, mais création de la vie telle qu'elle pourrait être (LANGTON, 1989).

Cette complexité liée à la vie, nous la retrouvons dans d'autres œuvres numériques. En utilisant des algorithmes génératifs, Karl Sims dans *Panspermia* (1990) a simulé la croissance d'un univers tridimensionnel de plantes les plus diversifiées. La panspermie est une théorie scientifique formulée dans l'antiquité puis développée plus récemment à la fin du XIXe siècle, qui envisage que la vie sur terre aurait été apportée de l'extérieur, sous forme de germes, de graines, de spores, qui auraient voyagé à travers l'espace. Dans un univers utopique, sans lieu déterminé, une profusion

5 «The project juxtaposes the simulated (A-Life) trees and their biological counterparts, so doing demonstrate what simulation don't represent as much as what they do».

de plantes de diverses variétés éclot d'une graine projetée sur la terre pour elle-même générer la vie en direction de la galaxie. Cette œuvre dévoile la complexité de la vie qui se développe par des processus d'autoreproduction ainsi que ses incommensurables capacités d'adaptation. Ces morphogénèses, nous les retrouvons dans l'installation interactive *Transplant* (1995) de Laura Sommerer et Laurent Mignonneau. Des plantes aux multiples configurations et multiples couleurs croissent diversement à l'écran, véritable biotope dans lequel le spectateur est intégré. Les combinaisons sont variées et l'image dépend de la relation au spectateur, de ses déplacements et de sa vélocité.

Au-delà la dimension plastique des œuvres de Natalie Jeremijenko, la question de l'écologie, science des relations que les organismes vivants entretiennent entre eux et avec leur environnement, est bien entendu au cœur du sujet; la pollution, le réchauffement climatique, la hausse du niveau des mers, l'accroissement des émissions de gaz à effet de serre, la raréfaction des ressources naturelles, la disparition d'espèces animales et végétales… interfèrent sur les potentialités évolutives de la nature et de fait affectent l'humain. L'arbre est un testeur de la dégradation de l'environnement. Natalie Jeremijenko mène des expérimentations qui, détournées des visées utilitaristes des technosciences, interrogent l'impact de l'usage que nous avons des technologies sur notre évolution et donne corps à un certain nombre de leurs conséquences. L'homme est intimement lié à son environnement. De fait, lorsqu'il se retourne contre cet environnement, l'exploite à outrance ou sans précaution, lorsqu'il l'épuise sans souci du lendemain, c'est contre lui-même qu'il se retourne.

Elle rejoint dans sa démarche, d'une certaine façon, Yves Michaud, dans l'interprétation qu'il fait de l'œuvre de Sloterdijk précisant en effet que «les philosophes et spécialistes d'éthique raisonnent toujours subtilement *à priori* sur ce qu'il faudrait faire ou ne pas faire, mais il se pourrait bien qu'on dût considérer aussi *à priori* le jugement à porter *a posteriori* sur les errements du monde et des hommes» (MICHAUD, 2002, p. 82).

Repenser le réel à l'aune du virtuel

En prenant en compte l'impact de facteurs physiques sur l'environnement virtuel (en l'occurrence la captation du CO_2), *A-trees* procède d'une liaison hybride entre l'espace tangible d'une part et l'espace du virtuel d'autre part. Cette mise en relation permet de percevoir des phénomènes invisibles et de repenser le réel à l'aune du virtuel; notre perception du réel est transformée, voire augmentée à l'aune du virtuel. C'est en ce sens qu'elle peut nous permettre d'anticiper même si la prise de conscience est parfois en deçà de la réalité des évènements réels.

Ce continuum actuel-virtuel, tangible-virtuel nous amène à nous interroger sur la limite entre vie naturelle et vie artificielle ainsi que sur la responsabilité que l'homme a envers l'évolution de son milieu. Aujourd'hui, le terme innovation est souvent restreint à des champs réduits de la connaissance et de la recherche la plupart du temps appliquée, à courte vue, utilitariste, qui exclut d'emblée les disciplines que sont les sciences humaines, les lettres et les arts. C'est de fait rejeter tout un pan de la recherche et de l'évolution des connaissances dans les domaines notamment de la création, qui pourtant permettent constamment de redéfinir le réel, tant dans ses dimensions sensibles et intellectuelles et à faire émerger de nouveaux concepts. La création artistique ajoute une pierre à la connaissance, et en ce sens elle participe aussi à l'innovation.

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À l'ère des NBIC (Nanotechnologies, Biotechnologies, Informatique et Sciences Cognitives), les recherches interrogent les transformations fondamentales qui s'inscrivent dans les pratiques artistiques contemporaines notamment lorsqu'elles convoquent les problématiques liées au corps et à la corporéité. De nombreuses publications en France, Belgique, Canada, Tunisie.

THE GROWING UTILITY OF ZEBRAFISH MODELS IN ECOTOXICOLOGY: A NEUROBEHAVIORAL PERSPECTIVE

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ABSTRACT

The zebrafish (*Danio rerio*) is a valuable model system in ecotoxicological research. The small size, external fertilization, rapid development, well-characterized genes, neurotransmitter systems, and behaviors foster the use of zebrafish-based models to investigate how a wide range of environmental contaminants affect the CNS homeostasis. In this chapter, we will discuss the advantages and limitations of using both larvae and adult zebrafish as an alternative model organism to assess the effects of selected potential contaminants (*e.g.*, pharmaceuticals, heavy metals, and pesticides) on neurochemical and behavioral responses. The presence of neuroactive chemicals in aquatic environments may predict putative risks for the aquatic ecosystem and the characterization of potential biomarkers in zebrafish is of great importance in the field.

KEYWORDS

Zebrafish; Aquatic toxicology; Environmental contaminants; Neurochemicals; Behavioral phenotypes.

Global problems of aquatic contamination

Good quality water is indispensable for animals, plants and humans. Water is one of the natural resources needed for sustainable economic growth (UNITED NATIONS, 2018). Unfortunately, the reserve of this essential resource is not only unevenly distributed in nature but also under several pressures. For instance, almost 68.9% of freshwater exists in the frozen form, 30.8% as groundwater whereas the remaining 0.3% which exists in the surface and atmosphere is primarily available to satisfy animals, plants and humans needs (RAVIKUMAR et al., 2011). Populations resident in arid and semiarid regions have resulted to drilling of boreholes for drinking water, irrigation and industry because of inadequate rainfall and surface water. Indeed, about 2.1 billion people worldwide do not have access to safe drinking water (United Nations Children's Fund (UNICEF and WHO 2019).

Moreover, the growing industrialization and urbanization have been associated with increased competition for water resources and degradation of groundwater. Environmental pollution resulting from anthropogenic activities poses a grave impact on the quality and quantity of available water resources. Several potentially toxic substances are frequently used by humans in different situations ranging from manufacturing processes to household applications during which accidental spillage or misuse may occur, leading to environmental pollution of surface or groundwater through overtly or covertly routes (REBELO et al., 2014). Indeed, the extensive use of chemicals and their persistence in the environment is a global problem due to their potential detrimental effects on the food chain and the ecosystem. Nearly 80% of all illnesses in developing nations are related to consumption of drinking water of poor microbiological quality (WHO, 2002).

The prevalence of hazardous contaminants in aquatic environments adversely affects resident organisms (BILAL et al., 2019, RASHEED et al., 2019). In general, environmental pollutants commonly found in the water include heavy metals, pesticides, fertilizer-based chemicals, pharmaceuticals, microplastics, and nanoparticles. Pollutants can be classified based on the mechanisms of toxicity (*e.g.*, neurotoxic, teratogenic, endocrine disruptors, and immune modulators).

Based on the aforementioned deleterious impact of pollutants, it is imperative to develop economical, thermal efficient, accessible, portable, chemical stability, sustainable technologies, and tools to ensure appropriate quality of water (WAHEED et al., 2021). Here, we discuss the mounting utility of zebrafish as an invaluable model organism in ecotoxicology to understand how pollutants (e.g., pharmaceuticals, heavy metals, and pesticides) may cause neurobehavioral and neurodevelopmental alterations in vertebrates (Figure 1).

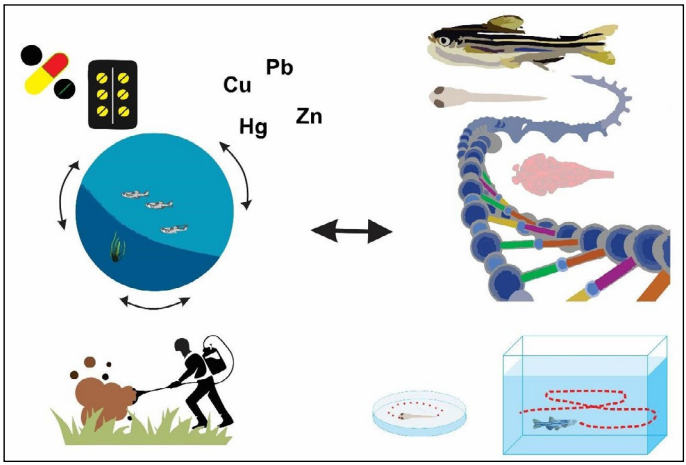

Figure 1. Schematic overview of the growing utility of zebrafish models in experimental ecotoxicology research. Pharmaceuticals, heavy metals, and pesticides can reach aquatic environments and cause deleterious effects on the organisms. Due to the well-conserved genome and brain physiology, larvae and adult zebrafish are suitable tools to elucidate how contaminants (alone or in combination) affect neurobehavioral responses in both ecotoxicological and translational perspectives.

General features of the zebrafish

During the last decade, the zebrafish (*Danio rerio*) has been considered an emergent model system in experimental research (FONTANA et al., 2018). As a vertebrate, zebrafish share a high degree of genomic and physiological conservation across taxa. Notably, 70% of zebrafish genes have at least one corresponding human ortholog (HOWE et al., 2013), which makes this species an invaluable system to elucidate how a wide range of contaminants modulate gene functions. Among the advantages of zebrafish, the high fertility rate, rapid development, and external fertilization are important features allowing an in-depth investigation of how aquatic contaminants may interfere in early developmental stages with a higher control over potential experimental interferences (SINGLEMAN and HOLTZMAN, 2014). Moreover, the presence of translucent embryos fosters the investigation of how genes

respond to environmental pollutants using a wide range of molecular tools (*e.g.*, fluorescent probes, whole-mount *in situ* hybridization, or morpholino antisense oligonucleotides) (JOWETT et al., 1996; YUAN and SUN, 2001). Among these features, zebrafish also express the main neurotransmitter systems found in humans (RICO et al., 2011). Although there are considerable anatomical differences between human and zebrafish central nervous system (CNS), this aquatic species shows analogous brain structures with conserved brain functions and sophisticated behaviors (KALUEFF et al., 2013) These aforementioned aspects foster the use of zebrafish models to assess how different compounds impair brain and behavior using medium-to-high throughput screens (ROPER et al., 2018). Paralleling humans, cortisol is the main stress hormone in zebrafish (ALSOP and VIJAYAN, 2009) and the homologous function of the stress-related axis is determinant to elucidate the influence of several contaminants as endocrine disruptors. Overall, the assessment of behavioral and neurochemical endpoints in zebrafish constitutes an important *in vivo* platform to understand the impact of potential neurotoxic substances in aquatic environment. Fish show cognitive abilities, in which learning, memory, and social recognition behaviors play pivotal roles for survival and reproduction in their natural habitat (PATTON and BRAITHWAITE, 2015). Thus, changes in these neurobehavioral responses due to exposure to environmental pollutants pose grave risks to fish population and other vertebrates because some of these pollutants may be bioaccumulated and magnified along the food chain.

Selected models of exposure to environmental contaminants at relevant concentrations

Pharmaceuticals

The detection of human pharmaceuticals in various effluents from industries and wastewater treatment plants has become an environmental issue because of the possible toxic effects in non-target aquatic species (DAUGHTON and TERNES, 1999; HALLING-Sorensen et al., 1998). The incidence of pharmaceuticals in the aquatic environment can also lead to development of

serious health consequences in humans (YADAV et al., 2021). Recently, the international regulatory bodies have focused on the regulation of the various micro-contaminants commonly detected in the environment (KHAN et al., 2020). To study the involvement of pharmaceuticals in aquatic toxicology, the zebrafish is a widely accepted model in ecotoxicological assays (GUNNARSSON et al., 2008; KALUEFF et al., 2014).

Both larvae and adult zebrafish are often used to clarify the acute and chronic effects of pharmaceuticals in the aquatic environment. Examples of such pharmaceuticals include fluoxetine, ibuprofen, hydroxychloroquine, azithromycin, venlafaxine, and carbamazepine. Fluoxetine, an antidepressant, acts as a selective inhibitor of serotonin reuptake (BROOKS et al., 2003; WINDER et al., 2012). In general, concentrations ranging from 1 to 600 ng/L are detected in surface waters (SAARISTO et al., 2017). Adult zebrafish treated with 500 ng/L fluoxetine for 7 days show decreased swimming speed (AL SHURAIQI et al., 2021). Conversely, chronic treatment (2 weeks) evokes anxiolytic-like effects, without changing locomotion (Stewart et al., 2014). Fluoxetine at 50 ng/L and 500 ng/L decrease the swimming activity of larvae 96-hour post-fertilization (hpf), supporting a modulatory role of fluoxetine on zebrafish behaviors. Venlafaxine, another antidepressant which selectively inhibits serotonin and norepinephrine reuptake (BIELSKI et al., 2005), has been detected in aquatic environment (METCALFE et al., 2010). Zebrafish larvae exposed to venlafaxine were less active in the light-dark test (THOMPSON et al., 2017). In fact, 1 and 10 ng/L venlafaxine reduce the distance traveled in the dark compartment, supporting a hypolocomotor effect (THOMPSON et al., 2017).

As a non-selective cyclooxygenase inhibitor, ibuprofen displays antipyretic, anti-inflammatory, and analgesic properties (BLACK and HILL, 2003; CLEUVERS, 2004). Ibuprofen was detected at 10 ng/L in the Yangtze River Estuary, China (XIA et al., 2017), suggesting potential negative effects on aquatic animals. In zebrafish larvae (120 hpf), 500 µg/L ibuprofen reduces locomotion and the free-swimming activity under dark stimulus is decreased at 50 and 500 µg/L (XIA et al., 2017).

Recently, during the coronavirus pandemic, the indiscriminate use of certain drugs, such as azithromycin (AZT) and hydroxychloroquine (HCQ) has also contributed to aquatic contamination (YAQIU et al., 2021b). AZT is a

macrolide antibiotic which inhibits bacterial protein synthesis, commonly indicated for respiratory, urogenital, dermal, and other bacterial infections, exerting immunomodulatory effects (MICHAEL et al., 2014). HCQ is also frequently used in the prevention and treatment of malaria (SHIPPEY et al., 2018) and is considered a pharmacological alternative in the treat of rheumatoid arthritis (LANE et al., 2020), and lupus erythematosus (DEEPAK and ISHMEET, 2020). Zebrafish exposed for 72 h to AZT (2.5 µg/L) and HCQ (2.5 µg/L) show decreased total protein levels, and increased levels of thiobarbituric acid reactive substances, hydrogen peroxide, reactive oxygen species, and nitrite levels, supporting a redox imbalance that culminate in oxidative stress (MENDONCA-GOMES et al., 2021). Moreover, the mixture of AZT + HCQ increased brain AChE activity (MENDONCA-GOMES et al., 2021). These data suggest potential neurotoxic effects of AZT and HCQ that may impair aquatic organisms. Future studies on the behavioral impact of these drugs using zebrafish are needed to investigate how these drugs can negatively impact neurobehavioral functions.

Diazepam (DZP) which is used to treat anxiety, tension, fear, somnipathy, and epilepsy, can also be commonly observed in aquatic environments (KOSJEK et al., 2012; VALDES et al., 2016). Juvenile zebrafish exposed to 12, 20, and 1200 µg/L DZP show significant changes in the swimming activity. Indeed, DZP causes hyperactivity (12 and 120 µg/L) and hypoactivity (1200 µg/L), depending on the concentration tested (WU et al., 2020). Chronic (120 days) exposure to 120 and 12 µg/L DZP also decreases the locomotor activity in female and male zebrafish (CHEN et al., 2021).

Carbamazapine (CBZ) is an antiepileptic which has a high half-life in the environment (BRANDÃO et al., 2013). CBZ concentrations commonly found in aquatic environments range from 0.03 to 11.6 µg/L (FERRER et al., 2012; Miao et al., 2003). In larval zebrafish (28-29 hpf), an inhibition in the spontaneous locomotion is observed following 10 µg/L CBZ exposure (HUIHUI et al., 2020).

Due to the increasing use of contraceptive pills, the synthetic estrogen, 17-α-ethinylestradiol (EE2), can also be commonly found in aquatic environments. As an endocrine-disrupting chemical, EE2 impairs stress responses by regulating the levels of specific hormones, as well as induces

anxiolytic-like behaviors, increases aggression, and reduces social preference in adult zebrafish (FENSKE et al., 2020). Collectively, these data suggest that exposure of zebrafish embryos or adults to different pharmaceuticals, at ecologically relevant concentrations impairs a wide range of behaviors, which may exert a deleterious influence on the dynamics of the prey-predator relationship in aquatic biota.

Metals

Larvae and adult zebrafish have been used in both acute and chronic studies to elucidate the neurobehavioral outcomes of exposures to numerous metals including lead (Pb), copper (Cu), cadmium (Cd), mercury (Hg), nickel (Ni), arsenic (As) and silver (Ag). Exposure of zebrafish embryos to 0.05-0.7 mM of Pb-acetate from 0 to 6 days post fertilization (dpf) elicits neurological deficits characterized by sluggish-like phenotype, evidenced by reduced swimming and escape reactions (DOU and ZHANG, 2011). Exposure to Pb at 0, 5, 10, and 20 µg/L from 6 to 144 hpf significantly impairs locomotion, turning activity, while increases social behaviors (ZHAO et al., 2020). The neurobehavioral deficits induced by Pb in zebrafish larvae reportedly involves N-methyl-D-aspartate glutamate receptor (NMDAR)-dependent brain-derived neurotrophic factor (BDNF) signaling pathway (ZHAO et al., 2020). The trans-generational impact of embryonic Pb exposure in zebrafish has been reported using the avoidance conditioning paradigm. Developmental Pb exposure causes learning impairments that persist to three generations in zebrafish (XU et al., 2016). Moreover, neurobehavioral fingerprints of zebrafish exposed to Pb have been characterized via a "behavior-based and physiology-supplemented" method. For example, exposure of adult male zebrafish to ecologically relevant concentrations (1, 10, and 100 mg/L) Pb for 14 days significant impairs exploratory activity, spatial working memory, and disrupts scototaxis (LI et al., 2019).

Regarding the effects of Cu, exposure of zebrafish to 1.6, 3.9, 7.8, 11.7, or 15.6 µM during embryogenesis dose-dependently impairs hatching and locomotion by affecting myogenesis and neurogenesis (ZHANG et al., 2015). Similarly, Cu alone or combined with microplastics markedly impairs

locomotor-related endpoints with concomitant decrease in avoidance behaviors and AChE activity in zebrafish larvae (SANTOS et al., 2021). Acute (24 h) exposure of adult zebrafish to copper sulphate ($CuSO_4$) at 15 µg/L significantly decreased ATP hydrolysis without affecting ADP and AMP hydrolysis in brain membranes, whereas sub-chronic (96 h) exposure markedly inhibited both nucleoside triphosphate diphosphohydrolase (NTPDase) and ecto-5-nucleotidase activities. These findings implicate the involvement of purinergic signaling in Cu-mediated neurotoxicity, supporting the use of zebrafish to elucidate the influence of Cu on different neurotransmitter systems (ROSEMBERG et al., 2007).

Exposure of zebrafish larvae to low Cd concentrations (1.25, 2.5, and 5 mg/L) for 7 days causes hypolocomotion during a light-dark photoperiod stimulation test with marked alterations in serotonergic system and BDNF signaling (Xia et al., 2020). Separate or combined exposure of zebrafish exposed to 0.01 mg/L Pb or 0.005 mg/L Cd reportedly causes an antagonistic effect on their behavioral activities. Precisely, Cd disrupts the behavioral rhythm, whereas Pb reduces locomotion (LIAO et al., 2021). The antagonistic impact of their co-exposure was evident on the locomotor activity, internal exposure level, and circadian behavioral rhythm. *In vitro* assays using zebrafish brain membranes incubated with Zn or Cd at 0.05 – 1 mM show pronounced changes in the purinergic, but not in the cholinergic system. Precisely, Zn increases ATPase and AMPase activities, whereas Cd increases ATP catabolism, but inhibits ADP and AMP hydrolysis (SENGER et al., 2006). Parental co-exposure of adult zebrafish to tributyltin (TBT, 100 ng/L) and Cd (100 ng/L) Cd for 90 d promotes developmental inhibition, nervous system damage, and endocrine disruption in two-generations larval offspring evidenced by the reduction in dopamine and serotonin levels, AChE activity, thyroid hormones, and down-regulation of hypothalamus-pituitary-thyroid axis genes (LI and LI 2020).

Neurotoxicity assessment using the novel tank and the light-dark preference tests demonstrated that acute exposure to methylmercury (MeHg) at 1.0 or 5.0 µg/g (i.p.) increases anxiogenic profile in both tests, whereas hyperlocomotion is only observed in the novel tank test. Moreover, MeHg exposure has been associated with reduced serotonin and dopamine levels with induction of mitochondrial oxidative

damage (MAXIMINO et al., 2011). Neurobehavioral patterns associated with exposure to $HgCl_2$ at 0.077 mg/L include surfacing and darting movements, loss of balance, aberrant swimming patterns, and reduced protein and glycogen content (VUTUKURU and BASANI 2013). Moreover, 100 nM $HgCl_2$ embryonic exposure chronically induces motor deficit, disrupts anxiety-related behavior and affects the structure of several biomolecules (*e.g.*, lipids, nucleic acids, and proteins) in zebrafish larvae (BAKAR et al., 2017). Zebrafish exposed to 5 mg/L arsenic show decreased locomotion and increased anxiety-like behaviors, whereas ATP, ADP, and AMP hydrolysis is reduced at 0.05, 5, and 15 mg/L, suggesting the involvement of purinergic system in arsenic-mediated neurotoxicity (BALDISSARELLI et al., 2012). Exposure to 0.025, 2.0, 5.0, and 15.0 mg/L $NiCl_2$ alters morphology, promotes locomotor deficits and impairs avoidance response in larvae. Conversely, both acute and subchronic exposures induce memory deficit and reduce aggression in adult specimens (NABINGER et al., 2018).

Pesticides

Zebrafish are sensitive to a wide range of contaminants, being a suitable model species to assess the main effects of pesticides on multiple behaviors. For example, larvae exposed to glyphosate at varying concentrations of 0.05 to 10,000 µg/L (from 1.5 to 120 hpf) exhibit significant motor deficits at 1000 µg/L and above, whereas concentrations below 10 µg/L do not promote behavioral alterations (FORNER-PIQUER et al., 2021). Adult zebrafish exposed to glyphosate at ecologically relevant concentrations (0.3 and 3 µg/L) for 14 days exhibit robust impairments in exploratory and social behaviors, paralleling increased anxiety-like responses. Glyphosate-exposed fish show increased brain oxidative stress and elevated serotonin and dopamine levels, turnover ratios of DOPAC/dopamine and homovanillic acid/dopamine, catalase and superoxide dismutase activities in the CNS (FARIA et al., 2021).

Exposure to chlorpyrifos (CPF, 0.001, 0.01, 0.1 and 1.0 µM) until 7 dpf causes adverse effects on behavior. At 0.01 or 0.1 µM, CPF elicits developmental toxicity, characterized

by decreased swim speed and thigmotaxis without affecting avoidance behavior. CPF at 1 µM promotes behavioral and larval morphology defects with marked inhibition of anxiety-like behaviors in zebrafish (RICHENDRFER et al., 2012). Moreover, CPF at 300 µg/L occasioned developmental toxicity associated with diminished swimming distance and speed and increased oxidative stress in zebrafish larvae (JIN et al., 2015). Developmental exposure of zebrafish to 10 ng/mL CPF do not affect locomotor activity whereas 100 ng/mL CPF reduces swimming activity and impairs spatial discrimination in adulthood, supporting persistent effects of the pesticide on different behavioral domains (LEVIN et al., 2004). Interestingly, concentrations ranging from 180 to 750 ppb CPF significantly increased locomotion of zebrafish larvae (KIENLE et al., 2009), suggesting a concentration-dependent effect during ontogeny. The impact of co-exposure to CPF and metals has also been reported elsewhere. Exposure of zebrafish to CPF elicits an anti-predator behavior (*e.g.*, increased freezing) and reduces swimming rates and muscle AChE activity, whereas Cu exposure decreases freezing. However, exposure to binary mixtures of Cu and CPF neither showed a modulatory effect on CPF-mediated swimming impairment via AChE and non-AChE mechanisms (Tilton et al., 2011). Acute CPF exposure causes a slight concentration-dependent increase in locomotor activity whereas a reduced locomotion is often associated with increasing Ni concentration. Thus, combined exposure to CPF and Ni mixtures is known to elicit an antagonistic interaction in zebrafish (KIENLE et al., 2009). These data clearly support that co-exposure to different contaminants may even potentiate or antagonize specific responses in zebrafish models.

Evidence also shows a role of the herbicide atrazine (ATZ) in modulating neurobehavioral functions of adults and larvae. For example, developmental exposure (from 1 to 120 hpf) to 0, 0.3, 3, or 30 ppb ATZ causes hyperactivity in the larvae at 0.3 ppb. However, 30 ppb ATZ markedly decreases the distance traveled by the exposed zebrafish (AHKIN CHIN TAI et al., 2021). Additionally, 30 ppb ATZ exposure reduces swimming activity during a larval visual motor response test at 120 hpf (HORZMANN et al. 2018). The influence of ATZ on defensive behaviors (*e.g.*, shoaling, thigmotaxis, and

depth preference) of adult zebrafish was investigated in a split depth tank. Fish exposed to 1000 µg/L ATZ for 14 days show reduced social interaction when four conspecifics are simultaneously tested. Although 1000 µg/L ATZ does not alter muscular AChE, the respective concentration reduces brain AChE activity (SCHMIDEL et al. 2014). Thus, we suggest that inhibition of brain cholinergic neurotransmission may play a role in the modulatory effect of ATZ on defensive behaviors.

Other pesticides reported to modify the zebrafish behaviour include fipronil, mancozeb, and imidacloprid. Fipronil significantly reduces the survival rate and locomotion via induction of inflammation, oxidative stress and apoptosis at 100, 500, 1000, and 2000 ppb (WU et al., 2021). Interestingly, behavioral alterations may occur even before the manifestation of biochemical changes in zebrafish embryos exposed to fungicide mancozeb at 5 and 20 µg/L. While 5 µg/L mancozeb increases distance moved and turn angle, these endpoints are decreased following 20 µg/L mancozeb. Similarly, 5 µg/L reduces immobility, but 20 µg/L mancozeb causes the opposite effect (LEANDRO et al., 2021). Exposure of zebrafish embryos during the blastula stage (about 2 hpf) to mancozeb at 0.5, 5, and 50 µg/L dose-dependently reduces locomotion, aversive stimulus response, and light-dark transition as well as impairs AChE activity (VIEIRA et al., 2020).

Exposure of zebrafish to imidacloprid, a neonicotinoid insecticide, at 0.15, 15, and 45 µg/L for 96 h resulted in significant decrease in distance traveled by the fish 45 µg/L only whereas transitions to and time spent in the top area of the tank were significantly decreased in all imidacloprid concentrations tested. Moreover, 15 and 45 µg/L of imidacloprid markedly reduced erratic movements, brain AChE activity, and increased carbonyl protein levels, without affecting lipid peroxidation and non-protein thiols (GUERRA et al., 2021). In sum, zebrafish exposed to a wide range of pesticides at different developmental stages show impaired CNS homeostasis, which lead to substantial modulation of ecologically relevant behaviors. These findings reinforce the importance of zebrafish-based models to investigate how environmental contaminants can affect brain physiology and behavior in aquatic organisms.

Conclusion

In conclusion, the behavior of an organism is the outcome of its interaction with the environment. Changes in environmental conditions due to the presence of pollutants can negatively modulate neurochemical levels, cellular metabolism and, consequently cause significant impacts on multiple behavioral domains. Considering the intrinsic advantages of zebrafish in the selected examples briefly discussed here, we strongly support the growing utility of this aquatic species in experimental ecotoxicology **(Figure 1)**. Indeed, behavioral neurophenotyping in zebrafish-based models is a very sensitive and non-invasive approach in ecotoxicology and translational toxicology because it reflects the relationship between ecological, molecular, and physiological processes, thereby representing a valid tool in biosafety appraisal.

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MECHANISMS INVOLVED IN NEUROPATHIC PAIN OBSERVED IN MULTIPLE SCLEROSIS: ROLE OF TRPA1 ACTIVATION

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ABSTRACT

Neuropathic pain is a common symptom observed in multiple sclerosis (MS) patients, however, the pain mechanisms are not entirely understood. In this view, one possible mechanism is the oxidative stress by-products generation and consequently nociceptor activation. In that sense, inflammation and oxidative compounds can activate different proteins such as the transient receptor potential ankyrin 1 (TRPA1). TRPA1 channel is a potential target for future investigation to treat neuropathic pain in MS. Therefore, in this book chapter we will discuss the TRPA1 activation and its relation to nociceptive behavior related to neuropathic pain in the MS models in mice. Furthermore, we will describe the TRPA1 modulation as a possible new target for MS neuropathic pain treatment.

KEYWORDS

Neuroinflammation; Demyelination; Chronic Pain; TRP Channels; Oxidative Stress.

Multiple Sclerosis and Pain

Multiple sclerosis (MS) is an autoimmune and inflammatory chronic disease of the central nervous system, and its physiopathology is still unclear (DOBSON, 2019, p.30). Furthermore, the development of MS is multifactorial, including genetic and environmental factors (KATSARA, 2018, p.104). Some environmental factors as ultraviolet B light, Epstein–Barr virus infection, obesity, smoking, and vitamin D can play the leading role in the disease development (DOBSON, 2019, p.30). In addition to environmental factors, the genetic susceptibility in MS involves genomic alteration on the peripheral immune cells and microglia. These genetic alterations could cause an autoimmune process leading to oligodendrocyte death and demyelination (DOBSON, 2019, p.32). Besides, several other mechanisms are involved in MS development, including inflammation, demyelination, neurodegeneration, and oxidative stress process (TOBORE, 2021, p.509).

Due to pathological alterations, different symptoms are presented in MS, such as fatigue, motor impairment, neurological deficits, and pain. The clinical forms of MS can differ according to symptomology and severity. Hence, MS patients are classified into three subtypes (LUBLIN, 2014, p.282). The most common clinical form is relapsing-remitting MS (RRMS), representing 85% of the disease diagnosis (KATSARA, 2018, p.104). RRMS patients present an inflammatory process characterized by neurological dysfunction episodes and partial recovery periods (LUBLIN, 2014, p.280). The RRMS prevalence is three females for each male, and the mean age of the disease onset is 20-30 years (ZEYDAN, 2020, p.10).

In contrast, the two progressives' MS subtypes are characterized by intense neurodegenerative processes and progressive worsening of physiological functions (LUBLIN, 2014, p.282). The secondary progressive MS develops in about 65% of the RRMS patients after 10–15 years. In these cases, the patients presented the progression with relapses without complete recovery. The primary progressive clinical form is characterized by a gradual increase of progressive disability (DOBSON, 2019, p.31). Another significant difference for the progressive MS forms is the incidence of one female for each male, and the disease onset occurs in middle-aged patients (ZEYDAN, 2020, p.12).

Despite different clinical forms of MS, the pain symptoms are reported in 62.8% of the patients (DRULOVIC, 2015, p.1599). Furthermore, the prevalence of pain in progressive clinical forms is higher than in relapsing-remitting patients, probably due to progression mechanisms (FOLEY, 2013, p.635). Nevertheless, the pain mechanisms involved in MS are not entirely understood (DRULOVIC, 2015, p.1600). The most prevalent types of pain in MS are headache (42.5%) and neuropathic pain (26.6%) (FOLEY, 2013, p.634). Accordingly, about 86% of the MS patients presented neuropathic pain (DRULOVIC, 2015, p.1599). The spinal cord can be affected by demyelination and inflammatory process in MS (BENEDICT, 2017, p.832-842). Due to the spinal cord signaling, the demyelinating injury is likely to be responsible for neuropathic pain in MS patients (JAWAHAR, 2013, p.1714).

Additionally, neuropathic pain is associated with a higher extended disability status scale (EDSS) in MS patients (DRULOVIC, 2015, p.1600). EDSS classifies the MS patient's neurological status according to functional impairment and disease severity of the central nervous system. The standard neurological status is related to a low EDSS (0 to 5), and the upper score (> 6) describes MS patients' walking inability (CINAR, 2018, p.71). Moreover, psychological comorbidities, such as depression, anxiety, and sleep problems, are closely related to chronic pain in MS (URITS, 2019, p.9). Consequently, the pain symptoms strongly affect the MS patient's quality of life and mental health (YILMAZER, 2020, p.8). However, pharmacological management is a challenge due to the MS types of pain high variability (URITS, 2019, p.8).

In this way, pain management in MS is an important research field to improve the quality of life for these patients. The pharmacological interventions included anticonvulsants, anti-depressants, cannabinoids, dextromethorphan/quinidine, and opioids or opioid antagonists. Promising treatments for chronic pain in MS included off-label use of drugs from anticonvulsants class and dextromethorphan or quinidine drugs (JAWAHAR, 2013, p.1716). Unfortunately, most pharmacological treatments with monotherapy reduce only about 50% of the painful symptoms (URITS, 2019, p.10). Besides, drug combinations could be used to treat MS pain, but this

clinical evidence-base is still lacking (JAWAHAR, 2013, p.1721). Thus, investigating the MS pathophysiological mechanisms is relevant to access new pharmacological alternatives to manage pain in this pathology.

The preclinical models of experimental autoimmune encephalomyelitis (EAE) have been used to study MS symptoms, such as neuropathic pain. The EAE models present a disbalance in reactive species generation possibly related to the nociception behaviors (DALENOGARE, 2020, p.11; RITTER, 2020, p.2430). Similarly, oxidative stress is related to disability progression in MS patients (RODRIGUES, 2021, p.17). Besides, the oxidative stress by-products can activate different proteins such as the transient receptor potential (TRPs), which are associated with hyperalgesia development (LOGASHINA, 2019, p.105). Therefore, this book chapter will discuss the activation of the TRP ankyrin 1 (TRPA1) and its relationship to nociceptive behavior related to neuropathic pain in the EAE model. Furthermore, we will describe the TRPA1 modulation as a possible new target for MS pain treatment.

TRPA1 channel and neuropathic pain

TRP channels can be classified in six subfamilies in mammals according to the amino acid sequence homology of each protein. Until now, 28 TRP members were described and distributed in TRPV (Vanilloid), TRPC (Canonical), TRPP (Polycystin), TRPM (Melastatin), TRPML (Mucolipin), and TRPA (Ankyrin) subfamilies. The TRP superfamily is formed by diverse ion channels widely expressed in tissues, which are relevant for physiological functions. In this view, TRP channels are activated by various stimuli, such as chemical, osmotic, and mechanical. Besides, some TRP expressed in peripheral sensory neurons act as thermoreceptors (thermo-TRP), being activated by cool temperatures (≤25°C, TRPM8) or noxious temperatures (≥43°C, TRPV1, the capsaicin receptor). These channels can also be activated or sensitized by inflammatory and oxidative compounds produced after tissue lesion. Some of these receptors, such as TRPV1 and TRPA1, are found in sensory nociceptive neurons, and play a crucial role in pain detection (KANEKO, 2014, p.2479; TALAVERA, 2020, p.732).

The TRPA1 protein is arranged in six transmembrane α-helices domains (TM1–6, pore-forming loop among TM5 and TM6) with intracellular N- and C- termini. It is the single member of the TRPA subfamily, and this channel was named because of the high number of ankyrin repeat domains (14-18 ARDs) found in the N- terminal region. The TRPA1 channel was initially described in fibroblast cell culture in 1999 and was called 'ankyrin-like protein with transmembrane domains protein 1', ANKTM1. However, it can be found in the peripheral and central nervous system, epithelial and glial cells, and other tissues (bladder, small intestine, and pancreas). This receptor functions as a non-selective calcium channel and was first described as a sensor for noxious cold (≤17°C). However, the TRPA1 is not considered a harmful cold indicator in all species but is relevant for cold hypersensitivity development in diverse pain models. Thus, more research needs to be performed to investigate if TRPA1 may works as a thermoreceptor in humans. Besides, TRPA1 antagonist treatment was not able to change the body temperature of rodents, and this protein is not involved in mice thermal preference (TALAVERA, 2020, p.729).

Moreover, the TRPA1 is commonly co-expressed with TRPV1 in peptidergic peripheral sensory fibers. Thus, the activation of these receptors may cause the release of neuropeptides (calcitonin gene-related peptide and substance P), leading to the phenomenon of neurogenic inflammation. The activation of TRPA1 could be mediated by exogenous irritant natural agonists, such as allyl isothiocyanate (AITC, found in wasabi and mustard oil), cinnamaldehyde (present in cinnamon), carvacrol (found in thyme and oregano), allicin (from garlic), thymol (present in thyme), and gingerol (found in ginger). Various environmental irritants gated this receptor, including formaldehyde, nicotine, acrolein and crotonaldehyde (present in cigarette smoke). As mentioned before, some reactive compounds produced after tissue damage can also activate the TRPA1, such as hydrogen peroxide (H_2O_2), 4-hydroxy-2-nonenal (4-HNE, formed after lipid peroxidation), methylglyoxal (produced during abnormal glucose metabolism), nitric oxide, and cyclopentenone prostaglandins (TALAVERA, 2020, p.736). Consequently, as TRPA1 agonists are involved in the induction of nociception and inflammation, this channel is a promising drug target to control pain (BENEMEI, 2019, p.54; CHEN, 2020, p.648; MEENTS, 2019, p.432).

Chronic pain is a significant health problem worldwide, affecting approximately 20% of the general population, and it will be defined in the International Classification of Diseases 11 (ICD-11). In secondary pain syndromes, chronic pain is mainly a symptom caused by a different disease, as observed in neuropathy. Neuropathic pain is a type of chronic pain defined by the International Association for the Study of Pain (IASP) as "pain caused by a lesion or disease of the somatosensory system". Neuropathic pain is often chronic (prevalence of 6.9 to 10% of the general population) and can be observed after a central or peripheral central system lesion or disease. It causes spontaneous pain and enhanced pain detection, reducing the quality of life of patients. Besides, most of the compounds used in the clinic to treat this type of pain are not fully effective or produce adverse effects (JENSEN, 2011, p.2204; KOSEK, 2016, p.1385; SCHOLZ, 2019, p.54; TREEDE, 2019, p.25).

Numerous studies using models of neuropathic pain caused by peripheral nerve injury (spinal nerve ligation, chronic constriction injury, or transection) in rodents described the role of TRPA1 in this type of pain. The first study was developed in 2005 using a model of L5 spinal nerve ligation in rats, which showed that the cold hyperalgesia was mediated by TRPA1 channel (OBATA, 2005, p.2395). A similar result was described by a distinct study, showing that TRPA1 is involved in L5 spinal nerve ligation-induced cold hyperalgesia in rats (KATSURA, 2006, p.116). Besides, using chronic constriction injury (CCI) of sciatic nerve authors also related the TRPA1 participation in mechanical and cold allodynia in mice (CASPANI, 2009, p.9; PINHEIRO, 2015, p.111). Mechanical allodynia associated with an L5/L6 spinal nerve ligation in rats was also reduced by a TRPA1 antagonist administration (EID, 2008, p.1746). Besides, in a model of partial sciatic nerve ligation in mice, it was shown that TRPA1 expressed in Schwann cells is relevant for the maintenance of macrophage infiltration nearby the sciatic injury (DE LOGU, 2017, p.10). In a similar way, using a model of infraorbital chronic constriction injury (trigeminal neuropathy), it was found that macrophage infiltration and the constant production of TRPA1 agonists cause neuropathic nociception in mice (TREVISAN, 2016, p.1375). Also, in a rat spinal cord injury (a model of central neuropathic pain) the TRPA1 was involved in nociception, and the production of acrolein seems to be a relevant mechanism in this type of pain (PARK, 2015, p.995).

Furthermore, cancer treatment with chemotherapeuticagents causes neuropathic pain. Chemotherapeutic-induced peripheral neuropathy is commonly detected after cancer management, and it was described that oxaliplatin, thalidomide, bortezomib, or paclitaxel-caused mechanical and cold allodynia depends on TRPA1 activation and oxidative stress production (DE LOGU, 2019, p.5439; MATERAZZI, 2012, p.564; NASSINI, 2011, p.1628; TREVISAN, 2013, p.3125). Besides, some chemotherapeutic compounds activate the TRPA1 directly, including exemestane, letrozole, anastrozole, and dacarbazine causing hypersensitivity (BRUSCO, 2019, p.2804; FUSI, 2014, p.3).

Diabetes induces different long-term complications, such as diabetic peripheral neuropathy, which affects many patients with this disease. Previously, it was shown the role of TRPA1 in diabetic neuropathic pain models (HIYAMA, 2018, p.5; WANG, 2018, p.100; WEI, 2009, p.150). Besides, methylglyoxal by activating the TRPA1 in sensory fibers was involved in the induction of nociception in a diabetic neuropathic pain model (ANDERSSON, 2013, p.6; HUANG, 2016, p.465). Another type of chronic pain that often induces neuropathic pain is complex regional pain syndrome type I (CRPS-I), which can be observed after limb trauma with no detectable nerve injury. The TRPA1 participation was detected in mouse and rat models of CRPS-I using TRPA1 antagonists or TRPA1 genetic deletion in mice. In this way, in this model, the macrophage accumulation and the production of TRPA1 agonists leads to TRPA1 activation in Schwann cells and peripheral neurons, sustaining allodynia and neuroinflammation induction (DE LOGU, 2020, p.544; DE PRA, 2019, p.10; KLAFKE, 2016, p.234). Thus, the TRPA1 has been studied in different models of peripheral or central neuropathic pain and represents a new strategy to manage this painful condition.

TRPA1 and neuropathic pain in multiple sclerosis models

The lack of specific treatments and clinical drug efficacy makes neuropathic pain symptoms a challenge in MS (FOLEY, 2013, p.640; SEIXAS, 2014, p.326; SOLARO, 2011, p.521). In this view, different rodent models have been used to investigate new targets for neuropathic pain treatment in MS, such as the

mouse model known as EAE (BJELOBABA, 2018, p.1040). The EAE models in rodents are extensively used in MS research because of the induction of similar clinical and pathological features of MS: neuroinflammation, demyelination, and neuron loss (BJELOBABA, 2018, p.1039). The induction of EAE involves the subcutaneous injection of the antigen Myelin Oligodendrocyte Glycoprotein$_{35-55}$ (MOG$_{35-55}$), which produces an inflammatory and demyelination process when administered with an adjuvant (BJELOBABA, 2018, p.1039). The adjuvants can differentiate the clinical manifestation of the models; for example, the Complete Freund's Adjuvant (CFA) is used to mimic a progressive MS mouse model induced by EAE (PMS-EAE) (OLECHOWSKI, 2013, p.115; OLECHOWSKI, 2009, p.158). On the other hand, the use of Quil A saponin develops a mild clinical score, similar to RRMS time courses in the induced mouse (KHAN, 2014, p.19).

In both models, PMS-EAE or RR-EAE, the nociception tests were performed and demonstrated spontaneous and evoked hypersensitivity development (DUFFY, 2016, p.10; KHAN, 2014, p.18; OLECHOWSKI, 2009, p.158). The development of cold and mechanical allodynia emerged before the clinical signs of the induction, according to the first studies that evaluated nociceptive behaviors in the PMS-EAE model (OLECHOWSKI, 2009, p.160). These results suggested a role of the inflammation process, such as astrocytes and microglia/macrophages activation, during the development of neuropathic pain-like behaviors in this model (OLECHOWSKI, 2009, p.160). However, the EAE induction, using CFA, causes severe motor impairment in the animals and makes it difficult to assess the pain behaviors from over the disease course (BJELOBABA, 2018, p.1040; OLECHOWSKI, 2009, p.161). Otherwise, the RR-EAE mouse model leads to neuropathic pain behaviors from 28-30 days after induction, without significant changes in locomotor activities (KHAN, 2014, p.18). Despite the long time for the allodynia development, the RR-EAE presents an essential advantage to the PMS-EAE model, permitting a better investigation of chronic pain behaviors (KHAN, 2014, p.19).

Previously, two models of MS were used to investigate the relationship between TRPA1 activation and pain behaviors (DALENOGARE, 2020, p.12; RITTER, 2020, p.2432). Using the PMS-EAE mouse model, the development of mechanical, cold, and heat hyperalgesia was detected on days 3, 5, 7, 9, 11, 13, and 14 after the

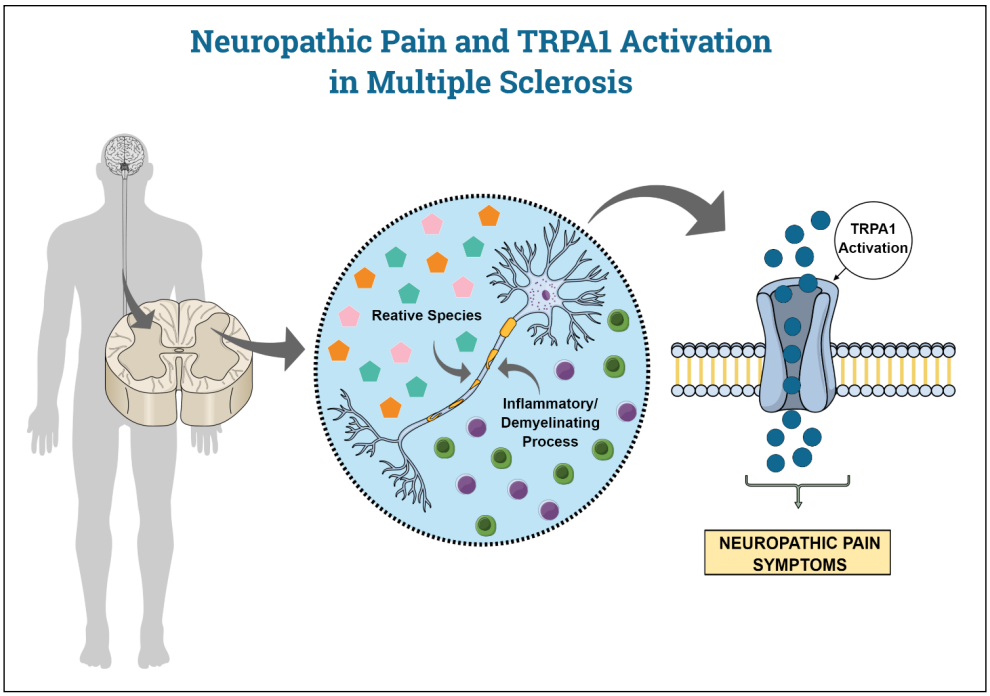
induction. The peak of allodynia was at 14 after induction. The worsening of clinical signs and motor impairments was observed from 15 days post-induction. Thus, we chose the 14th day post-induction to evaluate the antiallodynic effect of all compounds (RITTER, 2020, p.2429). The treatments with selective (HC-030031 or A-967079) and non-selective TRPA1 antagonists (metamizole or propyphenazone) by intragastric route showed an antiallodynic effect in the induced mice. Moreover, TRPA1 antisense oligonucleotide and HC-030031 demonstrated antiallodynic effects for the mechanical, cold, and heat hyperalgesia when used the intrathecal route (administration in the spinal cord). Furthermore, it was shown an enhancement of 4-hydroxynonenal (4-HNE), a by-product of oxidative stress and a TRPA1 agonist, in the spinal cord tissue for PMS-EAE mice. The treatment with antioxidants (apocynin and α-lipoic acid), by oral and intrathecal via, were able to reduce also the nociceptive behaviors caused by this MS model (RITTER, 2020, p.2430).

The development of allodynia was observed on days 7, 14, 21, 28, and 35 after induction in the RR-EAE model. Thus, the 35th-day post-induction was chosen as a nociception peak day to treat the animals with all the compounds (DALENOGARE, 2020, p.12). Two TRPA1 agonists, 4-HNE and hydrogen peroxide are increased in the spinal cord of the RR-EAE induced-mice. Moreover, the treatments with TRPA1 antagonists (HC-030031 and A-967079) and antioxidants (α-lipoic acid and apocynin) by intragastric route reduced the nociceptive behaviors in the RR-EAE mice (DALENOGARE, 2020, p.10). Here, it was also tested HC-030031, α-lipoic acid, apocynin, and the TRPA1 antisense oligonucleotide that presented transitory antinociceptive effects in the induced-mice by intrathecal administration (DALENOGARE, 2020, p.12). These results suggest the main role of TRPA1 channels activation by oxidative stress agonists in the hypersensitivity development by these two MS induction models. Thus, TRPA1 channels are potential targets for future investigation to treat neuropathic pain in MS (Figure 1) (DALENOGARE, 2020, p.13; RITTER, 2020, p.2432).

Another important rodent model for investigating the link between TRPA1 and MS development is the cuprizone-induced model (SAGHY et al., 2016, p.2166).

Cuprizone is known to be toxic for the myelin sheath, and induction consists of feeding mice with a diet containing 0.2% cuprizone mixed into standard food for some weeks (BJELOBABA, 2018, p.1025; SAGHY, 2016, p.2168). The markable signs of this induction are the demyelination process and oligodendrocyte dysfunction, which permit the MS development investigations without focus on the immune aspect of the disease (BJELOBABA, 2018, p.1027). As first findings, it was shown the TRPA1 expression in the central nervous system of mice and attenuation of cuprizone-induced demyelination in TRPA1 genetic deficient mice (SAGHY, 2016, p.2168). The proposed mechanism shows that the attenuation of the demyelinating process occurs due to a reduction in calcium influx in the absence of this receptor. The TRPA1 channels activation elicits the calcium influx and consequently modulates the pro or anti-apoptotic pathways in oligodendrocytes by astrocyte functions (SAGHY, 2016, p.2168). These findings suggest the role of TRPA1 in MS beyond the nociception behaviors, which its inhibition appears to attenuate the degenerative process in MS.

Figure 1. Neuropathic pain and TRPA1 activation in Multiple Sclerosis. Multiple sclerosis is an autoimmune and inflammatory disease, and oxidative stress is also present during its development. The TRPA1 endogenous agonists, such as reactive species of nitrogen, oxygen, and carbonyl are produced and cause an extensively oxidative stress process contributing to the demyelinating process. The by-production of oxidative stress could lead to the activation of some proteins as TRPA1 channels causing neuropathic pain symptoms.



Unfortunately, the TRPA1 antagonists are not available for clinical use in humans yet. The low solubility and non-total elucidated pharmacokinetics of these compounds leads to the discontinuation of the clinical tests (KOIVISTO, 2018, p.116). The TRPA1 antagonist, GRC 17536, has been tested in Phase 2 of a clinical trial to treat diabetic neuropathy (GLENMARK, 2014; KOIVISTO, 2018, p. 117). These results demonstrated a positive effect on reducing the pain scores in a specific group that presents moderate-to-severe pain. Moreover, GRC 17536 offers a good response for the diabetic neuropathic pain treatment well tolerating without significant side effects (GLENMARK, 2014; KOIVISTO, 2018, p.116). Nevertheless, concluded data is still not published. Regardless, the development of TRPA1 antagonists' is also a challenge due to species-dependent variations (humans, rats, and mice) and their different efficacy (KOIVISTO, 2018, p.116).

Conclusion

In such a way, the TRPA1 channel investigation and its involvement in MS is a new research field that demands a large study. Another claim is the search for new molecules or formulations to be used as TRPA1 antagonists. The PMS-EAE model also caused the development of facial allodynia and the relation with the activation of the TRPA1 channels (DALENOGARE, 2021, p.13). This newest data reinforces the leading role of TRPA1 channel activation in the most prevalent types of pain in MS patients: the central neuropathic pain and headache (FOLEY, 2013, p.640). Thus, more investigation is necessary to clarify the involvement of TRPA1 channels activation and the MS physiopathology and symptoms, such as chronic pain.

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ENZYMATIC MECHANISMS FOR THE GENERATION OF BIOACTIVE PEPTIDES

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ABSTRACT

Bioactive peptides can modulate different physiological systems by influencing some biological mechanisms. They are small fragments of proteins that can be generated from foods through different mechanisms as (i) during gastrointestinal digestion after consumption due to the action of gastric and intestinal enzymes, (ii) during the processing of foods as in ageing, fermentation or curing, or (iii) using commercial enzymes and controlled hydrolysis conditions in reactors or microbial fermenters. In order to exert their activity in target organs, bioactive peptides must be able to cross the intestinal barrier and arrive to blood stream. Many types of foods have been described as a good source of bioactive peptides, and dry-cured ham is a good example for the natural generation of this compounds during the long curing conditions of its processing.

KEYWORDS

Bioactive peptides; food; enzymes.

Introduction

Bioactive peptides are small protein fragments between 2 and 20 amino acids length that are able to exert a positive function in the organism although they are inactive before being excised from their parent protein. Their bioactivities can modulate different physiological systems such as the cardiovascular system such as antihypertensive, antithrombotic, antioxidant, and hypocholesterolemic peptides; the gastrointestinal system such as anti-inflammatory, antidiabetic, and satiety peptides; the immune system such as antimicrobial, cytomodulatory, and immunomodulatory peptides; and the nervous system, such as opioid peptides.

Although sometimes bioactive peptides may already be present in the food as part of its composition like carnosine and anserine, they can be generated through different mechanisms: (i) peptides generated during the gastrointestinal digestion mainly due to the action of gastrointestinal enzymes such as pepsin, trypsin, and chymotrypsin, (ii) peptides generated through controlled hydrolysis conditions using commercial enzymes in reactors or fermenters, and (iii) peptides generated during the processing of food such as during fermentation treatments, curing, or ageing processes (MORA et al., 2016, p.395).

Generation during gastrointestinal digestion

The gastrointestinal digestion of foods is the last step for the generation of bioactive peptides. It occurs in the organism and results in the liberation of peptides from the ingested proteins contained in foods where they were encrypted. Main enzymes participating in gastrointestinal digestion are saliva amylase, pepsin, trypsin, and chymotrypsin although also the activity of peptidases situated in the gut and involved in transport mechanisms are very important (see Figure 1). In this sense, in order to be able to exert their activity, bioactive peptides must arrive intact to the target organs, surviving the mechanisms of gut and blood stream transport, where different enzymes might degrade their structure and sequence. The concentration of a bioactive peptide necessary to exert the 50% of inhibition is defined as the

IC_{50} value. However, the *in vitro* calculated IC_{50} values do not always correlate with the observed physiological effect in the body. Regarding this, some peptides have been reported as strongly bioactive after *in vitro* assays but no positive results were observed after oral administration. This fact could be due to a partial hydrolysis occurred in the peptides during gastrointestinal digestion, resulting in smaller sequences not able to exert the biological activity.

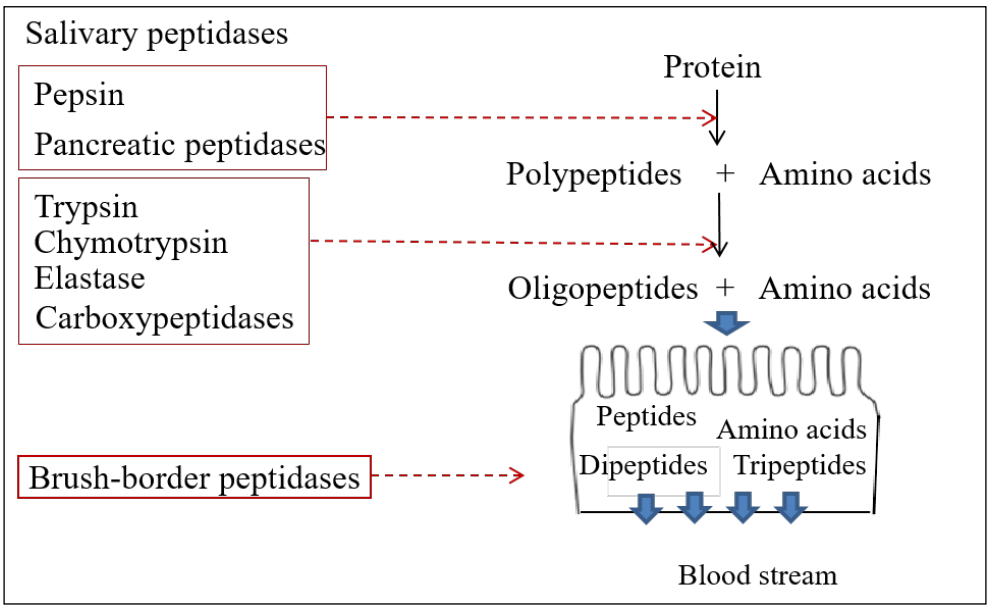

Figure 1. Scheme of main peptidases involved in gastrointestinal digestion and the generation of bioactive peptides and free amino acids.

As an example, pepsin and pancreatin enzymes were used at simulated gastrointestinal conditions in pork meat and some of the generated peptides were assayed for Angiotensin-I converting (ACE-I) inhibitory activity, related with antihypertensive activity. The peptide KAPVA (IC_{50} of 46.56 μM) followed by the sequence PTPVP (IC_{50} of 256.41 μM) showed the strongest ACE-I inhibitory activity (ESCUDERO et al., 2010, p.2895). These peptides were further tested *in vivo* to prove their antihypertensive activity in spontaneously antihypertensive rats (SHR). The obtained results suggested that pork meat could constitute a source of bioactives generated after gastrointestinal digestion that could exert their activity in the cardiovascular system (ESCUDERO et al., 2012, p.382).

Hydrolysis treatments with commercial enzymes

The use of commercial proteolytic enzymes for the production of bioactive peptides is another interesting area highly developed in recent years (CRUZ-CASAS et al., 2021, p.100047). Different peptidases obtained from animal, plant, and microbial sources such as Alcalase, Neutrase, Papain, Thermolysin, Bromelain, etc. can be used for the generation of bioactive peptides from different food sources.

These treatments are mainly applied when the objective is to obtain high amounts of bioactive peptides for commercialization as this procedure is much more efficient and results in higher yields. Hydrolysis treatment is usually optimised in a laboratory and later scaled to pilot plant and for industrial production, and it is developed in reactors or fermenters depending on the use of peptidases or microbial peptidases, respectively (Figure 2).

Those enzymes providing from microbial sources are more accessible, easier to produce, and cheaper. Some examples are Subtilisin from *Bacillus licheniformis*, Flavorzyme from *Aspergillus oryzae*, or Neutrase from *Bacillus subtilis*. However, enzymes isolated from animal or vegetal sources such as bromelain from *Ananas comosus*, papain from *Carica papaya*, or trypsin enzyme obtained from the digestive system of many vertebrates, are more difficult to be obtained in high amounts and so are more expensive.

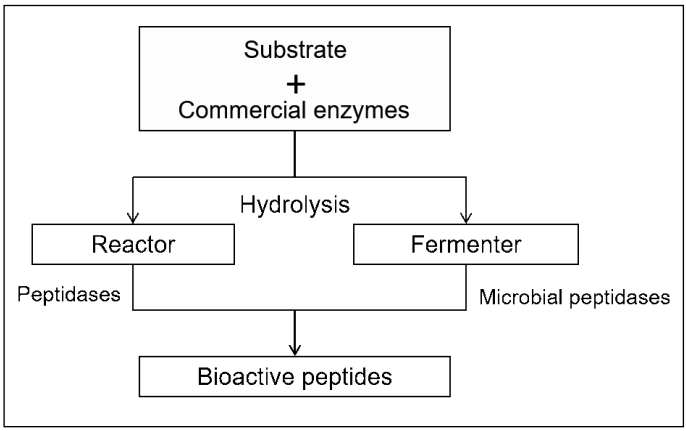

Figure 2. Production of bioactive peptides using commercial enzymes under controlled hydrolysis conditions in reactors or fermenters.

The main application of these studies is focused on getting the maximum use of industry by-products, obtaining an added-value, increasing economic benefit, and reducing the environmental impact. Thus, the bioactive peptides obtained from food by-products have been used for edible uses such as feed and food ingredients, sport supplements, or as functional ingredients, as well as for non-edible uses such as in the cosmetic or pharmaceutical industry (RADENKOVS et al., 2018, p.64).

Generation during the processing of food

The processing of food can also be an important mechanism for the generation of bioactive peptides. In this sense, the ageing of meat, curing processes of meat and fish, and fermentation occurred during the preparation of some beverages, vegetables, or dairy products, are clear examples for the hydrolysis of proteins in smaller fragments or peptides.

Main responsible for this generation of bioactive peptides are endogenous enzymes in ageing and curing processes, and their combination with microbial peptidases in fermentation processes. The phenomena of proteolysis influence the final characteristics of the products affecting the texture specially due to the action of endopeptidases (calpains and cathepsins) and the generation of small peptides and free amino acids due to action of exopeptidases. Thus, some of the generated small peptides have been describes as bioactive exerting ACE I-inhibitory activity, antioxidant activity, DPP IV-inhibitory activity, etc. (TOLDRÁ et al., 2018, p.395).

Dry-cured ham as a source of bioactive peptides

Dry-cured ham is a high-quality product which processing is based on the action of endogenous enzymes and the generation of hundreds of protein fragments, peptides, and free amino acids. Some of these peptides have been described to exert different biological activities such as antihypertensive, antioxidant, hypocholesterolemic, antidiabetic, anti-inflammatory, or antimicrobial. In fact, previous studies showed that a daily intake of 80 g of dry-cured ham/day did not impair blood pressure whereas total cholesterol, low-density

lipoproteins (LDL), and basal glucose levels, dropped as well as a regulation of the thrombogenic status (MONTORO-GARCÍA et al., 2017, p.160; MARTÍNEZ-SÁNCHEZ et al., 2017, p.321). More recently, the mechanisms of identified antihypertensive and antiinflammatory peptides were elucidated *in vitro* and *in silico* (MARTÍNEZ-SÁNCHEZ et al., 2019, p.4204).

ACEI-inhibitory peptides are related to the antihypertensive activity as they have the ability to control and prevent hypertension. As an example of antihypertensive activity, the pentapeptide AAATP was identified in an ACEI inhibitory extract of dry-cured ham after separation by size-exclusion chromatography, and its activity was tested *in vitro* obtaining an IC_{50} of 100 μM. The antihypertensive effect of AAATP peptide was later confirmed in spontaneously hypertensive rats (SHR), showing a decrease of systolic blood pressure by 25.6±4.5 mmHg after 8 h of oral consumption (ESCUDERO et al., 2013, p.499). More recently, the most abundant sweet dipeptide identified in dry-cured ham named Ala-Ala (AA) was quantified at different times of curing using mass spectrometry in tandem. Its IC_{50} was determined from the *in vitro* inhibition of ACE-I enzyme and resulted to be 110.824 μM. The oral administration of 1 mg of AA per kg of body weight in spontaneous hypertensive rats resulted in a decrease of systolic blood pressure of -16.5 ± 4.5 mmHg after 8 hours of consumption, which is very good value considering the abundance of this dipeptide in dry-cured ham (HERES et al., 2021, p.104818). These results confirm the antihypertensive potential and role in cardiovascular health of dry-cured ham, and also support the previous findings in human clinical trials.

The antioxidant activity of bioactive peptides derived from food has also been widely studied, and dry-cured ham also resulted to be a good source of antioxidant fragments. In this sense, an aqueous extract of dry-cured ham peptides was separated in fractions using size-exclusion chromatography and some of the isolated fractions showed antioxidant activity in the analysis of DPPH radical-scavenging and ferric-reducing capacity. Some of the identified peptides in this fractions were further synthesized and tested *in vitro* to determine their antioxidant activity. From this study, specific peptide sequences derived from dry-cured ham were studied and the pentapetide SNAAC showed an IC_{50} value of 75.2 μM in DPPH radical-scavenging activity and 205 μM in ferric-reducing capacity. This antioxidant capacity was tested and

confirmed using a linoleic acid solution, showing a 33 % less lipid oxidation in those samples with SNAAC peptide as indicated in Figure 3 (GALLEGO et al., 2018, p.873).

Also some peptides derived from dry-cured ham have been reported to exert antidiabetic properties (MORA et al., 2020, p. 103840). In this sense, *in vitro* assays such as the determination of α-amylase and α-glucosidase inhibitory activity or DPP IV inhibitory activity are key tests to elucidate potential sequences to exert hypoglycaemic properties. Previously identified ACEI-inhibitory peptides in dry-cured ham (AEEEYPDL and LGVGG) have been recently identified in Iberian dry-cured ham as α-glucosidase inhibitory peptides, confirming their multifunctionality.

Finally, peptides FNMPLTITITPGSKA, HCNKKYRSEM, MDPKYR, and TSNRYHSYPWG, were identified in dry-cured ham using mass spectrometry in tandem and later synthesized and assayed *in vitro* at a concentration of 1 mM. They showed different percentages of inhibitory activity for platelet-activating factor acetylhydrolase (26.06% in FNMPLTIWITPGSKA, and 16.30% in TSNRYHSYPWG), autotaxin (18.93% in TSNRYHSYPWG), and lipoxygenase (23.33% in HCNKKYRSEM) (GALLEGO et al.,2019, p.110).

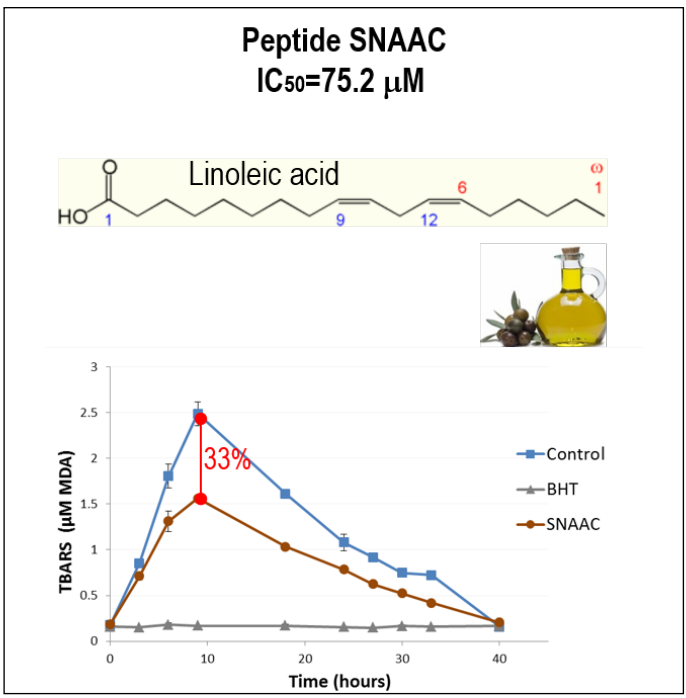

Figure 3. Scheme of the effect of peptide SNAAC in a linoleic acid solution.

Also abundant by-products are generated during the deboning of dry-cured ham such as bones, skin, fat and trimmings, which could be used for several applications by giving them an extra value (TOLDRÁ et al., 2016, p.54; TOLDRÁ et al., 2021, p.108608). As an example, skin can be consumed as fried skin tapas, fat can be used for cooking, bones can be boiled to obtain nutritional and tasty broths and ham trimmings can be consumed as snacks. In a previous study, dry-cured ham bones were traditionally used to prepare broths, and the obtained peptide extract was assayed for ACEI-inhibitory activity, endothelin-converting enzyme (ECEI) inhibitory activity, platelet-activating factor-acetylhydrolase inhibitory activity (PAF-AH), DPP-IV inhibitory activity and antioxidant activity. Main results showed that active peptides were stable after cooking and *in vitro* gastrointestinal digestion as it is indicated in Figure 4. This results probed that dry-cured ham bones broth could positively contribute to cardiovascular health of consumers (GALLEGO et al., 2019, p.1115; GALLEGO et al., 2017, p.296).

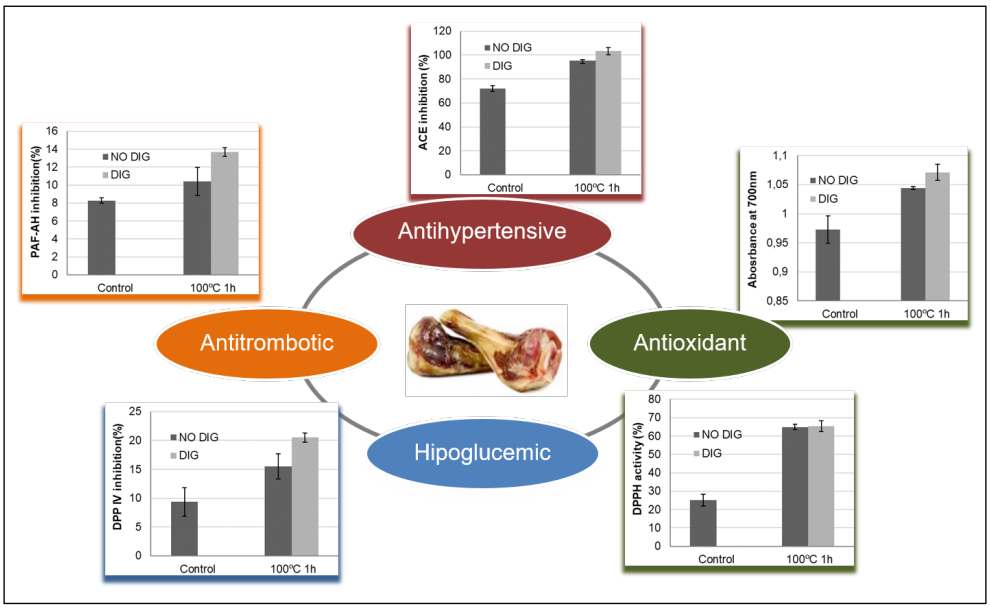

Figure 4. Scheme of main activities tested in dry-cured ham bones broths. Graphs indicated simulated gastrointestinal digestion (DIG) and NO DIG are samples before gastrointestinal simulation. Broths were cooked at 100 ^{o}C during 1 hour. Control are bone samples with no cooking.

Current status and future trends

In summary, bioactive peptides can be generated from foods through different mechanisms as during gastrointestinal digestion after consumption, during the processing of foods, or using commercial enzymes and controlled hydrolysis conditions. Small peptides are more bioavailable and their chance to arrive to target organs and exert biological activity is higher than longer peptides. Main described bioactive peptides are antihypertensive, antioxidant, anti-inflammatory, hypoglycemic, hypocholesterolemic, or antimicrobial, among others. Also advanced proteomic and peptidomic tools are necessary for the accurate identification and quantitation of bioactive peptides although more studies are still necessary for a better understanding the specific mechanisms of action.

The current trends in the study of bioactive peptides are mainly focused in the study of food matrix interaction and its effect on the hydrolysis, bioactivity, and/or bioavailability of peptides. Also the optimization of methodologies for bioactive peptides quantitation is of special interest. The knowledge about the concentration of peptides in food, especially when peptides have been naturally generated, the calculation of the dose/effect in *in vivo* assays, as well as the concentration of bioactive peptides in blood stream and target organs, is necessary for a better understanding of the mechanisms of action.

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NON-ANIMAL MODELS FOR THE STUDY OF CANCER

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ABSTRACT

Traditionally, studies on cancer research have prioritized animal models, however they are expensive, require long investment of time and frequently they fail to predict drug anticancer activity when translated to clinical assays. In this scenario, non-animal models (NAMs), although not absent of problems, offered some advantages such as direct relevance to humans, test much more quickly or cost reduction. Here, we summarize NAMs currently used to study solid cancer tumours with special attention to its applications in drug screening and development besides pointing out their advantages and disadvantages.

KEYWORDS

In vitro; *in silico*; Three-dimensional models; Organ-on-a-chip; Zebra fish.

Introduction

Cancer metastases is the main cause of cancer deceases and thus being the second major cause of death worldwide. Neoplastic disease provoked about 10 million deaths in 2020 being lung cancer the most frequent cause (1.80 million). Regarding new cancer cases, breast cancer is the most common in women whereas lung cancer in men (WHO, 2021).

According to epidemiological data, an important increase in cancer prevalence is expected worldwide for the next decades (MATTIUZZI and LIPPI, 2019). For this reason, several countries and continents have included in their agenda battling against cancer by improving public health policies or defining carcinogen biomarkers of exposure among others (MADIA et al., 2019). Therefore, understanding carcinogenesis biology and pathogenesis is a pivotal key to fight against the disease and facilitates to design and discover novel and more effective treatments (INGLE, 2019). To achieve the goal appropriate preclinical cancer models that closely resemble human physiology are required (SOLEIMANI et al., 2019).

Traditionally, studies on cancer research have prioritized animal models, being transgenic and "humanized" rodents the preferred ones, by justifying the interactions of neoplastic cells to their environment and other tissues, besides to include metastatic model. The usefulness of these experimental models is clear, but they are expensive and require a long investment of time (LEE and LEE, 2020). Moreover, PISTOLLATO et al. (2020) have reported that 97% of potential anticancer drugs fail when assessed in clinical trials although previous positive findings in animal models. In this scenario, non-animal models (NAMs), although not absent of problems, offered some advantages such as direct relevance to humans, test much more quickly or cost reduction. Here, we summarize NAMs currently used to study solid cancer tumours with special attention to their applications in drug screening and development besides pointing out their advantages and disadvantages.

Non-animal models

Non-animal models are represented by numerous and diverse methods and techniques that include from *in silico* models (computer modelling or databases) to complex models mimicking organ or systems functionality. Scientists use these NAMs to refine, reduce and when possible, replace the use of experimental animals to evaluate, analyse or predict chemical behaviour when entered in human organism. In the case of cancer studies, they offer previous evidence of the complex mechanisms involved before addressing the preclinical experimental assays necessary and reducing experimental animal number and suffering besides cost expenses.

In silico models

Computational data and simulations are used in drug discovery and pharmacology but also in predicting and understanding physiological and pathophysiological processes. Briefly, they include all techniques based on the use of software starting from basic databases or quantitative structure-activity relationships to machine learning or network and data analysis tools that have been the basis to develop computational models to predict, hypothesise, and even contribute to new findings in medicine and therapeutics (EKINS et al., 2007).

In the case of cancer, *in silico* models are applied for understanding cancer biology but also in preclinical and clinical investigation of novel therapies and treatments (reviewed in EDELMAN et al., 2010). According to the information provided:

1- Databases can be considered as a basis in silico models to predict carcinogenic potential of chemicals based on their characteristics and properties. One example is OncoLogic® (BENIGNI et al., 2012).

2- Statistical models. Used for diagnosis and targeted therapies because they allow to identify altered genes by molecular signatures. Examples are Imatinib (Gleevec) (EDELMAN et al., 2010)

3- Biochemical networks and relationships among genes, proteins and metabolites that characterize cancer biology.

4- Microenvironment-tissue level models that include continuum-based models, agent-based models, and hybrid models.

Continuum-based models allow studying interactions between intracellular and extracellular factors in tumour biology to study avascular tumour growth. This modelling considers cells as a concentration of cell types in contrast to discrete models. Depending on the phases defined besides the tumour progression growth and cell turnover, it can be assessed the effect of nutrients and the biomechanical influence of the tumour microenvironment on its growth over the time. (SOLEIMANI et al., 2014). Few studies also have utilized continuum-based simulations to assess aspects of vascular tumour growth and drug testing.

Agent-based models where tumour cells appear as individual entities that interact with other entities and external factors in defined specific times and according to pattern rules. There are models that represent three-dimensional tumour cell patterning, immune system surveillance, angiogenesis, and the kinetics of cell motility (EDELMAN et al., 2010).

Hybrid models are based on multiscale settings and combine the two models. This model allows to study the interactions between individual cells and between the cells and their microenvironment and thus, it is a better approach for the multicellular complexity of cancer tumour system. Recently, ROCHA et al. (2018) have reported a multiscale model of tumour dynamics validated by integrating *in vivo* and *in vitro* experimental data for a selected type of cancer, which can emerge as a valuable tool to investigate cancer biology at the various levels but also to evaluate novel oncological treatments.

In silico models are powerful tools to obtain information related to oncogenic processes, however extrapolation of data is still difficult because this methodology is in early stages of development. Historical data from patients besides data obtained from experimental animal models are fundamental to generate mathematical and computational models that can mimic diverse aspects of cancer process for assessing novel therapies and drug treatment, which in turn could reliable be translated to the clinic. Therefore, their use contributes to reduce the number of experimental animals, money and time invested.

In vitro models: from cell lines to organ-on a chip

Two-dimensional models

The use of two-dimensional or monolayer *in vitro* cell culture is a valuable simple model used in non-clinical research studies because it is relatively cheap, easy to implement and large amounts of basic data can be generated. This model besides contributing to define the starting point for testing hypotheses, helps to reduce the animal numbers in posterior preclinical analyses, and subsequently the economic expenses related to further investigations (JACKSON and THOMAS, 2017). The use of this model in cancer therapeutics screening is still the most frequently used *in vitro* method for its simplicity, reproducibility, and low cost (NUNES et al., 2018).

Cell origins include primary cultures, obtained from patient samples or animal models, and tumour cell lines both providing useful knowledge to understand cancer biology or to address therapeutic approaches (VAN MARION et al., 2016). Limitations of primary cells include their limited life span and notable heterogeneity while tumour cell lines grow rapidly but suffer genetic differentiation with time that can diminish their ability to respond in front of a specific drug chemical. Nevertheless, identification of factors (CXCL12) and receptors (CXCR4 and oestrogen receptor GPER) related to cancer progression and thus its growth and metastasis, have been identified using ovarian cell lines (VAN MARION et al., 2016).

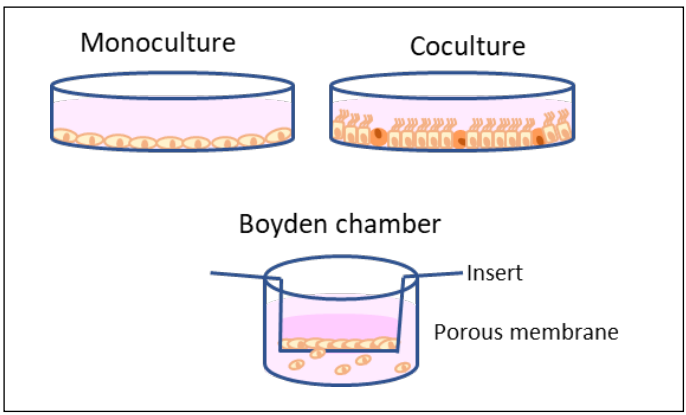

Figure 1. Schematic representation of two-dimensional models.

Main problem of monolayer culture models is they cannot replicate the complex three-dimensional architecture of solid tumours, oxygen and nutrient gradients and cell-cell and cell-matrix interactions. Failing to reproduce the native solid tumour microenvironment and properties, they have been unable to respond appropriately to predict the performance of novel oncological drugs with discrepancies with the *in vivo* assays (NUNES et al., 2018). One-step further is coculturing different types of cells together that includes cell-cell interactions, as for example a Boyden chamber (Figure 1) to study chemotaxis, cell migration and invasion (KITAEVA et al., 2020). However, the complex architecture and organization of the *in vivo* tumour is not reproduced in these plastic devices where cells interact (VERJANS et al., 2017).

Three-dimensional models

Currently cancer research can choose among a great variety of three-dimensional models based on different approaches. These models present several advantages in front cell monolayers such as complex network of cell-cell contacts, advanced extracellular matrix, presenting different cell types or even exhibiting migration and invasion mimicking the cancer microenvironment *in vivo* (FROEHLICH et al., 2016).
Technological progresses in cell culture techniques, including 3D bioprinting and microfluid technologies, allowed the development and improvement of complex culture models such as organoids, spheroids, reconstructed tissues, and the more advanced organ-on-a-chip models. These models, although not exempt of limitations, may mimic human organs, and allow assaying safety and efficacy of novel drug treatments making drug screening more competitive and affordable (LEE and LEE, 2020). According to LANGHANS SA (2021), these complex models can be classified as non-scaffold or non-anchored based models (spheroids, organoids and tumoroids), scaffold or anchored base-models (such as hydrogels) and complex culture platforms (organ-on-a-chip, tumours-on-a chip).

Spheroids

Spheroids are defined as sphere like structures with well-rounded morphology, presence of cancer cells, and capacity to be maintained as free-floating cultures. Technological developments during five decades result in a variety of spherical models, protocols, and applications (WEISWALD et al., 2015; ISHIGURO et al., 2017) but standardized and optimized protocols are still required (FROEHLICH et al., 2016). Currently, spherical cancer models are classified in four groups depending on the cell source, cell handling and time required to obtain the three-dimensional architecture (Table 1).

Table 1. Classification of spherical cancer models and main characteristics.

Spherical model	Cell source	Other characteristics	Clonality
Multicellular tumour spheroids	Cancer cell lines	Suspension	Monoclonal
Tumour Spheres	Dissociated tumour tissue	Systems to evaluate cancer stem cells *in vitro*	Polyclonal/ Monoclonal
Tissue-derived organoids	Dissociated tumour tissue	*Ex vivo* model of organogenesis	Polyclonal/ Monoclonal
Organotypic multicellular spheroids	Tumour tissue	Nontumor cells and stromal component included	Polyclonal

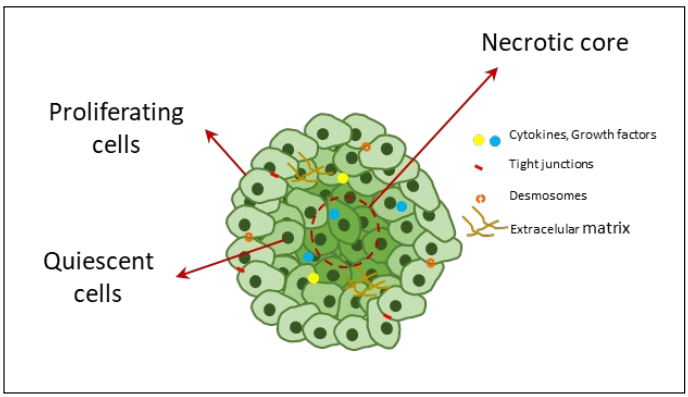

Figure 2. Schematic representation of a multicellular spheroid.

Multicellular spheroids or MTCS (Figure 2.) have demonstrated their usefulness in drug screening, both low- and high-throughput, because their architectural structure simulated better *in vivo* solid tumours drug behaviour than cell monolayers and present drug resistance reported in *in vivo* tumours. Moreover, MTCS facilitate the analysed of the cellular mechanisms related to drug resistance as alterations of cell cycle, permeability, or hypoxia. Nevertheless, no consensus is reached about the reason of anticancer drug resistance: influence of multicellular and three-dimensional architecture or cell intrinsic properties (WEISWALD et al. 2015)? MTCS are dense and compact spheroids as cell interactions stimulate secretion and deposition of extracellular matrix (ECM) proteins and proteoglycans, formation of intercellular connections and other cell and ECM interactions, which in turn rise interstitial fluid pressure and consequently difficult drug diffusion into the tumour mass. However, changes in the expression of specific target receptors as HER2 in breast cancer and other antidrug defence mechanisms are also reported (FROEHLICH, 2016).

Organotypic and tissue-derived spheroids seem suitable to study personalised drug treatments, for the heterogeneity they present and the diverse individual responses to the same drug treatment.

Organoids

Organoids or organ-like organisms are 3D cultures formed by aggregation of cells obtained from specific organ, stem cells or induced pluripotent stem cells (iPSCs) when cultured under special conditions that facilitate their ability to self-organise and display organic architecture, some organic functions and even form systems in a culture-dish (LEE and LEE, 2020).

Differentiation of pluripotent stem cells into organoids in each organ is reported in CLEVERS (2016) and LANCASTER and KNOBLICH (2014) reviews regarding organoid technologies to modelling organogenesis. Moreover, among the different pluripotent stem cell-derived organoids success in obtaining valid cancer pancreatic models have been described. In addition, building organoids from specific mature organs is also viable as reported elsewhere (DROST and CLEVERS, 2017) and their use to mimic cancer is named by LEE and LEE (2020), which include pancreatic, prostate, and small intestine organoids.

Finally, tumour organoids or tumoroids can be obtained directly from stem cells of cancer patients or animal models, as they have proven to maintain the original genomic profile. These tumour organoids are valuable tools to investigate the individual characteristics of cancer microenvironment, to identify specific genetic upregulation in front anticancerogenic drugs and to identify mutants (LEE and LEE 2020).

Scaffold models

In these models, cells are cultured on artificial three-dimensional structures where they attach, migrate and fill (Figure 3). Cells are seeded in an acellular three-dimensional matrix (such as inserts) or suspended in a hydrogel as the natural commercially available Matrigel®.

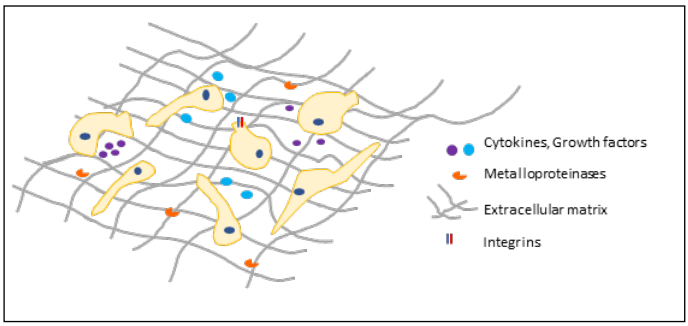

Figure 3. Schematic representation of a scaffold model.

NUNES et al. (2017) and LANGHANS (2020), review nature, source, and methodologies to obtain these three-dimensional structures, including bioprinting. In general, these structures are not a mere physical support and mimic as much as possible the original extracellular matrix (ECM). Moreover, polymers are the major material used presenting different advantages and disadvantages depending on their natural or synthetic origin. Natural ones are biocompatible and can contain chemokines, growth factors, ECM components and other soluble factors as well as cell adhesion sites that synthetic polymers lack. However, natural polymers are more easily degraded, and their composition varies among batches compromising the reproducibility needed in drug screening.

These three-dimensional models have proved their usefulness to simulate tumour ECM and interactions with tumour cells but nowadays are still not useful in drug research and screening. The fabrication of the artificial structure requires

expensive high-tech equipment to guarantee reproducibility and the materials can interfere in several determination by altering drug adsorption and adhesion. Moreover, cells cannot be recovered or isolated for other evaluations (GUPTA et al., 2016). Generally, these three-dimensional models are good candidates for tissue engineering purposes.

Organs-on-a-chip

Organs-on-a-chip are based on microfluidic technologies that simulate dynamic fluid flow and pressure (Figure 4). They are formed by cell culture devices of flexible polymers or plastic glass that contain microchannel where living cells are found (VAN MARION et al., 2016; SONTHEIMER-PHELPS et al., 2019). These devices can outline the *in vivo* tissue or organ structures in physiological or pathophysiological situations by combining different compartments, increasing the number of microchannel and including ECM. Complex organ-on-a-chip recreate design blood and lymphatic vascular networks as well as contain parenchymal microchannel for studying systematic interactions (SOLEIMANI et al., 2018).

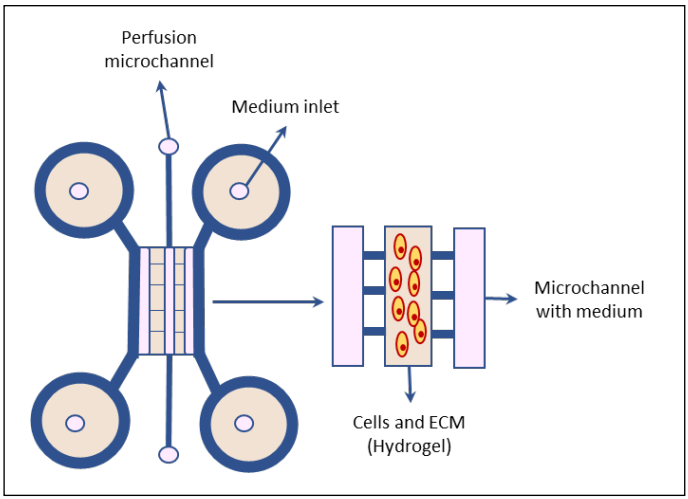

Figure 4. Schematic representation of a microfluidic device.

These devices have proven to be very useful in front other models by identifying how local microenvironment affects tumour growth, for example. Other aspects of tumour biology as angiogenesis or neovascularization, cancer

progression and steps in metastasis (migration, invasion, ...) have also been reported and can be reviewed in SONTHEIMER-PHELPS et al. (2019). Regarding efficacy of cancer therapies, these models may help to understand some cancer chemical resistance, design personalized therapies of single or combined drugs, evaluate toxicity and efficacy of antitumour treatments and drug delivery in nano-based therapies and in general drug screening.

Organ-on-a-chip offers some advantages in front the static models including three-dimensional models by including perfusion, an aspect fundamental to understand neovascularization and metastasis. However, they still need validation studies to demonstrate their reliable predictivity, in addition expert staff are required to perform the assay and prevent technical errors during performance.

In vivo non-animal model systems

Some complex systems are considered non-animal models and include zebrafish embryos (*Danio rerio*), *Drosophila melanogaster* and the chick chorioallantoic membrane (CAM) of fertilised eggs (VAN MARION et al., 2016). They present a microenvironment that lacks *in vitro* methods where the cancer cells will grow.

Drosophila melanogaster was employed to study early steps of cancer metastasis and allows simultaneous genetic manipulation and inclusion of markers. *Drosophila* genome is 60% homologous to that of humans, and about 75% of the genes responsible for human diseases have homologs in flies. Its brief generation time and low maintenance costs, allows the use of *Drosophila* to study complex pathways including cancer. Despite some limitations, due to the anatomical differences between flies and humans, the use of *Drosophila*'s cancer models has been fundamental to understand some basic processes that regulate human cancers (MIRZOYAN et al., 2019).

Zebrafish presents genes involved in the malignant tumourogenic process well conserved, as the Myc family. Also, overexpression of specific genes is quite affordable by genetic modifications in the early stages of its development. Finally, zebrafish is a good model for induction of orthotopic tumour

as fish solid tumours present some histological similarities with human solid tumours and are also similar at molecular level which allows the study of tumor initiation, progression, and heterogeneity (INGLE AD, 2019; VÖLKEL et al., 2018). Moreover, it is transparent during embryonic stage, and it is easier to follow the tumor process.

CAM of fertilized chicken eggs has been used to study cancer angiogenesis and metastasis, because tumour cells can escape to the tumour site and reach the embryo through the continuous circulatory system. CAM tumour models provide a reliable tumour and tumour microenvironment because it contains blood vessels, incorporates fluid flow dynamics, and the tumours are very accessible for treatment administration. Furthermore, CAM tumour models may provide information on metastasis, drug pharmacokinetics, and toxicology (MAPANAO et al., 2021). The use of CAM has continued to increase in the last decade as it has been shown to be a good growth platform for a wide range of cancer cell lines, Moreover, it is a rapid cost-effective drug-testing model that reproduces many aspects of human cancers and allows the recreation of the hypoxic environment of tumours. The microenvironment of the tumour is crucial for its development and progression (HARPER et al., 2021).

Conclusions

Currently there exists different non-animal methods to understand cancer biology and progress besides to drug screening, safety and efficacy. Although not absent of limitations, technological advances and increasing knowledge will lead NAMs to be more effective tools to replace animal-based models for cancer research and cancer preclinical studies, thus contributing to reduce the number of experimental animals in such studies and happily replace them.

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IMPROVED HEALTH AND HEALING OF FISH SKIN BY NATURAL ADDITIVES IN DIET

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ABSTRACT

This chapter begins with a brief introduction to the fish skin followed by the several steps that take place just after a skin injury occurs. The interest of these studies in farmed fish will be justified and the different experimental models of skin lesions and the parameters studied in fish to know the progression of the wounds revisited. The importance of the indirect damages originated in other parts of the fish body after a skin wound will be also considered. Finally, a review of the natural products used to date as a strategy to improve the healing and regeneration of skin wounds is presented. In this chapter, the gilthead seabream (*Sparus aurata*) has been selected as a model species because it is one of the most important farmed fish in the Mediterranean aquaculture.

KEYWORDS

Skin; Mucosal immunity; Wound healing; Nutraceuticals; Gilthead seabream.

A singular epithelium: the fish skin

All the body surfaces of multicellular organisms are recovered by epithelia, which provide a physical, chemical, and biological barrier between the internal milieu and the external milieu. The skin is the first barrier that protects against any hazard present in the environment. Fish encounter all possible aquatic conditions and therefore, the skin has to perform several activities to cope with the various situations. In fact, the fish skin is an organ involved in varied roles related to body protection, locomotion, communication and sensation, respiration, ion regulation, excretion, reproduction, secretion/absorption of molecules, reservoir of substances, and immunity (reviewed by ELLIOT, 2000; MARSHALL & BELLAMY, 2010).

Vertebrate skin is a very complex structure made up of several layers. The layers present in the integument of adult teleosts from the external environment to the interior of the body of the fish are the cuticle or mucus layer, the epidermis and the dermis (HAWKES, 1974). The mucus layer is formed by a lubricating secretion that forms a viscous layer with a very complex composition and in which there is a large population of bacteria that form the skin microbiota. The physico-chemical parameters have been analysed, as well as the humoral components related to immunity (e.g., lysozyme, complement, immunoglobulins, or many enzymes) present in the mucus of seabream skin (GUARDIOLA et al. 2014a, b; 2015). Furthermore, the composition of mucus has been of great interest and its metabolites and proteins have been studied. To date, more than 2000 proteins were identified in seabream mucus and classified into structural, metabolic and protective functions (JURADO et al.,2015; SANAHUJA & IBARZ, 2015; PÉREZ-SÁNCHEZ et al. 2017, FERNÁNDEZ-ALACID et al., 2018).

The epithelium of the skin is squamous and stratified and is made up of epithelial cells (keratocyte cells) and between them appear isolated mucous cells (goblet) that are unicellular glands. The epidermis is approximately 5-10 cell layers thick and consists exclusively of living cells (ZHAO et al., 2008). The number of cell layers varied, not only between

different species of fish but also according to the body region of the same fish (CORDERO et al., 2017a). Keratocyte cells are characterized by being polygonal cells closely linked by desmosomes with neighbouring cells. The apical surface of the cells located in the superficial layer of the epithelium presented actin microridges whose function is to retain the external mucosa layer (CORDERO et al., 2017a).

The mucus is mainly secreted by goblet cells which are mostly placed in the intermediate layers of the epidermis. Goblet cells release mucous granules containing high molecular weight glycoproteins called mucins. The use of peroxidase-conjugated lectins and conventional histochemical techniques (such as periodic acid of Shiff, saponification, alcian Blue, chlorhydric hydrolysis, sialidase, bromophenol blue, thioglycolate reduction and ferric-ferricyanide-FeIII) allowed the establishment of the content and distribution of carbohydrates, proteins and glycoconjugate sugar residues on the skin of gilthead seabream. Variable amounts of glycoproteins containing sialic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, mannose and/or glucose residues were observed in the cuticle and mucous cells of the corporal skin. While epithelial cells contained glycogen, proteins, carboxylate groups, as well as glycoproteins with mannose and/or glucose and N-acetyl-D-galactosamine residues (SARASQUETE et al., 1998). Furthermore, the capacity and rhythm of production and exudation as well as renewal of the skin mucus have also been studied by using stable isotope analysis with gelatine capsules of labelled meal (IBARZ et al., 2019). The dermis is mainly composed of dense or fibrous connective tissue with a large number of collagen fibres, and it has two layers, the hypodermis or stratum spongiosum, a frequent site of development of infectious processes and the innermost layer or stratum compactum. The dermis has cells with pigment (chromatophores) that give fish their colour. The dermis separates the epidermis from the underlying muscle and is related to lipid storage and it is the layer that the nerve terminals and blood capillaries reach that support the entire skin (ESTEBAN & CEREZUELA, 2015).

More recently, the whole body of gilthead seabream was scrutinised using X-ray computed tomography and segmentation, measurements, and three-dimensional reconstructions were carried out by means of a combination

of software. The density ranges that topographically coincide with the skin were determined and it was demonstrated that it is possible to apply a fully automated image analysis technique to study and quantify the fish skin, in particular, and the body composition as a whole. More concretely, the skin and the subcutaneous fat had a density range of −400 to −50 Hounsfield Units and represented 31.00% of the gilthead seabream total volume (CEBALLOS-FRANCISCO et al., 2020). Much more efforts have to be done in using in vivo image analysis to study in farmed fish because it offers the opportunity to analyse the whole fish body in live animals.

Coordinated steps triggered after a skin injury: the process of wound healing

Several types of lesions, abrasions, or ulcers are usually seen in farmed fish as a result of physical processes among fish, poor husbandry practices, or traumatic processes due to the high densities at which they are confined (LAW, 2001). Damage to the skin may threaten the barrier function of the fish resulting in reduced animal welfare or increased morbidity and mortality (NOBLE et al., 2012). A better understanding of the tissular, cellular and molecular mechanisms underlying wound healing will ultimately allow us to influence and accelerate the wound repair/regeneration process. Understanding how fish skin responds during the healing process will allow having efficient protocols of prevention or treatment of acute or chronic injuries for farmed fish. Furthermore, because of the intimate contact of fish with the environment, cutaneous diseases are relatively more common in fish than in terrestrial vertebrates (ELLIS et al., 2002; TAKEO et al., 2015).

When the skin is wounded, a well-conserved wound healing cascade is initiated in order to restore tissue integrity and three main phases (that overlap spatially and temporally) can be established: 1) a haemostasis/coagulation phase in which the fibrin clot is formed and subsequently dissolved; 2) an inflammatory phase, involving the recruitment of immune cells that fight potential pathogens and secrete cytokines to trigger the tissue repair processes. This phase is evident 1-3 h post-injury and is observed for 3-4 days; and 3) a final phase

of re-establishing tissue integrity and functions. In general, the wound healing process differs between species but, in aquatic ones, is faster and more efficient compared to that of terrestrial vertebrates (FONTENOT & NEIFFER, 2004), making the regenerated skin almost indistinguishable from the original one, without scarring (COSTA & POWER, 2018).

Three main types of experimental wounds have been studied in seabream (Figure 1). In the first one, round wounds were made with a biopsy punch (CORDERO et al., 2017b). The second types of wounds were produced by mechanical scale loss of almost half of the fish skin (MATEUS et al., 2021). Finally, another way is to make chronic wounds or ulcers by abrasion of the skin with an electric tooth brush for several times (CORDERO et al., 2017a).

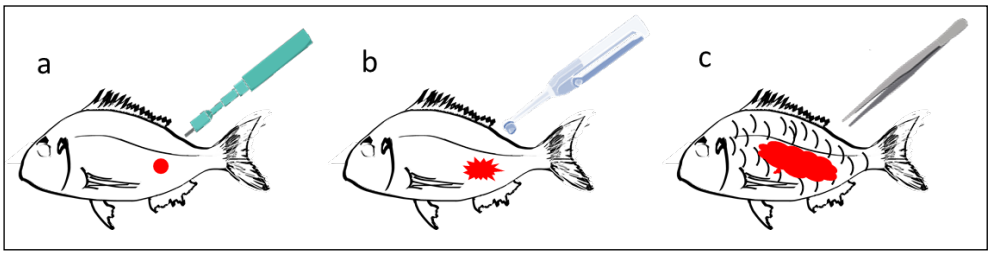

Figure 1. Schematic representation of the different experimental wounds (red colour) made on gilthead seabream by using: a) a biopsy punch, b) a toothbrush (usually several times on the same place) or c) tweezers (for removing scales).

The injuries made with the biopsy punch arise more reproducible injuries (similar wounds regarding size and profundity) and have been used in several studies. The area and perimeter of the wounds can be determined by image analysis using macroscopic photographs where the healing progression can be evaluated by means of computerized software using macroscopic images. Usually, the wound area starts to fall by 4 days post-wounding. When the wound healing rate over the time was compared between wounds made above or below regions of the lateral line, a higher ratio of wound closure was observed in the region below the lateral line, whose wounds were closed after 15 days (CORDERO et al., 2017b).

Microscopically, histological observations (from 0 till 6 days after wounding) suggested that tissue re-epithelialization was independent of inflammation (COSTA & POWER, 2018). During the healing process the modulation of gene expression have been studied in many genes including those related to extracellular matrix, epithelial and mesenchyme interactions,

inflammation, as well as those involved in immunity (because the mucosal immune response has a crucial impact on the quality of the tissue response to an injury), in oxidative stress and in the skin and tissue regeneration. All the available results indicate that after a superficial skin damage, the tissue reconstitution begins with re-epithelialization, which is followed by active deposition of extracellular material and finally by the tissue repair. In other words, the reconstitution of the skin's physical barrier is the priority over the many other functions of the integument, including immune surveillance (CEBALLOS-FRANCISCO et al., 2017; COSTA & POWER, 2018).

Changes originated by a superficial wound: beyond the skin

There is very little information about the changes produced by the wounds not only in the skin mucosae but also in other parts of the body. Till present, notable changes have been demonstrated in the skin mucus, in the microbiota and in the skin tissue, as well as in the immune components present in serum and, more recently, in other very important mucosa, in the gut.

In general, the presence of skin damage provides microenvironments that alter the mucus composition, the microbial biodiversity of this mucosa and the skin cells and, all the changes indicate that fish seem to be more vulnerable to any adverse situation and diseases. The histological and molecular changes underlying skin and scale regeneration in fed and fasted seabream were studied using a microarray 3 and 7 days after scale removal in order to provide a comprehensive molecular understanding of the early stages of these processes (VIEIRA et al., 2011). Besides this, alterations in the proteome map of skin mucus of gilthead seabream during healing of experimentally produced chronic wounds identified 21 differentially expressed proteins. The altered proteins were involved in immunity and stress as well as in other metabolic and structural proteins which were downregulated in the skin mucus of wounded specimens. Similarly, some transcripts were also under expressed at mRNA levels in wounded fish (CORDERO et al., 2017a). Furthermore, the mucosal glycosylation pattern (by determining the abundance of terminal carbohydrates), some immune parameters in skin mucus and the bacterial microbiota associated to the ulcered

skin has also been studied (TAPIA-PANIAGUA et al., 2018). Significant decreases of terminal abundance of α-D-mannose, α-D-glucose and N-acetyl-galactosamine were observed in skin mucus of ulcered fish, compared to non-ulcered fish. The levels of several enzymes (protease, antiprotease, peroxidase, lysozyme) and total immunoglobulin M in mucus were decreased in ulcered fish (compared to control fish) although the observed decreases were only statistically significant for proteases and antiproteases. Regarding the skin microbiota composition, clear differences between ulcered and non-ulcered areas were observed. The genus taxonomic analysis showed that *Staphylococcus* and *Lactobacillus* were more abundant in non-ulcered skin whereas in ulcered area were *Streptococcus* and *Granulicatella*. In ulcered skin slight increases of sequences related to *Flavobacterium*, *Chryseobacterium* and *Tenacibaculum* genera were encountered (TAPIA-PANIAGUA et al., 2018).

Very recently it has been demonstrated that a skin wound in a specific flank of the fish induced a global response that affected the entire skin surface, not only the wounded area, as it was usually supposed and for that, the most studied site. Samples of the mucus and skin of the two flanks of the fish (one wounded and another intact) were taken separately from each studied fish. The antioxidant defences in the skin mucus were altered while the expression of genes related to stress and regeneration remained upregulated in the skin of the non-wounded flank and non-changed or downregulated in the wounded flank even at 7 days post-wounding (ESPINOSA-RUIZ & ESTEBAN, 2021).

Important changes have also been demonstrated in the serum of wounded fish and the highest activities verified concurred in the inflammatory healing phase. More concretely, immunoglobulin M level, protease, antiprotease, peroxidase and lysozyme) and bactericidal activity (against *Vibrio harveyi* and *Photobacterium damsealae*) showed statistically significant variations depending on the site of the skin wound (above or below the lateral line and more significant variations were observed in fish wounded below) and the time post-wounding (from 0 to 7 days). However, antiproteases or lysozyme remained unaltered (CEBALLOS-FRANCISCO et al., 2018).

More recently, it was demonstrated that skin damage (caused by biopsy punch or scale loss) provokes changes in other mucosal, in the gut. Changes in the morphology of the intestine (enterocyte height and the width of the lamina propria, submucosa

and muscle layer) revealed that scale loss was associated with intestinal inflammation. Curiously, this inflammation was associated with a significant up-regulation of gene transcripts for cell proliferation and anti-inflammatory cytokines while a down-regulation of gene transcripts for the pro-inflammatory cytokines was recorded. Besides this, an up-regulation of a marker of mast cells in the intestine was also detected, which seems to suggest the crosstalk between immune barriers. Skin damage induced an increase in cortisol levels in the anterior intestine which suggest that glucocorticoid levels in fish gut are modified by skin wounds and modulates intestine inflammation (MATEUS et al., 2021). All the changes induced in a fish as a consequence of an experimental wound are summarized in Table 1.

Table 1. Changes which have been demonstrated which are induced in a healthy gilthead seabream specimen as a consequence of a skin experimental wound.

LOCAL RESPONSE	MUCUS	Composition Glycosylation pattern Antioxidant defences Immune parameters
	SKIN	Mucus exudation Histology Microbiota Gene expression
SYSTEMIC RESPONSE	SERUM	Biochemical activities Immune parameters
OTHER ORGANS	GUT	Histology Inflammation Gene expression

Use of dietary additives to improve gilthead seabream skin

The modern aquaculture industry requires easy-to-implement preventive solutions that can help maintain optimal animal welfare, while also thinking about the environment. This would contribute to reducing chemical products and improving production, which would also translate into higher profits for the sector. Of particular interest is to have a good knowledge

of the biology of the farmed fish, and to develop specific feeds known as health-diets or functional foods.

More intense flesh and skin coloration has traditionally been associated with superior and healthy aquatic products. For this fact, farmed fish skin or muscle colour are increasingly important for consumers' acceptance of aquatic food products, mainly for species that are commercialized in their entirety, as is the case of gilthead seabream (COLIHUEQUE & ARANEDA, 2014). The relationship between the dietary carotenoid level and the external skin pigmentation of seabream has been investigated. The highest pigmentation was obtained when seabreams were fed a diet containing microalgae, the medium when fish fed poultry by-product meals and the lower values in fish fed diets containing insect meal (PULCINI et al., 2020).

More recently, a comprehensive study of an interactome based on multiple omics has helped to understand somewhat better the biological response that takes place in seabream skin as a consequence of a dietary stimulus (supplementation with spray-dried porcine plasma). The results suggest that there is a very intimate relationship between the skin tissue and its exudate mucus, which resulted in accelerating the turnover of cellular proteins and activating the mucus exudation process (REYES-LÓPEZ et al., 2021).

Till present, only two feed additives (arginine and the probiotic *Shewanella putrefaciens*) have been probed with the aim of improving the healing process of gilthead seabream skin. In both studies a similar study strategy was followed. Two groups of fish were done: one was fed with control diet (non-supplemented) while the other was fed with control diet enriched with the selected additive. Fish were fed for 30 days and, at that time, half of the fish were sampled while the other half were injured with a biopsy punch and continued being fed the same diet for an extra week, at which time the remaining fish were sampled. Regarding arginine, it was selected because it is an amino acid that has been known to support wound healing in mammals. Diets containing 1 % and 2 % of arginine were tested. Macroscopically, the wound closure rate was significantly improved in fish fed the arginine diet, compared

with those in the control group. Increased activities in serum (aminotransferase, creatine kinase and immunoglobulin M levels) and in skin mucus (protease and peroxidase activities) were down-regulated, but the immunoglobulin M level in the skin mucus was up-regulated in the wounded fish fed arginine, compared with the fish fed non-supplemented diet. By studying the gene expression of notable genes, it was demonstrated that the dietary amino acid facilitated the skin wound healing and alleviating the inflammatory response and enhancing the re-epithelialization and extracellular matrix biosynthesis in skin wound sites (CHEN et al., 2020a). Curiously, the gut histology demonstrated an intense infiltration of leukocytes in the submucosae of the wounded fish, which was remarkably reduced in fish that were fed the 1% arginine diet. Furthermore, the amino acid diet downregulated the gene expression of toll like receptors (TLR), and some proinflammatory cytokines, but significantly enhanced the gene expression of anti-inflammatory cytokines, and tight junction proteins (tricellulin and occludin) in the gut of wounded fish. In summary, dietary administration of 1% arginine significantly inhibited intestinal inflammatory response and tight junction disruption in skin-wounded gilthead seabream by modulating TLR signalling in the intestine. Contrarily, adverse effects were demonstrated in both skin and gut of fish fed 2% arginine (CHEN et al., 2020b).

The effects of *Shewanella putrefaciens* (known as SpPdp11) a probiotic isolated from the skin of healthy gilthead seabream, were also studied because all the beneficial properties demonstrated for this fish species (CÁMARA-RUIZ et al., 2020). For these studies the control diet was enriched with 10^9 cfu g^{-1} SpPdp11 (SP diet). In the skin, results from image analysis of wound areas showed that SP diet accelerated the wound closure, compared with fish from the control group. SP diet increased the protease and peroxidase activities in skin mucus from wounded fish fed SP diet. Additionally, SP diet up-regulated the gene expression of antioxidant enzymes, anti-inflammatory cytokines, and re-epithelialization related genes in the fish skin. Besides this, significant decreases in pro-inflammatory cytokines expression were observed in fish from SP group, respect to control ones. Inclusion of SpPdp11 in diet

facilitated the wound healing and the re-epithelialization of the injured skin. It provoked a decrease in the inflammatory response in the wound area because an intensification of the antioxidant system was demonstrated together with a synthesis of matrix proteins and neo-vascularization in the skin wound sites of fish (CHEN et al., 2020c).

The results on gut demonstrated that wounded fish had a disordered enterocyte nucleus disposition, a more intense infiltration of leukocytes and a thicker lamina propria in the gut compared to non-wounded fish. However, the fish fed the SP diet did not present these pathological symptoms in the gut. No significant variations in the number of goblet cells were detected among the different experimental groups. The expression of pro-inflammatory cytokines, mucins, and immunoglobulin T heavy chain (IGHT) was up-regulated, while tight junction protein occludin was down-regulated in the gut from fish wounded and fed the control diet. However, wounded fish fed the SP diet showed an increased gene expression of anti-inflammatory cytokine transforming growth factor-ß1 and the tight junction proteins tricellulin and occluding and a strongly depressed gene expression of pro-inflammatory cytokines, MUC2. In brief, the skin wounds provoked an intestinal inflammatory response that included changes in the mucus layer and tight junction disruptions and administration of SpPdp11 alleviated these intestinal dysfunctions caused by skin wounds in gilthead seabream (CHEN et al., 2020d).

Conclusions and future remarks

The presented results demonstrate the importance of the skin integrity in the maintenance of fish body homeostasis. All these changes evidenced in the fish body as a result of a wound, evidence that a simple wound is capable of producing notable alterations throughout the entire animal's body, even in a healthy fish, underline the maintenance of a healthy skin as a guarantee of healthy animals. Fish have a very good and fast skin healing process which can be improved if adequate nutrients are provided to the fish. The concentrations and period of administration have to be studied for each nutrient and fish species.

At present fish skin is also investigated as an origin of molecules with commercial interest. For example, skin was the most significant protein source of different selected gilthead seabream by-products (head, gills, guts, trimmings, bones, skin) (KANDYLIARI et al., 2020). New researches will possibly know much more about such proteins although some of them started to be studied. For example, collagen is extensively used as a biomedical material, carrier for controlled drug release, as a nutraceutical supplement, also an active ingredient in cosmeceuticals and food additive. Collagen has been obtained from discarded gilthead seabream skin and a 3D wound dressing was prepared. The results demonstrated that this material could be used as novel efficient material for skin repair due to its healing properties (GASPAR-PINTILIESCU et al., 2021). All these results make very interesting to explore new marine bioresources in order to make a valorisation of many products which could be obtained from farmed fish and, at the same time, to contribute to the advance in many other disciplines such as veterinary or human health, among others.

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SANITIZATION TO PREVENT THE FUNGAL SPOILAGE OF PROCESSED FOOD AND CONSUMER EXPOSURE TO MYCOTOXINS

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ABSTRACT

Problems of fungal spoilage occurs in the food industry sector around the world. Due to their intrinsic parameters, some foods like bread, cheese and cured meat product are widely affected. Both raw materials and processing environment, especially the air, are the main source of fungal spores. After the product contamination, the ability of the species reaching the fresh produced food to overcome the technological barriers imposed by industries (i.e., preservatives, reduced water activity, low pH, oxygen restriction) will determine spoilage. Through the adoption of hygiene procedures, with emphasis in the selection of sanitizers with antifungal activity, the load of fungal spores in the production environment could be reduced, and consequently also the spores reaching the food. The prevention of fungal spoilage also can impact the consumer exposition to mycotoxins.

KEYWORDS

Sanitizer; Bread; Cured meat; Cheese; Mycotoxin.

Introduction

Filamentous fungi are ubiquitous in nature, and some have the capability to produce mycotoxins when infecting agricultural crops. Besides, some species can contaminate products at post-harvest and storage, and even after food processing, starting food spoilage when growing and presenting a real possibility to secrete mycotoxins during their metabolism (PITT & HOCKING, 2009). In a certain extent, both fungi and mycotoxins can be considered natural and unavoidable.

Usually investigations are concerned in pre-harvest, since as soon as the fungi starts infecting the crops, mycotoxins can be produced and will remain in the product throughout the production, storage, and supply chain. The post-harvest period, especially from harvest to storage, is also critical for mycotoxin producing species growth and release of toxic compounds in the raw material substrates. Studies focused in these moments have a high relevance, since even the fungi are inactivated in further processing steps, the mycotoxins can be spread to many different foods when using a contaminated raw material. However, the spoilage problems by fungi after processing are also a worldwide issue (HOCKING, 2014), leading to food waste and losses up to 5-10% in some food industry segments and should not be neglected.

Considering the one health concept, food is a key point, because it will be the source of human exposition to these toxic compounds once contaminated food become part of animal and human diet. So, different competencies are needed to prevent and control these contaminants along the food chain (VENÂNCIO & PATERSON, 2007). The problematic involved with the spoilage of some processed foods will be accessed, and intervention strategies to prevent and control the fungal growth and food spoilage with the focus in the hygiene of producing environment through the use of effective sanitizers will be discussed.

Problems in bread industries

Bread and bakery products are one of the most common food consumed around the world, and due to their characteristics (water activity 0.95 – 0.98, pH 5.5 – 6.0 and high carbohydrate

content), molds are the main agents making them not adequate to consumption due to spoilage (LEGAN, 1993; SARANRAJ & SIVASAKTHIVELAN, 2015).

In Brazil, bread spoilage is the most common cause of complaints by consumers due to the presence of fungi (LEMOS et al., 2018). The occurrence of moldy breads is a relevant economic problem and this scenario can be worst in tropical warm and high moisture countries (FREIRE, 2011). Moreover, mycotoxins can be produced during the metabolism of some species and released into the food, leading to a risk of consumer exposition to these toxic compounds. A study demonstrated that the diffusion of citrinin was restricted to the first slice of bread, but aflatoxins and ochratoxin A were able to contaminate up to 5 consecutive slices (REIß, 1981). This is a cause to concern because consumers are used to remove the moldy part (or slice), consuming the remaining bread [Unpublished], which presents dubious quality.

Sources of contamination

The species responsible by bread and other bakery products spoilage usually originates from cereals and grains used to produce the flours. These raw materials are commonly infected by fungi, which can occur both in the field or during the storage. A diversity of fungal species contaminates the different flours (SANTOS et al., 2016; GARCIA et al., 2019a) and can easily reach the bread processing area, where usually the problem starts.

During the bread manufacture a high load of fungal spores derived from cereal and grains contaminate the dough and are also released in the air of processing area as aerosols, attached to flours. The concomitant presence of bread spoilage species in the raw materials and facilities air in industries were reported in bread industries having spoilage troubles (SANTOS et al., 2016; GARCIA et al., 2019a).

The time and temperature usually adopted during the baking step have been demonstrated as enough to inactivate spores of the main species related do bread spoilage present in the dough (GARCIA et al., 2019b). Nevertheless, levels above 10^3 UFC/m^3 had been reported in the air of bakeries and bread industries (GARCIA et al., 2019b; MAGAN et al., 2003), mostly released during the ingredient mixing.

Eventually, fungal particles dispersed in the air will gradually sediment by gravity, reaching both the equipment surface and the surface of freshly produced breads while they are cooling, slicing or packed (GARCIA et al., 2019d).

Even many species can reach the processing environment through raw material, those species tolerant to preservatives are more capable to overcome the barriers and spoil the product.

Spoilage agents

Preservatives, mostly organic acids and theirs salts, are hugely used by bread industries to restrict the growth of most fungi. For this reason, only few fungal species more tolerant to this kind of preservatives are responsible for the spoilage of baked goods (MAGAN et al., 2003; SUHR & NIELSEN, 2004; GARCIA et al., 2021; MORO et al., submitted).

The *Penicillium* spp. most related to bread spoilage are *Penicillium roqueforti*, *Penicillium paneum*, *Penicillium brevicompactum*, and *Penicillium chrysogenum*, the first two species with known preservative resistance (Garcia et al., 2021; Moro et al., submitted). This genus usually produces greenish-blue colonies in the products surface (GARCIA et al., 2019d).

Another common defect seen in breads is the 'chalk mold', which occurs due the growth of the white yeast-like *Hyphopichia burtonii* or *Saccharomycopsis fibuligera* on the product surface (LEGAN, 1993; MAGAN et al., 2003; GARCIA et al., 2019d), releasing a typical off-flavor which is perceived even before the observation of colonies. The *H. burtonii* is also tolerant to propionic acid, commonly used as bread preservative (GARCIA et al., 2021; MORO et al., submitted).

Xerophilic *Aspergillus* spp., mainly *Aspergillus chevalieri* and *Aspergillus pseudoglaucus*, are also commonly present in spoiled breads and panettones, forming a bluish or yellowish layer on the product's surface (LEGAN, 1993; MAGAN et al., 2003; GARCIA et al., 2019d).

The heat resistant *Paecilomyces variotii* can also spoil certain type of bread, forming dark yellow-brown colonies and *Chrysonilia sitophila* produces red spots (LEGAN, 1993; MAGAN et al., 2003; GARCIA et al., 2019d).

Rhizopus spp., *Mucor* spp., *Cladosporium* spp. and black *Aspergillus* can also be observed forming dark colonies in breads without use of preservatives (LEGAN, 1993; HOCKING, 2014).

Hygiene control measures

Clearly, the quality of raw material (as source of contaminating spores) and the hygiene of the production environment are determinant for the occurrence of early spoilage problems in bread industries.

Successful control will be achieved if the contact of spores with freshly produced bread is prevented or minimized. The design of facilities, segregating the areas of ingredient mixing from the one where breads stay for cooling is of extreme relevance. The time of colling is also critical, since as longer the breads stay there, higher is the chance of deposition/sedimentation of spores dispersed in the air.

Adoption of hygienic-sanitary measures is very important for reducing the fungal load in the bread processing environment, and the selection of sanitizers effective against the species will be a determinant of the success. Peracetic acid was demonstrated as the most effective agent, followed by benzalkonium chloride against bakery spoilage fungi (*Penicillium roqueforti*, *Penicillium paneum*, *Hyphopichia burtonii*, and *Aspergillus pseudoglaucus*), while sodium hypochlorite was only effective in very high concentrations (>5000ppm) (BERNARDI et al., 2019a). Furthermore, a short contact time and the presence of organic load can reduce the sanitizer efficacy, as well as the temperature influences in the agent activity, with higher temperatures increasing the efficacy of peracetic acid and lower temperatures rising the benzalkonium chloride effectivity (STEFANELLO et al., 2021).

Problems in meat industries

Fermented meat products are widely consumed around the world and the presence of typical molds on the surface of meat products can be considered normal and even a quality factor if the developing species are not able to produce mycotoxins or antibiotics (PERRONE et al., 2019). The technological action of fungi occurs mainly due to the secretion of fungal enzymes which could improve sensorial characteristics of a cured product by impressing peculiar flavors (ASEFA et al., 2010). Though, sometimes undesired toxin producing species can also develop on the product surface, representing a cause

of concern due to the risk of consumer exposure to these deleterious compounds (PARUSSOLO et al., 2019a; PERRONE et al., 2019).

Sources of contamination

The ability to grow in acidic pH and tolerance to low pH and high salt concentration of cured meat products (PITT & HOCKING, 2009; PERRONE et al., 2019), favors the development of filamentous fungi despite other microbial groups. The load and diversity of species present in the raw materials and in the environment where the production occur will influence the product mycobiota (BATTILANI et al., 2007; SØRENSEN et al., 2008; PARUSSOLO et al., 2019b).

Ochratoxin A producing species are among the most undesired fungi occurring in cured meat products. Both fungi and mycotoxin presence in these products is attributed to contamination coming from raw materials, mainly spices as well as the air of the maturation chamber, where commonly lots of different ages are ripening together (PARUSSOLO et al., 2019b; ALMEIDA et al., 2021, *submitted*; BATTILANI et al., 2007; ASEFA et al., 2010; SCARAMUZZA et al., 2015). A study carried out by ALMEIDA et al. (2021, *submitted*) revealed an increase of 2 log in the fungal population when comparing cured sheep ham with spices when compared to the ones manufactured without the addition of this raw material.

Spoilage agents

In general, in temperate climates like in Europe, the *Penicillium* genus is the responsible for most spoilage episodes in cured meat, highlighting *Penicillium verrucosum* and *Penicillium nordicum* as the main ochratoxin A-producing species occurring in these products (Perrone et al., 2019; PITT & HOCKING, 2009). However, species of *Aspergillus* from Section *Circumdati* (mainly *A. westerdijkiae* and *A. ochraceus*), have been described in cured meat products both in South America and Mediterranean countries (PARUSSOLO et al., 2019; PERRONE et al., 2019).

Hygiene control measures

As previously mentioned, the hygiene of the producing and maturation environment, including air, and work surfaces is crucial for controlling the problem. Furthermore, controlling

the quality of the spices used for manufacture will reduce the contamination load in the products of surfaces and will impact the microbial load of freshly prepared meat products.

Is important to highlight a study carried out by Bernardi et al. (2021), which revealed a high resistance of fungal strains isolated from cured meat products to the sanitizers used in food industries, even in the higher dosage recommended. This study reported that the lowest concentration specified in the product label was the closest to the adopted in the hygiene routine of meat industries and it was never effective against the spoilage species tested. Even with limited efficacy, peracetic acid was the most effective sanitizer among the tested.

According to Stefanello et al. (2021), to improve the sanitizer action is very important to proceed with an effective cleaning step before the sanitization procedure, since the presence of organic load reduced the efficacy of most sanitizers. Still, the contact time of sanitizer is also relevant and a minimum of 15 minutes of product action should be respected. Regarding the temperature of the process, peracetic acid was more effective in higher temperatures (40°C), while the best action of benzalkonium chloride tends to occur in lower temperatures (10°C). These observations were corroborated by Silva et al. (2021, *submitted*) when evaluating *Penicillium verrucosum*, *Penicillium nordicum* and *A. westerdijkiae* the most relevant species spoiling cured meat products due their ability to produce ochratoxin A. This author observed a higher sensitivity of the *Penicillium* species studied when compared to the *Aspergillus* one.

Problems in the cheese industry

Cheese spoilage by fungi is an important issue for the dairy industry cheeses because of the intrinsic characteristics of this product, such as acidic pH (4.3-5.0), intermediate water activity, and high lipidic content (HOCKING & FAEDO, 1992; PITT & HOCKING, 2009). In Brazil, cheese spoilage by fungi is among the three most frequent consumers complains about spoiled food by fungi (LEMOS et al., 2018).

The development of fungi reduces the product quality both due to invisible defects, such as production of off-flavors; as well as visual defects, such as the perception of the fungal

colony (KURE & SKAR, 2019). Some of the molds growing on cheese may also produce mycotoxins, which lead to a food safety issue.

Sources of contamination

The molds related to the spoilage of dairy products can originate at different stages, from dairy farms to dairy processing units and at consumers' homes. Since the mycobiota of raw milk is dominated by yeast, and this group of micro-organisms together with the molds are usually inactivated by pasteurization, the product contamination occurs after the milk heat treatment (GARNIER et al., 2017). Therefore, contamination with fungal spores typically originate from the production environment, which includes the air, work surfaces, equipment, food handlers, and ingredients (BARRIOS et al., 1998; LEDENBACH & MARSHALL, 2009; JAHN et al., 2017; KURE et al., 2004; VACHEYROU et al., 2011).

According to Kure & Skar (2019) the air in the production plant represents one of the major contamination sources. Air borne spores can contaminate the curd in the vessels and vats. After settling in the surface of the cheese, mold spores can grow on the cheese later in the process if conditions are suitable. Also, the cheese will normally be immersed in brine for a certain period, and the brine can be a reservoir of fungi.

In some dairies moldy cheeses are 'cleaned' for surface molds (BARRIOS et al., 1998; JAHN et al., 2017), which allows to spread that problem fungus in the air, contaminating the fresh produced cheeses through the air and the smear.

As the ordinary semi-hard cheese is maintained under refrigeration, the species related to their spoilage have a psychrotolerant behavior, and usually tolerates low oxygen tension, since vacuum is a technology commonly applied aiming to extend this product shelf life (KURE & SKAR, 2019; HOCKING & FAEDO, 1992). Due to the high fat content of this product, spoilage agents also tend to present high lipolytic ability.

Spoilage agents

Fungi responsible for problems in cheese production are varied, and *Aspergillus*, *Candid*a, *Cladosporium*, *Mucor*, *Penicillium*, *Geotrichum* and *Trichoderma* are frequently mentioned (LEDENBACH & MARSHALL, 2009; PITT &

HOCKING, 2009; JAHN, GARCIA & COPETTI, 2017; GARNIER et al., 2017). However, by far the genus most frequently isolated from spoiled cheese is *Penicillium* (responding for about 90% of cases around the world) followed by *Aspergillus* (KURE & SKAR, 2019). *Penicillium commune* is particularly well adapted to cheese and responsible for most spoilage episodes. Xerophilic *Aspergillus* (mostly from *Eurotium morph*) are commonly causing defects in the rind of matured hard cheeses.

Hygiene control measures

As extensively mentioned above, among the most effective ways to reduce the spoilage problem in cheeses is preventing the fungal spores to reach the manufactured product through an adequate sanitization of the production environment. This measure will reduce the number of spores contaminating the product, increasing their shelf life, and reducing losses due to early spoilage.

Is important to highlight that the fungal strains originated from culture collections were more sensitive to sanitizers than the strains from the same species but isolated from spoiled cheeses, what could be related to a selective pressure due to the intensive usage of sanitizers in the food industry sector (BERNARDI et al., 2021). Furthermore, only very high concentrations of benzalkonium chloride, peracetic acid and sodium hypochlorite where effective against the strains isolated from spoiled cheese, usually above the concentration applied by the dairy industries (BERNARDI et al., 2021).

Conclusion

Since fungal growth can also occur in processed food, is important to consider all the food chain and not only the pre- and post-harvest steps when aiming to reduce losses and prevent the consumer exposure to mycotoxins. The fungal control in processed foods depends on understanding the problematics of each food industry, adopting hygiene measures, and choosing sanitizers with good antifungal action against the species causing trouble.

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IMPACTS OF CLIMATE CHANGE ON MYCOTOXIN CONTAMINATION OF FOOD COMMODITIES BY MOULDS IN TROPICAL/ DEVELOPING COUNTRY SCENARIOS

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ABSTRACT

Climate change abiotic factors are implicated in significant impacts on food production chains and thus food security/safety on a global basis. Parts of Asia, Africa and Latin America are considered hot spots for such impacts via extreme weather events. Elevated CO_2, temperature and wet/dry fluxes are changing the pre-harvest fungal diversity in staple crops such as rice and maize influencing the microbiome and contamination with mycotoxigenic fungi and mycotoxins. This has reduced yields and nutritional quality and exacerbates toxin contamination during drying and storage. Mycotoxigenic fungi have been shown to be very resilient to climate change factors and contamination with aflatoxin B1 in maize and nuts and ochratoxin A in coffee has occurred. Temperature/drought stress reduced nutritional value and increased contamination with aflatoxins and fumonisins in rice and maize. It is critical that minimisation strategies, including biocontrol, have the resilience to reduce risks to consumers.

KEYWORDS

Climate Change; Food Safety; Spoilage; Mycotoxins; Resilience.

Introduction

Climate change scenarios are now very high on the agenda in terms of both policy makers at a governmental level as well as food safety authorities. This is because it has become clear that climate change affects the sustainability of food and feed production chains and thus will have profound impacts on food security and food safety for consumers in the next few decades. There has been particular interest in how climate change abiotic factors of increased atmospheric CO_2 (2-3X the existing value of approx. 400 ppm), associated with an increase in temperature (+1.5-3°C) and extreme fluxes in wet/dry episodes on a regional and global basis will impact of food security and safety. Food raw commodities are traded on a global basis, and the impacts that climate-related abiotic factors may have on staple food production systems, including cereals, fruit, nuts, coffee, cocoa and feed ingredients needs to be quantified. They are often transported over long distances to markets through different climatic zones and can re-absorb moisture resulting on initiation of spoilage and potential for mycotoxin contamination (MEDINA et al., 2017; WU et al. 2011; BOTANA and SAINZ, 2015). Indeed, it has been shown that post-harvest losses alone are consistently in the range of 20-25% due to spoilage moulds and mycotoxins and would probably increase under climate change scenarios (ESKOLA et al., 2020).

Although sometimes bioactive peptides may already be present in the food as part of its composition like carnosine and anserine, they can be generated through different mechanisms: (i) peptides generated during the gastrointestinal digestion mainly due to the action of gastrointestinal enzymes such as pepsin, trypsin, and chymotrypsin, (ii) peptides generated through controlled hydrolysis conditions using commercial enzymes in reactors or fermenters, and (iii) peptides generated during the processing of food such as during fermentation treatments, curing, or ageing processes (MORA et al., 2016, p.395).

The quality and yields of staple foods are influenced both by climate change factors as well as the diversity of pests, diseases and presence of weeds. The relative proportion of the impacts that these groups have of food/feed raw commodities is shown in Figure 1.

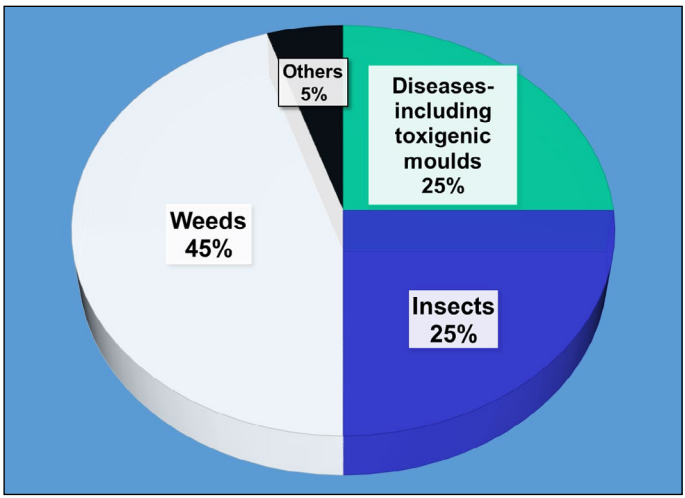

Figure 1. The relative contribution of different biotic components to food quality and safety issues.

Indeed, studies of the impact of climate change-related abiotic factors including predictive modelling have shown that pests and diseases may be moving towards the poles at about 1-2 km per annum. In addition, the diversity of the pests and diseases is changing because of climate change abiotic factors (BEBBER et al., 2013, 2014, 2015). This is having significant impacts on relative pest damage and pathogenic infections as well as contamination with mycotoxins. There are global hotspots for impacts of climate change scenarios and these include a significant number of lower middle income countries (LMICs) and emerging economies in Africa, Asia, and South America as well as the Mediterranean region of Europe (BATTILANI et al., 2016).

Mycotoxins are important naturally occurring toxic secondary metabolites in a range of raw staple commodities which are heat stable and thus once present difficult to destroy even during processing and cooking. They are predominantly produced by species in the *Aspergillus*, *Penicillium*, *Fusarium* genera. Contamination with these toxins can occur at any time: in the field pre-harvest, harvesting, drying, transport and storage, milling and in finished products. The potential for contamination depends on how efficient these processes are carried out to minimise contamination (MAGAN et al., 2020a). They are of particular concern as studies have shown that aflatoxin B1 (a class 1a carcinogen) and other toxins (e.g. ochratoxin A, trichothecenes, fumonisins) in staple commodities such as

maize and peanuts are responsible for stunting in infants and young children in LMICs, especially Sub-Saharan Africa (IARC, 2016).

The ecology of the key genera involved is valuable in appreciating whether there is a high or low risk of contamination with specific mycotoxins or mixtures of mycotoxins. Figure 2 shows the diagrammatic relationship between temperature and water availability for these three key genera. The marginal conditions at 0.70 water activity (a_w) are critical in appreciating the relative level of risk of toxin contamination under existing and future climate-related abiotic factors (PERRONE et al., 2020). The safe conditions of <0.70 aw are directly related to moisture contents of different commodities. These are: wheat 14.5%; maize 15%, rice 12-15%; coffee beans 12-13%; Soya 9-13%; tree and ground nuts 4-8%.

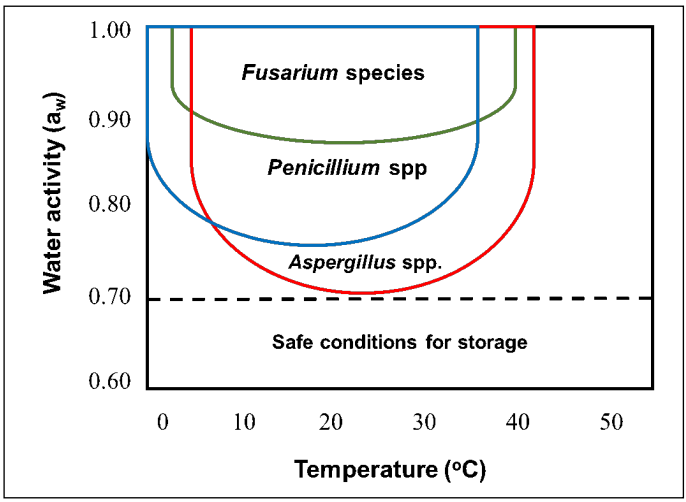

Figure 2. Diagrammatic profiles of the three key genera of fungi responsible for mycotoxin contamination of different staple commodities.

Relationship between mycotoxin contamination and dry matter losses under different abiotic interacting conditions

It should be noted that different commodities have an inherent calorific value. Thus, rice, wheat and maize have values of 5.4 kJ/g, 15.1 kJ/g and 14.2 kJ/g respectively. Thus, infection by mycotoxigenic fungi can not only result in contamination with toxins but also result in significant nutritional losses. Thus, under climate change conditions, this may be exacerbated, resulting in multi-mycotoxin contamination as well as having nutritional

impacts on commodities destined for food and feed use. Figure 3 shows the relative contamination of paddy and brown rice with fumonisin B1 under different temperature x water activity (a_w) storage conditions when inoculated with *Fusarium verticillioides*. This also shows that the calculated dry matter losses can be very small before exceeding legislative limits in place for other cereals such as maize. The levels of dry matter losses occurring represent 500-3000 kJ/kg. Similar results were also obtained with aflatoxin B1 contamination of rice (MARTIN CASTAÑO et al., 2017a). Very small changes in dry matter losses can result in exceeding the legislative limits for raw commodities such as maize, wheat and nuts (MARTIN CASTAÑO et al., 2017b; MYLONA et al., 2012, 2021; GARCIA-CELA et al., 2019, 2020).

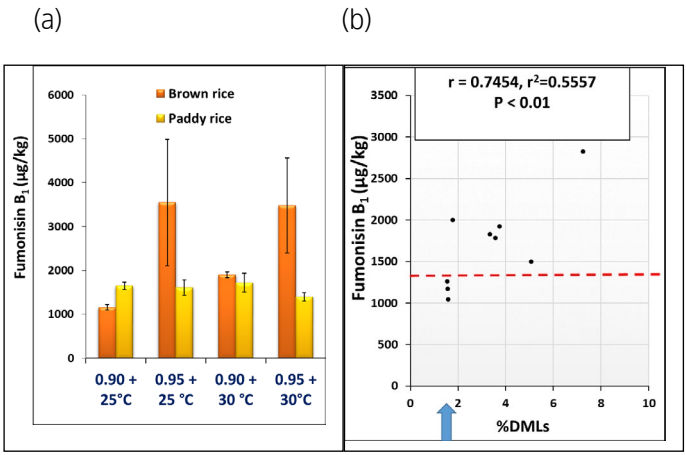

Figure 3. (a) Relationship between storage temperature and water availability on fumonisin B_1 contamination of paddy and brown rice. (b) Relationship between dry matter losses and fumonisin B_1. The dotted line indicates the EU legislative limits for this mycotoxin in cereals; arrow indicates maximum dry matter loss before exceeding limit (adapted from Martin Castaño et al., 2017b).

Climate change abiotic factors and impacts on mycotoxin contamination of cereals

We have carried out extensive studies to understand the relative resilience of *Aspergillus flavus* and aflatoxin B_1 contamination of maize. These have included studies on maize-based media and on maize grain under existing and future climate-related conditions (30 vs 37°C, 350 vs 650/1000 ppm CO_2, wet/dry conditions). For example, studies with maize cobs of different ripening stages (milk, dough and dent) showed that *A. flavus* could rapidly colonise the cobs and produce high AFB_1 contamination at both 30 and 37°C, especially at the dough and dent stages when the aw levels of the kernels were 0.976

and 0.958 respectively (GASPERINI et al., 2019). The resilience of *A. flavus* strains to elevated temperatures and a range of a_w conditions were also confirmed with significant DMLs at >0.90 aw over a range of temperatures from 20-35°C. This represented losses in nutritional value of up to 2,325 kJ/kg.

Subsequent studies examined the effect of exposure to three-way interacting climate-related abiotic factors when *A. flavus* was colonising maize grain. This showed that colonisation of maize grain at elevated temperatures and CO_2 conditions was very similar. However, there were significant impacts on AFB_1 contamination (MEDINA et al., 2017; GILBERT et al., 2018). Table 1 summarises the effect of climate-related abiotic factors on both a regulatory gene (*aflR*) involved in the biosynthesis of aflatoxins and the relative stimulation of AFB_1 contamination of maize grain.

Table 1. Summary of the effect of interacting climate-related abiotic factors on both a regulatory gene (*aflR*) and aflatoxin B1 contamination of stored maize grain under different wet/water stress conditions (adapted from GILBERT et al., 2018).

Temp (°C)	a_w	CO_2 (ppm)	*aflR*	AFB_1
30	0.99	650	=	=
		1000	=	=
	0.91	650	(x2)	(x2.7)
		1000	=	=
37	0.99	650	(x2)	(x3.6)
		1000	(x3)	(x5.0)
	0.91	650	(x2)	(x1.8)
		1000	=	(x1.5)

Subsequent studies used molecular approaches to understand the kinetics of growth, and of metabolomics of *A. flavus* when colonising maize grains when exposed to three-way interacting climate-related abiotic factors, both *in vitro* and *in situ* (GILBERT et al., 2018; GARCIA-CELA et al., 2020). These showed that CO_2 exposure affected secondary metabolite clusters including aflatoxins, cyclopiazonic acid

(CPA) and other secondary metabolites. Exposure to elevated CO_2 (400 vs 1000 ppm) also affected the molecular responses to temperature (30 vs 37°C) and water stress (0.91 vs 0.99 a_w). There were also interaction effects of the 3 variables which had a significant effect on cell division/DNA replication. In addition, oxidative stress responses, sugar transporters/stress related gene expression were all upregulated (GILBERT et al., 2018). This certainly suggests that *A. flavus* is very resilient when exposed to these three-way interacting abiotic climate-related factors when colonising maize.

Studies by Vaughan et al. (2014) examined the exposure of maize to *Fusarium verticillioides* and examined effects on both infection and on fumonisin B_1 production. They found that while there was an increase in *F. verticillioides* fungal biomass in ripening maize there was less effect on FB1 contamination. Subsequent studies which included 400 vs 800 ppm CO_2 and drought stress treatments were carried out. These showed that *F. verticillioides* fungal biomass significantly increased as well as levels of FB_1 in ripening maize cobs when exposed to interacting climate-related abiotic factors (VAUGHAN et al., 2017).

More recently studies were carried out on oats which are susceptible to *Fusarium langsethiae* and contamination with T-2/HT-2 toxins (type A trichothecenes). These studies showed that under interacting conditions of elevated CO_2 (1000 ppm), elevated temperature (30°C) and intermediate drought stress there was an increase in expression of biosynthetic genes including the *Tri5* and *Tri6* genes and a stimulation of T-2/HT-2 contamination in stored oats (VERHEECKE-VAESSEN et al., 2019).

Very few studies have been conducted to examine the impact that acclimatization of mycotoxigenic strains may have on resilience and on relative pathogenicity and toxin production. Studies by VARY et al. (2015) showed that *Fusarium graminearum*, when cultured for 10 generations in elevated CO_2, the pathogen became more pathogenic when infecting ripening wheat ears based on both symptoms and on fungal biomass of the pathogen using q-PCR. Studies of *A. flavus* strains from pistachio nuts cultured for 5 generations in elevated CO_2 (1000 ppm) were found to grow similarly in existing and climate change abiotic factors although the mycotoxin production was sometimes stimulated (MEDINA

et al., 2017). More knowledge is needed of the potential for increased resilience and toxin production when acclimatised over a number of generations which simulates what may happen in the future in staple crops and which may affect staple food security and safety.

Coffee and contamination with ochratoxin A under existing and future climate related scenarios

Coffee is an economically important crops in many regions of the world where climatic conditions are suitable including South and Central America, South East Asia (e.g. India, Vietnam, Indonesia) and East Africa. Ripe coffee cherries are harvested and fermented prior to drying and subsequent roasting. Poor hygiene and uneven drying during these phases can lead to colonisation by *Aspergillus* section *Circumdati* species (e.g. *Aspergillus westerdijkiae*, *A. steynii* and *A. ochraceus*) and *Aspergillus* section *Nigri* species (*A. carbonarius*). These all can produce ochratoxin A under conducive temperature and water availability conditions (ABDEL-HADI et al., 2011; Akbar et al., 2016). Recent studies have suggested that ecologically the growth and ochratoxin A production by strains of *A. westerdijkiae* on green coffee or roasted coffee –based media is slightly different. Overall, the colonization of roasted coffee-based media was slower than that of green coffee-based media (AKBAR et al., 2020a). Optimum growth was predominantly at 25°C and 0.98 a_w for four different strains on both nutritional media. Optimum colonization was over the aw range 0.95–0.99 on both media. However, growth was very slow at 0.90 a_w when compared to the other a_w levels examined. Indeed, the type strain (CBS 121986) was unable to grow at 35°C and 0.90 a_w.

Subsequent studies of the impact of climate change interacting factors suggest that while growth is relatively unaffected by such interacting abiotic factors (30 vs 35°C; 400 vs 1000 ppm CO_2; drought stress) there were effects on ochratoxin A production, especially by strains of *A. westerdijkiae* (AKBAR et al., 2020a) although this was not found for strains of A. carbonarius (AKBAR et al., 2016). These studies were carried out on both coffee-based media and on stored green coffee, inoculated with spore inoculum of this species. Table 2 shows

the effect of exposure of green coffee beans to climate change abiotic factors on ochratoxin A contamination when inoculated with *A. westerdijkiae* and stored under different temperature x a_w x CO_2 concentrations.

Table 2. Effect of interacting climate-related factors of water activity (a_w), temperature (30 vs. 35°C) and CO_2 (400 vs. 1000 ppm) on the mean ochratoxin A contamination (ng g^{-1} ± SE) of green coffee beans stored for 10 days after inoculation with each of the strains (from AKBAR et al., 2020).

Temperature (°C)		**30**		**35**	
CO_2 (ppm)		**400**	**1000**	**400**	**1000**
Strains	**A_w**				
A. westerdijkiae (strain B2)	0.97	3977±603	2760±53	176±0.2	14±4
	0.95	4243±571	4767±372	1298±32	63±21
	0.90	1644±545	4599±426	8±0.2	680±187
A. westerdijkiae (strain CBS 121986)	0.97	2681±347	3396±199	8±0.4	13±0.9
	0.95	2842±325	30889±225	8±0.6	51±29
	0.90	2679±391	3974±1022	7±0.7	70±21

The available information certainly suggests that there are differential effects of climate change abiotic factors on different species of mycotoxigenic fungi colonising coffee beans especially during the drying process (AKBAR et al., 2016; 2020). Thus, it is possible that there are differential effects of such interacting abiotic factors on mycotoxigenic species and this may also perhaps affect the ratio of related toxins produced by specific species. This needs to be taken into account when examining the relative risks associated with specific commodities (MAGAN et al., 2011; PERRONE et al., 2020).

Minimisation strategies in the context of climate change

Minimisation strategies for mycotoxin contamination are critical for the global trade in staple foods. Chemical, physical and biological approaches have been used to try and address mycotoxin contamination in staple food chains (MAGAN et al., 2020a; WU and KHLANGWISET, 2010). The rejection of batches

of food commodities from LMICs and emerging countries exported to developed regions results in significant economic impacts (WU et al., 2010). Indeed, some have argued that the legislative limits in Europe, for example, are too stringent and rejection leads to economic hardship in many LMICs. However, significant progress is being made to develop appropriate pre- and post-harvest technologies for reducing mycotoxin contamination of such staple food and feed commodities. Thus, solar drying systems (both passive and active) are now being utilised more effectively for drying commodities prior to storage. The use of different innovative bag systems with different layers are more widely being applied for conservation of quality and preventing ingress by pests and the activity of spoilage and mycotoxigenic moulds (MAGAN et al., 2020a).

The use of exposure of cereals, nuts and coffee and cocoa to gaseous ozone has attracted significant interest in the last decade. However, because of the resilience of many mycotoxigenic fungi to such gaseous exposure, the concentrations necessary are often high (AKBAR et al., 2020b; SULTAN and MAGAN, 2021). Ozone has been used to treat cereals in silos and also to control mycotoxigenic fungi in different tree nuts and groundnuts. However, this is not a very user-friendly treatment and care is needed when very high concentrations are necessary for achieving the level of control necessary. The concentration x time of exposure x moisture content of the commodity needs to be optimised. It is also a very corrosive gas which required the use of stainless steel or non-corrosive infrastructure for use. In addition, high concentrations of ozone can lead to tainting of lipid rich commodities, such as nuts, and thus flavour/taste can become significantly altered.

Biological control, especially of aflatoxins in maize and groundnuts have attracted significant attention and been very successful. This has mainly been via the use of non-toxigenic strains of *A. flavus* which do have some or all of the biosynthetic genes involved in mycotoxin production deleted (BANDYOPADHYAY et al., 2016; KAGOT et al., 2019; MAGAN et al., 2020b). Recent studies have shown that the efficacy of non-toxigenic strains for control of aflatoxin B_1 in both non-GM and GM maize cultivars may be reduced under climate change conditions (GASPERINI et al., 2019). Indeed, it has also been shown that entomogenous fungi used for pest control are less effective under climate change-related abiotic conditions (BORISADE and MAGAN, 2015). While

several commercial products are now available on the market, their resilience under climate-related abiotic factors have often not been tested. It is critical that resilient strains of biocontrol agents for control of both mycotoxigenic pathogens as well as for other fungal pathogens and pests are identified and used (MAGAN et al., 2020b; TUT et al., 2021). Improved formulations of such resilient strains would have a competitive advantage under climatic change abiotic conditions and either conserve or improve levels of control achievable in the future.

Conclusions and future perspectives

Under climate change-related abiotic factors plant diseases/pest reproduction and interaction with plants may increase mycotoxin contamination. In addition, significant impacts on the nutritional value of food/feed crops and on concomitant mycotoxin contamination is occurring, often with multiple mycotoxins being found. This suggests that the ratios of different toxic secondary metabolites may change under such climate-related factors. Indeed, new knowledge is being gained on the relative contamination with free and bound mycotoxins (so called masked mycotoxins) being common in many staple commodities. Thus, bound mycotoxins to plant components have been reported for deoxynivalenol, fumonisins, ochratoxin A and T-2/HT-2 toxins. There is little knowledge of whether the ratio of such mycotoxin form is changing. The relative increase in the resilience of mycotoxigenic fungi, which appear to be evolving tolerance to climate change abiotic factors could pose significant threats to the development of appropriate sustainable control/minimisation strategies. For example, will more resilient biocontrol candidates need to be screened for efficacy and different formulations be necessary for conserving control of mycotoxin contamination. Could, in the next 1-2 decades this result in existing legislation being out of step with the problems which will arise necessitating different food safety approaches and legislation. It is timely that we consider some of these issues now so that, in the context of global food chains which exist, we can ensure that food security and more importantly food safety can be conserved. This is important for consumer confidence in the staple food chains which we all rely on.

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SARS-COV-2 ENZYMES AS A DRUG TARGET: *IN SILICO* STRATEGIES FOR DRUG REPURPOSING AND DRUG RE-DESIGN

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ABSTRACT

The COVID-19 disease, caused by the SARS-CoV-2 coronavirus, has become a pandemic health emergency. The proteases from SARS-CoV-2, the main protease (Mpro) and papain-like protease (PLpro), are attractive targets for antiviral development due to their essential role in the viral life cycle. Here, we will discuss some structural and functional aspects of PLpro and Mpro, as well as, emphasis will be given on computational methods used in the search for effective antivirals. Classical and quantum methods are useful tools to understand the enzyme mode of action and providing insights into the drug design.

KEYWORDS

COVID-19; Protease; Computational Chemistry; Antiviral.

Introduction

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has created a global health crisis. The emergence of the SARS-CoV-2 pandemic between the end of 2019 and the beginning of 2020 prompted for an in-depth investigation of the SARS-CoV-2 molecular machinery, with the aim of finding strategies to inhibit its spread (AMIN et al., 2021; ANIRUDHAN et al., 2021).

After entering into the host cell, SARS-CoV-2 releases its genomic RNA that is translated to polyproteins pp1a and pp1ab. A critical step in the SARS-CoV-2 life cycle is the proteolytic processing of the pp1a and pp1ab leading to the non-structural proteins (nsp), which are essential for assembly during viral replication. This proteolytic action is due to two cysteines proteases, the papain-like protease (PL^{pro}), and 3C-like protease or main protease ($3CL^{pro}$ or M^{pro}) (ANIRUDHAN et al., 2021; FRANCÉS-MONERRIS et al., 2020). In this sense, M^{pro} and PL^{pro} enzymatic activities are crucial for the SARS-CoV-2 replication, indicating to be promising antiviral targets.

The search for effective therapies for COVID-19 includes (i) the designing of vaccines as protection, (ii) the discovery of new molecules as antivirals, and (iii) identifying existing drugs to repurpose them as effective anti-SARS-COV-2 (DURDAGI et al., 2021). Here we will discuss the structure and function of Mpro and PLpro, and *in silico* strategies currently used to find SARS-CoV-2 antivirals.

PL^{pro} and M^{pro}: structure and functions

PL^{pro} is responsible for the catalytic cleavage of 3 sites from the polyproteins pp1a and pp1ab, whereas M^{pro} cleaves 11 of them, releasing the nsp1-16 (ANIRUDHAN et al., 2021; FRANCÉS-MONERRIS et al., 2020). The PL^{pro} is a monomeric enzyme and has four domains: palm, thumb, finger, and the N-terminal ubiquitin-like domain (residues 1-60, 61-178, 179-240, and 241-315, respectively). The active site of PL^{pro} is located between the palm and thumb domains and it is composed of the triad Cys111, His272, and Asp286. In addition, a zinc ion is present in the finger domain, bound to four cysteine

residues, playing an essential structural role (BÁEZ-SANTOS; JOHN; MESECAR, 2015; OSIPIUK et al., 2021). PL^{pro} recognizes the Leu-(Gln or Arg)-Gly-Gly↓(Ala or Lys) sequence in the substrates and cleaves between Gly and Ala(or Lys) residues (indicate by ↓), releasing the nsp1, nsp2, and nsp3. The PL^{pro} is encoded in the large nsp3, thus it undergoes autocatalytic cleavage (ANIRUDHAN et al., 2021; FRANCÉS-MONERRIS et al., 2020; OSIPIUK et al., 2021).

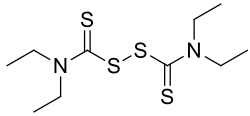
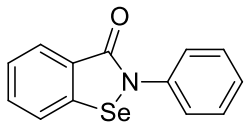
The M^{pro} is a homodimeric enzyme presenting the domains I, II, and III (residues 8-101, 102-184, and 201-306, respectively). The catalytic site is located between domain I and II, where the Cys145 and His41 residues form the catalytic dyad (GHOSH et al., 2020; MENGIST; DILNESSA; JIN, 2021). This enzyme cleaves the large polyprotein at 11 sites, recognizing the sequences (Leu, Phe or Val)-Gln↓(Ser, Ala or Asn), and releasing the nsp4-16. M^{pro} undergoes autocatalytic cleavage from the nsp5 (AMIN et al., 2021; ANIRUDHAN et al., 2021). Studies indicate that the structure of M^{pro} has conformational plasticity in the vicinity of the active site, challenging the search for antivirals (KNELLER et al., 2020).

The cysteine (Cys) residue from the catalytic dyad and triad of M^{pro} and PL^{pro}, respectively, is responsible for the peptide hydrolase activity. In both proteases, the histidine (His) residue acts as base promoting Cys' deprotonation and enhancing the sulphur nucleophilicity, necessary for the peptide bond cleavage (Figure 1) (GHOSH et al., 2020; SUÁREZ; DÍAZ, 2020).

Many studies have been done in the search for SARS-CoV-2 M^{pro} and PL^{pro} inhibitors (CHEN et al., 2021; DRAYMAN et al., 2021; JIN et al., 2020; MANGIAVACCHI et al., 2021), and here we highlight the organochalcogen drugs ebselen and disulfiram (Table 1), which can block the catalytic Cys residues and are on clinical trials studies (JIN et al., 2020; MA et al., 2020; SARGSYAN et al., 2020). They are promising candidates for drug repositioning.

Although several vaccines are been use worldwide, viral resistance remains a possibility, and therefore, there is a continued need for new treatment options for COVID-19, such as antivirals (DRAYMAN et al., 2021).

Table 1. PL^{pro} and M^{pro} inhibitors with potential use against COVID-19.

Compound	Chemical structure	IC50 (μM)		Clinical trials
		PLpro	Mpro	
Disulfiram		6.9-7.5	2.1-9.4	NCT04485130 NCT04594343
Ebselen		2.4-10.3	0.7-3.7	NCT04484025 NCT04483973

Source: data from (JIN et al., 2020; MA et al., 2020; SARGSYAN et al., 2020).

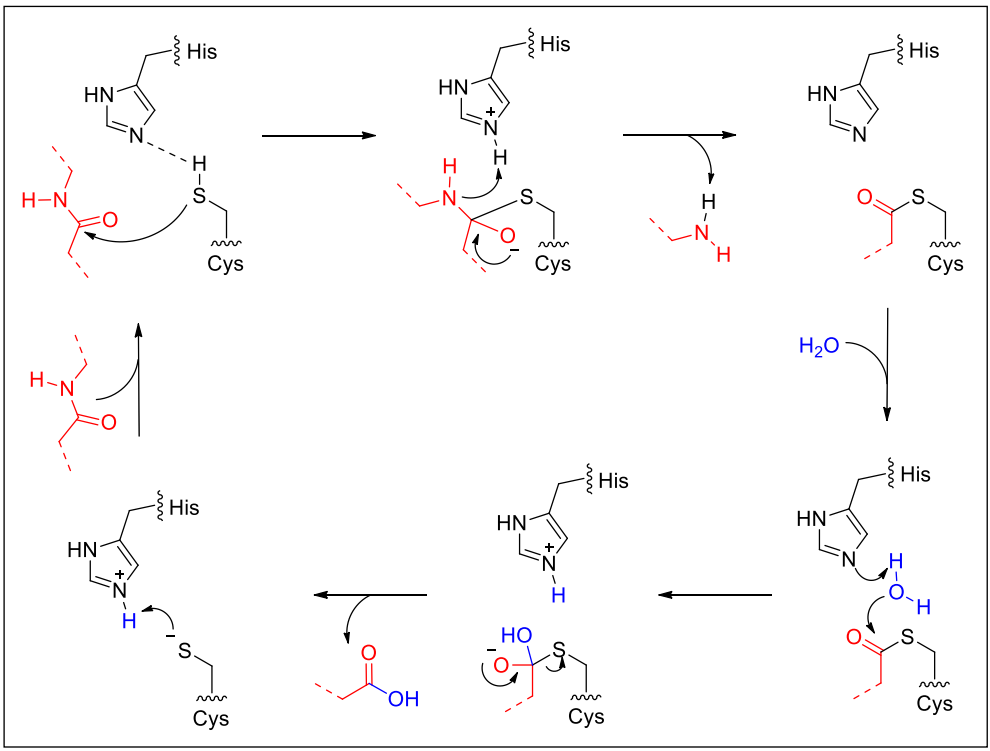


Figure 1. General catalytic cycle of Mpro and PL^{pro}. Source: authors.

Exploring SARS-CoV-2 Inhibition with Computational Chemistry

As outlined in several reviews (LEELANANDA; LINDERT, 2016; MCINNES, 2007; TIAN et al., 2015), in the last decades, computational methods in drug discovery have received great attention from both academia and industry. In this context, we will describe some examples of *in-silico* applications to the problem of SARS-CoV-2 inhibition, focusing mainly on canonical computational chemistry and molecular modelling methods; several other computational protocols, which fall more properly under the etiquette of cheminformatics, such as machine learning (VAMATHEVAN et al., 2019) and neural network (BASKIN; WINKLER; TETKO, 2016) techniques, will not be discussed, even if their importance cannot be denied. For sake of clarity, the computational methods here-on discussed will be divided into three categories: *classical methods* (i.e., based on classical mechanics), which are known to be relatively fast and readily appliable to large biomolecules and proteins, but do not provide information about bond-breaking and formation events; *quantum mechanical methods* (i.e., based on the solution of the (approximated) Schrödinger equation), which provide an atomistic accurate description of small-molecules, but cannot be applied to systems of protein-like complexity, and *hybrid methods*, which combine the best of both worlds by bridging classic and quantum descriptions of the system. These last two methods can provide a comprehensive description of chemical reactivity.

The SARS-CoV-2 M^{pro} has received most of the attention, given its importance in SARS-CoV-2 replication and thanks to the homology to SARS-CoV M^{pro}, which fastened the early resolution of its crystallographic structure. Indeed, already in early 2020 a high-throughput screening was combined with a computational protocol to identify several potential inhibitors of M^{pro}, focusing on clinically approved drugs or drug candidates in clinical and pre-clinical trials (JIN et al., 2020). This approach provided a fast insight into several promising inhibitors, which inspired further computational studies on their binding to M^{pro} and mechanism of action. Indeed, in less than two years, Jin *et al.* has obtained more than 900 citations, and many of the "potential inhibitors" found in this study have become the focus of computational as well as experimental studies.

Classic methods. Molecular Docking is the most widely applied method to study non-covalent inhibition, because of its speed and because it provides a clear-cut pictorial description of the binding event. The speed of Molecular Docking is granted by its nature as a classic *method based* on a suitably parametrized potential (force field), which provides a fast way to investigate many so-called binding poses of an inhibitor within a protein architecture, leading to a rough, *albeit* useful, idea of the binding energies and of the inhibitor arrangement. A wide number of studies are available in the literature (AL-JANABI; ELZUPIR; YOUSEF, 2021; AL-KARMALAWY et al., 2021; AOUAD et al., 2021; NGO et al., 2020; NOUREDDINE; ISSAOUI; AL-DOSSARY, 2021; WAKCHAURE; GHOSH; GANGULY, 2020) on several potential drug-like compounds that may act as M^{pro} inhibitors, ranging from small peptides (JIN et al., 2020), to well-known drugs such as non-steroidal anti-inflammatory drugs (NSAID) (ELMAATY et al., 2021b), N-heterocycle antivirals such as remdesivir and favipiravir (HAGAR et al., 2020) and glucocorticoid (ELMAATY et al., 2021a), as well as compounds in clinical trials such as ebselen, perhaps the most popular organoselenide with pharmacological activity (JIN et al., 2020; NOGARA et al., 2021). Extensive docking analysis of ebselen binding to M^{pro} (NOGARA et al., 2021), shown that docking can be used not only to provide a reliable binding energy of the drug-like system to the target, but also the possible activity of many metabolites of the drug, whose binding energy can sometimes be as favorite as that of the parent compound itself. While a negative binding energy does not necessarily translate into a strong inhibition, this approach still provides a fast overview of the potential activity of the investigated inhibitors in biologically reasonable conditions (i.e., considering metabolic modifications).

A way of improving the description of binding energies, without renouncing to the speed and robustness of a *classic approach*, is provided by molecular dynamics (MD) simulations, which were extensively applied in the investigation of non-covalent inhibitors against SARS-CoV-2 infection (HAN; KRÁL, 2020; LUAN; WANG; HUYNH, 2021; MIRZA; FROEYEN, 2020; TAHIR UL QAMAR et al., 2020; WANG, 2020). Indeed, already in mid-2020, the combination of molecular docking with MD simulations, has revealed the importance of an "anchor site" near the catalytic pocket of M^{pro} in designing non-covalent

inhibitors (HUYNH; WANG; LUAN, 2020a). Such anchor site was proved to be essentially an hydrophobic site within the enzyme, that can be targeted to strengthening the non-covalent binding and stabilize the adduct between M^{pro} and potential inhibitors bearing a non-polar function (HUYNH; WANG; LUAN, 2020b).

Another interesting approach is the one followed by Jorgensen and coworkers (GHAHREMANPOUR et al., 2020) who employed molecular docking for a fast virtual screening of around 2000 FDA approved molecules, narrowing down the range of interesting potential inhibitors to only 42. These systems were carefully analyzed by "visual inspection" in order to rule out from further investigation systems where docking yielded binding poses with unsatisfied hydrogen-bonding sites, electrostatic mismatches, and unlikely conformations of the ligand. Roughly half potential inhibitors survived the scrutiny and 14 of them were subjected to 70 ns MD simulation, to assess the time-dependent stability of the inhibitor – protein adduct. This protocol led to the selection of 17 potential inhibitors out of the initial set of 2000 compounds. Further experimental investigation confirmed that 14 of the drugs at a concentration of 100 μM decreases M^{pro} activity, and four out of fourteen have IC_{50} values below 20 μM. Such inhibitory capacity is rather weak, but it can serve as a starting point for further drug design.

Indeed, Jorgensen and coworkers proved that even a rather week inhibitor with an experimental IC_{50} in the range 100-200 μM, can be chemically designed into a strong inhibitor in the nanomolar range, by means of chemical intuition and computational predictions (ZHANG et al., 2021). Using a free energy perturbation/Monte Carlo approach, the difference in Gibbs free energy of binding ($\Delta\Delta G_{bind)}$ upon structural modification of a selected parent compound can be evaluated (such as Perampanel) (GHAHREMANPOUR et al., 2020). Some simple modifications were enough to obtain $\Delta\Delta G_{bind}$ ~ -4 Kcal mol^{-1}, to which corresponded experimentally a switch from an IC_{50} ~ 100-250 μM to an IC_{50} ~ 10 μM. Combining several basic modifications in order to better fit the enzymatic cavity, the authors were able to propose inhibitors with an IC_{50} as low as ~20 nM.

This approach, based entirely on classical methods, is a beautiful example of how the simple concept of the binding free energy, when combined with chemical intuition, can produce results ahead of the experiments in both drug

repurposing and drug redesign, leading to a new class of strong inhibitors starting from repurposed drugs with a low inhibitory potential.

Quantum and hybrid methods. Quantum mechanical methods represent the step forward in increasing the calculation accuracy. In the last two decades, the most applied quantum method has been density functional theory (DFT), whose advantages and drawbacks are described in many reviews (GRIMME, 2011; MARDIROSSIAN; HEAD-GORDON, 2017; VERMA; TRUHLAR, 2020). The most common and straightforward application of DFT within the field of SARS-CoV-2 inhibition, is the mere geometry optimization of the drug-like scaffold under investigation, with the aim to compute properties like the molecular electrostatic potential, partial charges or to study the frontier molecular orbitals (AL-JANABI; ELZUPIR; YOUSEF, 2021; BHARADWAJ et al., 2021; ELMAATY et al., 2021b; HAGAR et al., 2020; NOUREDDINE; ISSAOUI; AL-DOSSARY, 2021). This simple approach aims at rationalizing the (non-covalent) interaction between a potential inhibitor and the target, as predicted by a classical method, usually molecular docking, giving a more complete picture of the intermolecular binding event.

Besides these trivial, *albeit* useful, applications, the striking advantage of quantum methods over classic methods is their ability to properly describe bond-breaking and formation phenomena, thus allowing a full mechanistic investigation of enzymatic reactivity and covalent inhibition. Since proteins are too large to be fully investigated quantum mechanically, several approximations must be used, the most commonly applied being the cluster approach (SIEGBAHN; HIMO, 2009) in which a few selected residues mimic the enzyme catalytic pocket or the inhibitor binding site, and the so-called *hybrid methods* in which a portion of the system (i.e., the one where bond-breaking and formation occurs) is treated quantum mechanically (QM), usually via a DFT description, and the surrounding enzyme is treated classically with a molecular mechanics (MM) description, leading to the family of methods generally known as QM/MM. Several approximations exist to bridge the QM part to the MM one as described in recent reviews (CHUNG et al., 2015; SOUSA et al., 2017), and the QM/MM energy calculation can be even

combined with a MD framework (QM/MM MD) to provide insight into enzyme mechanisms and evolution. Indeed, the first QM/MM MD applications to SARS-CoV-2 have been in elucidating the chemical mechanism of the fully functional M^{pro} (RAMOS-GUZMÁN; RUIZ-PERNÍA; TUÑÓN, 2020; ŚWIDEREK; MOLINER, 2020), with a complete description of the steps involved in peptide bond breaking. Particularly, all studies agreed on that the enzyme works via a catalytic dyad composed by a Cys and a His residue, which first undergo a (slightly endergonic) proton transfer mechanism, enhancing the Cys nucleophilic strength. Then, the cysteinate can attack the peptide bond via a fast acylation reaction, followed by a rate-determining deacylation reaction in which the peptide bond is cleaved (Figure 1). Such insight is particularly interesting since the first part of the mechanism, i.e., the proton-transfer/acylation reactions, should be mimicked by potential M^{pro} covalent inhibitors. Similar conclusions were drawn also by employing canonical QM/MM methods, without accounting for the MD-like evolution of the adduct, thus providing a reliable and general principle for drug design (FERNANDES; SOUSA; CERQUEIRA, 2021).

In fact, essentially all the quantum investigation on covalent inhibitors followed a mechanistic pathway analogue to the first part of the fully functional M^{pro}, despite the strong chemical differences between the potential inhibitors. QM/MM MD investigations were done for Michael acceptor peptides (ARAFET et al., 2021; RAMOS-GUZMÁN; RUIZ-PERNÍA; TUÑÓN, 2021a), which were found strong M^{pro} inhibitors by molecular docking and high-throughput studies previously described, and for aldehyde warhead potential inhibitors (RAMOS-GUZMÁN; RUIZ-PERNÍA; TUÑÓN, 2021b). Conversely, a cluster approach was used to investigate the covalent binding of ebselen to the catalytic Cys of M^{pro} (MADABENI et al., 2021), with the ebselen scaffold being used a case study to describe the reactivity of selenyl amides towards M^{pro}. Moreover, the potential activity of the main ebselen metabolite was also investigated, to provide a more realistic, biologically relevant picture. These analyses provide atomistic insight into specific inhibitor classes, that can be used to rationalize experimental evidences or to suggest drug design strategies to improve the efficacy of covalent inhibition. Moreover, even if QM/MM MD methods show their complete potential when applied

in mechanistic investigations as outlined above, QM/MM methods can be used after a classic method in a sequential fashion, thus refining the results provided by a docking or MD calculation. A similar approach has been used to refine the binding energies of natural compounds, potential non-covalent inhibitors of Mpro (BHARADWAJ et al., 2021), by performing a QM/MM energy calculation on the final snapshot from a MD simulation. Such an approach includes electronic structure effects on the inhibitor – target adduct, otherwise neglected in classical MD and in general in classical methods.

Conclusion

These works highlight the usefulness of computational chemistry approaches in the drug design process, especially when a compromise between speed and reliability is strongly required, as happened in 2020 because of the SARS-CoV-2 pandemic emergency. Several different aspects were described, but mainly two main areas of competence can be distinguished: classical approaches dominate the panorama of non-covalent inhibitor design, by making use of different strategies to predict the binding free energy, while quantum and hybrid methods are the leading methods for the investigation of mechanistic aspects of covalent inhibitors. Both approaches can be combined, by using docking methodologies for a fast screening of non-covalent adducts, and QM/MM (MD) methods to refine the energetics or to properly describe the covalent bond formation. This synergy already provided useful insights that led to the design of new potential antivirals, and further developments in drug design can only be expected.

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NATURAL DEEP EUTECTIC SOLVENTS TO OBTAIN BIOCOMPATIBLE READY-TO-USE PHYTOCHEMICAL EXTRACTS: FROM FOOD TO PHARMA APPLICATIONS

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ABSTRACT

One of the challenges for integrating the food and pharmaceutical industries to the One Health concept concerns to the use of environmentally friendly practices for the production of foods, medicines and cosmetics that contribute for welfare and health promotion. Natural deep eutectic solvents (NADES) were first described 10 years ago as a third liquid phase existing in the cells of all living organisms, distinct from water and lipids. NADES have been proposed as promising ecofriendly solvents to substitute for conventional volatile organic solvents in the extraction of phytochemicals for the food and pharmaceutical industry. The safety issues related to the use of NADES for obtaining ready-to-use extracts for food and pharmaceutical applications were addressed.

KEYWORDS

Natural deep eutectic solvents; Green solvents; Bioavailability; Bioactivity; Biocompatibility.

Introduction

The One Health concept that was created less than 20 years ago, highlights the inseparable relationship between all living species and the environment, which are interconnected in a symbiotic way, requiring transdisciplinary approaches to address the challenges posed to human health (AMUASI et al., 2020, p. 1469-1471). The challenging climate changes and pandemic issues that occurred between 2020 and 2021 revealed the urgency to adopt this type of concept as well as to increase the efforts to achieve the Sustainable Development Goals of the United Nations (https://sdgs.un.org/goals). In this context, one of the challenges for integrating the food and pharmaceutical industries to the One Health concept concerns to the use of environmentally friendly practices for the production of foods or medicines that contribute for health promotion.

In addition to their relevant role in animal and human nutrition, plants are important raw material for the extraction of valuable phytochemicals for use as food and chemical additives, nutraceuticals and herbal medicines. However, conventional extraction methods employ organic solvents that need to be removed before use because of their toxicity, besides flammability, environmental pollution and occupational safety issues (SILVA et al., 2020a, p. 1). In this context, natural deep eutectic solvents (NADES) have been proposed as substitutes for conventional volatile organic solvents to meet the demands of biosustainability and safety.

This review will focus on safety issues related to the use of NADES for obtaining ready-to-use extracts for the food and pharmaceutical industries.

Natural deep eutectic solvents

Natural deep eutectic solvents (NADES) were first described by Choi et al. (2011, p. 1702) as a third liquid phase existing in the cells of all living organisms, distinct from water and lipids. They were postulated to allow the existence of high concentrations of bioactive compounds that have low aqueous solubility in plant cells. NADES are chemical mixtures containing two or more components that can be solid or liquid, being a hydrogen bond acceptor (HBA) (usually

quaternary ammonium salts, or amino acids), and a hydrogen bond donor (HBD) as organic acids or carbohydrates (MIŠAN et al., 2020, p. 2565). The interaction of these constituents via hydrogen bond produces eutectic mixtures that have much lower melting point than their individual components (SILVA et al., 2020a, p. 4). According to the chemical point of view, these solvents can be divided into five groups: ionic liquids, made of an acid and a base; neutral, made only from sugars or sugars and other polyalcohols; neutral with acids, made from sugars/polyalcohols and organic acids; neutral with bases, made from sugars/polyalcohols and organic bases; and NADES containing amino acids from a mixture of amino acids with natural organic acids/sugars (CHOI & VERPOORTE, 2019, p. 89).

NADES have attracted the interest of scientific community, not only due to its favorable physicochemical characteristics (e.g. low melting point, negligible volatility, chemical, and thermal stability, and non-flammability) but also due to its biocompatibility and eco-friendly properties (MIŠAN et al., 2020, p. 2565). Unlike the conventional organic solvents, NADES are composed of substances that are abundant in plant and animal cells, generally non-toxic and accepted for use in food formulations. For this reason, NADES mixtures apparently would not need to be removed before human consumption of a plant extract, thus providing a ready-to-use extract and a biosustainable alternative to conventional organic solvents (SILVA et al., 2020a, p. 5; MIŠAN et al., 2020, p. 2579).

Despite being composed of food grade ingredients, some important issues must be taken into account for their safe application in human diet. Some of these issues include the acceptable daily intake (ADI) of each ingredient and the possible changes in the biological properties of individual components due to the physicochemical interaction in the NADES mixture. The ADI is defined as the amount of a chemical that can be ingested daily over a lifetime without appreciable health risk (CODEX ALIMENTARIUS, 2021, p. 3).

The vast majority of NADES have choline chloride (ChCl) in their composition (CHOI & VERPOORTE, p. 90; SILVA et al., 2020a, p. 3). Choline is a natural ingredient and an essential nutrient for human development. The maximum safe intake level of choline was reported by the US Standing Committee on the Scientific Assessment of Dietary Reference Intake, as

amounting to 3500 mg of ChCl/person/day for adults (OECD, 2004, p. 107). Betaine, a methylated derivative of glycine that possess a quaternary ammonium group and is also frequently used in NADES mixtures, has an ADI of 6 mg/kg body weight/day for adults (EFSA Panel, 2019, p.4). Meanwhile, other HBA ingredients in NADES like L-proline and acetamide were not found in the databases issued by safety committees.

Glutamic acid, glycerol, citric acid, malic acid and lactic acid, which are also currently used in the composition of NADES, are approved by the Codex Alimentarius as food additives allocated an "ADI not specified", which means that they are allowed for food use with no other limitation than in accordance with the Good Manufacturing Practices (GMP) (CODEX ALIMENTARIUS, 2021, p. 464). When an additive is GMP, its use is limited to the amount necessary to obtain the technological effect, which means that it is usually authorized at a *quantum satis* (q.s.) limit (CODEX ALIMENTARIUS, 2021, p. 3). It is noteworthy that an "ADI not specified" does not mean acceptable unlimited intake but indicates that the total daily intake of the substance arising from its use under GMP conditions does not represent a hazard to health (CODEX ALIMENTARIUS, 2021, p. 12). The allowance for NADES-based extracts to be used as ready-to-use ingredients for food and pharma applications will be expected to increase the average daily dietary intake of NADES components. Despite existing toxicity studies on the isolated components of NADES, the safety evaluation of NADES mixtures must include a reassessment of risk taking into account the expected average daily intake of each substance, and the biological effects of the NADES mixture itself, i.e. of the combined NADES components.

Experimental evidence of NADES biocompatibility

The potential of NADES for extracting bioactive compounds has been explored in various plant matrices and the bioactivity of extracts obtained has been usually tested using antioxidant *in vitro* assays. The first studies on the NADES effects in cell cultures *in vitro* and in animal models were published in 2015 (Table 1). Radošević and collaborators (2016, p. 51) demonstrated that a number of ChCl-based NADES mixtures did not cause 50% cell growth inhibition up to 2000 mg/L in a cell culture citotoxicity assay, suggesting

low toxicity. Furthermore, this study was one of the pioneers to demonstrate that a grape skin extract obtained with NADES based on ChCl:malic acid (1:1, molar ratio, plus 30% water) showed greater antiproliferative activity than the extract based on conventional solvents in MCF cells -7 and HeLa. According to the authors, this effect may be partially associated with the composition of NADES, since organic acids, such as malic acid, have anti-inflammatory and antioxidant activities reported in the literature (RADOŠEVIĆ et al., 2016, p.50). Subsequently, Mbous and collaborators (2017, p.2) demonstrated that NADES (ChCl:fructose and ChCl:glucose) have lower citotoxicity than a deep eutectic solvent (DES) based on N,N-diethylethanol ammonium chloride:triethylene glycol (1:3, molar ratio), which are non-natural components (higher EC_{50} values; Table 1). However, NADES showed higher citotoxicity against a normal liver cell line (WRL-68) when compared to cancer cell lines indicating that these associations may not be completely safe and could harm normal liver cells (MBOUS et al., 2017, p. 4).

Hayyan and collaborators (2015, p. 1) were one of the first to investigate the biocompatibility of NADES mixtures *in vivo*. A single oral administration to impression control region (ICR) mice revealed that NADES (ChCl:glycerol, ChCl:urea) and other DES tested (ChCl:ethylene glycol and ChCl:trimethylene glycol) had lower LD_{50} values than their individual components, suggesting a greater toxicity of the eutectic mixtures (HAYYAN et al., 2015, p. 14). Later, another acute toxicity trial using the same protocol in ICR mice revealed that ChCl:fructose and ChCl:glucose had higher toxicity (LD_{50} values) than a DES based on non-natural compounds (MBOUS et al., 2017, p. 12). In both studies, markers of hepatic damage were increased in the animals but the dose associated to this effect was not clearly stated. Elevated viscosity has been suggested to be implicated in these toxicity issues.

Faggian and Sut (2016, p. 6; 2017, p. 6) have reported oral administration of NADES mixtures to mice in pharmacokinetic studies of NADES as delivery vehicles for bioactive compounds. Faggian (2016, p. 1) administered one oral dose (0.5 mL per mice) of a NADES based on proline:glutamic acid (2:1, molar ratio) as a vehicle for rutin. Similarly, three different NADES mixtures, namely proline:malic acid (2:1, molar ratio), proline:urea (2:1, molar ratio) and proline:malic acid:lactic acid:water (1:0.2:0.3:0.5, molar ratio) were tested as vehicles for a single oral dose of berberine in mice (SUT et al., 2017, p. 6). The dose/volume of NADES administered

was not clearly stated in this study, maybe because it was not focused on assessing safety issues.

The safety of NADES for the extraction of phenolic compounds and subsequent oral use of the extract with no solvent removal was evaluated initially by Benlebna (2018, p. 6209). In this study, the toxicity of a coffee extract obtained using NADES based on betain:glycerol (2:1, molar ratio, plus 10% water) was investigated after oral administration to Wistar rats during 14 days compared to a group that received water alone. The administration of the coffee extract with NADES was hampered by the high viscosity of the mixture and induced 25% mortality. Reduced food intake and body weight were also found in animals treated with the NADES-based coffee extract along with increased water consumption, blood lipid levels, plasma oxidative stress and hepatomegaly (BENLEBNA et al., 2018, p. 6202). However, controls of NADES vehicle alone or coffee extract in water vehicle were not included in this study. This way, it is uncertain whether the observed effects were due to the bioactive compounds from coffee, NADES itself, or an interaction between both factors (MURADOR et al., 2019, p. 32). Recently, our research group studied the safety of a NADES-based ready-to-use extract in a rat model (SILVA et al., 2020b, p. 7). This study was conducted using a blueberry extract obtained using ChCl:glycerol:citric acid (0.5:2:0.5, molar ratio, plus 25% water) (SILVA et al., 2020b, p. 7). Biocompatibility was assessed in rats after daily intake of the NADES mixture itself or the NADES-based blueberry extract (2 mL/kg body weight, per gavage) for 14 days. In this study, the volume of NADES administered per day was carefully calculated to avoid toxic doses of their individual components. No overt toxic effects or increase in the activities of plasma glutamic oxalacetic transaminase (also known as aspartate aminotransferase) or glutamic pyruvic transaminase (also known as alanine aminotransferase) were observed, indicating hepatic safety (SILVA et al., 2020b, p. 7).

Food and pharma applications

The use of NADES in the food and pharmaceutical industries are among their most promosing applications. Various NADES mixtures have been demonstrated to allow faster and more efficient extraction of valuable phytochemicals from

fruits, medicinal plants and agri-food wastes compared to conventional organic solvents. The polarity of most NADES mixtures is similar to water and polar organic solvents such as methanol and ethanol. For this reason, phenolic compounds were the focus of most extraction studies using NADES. This diverse group of plant secondary metabolites includes many compounds that have interesting nutraceutical, pharmacological and cosmetic properties but also other compounds that are used as food coloring (anthocyanins) and flavouring (vanillin) agents. Additionally, NADES mixtures are also able to efficiently extract alkaloids, saponines, essential oils, proteins, carbohydrates, fatty acids, and photosynthetic pigments from diverse matrices as recently reviewed by MIŠAN et al. (2020, p. 2570).

The first NADES extract that was approved for commercial use belongs to the cosmetic field (CHOI & VERPOORTE, 2019, p. 90) that raises less safety concerns because topical use results in lower or no skin absorption rates for NADES components. The use of NADES in food and pharmaceutical products directed for oral intake comprises more complex safety issues but offer interesting opportunities. NADES can provide an unique approach to use a biorefinery concept based on green solvent extraction for integrating the agri-food by-products to the pharmaceutical industry.

In addition to their suitability as extractive solvents for plant phytochemicals, NADES have also been shown to increase the bioavailability of phenolic compounds (flavonols, isoflavones and anthocyanins) and alkaloids (berberine) when they were used as vehicle for the oral intake of these bioactive compounds. Faggian (2016, p. 6) demonstrated that a NADES based on proline:glutamic acid (2:1), in addition to increasing the solubility of rutin, increased the bioavailability of this flavonol by 2-fold, when compared to the water vehicle (Table 1). Similarly, berberine bioavailability was increased when NADES vehicles were used, namely proline:malic acid (2:1, molar ratio), proline:urea (2:1, molar ratio) or proline:malic acid:lactic acid:water (1:0.2:0.3:0.5, molar ratio) (SUT et al., 2017, p. 6)(Table 1). In these studies, the increased bioavailability was attributed to a greater solubility of these compounds in NADES solvents compared to water, suggesting that the interaction between vehicle and compounds may affect the absorption of these phytochemicals (FAGGIAN et al.,

2016, p. 6; SUT et al., 2017, p. 6). Accordingly, measurements of curcumin solubilization in simulated gastrointestinal fluids and quantum chemistry computation revealed that intermolecular interactions were responsible for increasing the solubility of this phenolic compound when NADES mixtures containing ChCl and glycerol were used as vehicle instead of water (JELINSKI et al., 2019, p. 116).

The ability to improve phytochemical bioavailability was also described for honey, a natural food that has NADES-like properties (DAI et al., 2020; p. 19). Calicosin-7-O-ß-d-glycoside solubilized in honey or in a honey-analogue NADES (glucose:fructose:sucrose, 32:32:5, by weight) exhibited higher plasma peak levels after oral intake by mice compared to the isoflavonoid that was solubilized in water (Table 1).

Our research group has recently studied a NADES based on ChCl:glycerol:citric acid (0.5:2:0.5, molar ratio, plus 25% water). We demonstrated that this NADES mixture increased by 140% the oral bioavailability of anthocyanins from blueberry extract in the plasma of rats when compared to the extract obtained using conventional organic solvent (SILVA et al., 2021, p. 6) (Table 1). In addition, we have described for the first time the buffering capacity of this NADES mixture within the acidic range, which delayed the neutralization of gastric content during intestinal digestion, leading to increased stability and absorption of phenolic compounds (SILVA et al., 2021, p. 9). This buffering effect can be explained by the presence of citric acid (pKa values 3.1, 4.71, 6.42) in the NADES mixture (SILVA et al., 2021, p.9). This mechanism can be expanded to pharmacokinetic studies conducted by other research groups helping to explain the increased bioavailability of compounds solubilized in other NADES mixtures. The NADES mixtures used as vehicles for rutin and berberine (FAGGIAN et al., 2016, p. 6; SUT et al., 2017, p. 6) also contained components that have buffering activity in the acidic range, namely glutamic acid (pKa values 2.19 and 4.25), malic acid (pKa values 3.51 and 5.03) or lactic acid (pKa value 3.86) (PubChem database).

Few studies have investigated the bioactivity/pharmacodynamic properties of NADES-based extracts *in vivo*. The studied NADES mixtures have been shown to potentiate the biological effects of phenolic compounds or exhibit protective effects per se *in vivo*. A ChCl:glycerol:citric

acid (0.5:2:0.5, molar ratio, plus 25% water) NADES has been shown to contribute to the gastroprotective effect of the crude blueberry extract in a rat model of gastric ulcer by mitigating gastric oxidative stress (SILVA et al., 2020b, p. 10) (Table 1). In addition, honey frying has been shown to improve the immunostimulatory properties of a chinese medicinal root (Astragali Radix) in a mouse model of leucopenia (DAI et al., 2020, p. 13). Such effect has been related to the NADES-like properties of honey that increased the dissolution and absorption of active isoflavonoids after oral administration. However, per se effects of honey were not investigated.

Perspectives

Current data reveals that NADES are promising ecofriendly solvents to substitute for conventional organic solvents in the extraction of phytochemicals for the food and pharmaceutical industry. Their extractive advantages often include higher extractive efficiency as well as reduced issues related to occupational toxicity and environmental impact. Although the biocompatibility of NADES components opens a great oportunity for preparing ready-to-use extracts, safety issues must be specifically addressed for each type of NADES mixture, especially when the extracts will be directed for oral intake. Future safety studies must advance from cell culture assays to animal models and must take into account the expected increase in the daily intake of each NADES component as well as the interactions between NADES components. Further comprehension on how each type of NADES mixture would affect the bioavailability and bioactivity of the extracted phytochemicals are also required before their use as extractive solvents and specially as vehicles for oral applications in the food and pharma fields.

Table 1. Biological effects of NADES mixtures.

Biological model	Type of study	NADES or DES studied	Study design/Observed effects	Reference
Cytotoxicity and toxicity on mice model	Safety of pure NADES	NADES: ChCl:glycerol; ChCl:ureia DES: ChCl:ethylene glycol; ChCl:trimethylene glycol All at 1:3 (molar ratio).	*In vitro* tests: MTT cell viability, cell membrane permeability, and reactive oxygen species assay in OKF6, MCF-7, PC3, A375 HepG2, HT29 and H413 human cell lines. EC_{50} values for NADES (18 to 81 µg/mL) ~= non-natural DES (12 to 69 µg/mL). *In vivo* test: DL_{50} values in ICR mice after single oral administration of NADES ranged from 5.3 to 6.4 g/kg b.w., values lower than their individual components, mild increase in markers of renal function, high increase in marker of hepatic damage (aspartate transaminase)	HAYYAN, 2015
Cytotoxicity and toxicity on mice model	Safety of pure NADES	NADES: ChCl:fructose and ChCl:glucose at 2:1 (molar ratio) DES: N,N-diethylethanol ammonium chloride:triethylene glycol (1:3, molar ratio)	*In vitro* tests: MTT cell viability, membrane permeability and oxidative stress assay in HelaS3, PC3, AGS, A375, MCF-7 and WRL-68 human cell lines. EC_{50} values for NADES (98 to 516 mM) > non-natural DES (34 to 120 mM). *In vivo* test: DL_{50} values in ICR mice after single oral administration of NADES were lower compared to non-natural DES. Increase in marker of hepatic damage (aspartate transaminase) after NADES exposure.	MBOUS, 2017
Toxicity on rat model	NADES as extractive solvent and vehicle for green coffee beans extract	Glycerol:betaine (2:1 mole ratio, plus 10% water)	*In vivo* test: Toxicity in rats after 14 days of administration of a NADES-based green coffee beans extract (3 mL of NADES extract/rat twice a day, per gavage)	BENLEBNA, 2018
Antiproliferative activity in vitro	Safety of pure NADES	ChCl:malic acid (1:1), Ch:fructose (1.9:1), ChCl:glucose (2:1), ChCl:xylose (2:1), ChCl:glycerol (1:2), (molar ratio) plus 30% of water	*In vitro* WST-1* assay and morphological assessment by light microscopy of HeLa and MCF-7 human tumor cell lines after exposure to NADES mixtures: EC_{50} values > 2000 mg/L for all NADES.	RADOŠEVIĆ, 2016
Bioavailability in mice model	NADES as a vehicle for isolated rutin	Proline:glutamic acid (2:1, molar ratio)	*In vivo* test: Pharmacokinetic assay after single oral administration of rutin (10 mg) dissolved in NADES in Balb/c mice (dose 0.5 mL of NADES/mice, per gavage). No NADES only control was studied. NADES vehicle resulted in higher rutin plasma level than water vehicle.	FAGGIAN, 2016
Bioavailability in mice model	NADES as a vehicle for isolated berberine	Proline:malic acid (2:1), Proline:urea (2:1), Lactic acid:proline:malic acid:water (1:0.2:0.3:0.5)	*In vivo* test: Pharmacokinetic assay after single oral administration of berberine (50 mg/kg b.w.) dissolved in NADES in Balb/c mice (dose of NADES not specified). No NADES only control was studied. NADES vehicle resulted in higher berberine plasma level than water vehicle.	SUT, 2017
Bioavailability in rat model	NADES as a vehicle for calycosin-7-O-ß-d-glycoside isolated	Glucose:fructose:sucrose (35:35:1, molar ratio), honey	*In vivo* test: Pharmacokinetic assay after a single oral administration of calycosin-7-O-ß-d-glycoside dissolved in NADES in Sprague-Dawley rats (dose of NADES not specified). No NADES only control was studied.	DAI, 2020
Bioavailability in rat model	NADES as extractive solvent and vehicle for blueberry fruit extract/phenolic compounds	ChCl:glycerol:citric acid (0.5:2:0.5 molar ratio) plus 25% of water	*In vivo* test: Pharmacokinetic assay after a single oral administration of crude blueberry extract obtained with NADES in Wistar rats (2 mL/kg, b.w of rat). No NADES only control was studied.	SILVA, 2021
Gastroprotective activity and safety in rat model	NADES as extractive solvent and vehicle for blueberry fruit extract/phenolic compounds	ChCl:glycerol:citric acid (0.5:2:0.5 molar ratio) plus 25% of water	*In vivo* test: Oral administration of crude blueberry extract obtained with NADES in Wistar rat for 14 days (dose 2 mL/kg, b.w of rat). A NADES vehicle control was included.	SILVA, 2021

ChCl, choline chloride; EC_{50}: concentration that causes 50% cell growth inhibition; ICR, Imprinting Control Region; MMT, [3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; WST-1, {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate}; MCF-7, human breast cancer cell line; PC3, human prostate cancer cell line; A375, human skin malignant melanoma cell line; HepG2, human liver hepatocellular cell line; HT29, human colon adenocarcinoma cell line; H413, carcinoma-derived human oral keratinocyte cells; HelaS3, human cervical cancer cell line; AGS, human gastric cancer cell line; WRL-68, human hepatocyte cell line; OKF6, human oral keratinocyte cell line.

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LE VIVANT COMME ESPACE ECOLOGIQUE DANS LA CREATION ARTISTIQUE

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Un écosystème est un complexe qui associe le vivant à son biotope (littéralement, le lieu de la vie) et les autres organismes vivants qui sont situés dans ce biotope. On sait depuis un certain temps déjà que le vivant n'est pas un donné immanent, qu'il faut un certain nombre de conditions pour qu'il apparaisse, et un certain nombre de conditions pour qu'il se développe et se diversifie, ou, plus exactement, qu'il se diversifie parce qu'il se développe. L'imbrication est très étroite entre un organisme vivant et son écosystème au point que l'ensemble forme système à travers des échanges permanents. Il y a donc un rapport interactif fondamental entre chaque organisme vivant et son écosystème. C'est ce rapport interactif qui permet aux organismes vivants de se développer dans un échange qui crée une situation de déséquilibre permanent, chaque organisme vivant étant lui-même régi par le principe de la néguentropie[1].

On sait par ailleurs que le vivant tend ontologiquement vers la complexité. Cette évolution implique une évolution concomitante du milieu systémique dans le cadre d'une co-évolution, avec les transformations réciproques que cela suppose, et que Varela a conceptualisée sous le terme de *enaction* (de *to enact*, que l'on peut traduire par «susciter», faire advenir»). Cela signifie donc que toute complexification des organismes vivants entraine nécessairement une transformation de son milieu et que, dialectiquement, toute transformation du milieu implique une transformation des organismes vivants (ou la disparition si la brutalité de cette transformation ne permet pas l'adaptation des espèces). Les travaux de Darwin, notamment, ont clairement démontré ces processus. Il semble, par exemple, que l'apparition du phénomène de prédation a constitué un facteur déterminant dans le développement de la complexité.

Sur terre, le vivant est constitué de composés carbonés et a besoin d'eau. Rien n'indique dans l'état actuel des recherches scientifiques que d'autres combinaisons soient possibles, même si des chercheurs comme Michel Morange[2] n'excluent pas, à

1 Si l'entropie peut être définie par la tendance d'un système fermé vers un maximum de désordre dans un maximum d'harmonie, la néguentropie est la capacité d'un système ouvert, le vivant par exemple, à reconstruire son organisation menacée tendenciellement par l'entropie.

2 Michel Morange, in «Le vivant», conception Jacques Vauthier, réalisation, http://www.canal-u.fr/producteurs/les_amphis_de_france_5/dossier_programmes/philosophie/sciences_et_philosophie/le_vivant, consulté le 28/08/2010

titre de pure hypothèse, que le vivant puisse se développer sur d'autres bases moléculaires comme le silicium par exemple, dans d'autres conditions systémiques. Il est défini par trois types de fonctions essentielles: l'autorégulation, l'autoconservation, l'autoreproduction. L'ensemble des interrelations qui s'effectuent entre le vivant et son écosystème est déterminé essentiellement par la satisfaction des exigences liées à ces trois fonctions dans le cadre d'un échange permanent de molécules et d'énergie entre l'organisme et son écosystème. On doit pouvoir considérer que cet échange s'effectue sur un mode informationnel. Par les molécules et l'énergie échangées, ce sont autant d'informations réciproques qui passent de l'un à l'autre, information étant à prendre ici comme un ensemble de données, y compris dans la dimension programmatique que cela suppose.

On sait que le vivant est fondamentalement déterminé lui-même par un système informationnel. Par exemple, les protéines sont fabriquées à partir des instructions transmises par la molécule d'ADN. Plus l'organisme est complexe et plus l'échange informationnel est complexe, on le voit notamment dans le cadre des réseaux neuronaux et de leur fonctionnement.

Ce qui est important, et qui nous intéresse plus particulièrement en relation avec les questions de la création artistique, c'est que ces systèmes informationnels sont modélisables. C'est-à-dire qu'il est possible de construire des modèles informatiques qui synthétisent ces dispositifs en les transformant en données numériques. C'est notamment tout l'objet des recherches qui sont menées autour de la «vie artificielle». Les progrès réalisés par l'informatique sont tels, notamment depuis ce qu'on appelle la seconde cybernétique, que le propos ne consiste pas à tenter de reproduire en tant que systèmes aboutis, mais à partir de ce qui permet le processus. La théorie, dans le domaine de la biologie, de la «complexité émergente» articulée aux recherches en informatique a permis la conception de systèmes informatiques autopoïétiques, pour reprendre le terme de Varela. C'est-à-dire de systèmes qui, à partir de données génésiques[3], sont capables d'effectuer par eux-mêmes des mutations de type darwinien. Ces programmes sont constitués d'agents logiciels qui, par

3 J'emploie ici le terme de génésique dans son acception générale de «qui a rapport à la génération».

rétroaction, sélection et réplication créent un système où, de génération en génération, survivent et ne se développent que les «espèces» qui sont les plus appropriées à résoudre la situation problématique, qui peut être très complexe, pour la résolution de laquelle le système a été programmé. Automates cellulaires, algorithmes génétiques, réseaux neuronaux artificiels..., sont autant de dispositifs programmatiques qui interagissent plus que métaphoriquement avec l'ordinateur comme un organisme vivant interagit avec son écosystème.

Panspermia (1990) de Karl Sims est une œuvre qui s'appuie sur la théorie de la «panspermie» selon lequel la vie existe dans l 'univers et se propage à travers lui sous la forme de germes ou de spores. *Panspermia* est donc l'histoire (en boucle) d'une espèce de plante primordiale sur une planète qui éjecte une coque à travers l'espace. Cette coque atterrit sur une planète et explose en une multitude de graines qui vont germer et donner lieu à des plantes très diverses. Ce qui est intéressant, du point de vue qui nous concerne, c'est que chaque type de plante a un type de croissance particulier. Chaque type de croissance est calculé par des algorithmes génétiques. Comme le précise Karl Sims: «Les techniques d'"évolution artificielle" ont été utilisées pour une sélection interactive à partir de changements aléatoires de formes de plantes jusqu'à ce qu'émerge une variété intéressante de structures[4].» Cela signifie donc que le programme passe en revue un grand nombre de modèles de croissance et crée à partir de ces données des processus spécifiques.

Autre exemple, de Karl Sims, *Evolved Virtual Creatures* (1994). Il s'agit là de «créatures» constituées de formes géométriques simples, généralement de type parallélépipédique, articulées entre elles. Karl Sims décrit ainsi le processus:

> «Une population de quelques centaines de créatures est constituée au sein d'un super ordinateur, et chaque créature est testée pour sa capacité à réussir une tâche donnée, comme la capacité de nager dans un environnement liquide simulé. Celles qui survivent avec le plus de succès, et leurs gènes virtuels contenant les instructions codées de leur croissance, sont

4 Karl Sims, «Panspermia», http://www.karlsims.com/panspermia.html, consulté le 02/09/10, trad. pers.

> copiées, combinées, et luttent pour donner naissance à une nouvelle population. Les nouvelles créatures sont testées à nouveau et certaines présentent des améliorations par rapport à leurs parents. Au fur et à mesure de ce processus de variation et de sélection, des créatures présentant de plus en plus de comportements appropriés peuvent émerger. Les créatures [...] sont le résultat de nombreuses simulations indépendantes à partir desquelles elles ont été sélectionnées pour nager, marcher, sauter, poursuivre [un signal lumineux], et entrer en compétition pour le contrôle d'un cube vert.[5]»

Les créatures sont dotées d'un réseau neuronal artificiel et placées dans un environnement virtuel solide (plan) ou liquide. À partir d'un principe d'action et rétroaction, elles élaborent des processus comportementaux qui vont leur permettre d'effectuer avec succès les opérations pour lesquelles elles ont été programmées. Il s'agit d'une véritable opération d'apprentissage où la créature doit prendre en compte les données liées à sa configuration morphogénétique et les confronter aux données fournies par le milieu (en termes de résistance et gravité, par exemple). «Le "système nerveux" des créatures est [...] complètement déterminé par l'optimisation: le nombre de nœuds internes, la connectivité et le type de fonction que chaque nœud neural est capable de réussir sont inclus dans la description génétique de chaque créature et peut croître en complexité comme un processus d'évolution[6].»

Les processus mis en œuvre procèdent de plusieurs générations de créatures. Pour les «Créatures Virtuelles Évoluées», Karl Sims parle d'une population de départ de trois cents individus. Par un processus de sélection, réplication, mutation à partir des caractères les plus performants de chaque génération de «parent», la population s'est réduite à 1/5. Le processus évolutif s'élabore sur une véritable mise en compétition des individus entre eux puisqu'il ne conserve que les individus les plus performants, à chaque génération, dans la perspective du but alloué.

5 Karl Sims, «Evolved Virtual Creatures», http://www.karlsims.com/evolved-virtual-creatures.html, consulté le 02/09/10, trad. pers.

6 Karl Sims, «Evolving Virtual Creatures», Computer Graphics, Annual Conference Series, (SIGGRAPH '94 Proceedings), July 1994, pp.15-22, trad. pers.

J'évoquais précédemment les principes de l'évolution darwinienne comme référence conceptuelle à ces processus. Il faut préciser que si l'évolution mise en œuvre est effectivement de type darwinien, elle ne procède pas à proprement parler des processus naturels d'évolution. D'ailleurs, Karl Sims parle plutôt d' «optimisation». Nous sommes ici davantage dans ce qui ressortit aux sélections génétiques effectués en biologie de façon à faire émerger des espèces performantes pour un objectif donné (vaches laitières, chevaux de course ...). L'évolution naturelle est une évolution qui n'a pas de but spécifique si ce n'est celui de maintenir la perpétuation de l'espèce. Les comportements adaptatifs développés par les individus et les espèces ne sont pas un but en termes d'évolution. Il y a sélection à l'intérieur d'une espèce par le fait que ne survivent dans un environnement donné que les individus qui inventent les comportements les mieux adaptés à leur survie et les transmettent génétiquement à leur descendance. Le problème est différent en ce qui concerne les créatures de Karl Sims. Chaque type de créature est programmé dans la perspective d'émergence de performances les plus élevées possibles dans le but de: marcher, nager, sauter, poursuivre un signal lumineux, entre en compétition avec une autre créature pour de la possession d'un cube.

Une autre œuvre de Karl Sims est intéressante en rapport avec cette problématique puisqu'elle fait intervenir un agent extérieur au dispositif informatique, le spectateur. Il s'agit de *Galapagos* (1997). C'est une installation interactive composée de douze moniteurs et ordinateurs disposés en arc de cercle. Chaque moniteur présente une créature virtuelle animée. Le spectateur choisit, en se plaçant sur un capteur situé devant chaque écran, les organismes qui lui semblent les plus intéressants d'un point de vue esthétique. Les organismes sélectionnés survivent, s'accouplent et mutent, tandis que les autres disparaissent et sont remplacés par de nouveaux organismes issus des survivants. Les successeurs sont des copies et des combinaisons de leurs parents mais leurs gènes sont altérés par des mutations aléatoires. L'évolution du cycle fait apparaître au fur et à mesure des organismes de plus en plus intéressants.

À travers ces trois exemples, il apparaît clairement que l'interaction entre les agents logiciels et le système informatique forme un véritable écosystème. À la fois à

l'intérieur des systèmes numériques autopoïétiques eux-mêmes puisque l'évolution des organismes virtuels entre eux se fait dans le cadre d'une compétition qui, dans leur interaction mutuelle, permet soit le développement des plus performants, avec ce que cela suppose comme mutations, soit l'élimination de ceux qui ne répondent pas, ou pas suffisamment, aux critères de performance, exactement comme dans tout écosystème «naturel». Ils sont soumis aux mêmes processus d'évolution que les organismes vivants, à ceci près que cette évolution est beaucoup plus rapide, que les organismes virtuels sont beaucoup moins complexes que des organismes vivants et que les situations auxquelles ils doivent répondre sont prédéterminées. Par exemple, les mêmes créatures n'ont pas, dans une même simulation, à apprendre à marcher, nager, poursuivre un objet ou être en compétition pour la possession d'un cube. Néanmoins, il ressort avec évidence que l'interrelation avec le milieu, aussi rudimentaire soit-il, et même si sa réalité physique n'est que virtuelle, est le facteur déterminant de cette évolution. Nous sommes bien dans un échange réciproque d'informations, non pas basé sur des échanges chimiques comme pour le vivant, mais sur des données numériques.

De la même façon, on voit bien comment cette interrelation conduit à une complexité grandissante des comportements dès lors qu'elle intervient de façon dynamique dans le processus relationnel. Le fait de poursuivre une lumière rouge, par exemple, pour les créatures qui nagent, signifie bien plus que prendre en compte les conditions physiques du milieu dans leur articulation aux données morphogénétiques des organismes. Cela suppose non seulement que les organismes maîtrisent ces données, mais qu'ils sont capables de les convoquer et de les organiser intentionnellement dans un but défini.

C'est encore plus évident pour ce qui concerne la lutte pour la possession du cube. Nous assistons là à l'émergence de comportements qui peuvent s'assimiler à des comportements prédateurs. Chaque créature doit à la fois construire des comportements liés à son déplacement, des comportements liés à l'objectif de ses saisir de cube, et, surtout, des comportements liés à l'évaluation des comportements de l'adversaire, ou, au moins, à leurs conséquences en termes d'occupation de l'espace que forme le cube. Il y a donc

nécessité, dans cette configuration, de faire émerger des comportements, si ce n'est stratégiques, au moins tactiques, et une certaine capacité d'opportunisme.

Mais l'écosystème qui se construit n'est pas seulement celui qui est lié au *soft*, le programme implémenté dans l'ordinateur qui permet cette génération comportementale. C'est aussi celui qui articule le *soft* au hard. Le dispositif d'évolution que permet le *soft* n'est pas pensable sans une interaction dialogique avec le programme fermé qu'est le logiciel système de l'ordinateur, celui qui lui permet de fonctionner en tant que tel, de mettre en œuvre les routines qui permettent au *soft* d'effectuer les opérations spécifiques qui permettent la génération comportementale des organismes ou de leur structure génétique. Le hard constitue une forme de biotope technologique, un *technotope*, qui, à partir de ses propres contraintes, va permettre à la vie artificielle de se développer dans des conditions d'échanges qui, du point de vue du processus, sont assimilables à celles qui lient le vivant à son biotope.

Le titre de l'œuvre *Galapagos* est une référence significative à Darwin qui, nous dit l'histoire, a élaboré sa théorie de l'évolution à partir des observations qu'il a faite de la singularité des espèces découvertes alors. En ce qui concerne cette œuvre, il y a un autre facteur déterminant dans le processus évolutif qui est le spectateur lui-même. L'ensemble écosytémique quitte les limites de la machine pour se développer dans l'espace d'exposition. Le spectateur, par son intervention interactive avec le dispositif intervient comme facteur d'évolution des organismes par le bruit[7] qu'il va apporter. Nous sommes dans la confrontation de deux logiques, une logique évolutionniste et une logique esthétique. Sims explique à ce propos que:

> «Le processus dans cette exposition est celui d'une collaboration entre homme et machine. Le visiteur fournit l'information esthétique en sélectionnant quelles formes animées il trouve els plus intéressantes, et l'ordinateur fournit la capacité à simuler les données génétiques, la croissance, et

7 «Bruit» est à prendre ici comme élément qui parasite un système et perturbe son fonctionnement.

> le comportement des organismes virtuels. Mais le résultat peut surpasser ce que chacun humain ou machine, peut produire seul. Bien que les choix esthétiques des participants déterminent le résultat, ils ne procèdent pas du dessin au sens traditionnel du terme. Ils utilisent plutôt la reproduction sélective pour explorer l' "hyperespace" d'organismes possibles dans ce système génétique simulé. Dès que les codes génétiques et la complexité des résultats sont gérés par l'ordinateur, les résultats ne sont plus contraints par les limites des capacités humaines à dessiner ou à comprendre.[8]»

On voit bien à travers ces exemples que les algorithmes évolutionnistes offrent une palette de possibilités importante pour la création artistique contemporaine. Jon McCormack, par exemple, utilise le principe des algorithmes évolutifs pour travailler sur le principe des niches. Un même écosystème peut constituer plusieurs niches pour plusieurs espèces différentes qui interagissent de façon complémentaire entre elles et avec l'environnement (pâturage, prédation, parasitisme ou symbiose par exemple). C'est ce qui permet la coexistence d'un grand nombre d'espèces différentes dans un même écosystème par la coexistence de niches différentes. C'est cette coexistence aussi qui induit à la fois une diversité et une spécialisation de chaque espèce à l'intérieur d'un même écosystème.

McCormack explique que:

> «La construction de niche est le processus par lequel les organismes modifient leur propre niche et celle des autres. Elles le font en modifiant ou en influençant leur environnement. [...] En modifiant leur niche, les organismes peuvent assurer un environnement transmissible à leur descendance. La construction de niche peut donc créer des formes de rétroaction qui modifient la dynamique du processus d'évolution.[9]»

8 Karl Sims, «Galapagos», http://www.karlsims.com/galapagos/index.html, consulté le 03/04/10, trad. pers.

9 Jon McCormack et Oliver Bown, «Life's What You Make: Niche Construction and Evolutionary Art», in M. Giacobini, et. al. (eds): EvoWorkshops 2009, LNCS 5484, pp. 528-537, 2009, document pdf téléchargé à partir de http://www.csse.monash.edu.au/~jonmc/research/publications.html le 02/09/10, trad. pers.

Les algorithmes évolutifs permettent de modéliser numériquement ces processus. Jon McCormack utilise par exemple la modélisation de ces processus comme outil de création artistique. Il décrit le principe initial de la façon suivante:

> «Le système consiste en un nombre d'agents haploïdes[10] dessinant qui évoluent à l'intérieur d'une surface à dessiner — à l'origine une toile blanche et vide. Les agents se déplacent sur la surface laissant un trait d'encre sur leur trajet. Si un agent entre en intersection avec un trait existant, dessiné par lui-même ou un autre agent, il meurt. [...]. Les caractéristiques de la trajectoire que choisit un agent pour dessiner est déterminée par ses gènes. Le trajet en cours est déterminé par un processus aléatoire.[11]»

Le modèle de construction de niches complète le dispositif de la façon suivante:

> «Pour ajouter une construction de niche au modèle de dessin, on alloue à chaque agent un allèle[12] additionnel dans son génome [...]. Cela définit la préférence de l'agent pour la densité des lignes déjà dessinées dans l'espace immédiat de sa position en cours, c'est-à-dire sa niche. Dans sa niche préférée, un agent est plus à même de donner naissance à une descendance et a de meilleures chances de survie. Comme les enfants héritent des gènes de leurs parents, ils sont mieux à même de survivre quand ils ont une préférence de densité similaire.[13]»

Les œuvres de Sims aussi bien que celles de McCormack, mais on pourrait citer encore bien d'autres artistes (M. H. Tramus et M. Bret, C. Sommerer et L. Mignonneau, A. S. Le Meur ...), ont ceci de particulier c'est que, comme nous l'avons vu, elles utilisent les processus modélisés du vivant et de son

10 Se dit de la constitution des gamètes qui, après la méiose, ne possèdent que n chromosomes (ce nombre correspondant à celui d'un élément de chaque paire), ce qui leur permet de s'apparier avec d'autres lors de la fécondation. L'ovule fécondé possède 2n chromosomes. Source: http://www.mediadico.com/dictionnaire/definition/haploide/1, consulté le 04/09/10

11 Jon McCormack ..., ibid.

12 Se dit de deux gènes qui ont une même fonction mais des effets dissemblables.

13 Jon McCormack ..., ibid.

évolution comme éléments générateurs d'œuvre d'art. Cela pose évidemment un certain nombre de questions quant à la dimension artistique de ces œuvres et à la façon dont elles interrogent spécifiquement les processus de création artistique.

Ces pratiques s'articulent essentiellement à un processus délégataire où l'artiste n'intervient pas à chacune des étapes du processus d'effectuation comme c'est traditionnellement les cas dans la production artistique, dans le champ des arts plastiques en particulier. L'intervention de l'artiste se situe en amont des processus d'effectuation par la conception des données génésiques[3] de cette effectuation. Et s'il y a œuvre d'art, ce n'est pas tant dans le résultat obtenu, comme les superbes dessins de McCormack par exemple, que dans la mise en œuvre du processus lui-même et les conditions de cette mise en œuvre. La relation de l'art avec la nature ne date pas d'aujourd'hui, il est même probable qu'elle en constitue une dimension intrinsèque. Le mythe de Pygmalion est là pour en attester. Mais ce rapport a longtemps été de type analogique, c'est-à-dire qu'il passait par une représentation formelle des objets de la nature (que ce soient les éléments qui la constituent, les phénomènes qu'elle produit, etc.) pris dans leur finitude et non dans leur dimension processuelle.

Aristote avait déjà émis l'hypothèse que la fonction de l'art n'est pas d'imiter les objets de la nature mais de tenter d'en représenter les processus. C'est ce qu'explique Paul Ricœur analysant Aristote: «… ce qui est imitable dans la nature ce ne sont pas les choses produites que l'on aurait à copier, mais la production même et son ordre téléologique, qui reste à comprendre et que la fable peut recomposer.[14]» À travers les œuvres qui ressortissent à l'art «évolutionnaire», nous atteignons un rapport à la création artistique qui prend la théorie aristotélicienne au pied de la lettre. L'œuvre se crée non pas à proprement parler par la mise en œuvre des processus génésiques de la nature elle-même, mais du moins à partir des éléments de modélisation de ces processus.

Toutefois, on pourrait établir des mises en parallèle, du point de vue de cette problématique, avec les œuvres qui ressortissent aux biotechnologies (Kac, TC&A, Art Orienté objet …) qui utilisent les processus même du vivant pour faire œuvre. Et cette concomitance n'est certainement pas le fait du

14 Paul Ricœur, La métaphore vive, Paris, Seuil/Points, 1975, p. 60 (n.b.p.)

hasard. Elle correspond à une volonté de repenser la création artistique à l'aune des paradigmes contemporains, y compris dans son essence même.

Louis Bec nous explique, à propos de la vie artificielle, que: «Sa niche techno-écosystémique se présente comme un "potentiel" de chimérisation.[15]» Je crois que nous touchons là le cœur du sujet qui fait qu'elle se présente comme un médium privilégié pour un certain nombre de créations artistiques. Ce que permettent les processus de modélisation, ce n'est pas seulement de reproduire les processus de la vie telle qu'elle est, mais de faire émerger des potentialités de la vie telle qu'elle pourrait être. C'est dans cet espace que se situe la fable dont parle Aristote, prise comme métaphore de l'œuvre d'art. Les œuvres que nous avons vues précédemment ne procèdent pas d'un protocole scientifique, même si elles en utilisent la méthode. Elles s'inscrivent dans un décalage critique[16] qui engage des possibles non nécessairement induits par les modèles dans le projet scientifique. Et c'est bien en cela qu'il s'agit d'œuvres d'art.

15 Louis Bec, «La vie artificielle sous tension», In Biomediale. Contemporary Society and Genomic Culture. Edited and curated by Dmitry Bulatov. The National Centre for Contemporary art (Kaliningrad branch, Russia), The National Publishing House "Yantarny Skaz": Kaliningrad, 2004, version française fournie par l'auteur.

16 Le terme est à prendre ici dans son sens d' «examen objectif».

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Xavier Lambert (Toulouse, Occitanie, France, 1952)

Intérêt depuis le début des années quatre-vingt-dix à la création artistique dans ses rapports aux technologies actuelles en tant que chercheur et en tant qu'artiste.

Participation en tant qu'artiste à plusieurs festivals nationaux et internationaux (ISEA 2000, ISEA 2002, Vidéoformes, Traverses vidéo, ...) avec présentation des œuvres numériques interactives. Une pratique artistique qui a conduit à s'interroger sur la façon dont les outils numériques pouvaient contribuer à définir de nouvelles postures quant au rapport qui lie l'artiste à son œuvre, notamment du fait de la dimension processuelle qui caractérise ce type de productions. Les développements des processus génératifs dans l'outil numérique ont contribué à élargir la recherche autour du concept de « paradigme cybernétique » comme paradigme induisant de nouveaux rapports au monde, de manière générale, et donc à la création artistique, prise dans sa dimension poïétique, en particulier.

C'est dans cette perspective que la recherche s'est orientée vers la question des systèmes en tant que processus et qu'outil conceptuel référent à un nombre de plus en plus important de pratiques dans le champ de l'art contemporain, celui des arts plastiques en particulier. Le domaine d'étude

recouvre en particulier les pratiques liées aux technologies contemporaines, qu'elles ressortissent aux technologies du numériques, aux bio-technologies, etc., en articulation avec un domaine théorique qui couvre des auteurs comme Couchot, Deleuze, Schaeffer, Goodman, Berthoz, Varela, … et, plus récemment mais dans le prolongement, sur la façon dont on peut approcher certains processus poïétiques à partir des sciences de la cognition, à partir d'une pratique sur une certaine forme de dessin automatique. Ce qui a donné lieu à la mise en place d'un programme de recherche avec des chercheurs en Intelligence Artificielle et en Sciences de la cognition et du mouvement.

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editora