UNIVERSIDADE FEDERAL DO PARANÁ

TAINÁ WILKE SIVEK

AVANÇOS NO DESENVOLVIMENTO DE SISTEMAS AUTOMATIZADOS PARA TESTES (ECO)TOXICOLÓGICOS: UMA REVISÃO BIBLIOGRÁFICA

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Monografia apresentada no Curso de Ciências Biológicas, como requisito parcial à obtenção do título de Bacharel em Ciências Biológicas, Departamento de Genética, Universidade Federal do Paraná.

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RESUMO

Continuamente, cresce a demanda pelo desenvolvimento de métodos alternativos ao uso de animais para avaliar segurança, para humanos (Toxicologia) e para o ambiente (Ecotoxicologia), de produtos e ingredientes. Dentre os tipos de métodos, é possível destacar os *in vitro*, que podem substituir totalmente a utilização de animais e que vêm evoluindo constantemente, com resultados mais significativos. Essa evolução se dá, principalmente, pela utilização de métodos tridimensionais (3D), como esferoides celulares, que, diferente dos métodos usualmente aplicados (bidimensionais, 2D), apresentam maior relevância metabólica, se assemelhando ao observado *in vivo*. Entretanto, o aumento de atividade metabólica pode apresentar desvantagens no cultivo de esferoides em ambiente estático, pois não há entrada de oxigênio e nutrientes, enquanto ocorre acúmulo de metabólitos. Para suprir essa desvantagem, é possível inserir os esferoides em sistemas automatizados, fluidos, o que permite constante renovação de meio. Contudo, não há indícios de desenvolvimento de uma revisão para avaliar a evolução da utilização desse tipo de metodologia para testes (eco)toxicológicos. Assim sendo, o objetivo do presente estudo foi realizar uma criteriosa revisão da literatura sobre sistemas de cultivo automatizados para esferoides celulares para estudos (eco)toxicológicos, de forma a mostrar os avanços, desafios e lacunas, principalmente para a área de estudos de ecotoxicidade. Utilizando bases de dados on-line, foram realizadas pesquisas para identificar artigos de estudos que utilizaram esferoides celulares, de células humanas e de peixes, em sistemas automatizados para ensaios toxicológicos. Adicionalmente, pesquisou-se também o desenvolvimento da construção de esferoides de peixes. O resultado mais surpreendente foi a ausência de estudos com esferoides de peixes em sistemas automatizados. Com isso, discutiu-se amplamente os atuais avanços com esferoides de peixe, além da possibilidade de utilizá-los em sistemas automatizados para células humanas encontrados na pesquisa, com indicações de possíveis adaptações. Concluiu-se que os sistemas automatizados para esferoides humanos podem ser utilizados com pouca ou nenhuma adaptação com esferoides de peixes. Espera-se que a revisão aqui apresentada contribua para o futuro desenvolvimento de sistemas automatizados para esferoides de peixes, bem como novos sistemas para esferoides humanos, para posteriormente serem acrescentados em regulamentações, como métodos a serem utilizados para avaliação de risco, como sistemas fisiologicamente relevantes.

Palavras-chave: Sistemas Automatizados. Esferoides Celulares de Peixes. Esferoides Celulares Humanos. (Eco)Toxicologia.

ABSTRACT

Continuously, the demand grows for developing alternative methods to use animals to evaluate the safety for humans (Toxicology) and the environment (Ecotoxicology) of products and ingredients. Among the types of methods, it is possible to highlight the *in vitro* ones, which can totally replace animal use and have constantly been evolving with the most significant results. This evolution is mainly due to the use of three-dimensional (3D) methods, such as cellular spheroids, which, unlike the methods usually applied (two-dimensional, 2D), have greater metabolic relevance, resembling what is observed *in vivo*. However, the increase in metabolic activity may have disadvantages in cultivating spheroids in a static environment, as there is no entry of oxygen and nutrients, while the accumulation of metabolites occurs. To overcome this disadvantage, it is possible to insert the spheroids in automated, fluid systems, which allows constant renewal of the medium. Although, there are no indications of the development of a review to assess the evolution of this type of methodology for (eco)toxicological tests. Therefore, the present study's objective was to conduct a thoughtful review of the literature on automated systems for cell spheroid cultivation for (eco)toxicological studies to show the advances, challenges and gaps, mainly in the area of ecotoxicity studies. Using online databases, a research was carried out to identify articles with studies that used cell spheroids, from human and fish cells, in automated systems for toxicological assays. Additionally, the development of the construction of fish spheroids was also assessed. The most surprising result was the absence of studies with fish spheroids in automated systems. With that, the current advances with fish spheroids were widely discussed, in addition to the possibility of using them in automated systems for human cells found in the research, with indications of possible adaptations. It was concluded that automated systems for human spheroids could be used with little or no adaptation for fish spheroids. It is expected that the review presented here will contribute to the future development of automated systems for fish spheroids, as for new systems for human spheroids, to later be added to regulations, as methods to be used for risk assessment, using physiologically relevant systems.

Keywords: Automated Systems. Fish Cell Spheroids. Human Cell Spheroids. (Eco)Toxicology.

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1 INTRODUÇÃO

A crescente preocupação em relação à associação de efeitos adversos, causados pela exposição química a seres vivos, tem resultado na busca por produtos mais seguros, tanto do ponto de vista ambiental como para saúde humana (CRAWFORD et al., 2017). No âmbito da saúde humana, uma das primeiras etapas de avaliação da segurança de produtos compreende a realização de testes toxicológicos. Inicialmente, esses testes eram conduzidos em animais, principalmente em ratos, camundongos e não-roedores (CRAWFORD et al., 2017; PRIDGEON et al., 2018). Entretanto, diferenças entre os efeitos observados nesses organismos e os possíveis efeitos em seres humanos (PRIDGEON et al., 2018), além da preocupação com o bem-estar animal, resultaram em uma série de medidas de substituição ou redução de testes com animais (BALLS, 2013; TANNENBAUM; BENNETT, 2015). Assim, testes com animais estão constantemente perdendo espaço na avaliação da segurança de produtos (ARAÚJO et al., 2014). Tal fato tem resultado em grandes incentivos de pesquisa na área de métodos alternativos, a qual é regida pelo princípio dos 3 R's (RUSSELL; BURCH, 1960), que determinam as regras dos métodos de substituição, redução e refinamento a testes com animais (do inglês *Replacement, Reducement, Refinement*).

Ações atuais da área de métodos alternativos não se restringem apenas à Toxicologia e incentivos para o desenvolvimento de métodos alternativos para testes de ecotoxicidade têm sido verificados, como forma de contribuir para a melhor compreensão dos impactos ambientais de substâncias químicas e no processo de sustentabilidade (CRAWFORD et al., 2017). Ainda assim, em comparação com a Toxicologia, os avanços são moderados, principalmente em relação aos desenvolvimentos de métodos *in vitro* (LILLICRAP et al., 2016; SCHOLZ et al., 2013), apesar da alta demanda desses testes, por serem utilizados não somente na avaliação de substâncias, em toxicidades aguda e crônica, mas também de efluentes (SCHOLZ et al., 2013), o que gera alta utilização de vertebrados (BURDEN et al., 2016). Para estudos de ecotoxicidade aquática, os peixes são amplamente utilizados, pois estão, virtualmente, em todos os ambientes aquáticos, percorrendo diferentes níveis tróficos, o que afeta a movimentação de substâncias, e tendo alta importância ecológica (KROON; STRETEN; HARRIES, 2017; VAN DER OOST; BEYER; VERMEULEN, 2003). Entretanto, o objetivo principal das análises é a comparação de efeitos tóxicos de substâncias em diferentes concentrações, em variados organismos,

sem considerar os possíveis modos de ação (HUTCHINSON et al., 2016). Assim sendo, é importante desenvolver métodos alternativos ao uso desses animais, o que é mais observado na área de análise de toxicidade aguda a peixes (BURDEN et al., 2016), o que permite redução do número de animais utilizados (NORBERG-KING et al., 2018) e aumento no número de substâncias avaliadas em um menor período de tempo (BURDEN et al., 2016). Ademais, a utilização de métodos *in vitro* e *in silico* pode contribuir para superar a limitação supramencionada referente ao estudo de modos de ação (HUTCHINSON et al., 2016).

Particularmente aos métodos *in vitro*, a maioria utiliza sistema de cultivo celular bidimensional (2D – células em monocamadas ou em suspensão) (CERIMI et al., 2022; JEONG et al., 2016). No entanto, dependo do tipo de efeito de toxicidade a ser analisado, sistemas de cultivo 2D podem apresentar limitações de similaridade com organismos vivos, frente à complexidade celular, estrutural e fisiológicas dos mesmos (DE SOUZA et al., 2021; JEONG et al., 2016; PRIDGEON et al., 2018). Além disso, em geral, estes sistemas celulares são cultivados na forma estática (sem fluxo de meio, em uma condição estável), que pode resultar em uma condição fisiológica não representativa da condição *in vivo* (ASHAMMAKHI et al., 2020; AZIZIPOUR et al., 2020; KHALIL; JAENISCH; MOONEY, 2020). Desta forma, modelos fisiologicamente relevantes têm sido desenvolvidos e muitos são baseados em técnicas de cultivo celular tridimensional, 3D (DE SOUZA et al., 2021; GUPTA et al., 2019; KAMMERER, 2021; PRIDGEON et al., 2018).

Um exemplo de sistema celular 3D amplamente utilizado é o esferoide. Esferoide celular consiste em uma esfera de células, formada sem a necessidade de dispositivo específico, apenas condições apropriadas, com inibição da possibilidade de ligação com a superfície (PRIDGEON et al., 2018), pela agregação das células, devido à matriz extracelular secretada pelas próprias (BHISE et al., 2016). Conforme o objetivo de aplicação do esferoide, ele pode ser construído em diferentes tamanhos, o que afeta a distribuição de oxigênio e nutrientes. Esferoides menores, utilizados para representar tecidos saudáveis, têm células saudáveis por toda sua extensão (BHISE et al., 2016; DE SOUZA et al., 2021; LAMMEL et al., 2019; LANGAN et al., 2018), enquanto esferoides maiores são utilizados em estudos de tumores, por permitirem a formação de um núcleo necrótico, o que se assemelha com a realidade tumoral (CABALLERO et al., 2017; DENG et al., 2019; MONTANEZ-SAURI; BEEBE; SUNG, 2015; MOSHKSAYAN et al., 2018). Em geral, os esferoides apresentam atividades

metabólicas maiores que culturas 2D (JEONG et al., 2016; LAMMEL et al., 2019), além de maiores sensibilidade e especificidade a diferentes substâncias (PRIDGEON et al., 2018). Ainda é possível destacar que é possível construir esferoides com mais de um tipo celular, em cocultura, para melhor reproduzir a realidade dos animais (LIN et al., 2020; MARIN et al., 2019; MOSHKSAYAN et al., 2018).

Os esferoides podem ser mantidos em cultivo por um período maior que as células cultivadas em monocamadas (FLOURIOT et al., 1993; LILLICRAP et al., 2016), podendo apresentar, inclusive, resultados mais satisfatórios após um maior período de tempo (CRAVEDI et al., 1996). No entanto, a manutenção a longo prazo de culturas de esferoides é desafiadora, pois o metabolismo aumentado de esferoides em relação as células 2D resultam em uma maior quantidade de metabólitos tóxicos, que, se não removidos com certa frequência, causam danos celulares (LIN et al., 2020; MOSHKSAYAN et al., 2018).

Como forma de contornar os problemas supracitados, diferentes dispositivos de cultivo celular 3D têm sido desenvolvidos. Dentre eles, os sistemas fluídicos são excelente opção (MARIN et al., 2019; PRIDGEON et al., 2018), pois, além de possibilitarem a realização de trocas constantes de meio, contribuem para a redução das manipulações das culturas pelo operador, proporcionando um sistema automatizado de cultivo (ASHAMMAKHI et al., 2020; MARX et al., 2016; MOSHKSAYAN et al., 2018). Devido à troca de meio no sistema, com constante renovação de nutrientes e oxigênio e remoção de metabólitos, os esferoides podem ser mantidos em cultura por mais tempo (BHISE et al., 2016; MOSHKSAYAN et al., 2018). Além disso, o fluxo de meio proporcionado pelos sistemas fluídicos pode proporcionar um microambiente *in vitro* com condições fisiológicas mais semelhantes ao *in vivo*, aumentando ainda mais a capacidade metabólica dos esferoides (BHATIA; INGBER, 2014; ESCH; BAHINSKI; HUH, 2015). Ainda simula a distribuição de substâncias em estudos toxicológicos (ELLIOTT; YUAN, 2011; SHARIFI; FIROOZABADI; FIROOZBAKHSH, 2019), em diferentes formas de absorção (MARIN et al., 2019). Outra vantagem dos sistemas fluídicos, relacionada ao estudo toxicológico, é a possibilidade de se realizar estudos de dose repetida e de toxicidade crônica (BOVARD et al., 2018; LIN et al., 2020; TOSTÕES et al., 2012).

Os sistemas fluídicos ainda permitem o cultivo de células de origens variadas, em esferoides ou em culturas biologicamente relevantes para o tipo celular avaliado, para representar diferentes tecidos. As respostas são obtidas em conjunto e de forma

sistêmica (MARIN et al., 2019; RAJAN et al., 2020), podendo alterar os efeitos obtidos em culturas isoladas, como estudado por BOVARD et al., 2018, em que os efeitos tóxicos da aflatoxina B1 foram reduzidos em células pulmonares cultivadas em sistema ar-líquido presentes em sistema fluídico conectado a esferoides hepáticos quando comparado ao sistema isolado. Os resultados obtidos indicam a importância e a necessidade de se desenvolver sistemas que mimetizem melhor os efeitos tóxicos observados *in vivo*, tanto para melhor compreensão quanto para futuras aplicações na triagem e avaliação de substâncias (CERIMI et al., 2022; CIDEM et al., 2020; MARX et al., 2016).

Considerando a relevância fisiológica de sistemas compostos por esferoides e fluidez, é importante saber o estado da arte desses modelos para estudos voltados à saúde humana e avaliações ambientais, principalmente ao se considerar que, nas análises ecotoxicológicas, os modos de ação acabam não sendo estudados, como abordado anteriormente. Sistemas automatizados, em conjunto com esferoides celulares, podem ser amplamente utilizados para determinar modos de ação e efeitos finais de substâncias, apresentando maior semelhança fisiológica com organismos, podendo ser aplicados em triagem de seus efeitos (eco)toxicológicos. Portanto, é necessário também identificar as lacunas observadas nos estudos fisiologicamente relevantes com esferoides de células de peixes, quando se compara com pesquisas utilizando células humanas e discutir possíveis movimentos direcionados a suprir essas limitações.

2 OBJETIVOS

O objetivo geral do presente trabalho foi mostrar os avanços, desafios e lacunas, principalmente para a área de estudos de ecotoxicidade aquática (com células de peixe), no desenvolvimento de sistemas de cultivo automatizados para esferoides celulares em estudos (eco)toxicológicos, por meio de uma criteriosa revisão da literatura.

3 DESENVOLVIMENTO

Este trabalho de monografia foi desenvolvido considerando apresentar como produto final uma revisão crítica da literatura sobre sistemas de cultivo automatizados para culturas de esferoides celulares. A revisão foi desenvolvida com a finalidade de reunir e comparar os avanços na área dos modelos empregados na área de Toxicologia (saúde humana) em relação à Ecotoxicologia (ambiental). Para tal, foi dividida, para pesquisa, em sessões que compreendem tópicos de utilização de sistemas automatizados no cultivo de esferoides de células de peixes, avanço no desenvolvimento de esferoides utilizando células (primárias ou linhagem) de peixes e utilização de sistemas automatizados no cultivo de esferoides de células humanas (primárias ou linhagem, de células saudáveis ou tumorais).

Sendo assim, as seções seguintes (metodologia, resultados, discussão, bem como a própria revisão da literatura) serão apresentadas na forma de manuscrito científico, redigido em inglês, que será posteriormente submetido a revista internacional indexada “Environmental Health Perspectives”.

4 AUTOMATED SYSTEMS FOR CULTURING CELLULAR SPHEROIDS: A COMPARISON BETWEEN SYSTEMS FOR HUMAN AND FISH MODELS

4.1 ABSTRACT

Background. Pressures related to developing new approach methodologies (NAMs) for toxicologic assessment are growing to reduce animal use and improve the results' reliability. For that, spheroids are viable three-dimensional (3D) *in vitro* models to better mimic *in vivo* responses. However, due to its increased metabolism, static systems can be toxic, reducing the time of culture. To overcome that, automated systems are a great option that better emulate animals' physiology and increase experimental advantages.

Objectives. This scoping review approaches the development of fish and human spheroids and their utilization in automated systems, the gaps related to ecotoxicology and assess possible adaptations to methods that can be used for (eco)toxicologic evaluations.

Design. Online databases were used to identify papers, with no time restriction, that used fish spheroids on toxicologic assays, on or off automated systems, and human spheroids on automated systems to assess toxic effects.

Results. No study with fish spheroid on automated systems was retrieved. Even with more filters related to human research, it was possible to find more articles with human spheroids on automated systems (33) than with fish spheroids for toxicologic tests (23). The methods for fish spheroid construction were first used for human spheroids. Research for automated systems with human spheroids already includes more than one system to replicate human physiology better.

Conclusion. As it was for the spheroid construction, it is possible to use the methods for human spheroid cultivation on automated systems for fish spheroids with little or no adaptation. Hopefully, this review will contribute to future developments of automated systems for fish spheroids and improvement for human spheroids.

Keywords: Automated systems; fish spheroids; human spheroids; NAMs

4.2 INTRODUCTION

In Green Toxicology (Crawford et al. 2017), toxicity evaluation of chemical substances is extremely important to identify the less harmful ones and, consequently, develop safe products for human health (Pridgeon et al. 2018) and the environment (Lillicrap et al. 2016). However, the toxicity evaluation needs to be performed in the early stages of product development. The conventional strategy to assess chemical toxicity is primarily fundamental in animal tests (*in vivo*), which are time-consuming and

costly, posing challenges to selecting safer chemical substances for green products. Fortunately, toxicity tests have gradually changed in the past years with the publication of Russell and Burch's book (The Principles of Humane Experimental Technique) in 1959 (Tannenbaum and Bennett 2015), which was written based on Charles Hume's concerns about animal welfare (Balls 2013). The book proposed applying the 3R principle (*i.e.*, Replacement, Reduction and Refinement of animal testing), thus moving forward the development of New Approach Methodologies (NAMs) for toxicity testing.

NAMs proposed are related to promoting the development of methods replacing animals, increasing their velocity and accuracy, and contributing with data for risk assessment (Parish et al. 2020). NAMs development is primarily observed in Toxicology, an area that accounts for the use of a substantial number of animals (mammalian models). Additionally, increasing progress in NAMs for Toxicology relates failing of animal models to predict effects on humans due to species-specific differences (Pridgeon et al. 2018). However, besides assessing effects on human health, chemical substances need to be evaluated regarding their hazards and risks to the environment, and within this context, a substantial number of non-mammalian vertebrates' models (*e.g.*, fish) are used. In aquatic toxicity, fish are used to evaluate acute and chronic toxicity of chemical substances or environmental samples (*e.g.*, effluents) and for bioaccumulation studies (Scholz et al. 2013). Thus, fish are the most used vertebrate in ecotoxicity studies (Burden et al. 2016) and the second group of animals most used for regulatory and scientific purposes.

Under these circumstances, efforts have been made to develop *in silico* and *in vitro* methods (NAMs) for aquatic toxicity with the central goal of improving environmental protection actions and reducing the number of fish in ecotoxicity studies. Particularly with the *in vitro* methods for ecotoxicity, although more than 850 fish cell lines are registered (with cell line repeats, depending on different information updates) on Cellosaurus (a global repository that summarizes the number of cell lines from various species, https://web.expasy.org/cellosaurus/) (Bairoch 2018), few *in vitro* methods for fish toxicity with regulatory acceptance are available today. The OECD TG 249 describes using the RTgill-W1 (Rainbow trout gill) cell line to assess acute toxicity (OECD 2021). Specifically, to the OECD TG 319A, this method is performed with primary fish hepatocytes (OECD 2018); thus, it is not a complete replacement method, which keeps the perspective of developing animal-free methods, especially for cosmetic industries. Three-dimensional (3D) culture systems of continuous piscine

cell lines may have great applicability as alternative tests for obtaining qualitative and quantitative information on the effects of chemicals in fish, especially for parameters of biotransformation, bioaccumulation and chronic toxicity (Lammel et al. 2019).

Conventional *in vitro* tests are usually performed with cell monolayer cultures (or two-dimensional (2D) cell cultures); however, 2D cell cultures may not reproduce the physiological condition required for certain types of evaluations (Pridgeon et al. 2018). Thus, 3D cell cultures have taken place to overcome such problems, better reproducing the complexity of an organ. In general, the cellular organization and physiology in 3D cell cultures better resemble the *in vivo* counterparts; and cell polarization, cell-cell and cell-microenvironment interactions, lumen formation, reduced proliferation, increased differentiation, and changes in RNA and protein expression are usually verified in this type of culture system (Pridgeon et al. 2018).

Among the 3D cell culture models, the spheroid is one of the models proposed to overcome the limitations of 2D models (Jeong et al. 2016), and the application of this type of cell culture has been verified in both contexts of toxicity studies, human health and the environment. Spheroids can be formed spontaneously under appropriate conditions (Pridgeon et al. 2018), generating an extracellular matrix (Jeong et al. 2016), and several efficient methods to construct spheroids have been developed in the past years (Bhise et al. 2014). Spheroids may present better cell differentiation phenotype and function, higher metabolic capacity, and morphological characteristics better resemble *in vivo* tissue/organ (Baron et al. 2012; de Souza et al. 2021; Jeong et al. 2016; Lammel et al. 2019; Pridgeon et al. 2018).

Considering their advantages, spheroids can be used in different assays to study human toxicity, with non-tumoral and tumoral human cells (Moshksayan et al. 2018) or cells from other mammals (Baron et al. 2012). For environmental health, with a focus on aquatic and fish toxicology, advances in the use of spheroids are more recent (de Souza et al. 2021). The spheroids can be formed with primary cell lines (Baron et al. 2012; Cravedi et al. 1996; Flouriot et al. 1993) or with immortalized cell lines from different organs of various fish species (de Souza et al. 2021; Faber et al. 2021; Jeong et al. 2016; Lammel et al. 2019). Although spheroids present several advantages over 2D cell cultures, challenges in using these models are specially related to their maintenance in culture.

Spheroids cultured under static conditions can present limitations regarding the transport of nutrients, culture oxygenation and waste elimination, which is limited by

diffusion, leading to the accumulation of toxic metabolites (Bhatia and Ingber 2014). These limitations may impair cell responses; however, automated systems can be used to overcome problems caused by static cultures of spheroids (Clarke et al. 2021). The main advantage of automated systems, such as chips, is the increased control of experiments and flow. In addition, these devices are developed to be used with different types of cells in single cultures or co-cultures and can support various environments (Bhatia and Ingber 2014; Moshksayan et al. 2018). Other advantages of automated systems are the possibility of forming chemical concentration gradients and requiring fewer reagents. Also, automated systems work on continuous perfusion, enabling long-term experiments with adequate viability (Moshksayan et al. 2018). An automated system can be combined with a spheroid model, allowing medium flow and maintaining a viable spheroid (Feng et al. 2020) that can be confined into a barrier to avoid its loss when the fluid is exchanged (Ching et al. 2021).

There are some examples of reviews with content similar to the explored here (exemplifying studies with spheroids and/or with automated systems): general ecotoxicology tests (Lillicrap et al. 2016); application of fish cell lines (Goswami et al. 2022); specific studies about hepatic cells from fish (Segner 1998); about endocrine disruption in fish (Navas and Segner 2006); drug testing (Khetani et al. 2015; Kunz-Schughart et al. 2004; Marx et al. 2016); hepatotoxicity (Kyffin et al. 2018); cancer studies (Asghar et al. 2015; Caballero et al. 2017); neurodegenerative disease research (Rocha et al. 2016); application of alternative methods in reproduction studies (Arck 2019); examples of different automated systems with various spheroids, for multiple applications (Osaki et al. 2018). Nevertheless, there is no review comparing the development of methods for cultivation on automated systems for cell spheroids for fish and human toxicologic studies. Furthermore, no study analyzes the possible adaptations for automated systems of human spheroids cultivation to be done for fish spheroids.

A scoping review was carried out to compare studies with cell spheroids for human and aquatic environmental (using fish cells) on automated systems and assess the gaps and delays in environmental health research. It was not our goal to draw conclusions about the quality of the studies in the sense of selecting one or more as the best. Our objective was to identify how progress is being made. In addition, we made recommendations for further development of methods for fish toxicologic research.

4.3 METHODS

The present scoping review was conducted following the reporting guidelines from Preferred Reporting Items for Systematic reviews and Meta-Analyses for Scoping Reviews (PRISMA-ScR) (Tricco et al. 2018).

4.3.1 Data Sources and Search Strategy

To identify relevant studies, the database used were Science Direct, PubMed (and PubMed Central) and Google Scholar, and the search was supplemented by scanning relevant articles and abstracts of events for fish cell(s) spheroids and their combinations and Science Direct, PubMed and PubMed Central for human cell(s) spheroid combination.

The search strategy included the following keywords: “fish cell(s) spheroid”, and the combination with “automated system”, “fluidic” and “perfusion”; “human cell(s) spheroid toxicology automated system”, “human cell(s) spheroid toxicology fluidic” and “human cell(s) spheroid toxicology perfusion”. The last searches were performed on 08 August 2022.

For Science Direct, the search was refined for Research and Review Articles and Scholar Google, it was not included Citations and Patents.

Only the selected articles were de-duplicated after inclusion.

4.3.2 Study Selection

Titles, abstracts and methodologies of the studies identified by electronic searches were screened by one reviewer (author T.W.S), and the full articles that met eligibility criteria were accessed.

The papers were included if they were written in English; used fish cell spheroids in a static system to evaluate the toxicological effects of chemicals; developed or applied different types of automated systems to maintain spheroids (from fish or human cells) to evaluate toxic effects of chemicals; applied the principles replacement or reduction of the 3Rs principle; constructed spheroids to applications different from (eco)toxicology but mentioned it as a possibility of use; research and review articles were considered.

The papers were excluded if they also had experiments conducted *in vivo*, even if the principle of refinement was considered; used only other mammal cell types to construct the spheroid (even for studies on human health); human cell spheroids were

not maintained on an automated system; did not even mention (eco)toxicology as a possible application.

Books, chapters of books, academic theses and dissertations and reports found by the database were also excluded. Time restriction was not applied. Using both cell lines or primary cells to construct spheroids was considered. Methodologies were analyzed to avoid screening articles that used animals and/or cells from organisms other than fish and humans.

4.4 RESULTS

4.4.1 Search Results for Fish Cell Spheroids

The search, considering both “fish cell spheroid” and “fish cells spheroid” of the four databases, with repetition and overlap of studies, resulted in 557 papers, with an additional five potential relevant from references of the analyzed papers, totalizing 562 papers, where 150 were potentially relevant. After the removal of copies, 67 studies were accessed for eligibility.

Of the 67 analyzed studies of “fish cell(s) spheroid”, 23 met the eligibility criteria (Figure 1), as detailed in Table 1.

Figure 1: Flow diagram of the sources of evidence and exclusion criteria, as described by Tricco et al. 2018, for the “fish cell(s) spheroid” search.

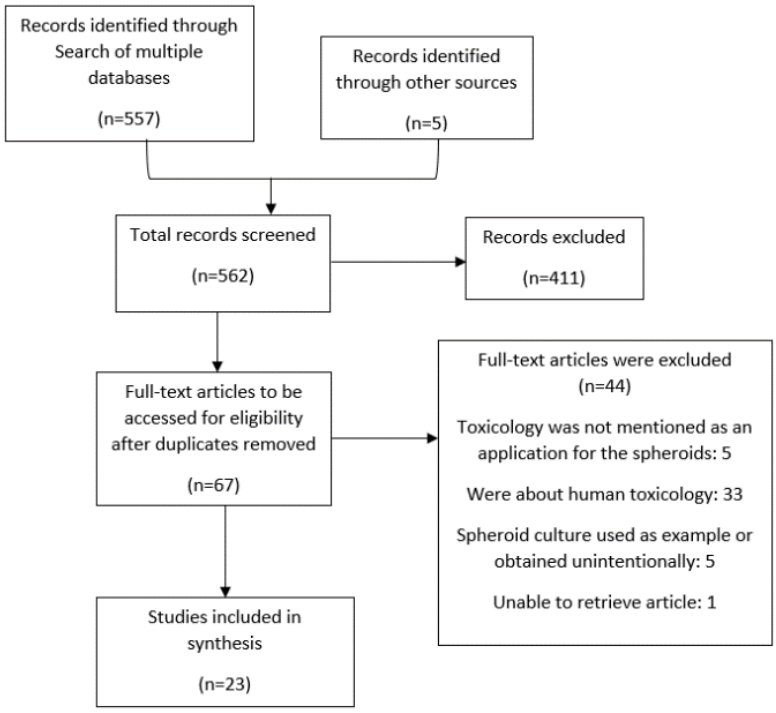


Table 1: Key results from studies that mentioned toxicology can be an application for the fish spheroid model they constructed.

Author	Type of Article	Cells used to construct spheroid	Brief explanation	Main conclusions	Application
(Flouriot et al. 1993)	Research	Rainbow trout primary hepatocytes	Comparison of vitellogenin and estrogen receptor RNA expression on spheroids and monolayers cultures	The monolayer was maintained for five days, with expression level lower than *in vivo*. Spheroids were maintained for one month (first-time long-term culture of trout hepatocytes) with expression levels similar to *in vivo*	mRNA expression of liver hormones (can be used in toxicology)
(Flouriot et al. 1995a)	Research	Rainbow trout primary hepatocytes	Comparison of spheroids and monolayer culture of hepatocytes to evaluate biotransformation enzymes through time	Hepatocyte spheroids culture is a promising system to study biotransformation pathways	Toxicology
(Flouriot et al. 1995b)	Research	Rainbow trout primary hepatocytes	Application of the system from Flouriot et al. 1993. Effects of xenobiotics on the expression of vitellogenin and estrogen receptor	Spheroids culture is a positive model to study vitellogenesis. (anti)estrogenic activities can be easily determined	Toxicology
(Cravedi et al. 1996)	Research	Primary hepatocytes of rainbow trout	Complementary study of (Flouriot et al. 1995a)	Long-term (30 days) maintenance of biotransformation enzymes for fish hepatocytes (spheroids). Better results after 30 days than after five days	Toxicology
(Pesonen and Andersson 1997)	Review	Primary hepatocytes	Promising culture model	Higher enzymes activities for a longer time (1 month)	Toxicology
(Segner 1998)	Review	Primary hepatocytes (Cravedi et al. 1996; Flouriot et al. 1993)	Alternative to monolayer culture for long-term	Prolonged survival and increased physiology. The system should be more explored	Toxicology
(Jung'a et al. 2005)	Research	Tilapia primary hepatocytes	Mixed monolayer and spheroid cultures to evaluate the	The system was maintained for three weeks, which means it is a suitable model. The	Stress response (can be used to

			production of albumin	ultrastructure of hepatocytes was consistent with *in vivo*	access toxicology)
(Navas and Segner 2006)	Review	Primary hepatocytes (Flouriot et al. 1993; Jobling and Sumpter 1993; Latonnelle et al. 2000; Pelissero et al. 1993)	Describe and discuss the advances in the studies that use vitellogenin as a marker to evaluate endocrine disruption.	The authors mention that the three-dimensional model is more similar to *in vivo* situations, expressing higher levels of vitellogenin when compared with the 2D model. Moreover, it can be maintained for longer periods	Toxicology
(Servili et al. 2009)	Research	SBB-W1 (neural cells from sea bass)	Formation of neurosphere as part of the characterization of the cell line.	The cells could form spheroids. And expressed nestin, characterizing the cells as stem, which is lost in monolayer	Toxicology and physiology (suggestion)
(Vo et al. 2011)	Research	ASP309 and ASP409 (pituitary of Atlantic salmon)	Development and partial characterization of the cell lines. Spheroid formation was one of the experiments.	ASP309 formed a better-packed spheroid, while ASP409 failed to	Mechanistic, endocrinology, toxicology, fish nutrition (suggestions)
(Baron et al. 2012)	Research	Rainbow trout primary hepatocytes	Construction on constant rotation (for 6-8 days). They were analyzed morphologically and biochemically	Sizes of spheroids had high variability, but biochemical markers showed that the spheroids provided realistic responses. A long time of maintenance (40 days) is possibly applied to study chronic toxicity and bio-accumulation	Toxicology
(Uchea et al. 2013)	Research	Rainbow trout primary hepatocytes	Metabolic assessment of the spheroids after their maturation and comparison whit S9 protein	The use of spheroids can increase the predictability of the metabolization and bioaccumulation of chemicals and it is more similar to *in vivo* conditions than S9	Toxicology
(Bury et al. 2014)	Review	Fish Hepatocytes (primary) (Baron et al. 2012)	Described the spheroids as recent (by the time) advance	The long-time viability of spheroids helps study metabolism. It can be combined with other techniques	Toxicology

(Uchea et al. 2015)	Research	Rainbow trout primary hepatocytes	Assessment of xenobiotic metabolism and efflux transportation through gene expression	Increased expression levels on spheroids than in monolayer culture. Better possibilities of application for different studies related to environmental health. More mature spheroids were more similar to *in vivo*	Toxicology
(Langan et al. 2016)	Research	RTG-2 (rainbow trout gonad)	Evaluation of the oxygenation of spheroids, according to their size	Smaller spheroids allow better control of stress, increasing the accuracy of response	Toxicology and biomedicine
(Lillicrap et al. 2016)	Review	Fish Hepatocytes (primary) (Baron et al. 2012)	Spheroids as a refinement of monoculture	Spheroids can be maintained for a long time to study metabolism	Toxicology
(Baron et al. 2017)	Research	Rainbow trout primary hepatocytes	Test of pharmaceuticals to access metabolization and clearance	Spheroids can be used as metabolically capable systems. The first time metabolic clearance was demonstrated in a 3D trout model	Toxicology
(Rodd et al. 2017)	Research	PLHC-1 (clearfin livebearer hepatocellular carcinoma)	Spheroids were constructed using a mold to evaluate the expression of metabolic enzyme	The 3D model remained available for longer and expressed a higher basal level of enzyme than the 2D model, showing that it is a sensitive system	Toxicology
(Langan et al. 2018)	Research	RTgutGC (rainbow trout gastrointestinal)	Construction of spheroids with different sizes (cell densities) to evaluate if it affects the metabolism	Smaller spheroids presented better metabolic responses. Bigger spheroids presented bigger hypoxic zones	Toxicology
(Hultman et al. 2019)	Research	Rainbow trout primary hepatocytes	Biotransformation of pyrene (compound highly metabolic active)	Spheroids were metabolic competent, exhibiting efficient biotransformation of pyrene	Toxicology
(Lammel et al. 2019)	Research	RTL-W1 (rainbow trout liver)	Establish a methodology to construct the spheroids with the cell line and determinate if it responds as	The authors were able to overcome the limitations of constructing the spheroids, which presented more	Toxicology

			primary hepatocytes	physiologically relevant results	
(Aarattuthodi et al. 2021)	Review	RTG-2 and RTS-11 (gonad and spleen of rainbow trout) (Faber et al. 2021)	Spheroids as an example of a 3D culture of fish cell lines	3D models are more similar to *in vivo*, which provides more reliable data	Viral infection (mentions that it can be used in other complex processes)
(de Souza et al. 2021)	Research	ZFL and ZEM2S (liver and embryo of zebrafish)	Comparison of methods to construct spheroids	Better results of orbital shaking	Future application to toxicology

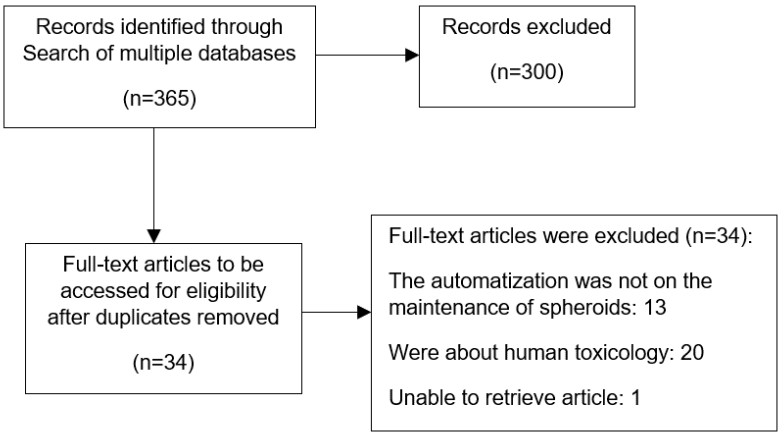
4.4.2 Search Results for Automated System with Fish Cell Spheroids

When considering automation, all the articles found were previously screened on “fish cell(s) spheroid”. That means that all articles of the three keywords (“automated system”, “fluidic” and “perfusion”) are present in the selection of the search for spheroids. Of the 558 papers previously found, 365 were results from the automated keywords (202 “automated system”, 42 “fluidic” and 121 “perfusion”). From the total 365 papers, 65 were considered potentially relevant. After the removal of copies, 34 studies were accessed for eligibility. PubMed did not present results for neither automation keywords. Therefore, only articles from Science Direct, PCM and Academic Google were evaluated for this analysis.

From the automation results, many papers were excluded after reading the methodologies because of different applications for an automated system (counting of cells, construction of spheroids and other specificities of each study) and perfusion (there is a technique called perfusion to obtain primary cells).

Of the 34 studies accessed on automation, none of them met the eligibility criteria (Figure 2). However, two reviews mentioned that both spheroids and fluidic systems are technologies to improve *in vitro* ecotoxicological tests (Lillicrap et al. 2016) and that three-dimensional models on automated systems better mimic structure and function as seen *in vivo* (Bury et al. 2014), even without examples from a spheroid of fish cells on automated systems.

Figure 2: Flow diagram of the sources of evidence and exclusion criteria, as described by Tricco et al. 2018, for the “fish cell(s) spheroid” automation search.



4.4.3 Search Results for Automated System with Human Cell Spheroids

Unlike the fish cell spheroids, it was chosen to filter the search for toxicology for human cells because of the larger quantity of studies on human health.

PubMed did not present results for neither automation keywords. Therefore, only articles from Science Direct and PCM were evaluated for this analysis. With repetition and overlap of studies, it was obtained 1302 papers from the two databases analyzed. Of them, 33 met the eligibility criteria (Figure 3) and are described in Table 2.

Figure 3: Flow diagram of the sources of evidence and exclusion criteria, as described by Tricco et al. 2018, for the “human cell(s) spheroid toxicology” automation search.

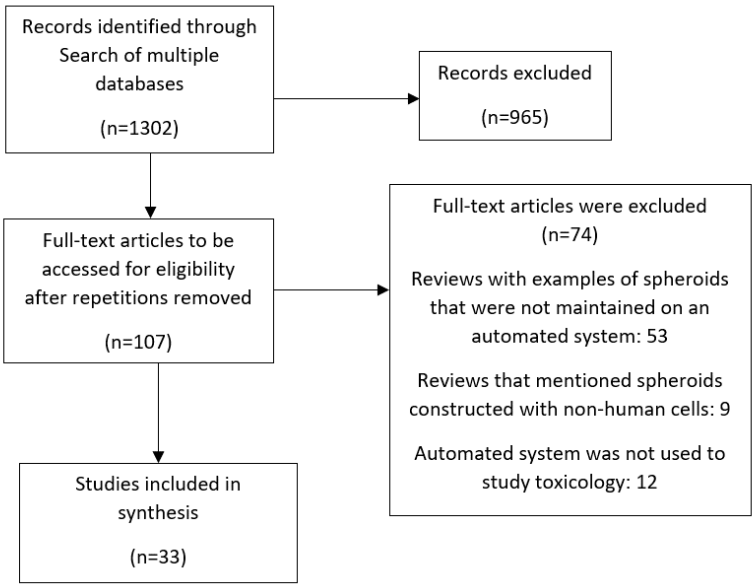


Table 2: Key results from studies that mentioned toxicology can be an application for the human spheroid model they constructed and used an automated system to maintain the spheroids.

Author	Type of Article	Cells used to construct spheroid	Brief explanation	Main conclusions	Application
(Elliott and Yuan 2011)	Review	MCF-7 (Wu et al. 2008).	Fluidic system to mimic drug delivery.	The spheroid system was more robust to determinate the IC_{50}.	Toxicology
(Bhise et al. 2014)	Review	HepaRG (Wagner et al. 2013).	Multi-organ-on-a-chip with HepaRG spheroids and skin biopsies.	It was possible to evaluate the dynamic between the liver and skin with the secretion.	Toxicology.
(Esch et al. 2015)	Review	MCF-7 (Aref et al. 2013).	Fluidic system to demonstrate its application to drug screening.	It was shown that the effective concentrations were closer to the human trials than to the 2D system.	Toxicology.
(Khetani et al. 2015)	Review	Primary human hepatocyte (Tostões et al. 2012).	Perfusion to maintain biomarkers expression.	Functions and expressions were maintained. Possibility to use the system for further analysis.	Toxicology.
(Montanez-Sauri et al. 2015)	Review	Colo205 (Agastin et al. 2011).	Fluidic and static systems compared drug resistance response.	Spheroids in fluidic systems presented more drug resistance.	Toxicology.
(Marx et al. 2016)	Review	-	Describes a platform that can be used for different spheroids without redesigning it.	It can be used for various applications, including more complexes, like metabolic interaction.	Toxicology is possible.
(Skardal et al. 2016)	Review	H2052 and primary adenocarcinoma cells (Ruppen et al. 2015).	The system was used to assess susceptibility to drugs.	Cocultures showed higher levels of chemoresistance.	Toxicology.
(Caballero et al. 2017)	Review	1. Breast tumor (Choi et al. 2015) and 2. MDA-MB-435 (Albanese et al. 2013).	1. Study of the effect of an anti-cancerous drug on metastasis (with 2D and 3D models). 2. Study of the delivery of drugs using nanoparticles.	1. The sensitivity of the 3D model was increased (the drug was more cytotoxic). 2. Alterations of the flow rate resulted in different nanoparticle accumulation.	Toxicology.
(Liu et al. 2017)	Review	HepaRG (Maschmeyer et al. 2015b).	Chip for multiple organs for long-term	Multi-organ fluidic systems can be used to	Toxicology.

			maintenance and assessment.	study different complex physiological processes.	
(Osaki et al. 2018)	Review	Multiples examples.	Chips for different applications.	-	Includes toxicology.
(Steffens et al. 2018)	Review	HepG2/C3A (Bhise et al. 2016).	Liver-on-chip.	Functionality for 30 days, toxic response, demonstrating it can be used for drug assessment.	Toxicology.
(Marin et al. 2019)	Research	HepaRG and HHsteC	The fluidic chip with co-culture analyzes the metabolism of “oral” and “intravenous” absorption.	The flow had a critical role in the metabolic response of the system. Potential to be used, with other systems, for preclinical assessment.	Toxicology.
(Deng et al. 2019)	Review	1. Primary human hepatocyte (Tostões et al. 2012), 2 and 3. HepaRG (Bovard et al. 2018; Maschmeyer et al. 2015a).	1. Spheroids in perfusion to study metabolism. 2. Co-culture with different cells to study systemic, repeated dose. 3. co-culture with lung system.	1. Expression of metabolic enzymes for 2-4 weeks. 2. 14-day performance to assess the co-cultures. 3. Detoxification by the liver protected the lung system	Toxicology.
(Oddo et al. 2019)	Review	U87 (Fan et al. 2016).	System to test drug effect on the tumor.	The system is reproducible and can potentially be used for drug screening and prolonged drug release.	Toxicology.
(Rogal et al. 2019)	Review	HepG2/C3A (Bhise et al. 2016).	Bioprint on the bioreactor.	Easy to disassemble, allowing access to the tissue.	Toxicology.
(Saglam-Metiner et al. 2019)	Review	A549 (Zuchowska et al. 2017).	Fluidic system for long-term evaluation.	The fluidic system is a valuable tool for studying anticancer therapy parameters.	Toxicology.
(Sharifi et al. 2019)	Research	HepG2.	Development of a system that can be used for drug evaluation and liver disease studies.	The results of oxygen level and spheroid size help to avoid hypoxic conditions and the system allowed metabolite transference. So it can be used to study drug effects.	Toxicology.

(Ashammakhi et al. 2020a)	Review	HepG2/C3A (Bhise et al. 2016).	Incorporation of bioprinted HepG2/C3A spheroids into a perfusable bioreactor.	The cultures were viable for 30 days. The secretion was evaluated for the culture period.	Toxicology.
(Ashammakhi et al. 2020b)	Review	Human liver (Maschmeyer et al. 2015b, 2015a).	Multi-organ-on-a-chip of liver spheroid and intestine.	A promising approach to studying the mechanism of drugs.	Toxicology.
(Azizipour et al. 2020)	Review	Neurospheroids (Kilic et al. 2016).	Comparison of the development of neurospheroids with and without flow.	Neurospheroids on the chip (with fluid flow) developed better than in the static condition.	Toxicology.
(Cidem et al. 2020)	Review	HepaRG (Bovard et al. 2018).	Lung/liver-on-a-chip to study the toxicity of an inhaled substance.	It was possible to evaluate the metabolites and the toxic profile.	Toxicology.
(Cong et al. 2020)	Review	HepG2/C3A (Bhise et al. 2016).	Liver spheroid in a chip to assess toxic effects.	Activity for 30 days, detecting markers changes.	Toxicology
(Khalil et al. 2020)	Review	1. Glioblastoma multiforme cells (Akay et al. 2018) and 2. MDA-MB-435 (Albanese et al. 2013).	Both were used to assess cancer drugs.	Both had a satisfactory response and might be further used in drug screening.	Toxicology.
(Klak et al. 2020)	Review	HepaRG and HHsteC (Lin et al. 2020).	System to multi-drug evaluation.	It was possible to obtain a satisfactory response.	Toxicology.
(Lin et al. 2020)	Research	HepaRG and HHsteC	Production of a fluidic chip to be used to assess multiple drug effects on liver-kidney co-culture.	The chip showed potential to be used in further preclinical tests.	Toxicology.
(Rajan et al. 2020)	Research	Primary hepatocytes, cardiomyocytes, A549, primary testicular cells and brain cells.	Multi-organs-on-a-chip to assess drug effects on the system.	It is more similar to *in vivo*, reproducible and can be used for preclinical screening in the future.	Toxicology.
(Kammerer 2021)	Review	HepG2.	Different examples of spheroids on fluidic systems.	All of them had better results for the automated 3D model.	Toxicology.

(Parihar et al. 2021)	Review	HepG2/C3A (Bhise et al. 2016).	Hepatic platform.	Possible to be used for preclinical assessment.	Toxicology.
(Picollet-D'hahan et al. 2021)	Review	HepaRG (Bovard et al. 2018).	Co-culture with lung system to study aerosols and chronic toxicity.	Coculture had a better response.	Toxicology.
(Serras et al. 2021)	Review	Primary hepatocytes (Tostões et al. 2012).	Repeated drug dose testing.	Maintenance of the markers contributes to determining the applicability of the system.	Toxicology.
(Cerimi et al. 2022)	Review	HepaRG (Bovard et al. 2018; Schimek et al. 2020).	Co-culture of liver spheroid and air-liquid interface lung to study mycotoxin effect.	The toxic potential was better managed in the system with the liver spheroid than in the culture without it.	Toxicology.
(Koyilot et al. 2022)	Review	Primary hepatocytes (Tostões et al. 2012).	Study liver metabolism.	The environment and functions of the liver were maintained.	Toxicology.
(Salimbeigi et al. 2022)	Review	1. HepG2/C3A (Bhise et al. 2016) and 2. HepaRG (Maschmeyer et al. 2015a).	Both are possible systems to be used in long-term studies.	1. Can be used in drug toxicity assays. 2. System capable of maintaining the co-cultures.	Toxicology.

4.5 DISCUSSION

The objectives of this scoping review were to identify the gaps related to the use of cell spheroids in automated systems for toxicologic studies for humans and fish, describe the current methodologies considerations and make recommendations for further studies, especially for fish toxicology. As it was not possible to retrieve studies using automated systems with fish spheroids, we delve deeper into the advances of studies with fish spheroids and the considerations of their characteristics for ecotoxicological application. We also discussed the possible utilization of the spheroids on the automated systems described.

4.5.1 Methods to Generate Spheroids

Spheroids can be constructed with different methods, depending on the properties of the used cells. For instance, macrophages cell lines have a better capacity to migrate and aggregate than fibroblastic cells (Faber et al. 2021), which in turn have more migratory ability than epithelial cells, which are packed and attached

to a surface tightly (de Souza et al. 2021). Besides the cell properties, there are other considerations to be taken when planning to form spheroids. First of all, the application of the spheroid is the most critical matter because it will affect all the choices of constructing a spheroid, including the cells used. Application, time of culture, size of the mature spheroid, apparatus used for the construction, culture condition and temperature are mutually affected.

Before considering all that matters in planning the spheroid construction, it is crucial to know the methods available. Here, they will be generally mentioned and the details will be explored later, individually, according to their use. Spheroids can be formed by self-assembling of cells, depending on the cell type, when attachment on the surface is prevented (Bloch et al. 2016; Xing et al. 2008). A standard method, that can be broadly applied to produce spheroids, especially with tumor cells, but also healthy cells, is the hanging drop (del Duca et al. 2004). This system consists of gravity as an external force to promote cell aggregation. Drops of the cell suspension are placed on a plate (with medium to avoid the drops from drying out) lid and the spheroids are obtained on the drop after the cultivation time (de Souza et al. 2021; del Duca et al. 2004). The most common method for fish spheroids construction is under rotation, where centripetal forces are responsible for cell aggregation (Baron et al. 2012; Faber et al. 2021). Briefly, this technique involves the cultivation of the cells on a plate or dish on an orbital shaker until the spheroids are mature (Hultman et al. 2019; Lammel et al. 2019; Langan et al. 2016). It is also possible to construct spheroids using different scaffolds (Bhise et al. 2016; Jeong et al. 2016; Napolitano et al. 2007; Timm et al. 2013). Additionally, it is feasible to promote spheroid formation already on the automated system further used for toxicity assays (Akay et al. 2018; Ruppen et al. 2015).

Now, to start the discussion about the alternatives related to spheroid construction, size is an important object because it is generated differently, depending on the choices made, and can have diverse consequences. Different parameters are related to spheroid size. The first to be mentioned is the size of the cell utilized. Mammal hepatocytes are much larger than fish hepatocytes, which generates larger mammal spheroids (Baron et al. 2012). Also, the used density of cells, where bigger densities produce larger spheroids (Langan et al. 2016, 2018) and are associated with cells size, as observed by de Souza et al. 2021, where different cells (ZFL and ZEM2S) with different densities (7000 cells and 3500 cells, respectively) resulted in spheroids

with similar sizes, after the same time of cultivation, which is the following parameter that influences on spheroid size. Spheroids keep growing while being cultivated, although depending on the cell used, the spheroid growth rate can decrease after five (de Souza et al. 2021) or seven days (Langan et al. 2018), and the size of the spheroid can be considered stable. Finally, the compactness of the cells impacts spheroid size, as observed by de Souza et al. 2021 and Langan et al. 2018. Epithelial cells, like ZFL and RTgutGC were observed to pack more tightly than fibroblastic cells, like ZEM2S and RTG-2, which their tight junctions can explain, packing the cells together and is also observed by the higher difficulty of detaching them from the flask.

The principal consideration of spheroid size is the interest in obtaining or avoiding a necrotic core. In general, ecotoxicology studies need healthy spheroids, to have better diffusion of oxygen and nutrients, while toxicology can be studied on healthy spheroids or tumor spheroids, where the necrotic core has physiological relevance. It is indicated that larger spheroids have more probability of having a necrotic core and the usual maximum diameter size described as acceptable is 150 µm (del Duca et al. 2004; Langan et al. 2016, 2018; Uchea et al. 2015). However, de Souza et al. 2021 and Jeong et al. 2016 obtained bigger spheroids (~230 µm and ~400 µm, respectively) with no indication of necrotic core and adverse effects on their function. Nevertheless, it is necessary to highlight that the absence of necrotic core related to a specific spheroid size should not be extrapolated to all cells because of the finds from Langan et al. 2016 and 2018, which states that different cell types have a variation in necrotic core proportion as the spheroid size is increased.

Another relevant consideration about spheroid size when studying (eco)toxicology is the increased metabolic rate of smaller spheroids, as studied by Hultman et al. 2019. There are three significant propositions related to this effect. The first is about the decreased diffusion rate of oxygen, which generates an increase in spheroid stress (Hultman et al. 2019; Langan et al. 2018); the second mentions the surface area, which is more extensive in smaller spheroids; and the third is about the lag time for the compound to access the biotransformation site on larger spheroids (Hultman et al. 2019). However, independent of the reasons, it is essential to mention the necessity of decreasing the heterogenicity and variability of sizes and shapes by increasing the standardization (Lammel et al. 2019; Langan et al. 2018). The possibilities of overcoming the variability will be further discussed.

Different possible items can be used to construct the spheroids, but, in general, they are plates and dishes. When the interest is in forming multiple spheroids together, it is possible to use untreated dishes (Cravedi et al. 1996; Flouriot et al. 1993, 1995b, 1995a; Uchea et al. 2013, 2015) or coated either with 2-methacryloxyloxyethyl phosphorylcholine (MPC) (Xing et al. 2008) or poly-(2-hydroxyethyl methacrylate) (p-HEMA) (Lammel et al. 2019), to reduce cell adhesion. Another material for spheroid construction is the 6-well plate, also coated with p-HEMA (Baron et al. 2012, 2017).

To produce isolated spheroids, 96-well plates are available. They can be coated with MPC (Xing et al. 2008) or p-HEMA (de Souza et al. 2021; Langan et al. 2016, 2018). To promote the spheroid formation, the plates can have a round-bottom, bought like that (de Souza et al. 2021) or produced (Faber et al. 2021). In addition, plates can be used as a secondary material for spheroid isolation and continued production. For example, it is possible to use non-coated 24- and 96-well plates (Jeong et al. 2016) or p-HEMA coated 48- (Lammel et al. 2019) and 96- (de Souza et al. 2021) well plates. Furthermore, Petri dishes can also form isolated spheroids, when used upside down, without coating, for the hanging drop method (de Souza et al. 2021; Servili et al. 2009).

The construction of the spheroids can be in still or rotating conditions, but to be successful, it depends on the used cell type. Some cells easily self-aggregate, while others need external forces. Nevertheless, to form spheroids in still cultivation, the cells need to be placed on treated dishes or plates to prevent cell adhesion (Bloch et al. 2016; Xing et al. 2008), even though some cells, like rainbow trout hepatocytes, have a strong affinity for each other, aggregating without external promotion and on non-treated material (Blair et al. 1990). Cells with migratory characteristics can produce spheroids under still conditions (Faber et al. 2021). Each cell type presents different behaviors related to cell adhesion and knowing their characteristics is crucial if it is viable to construct spheroids with them and to decide the better method to do so (de Souza et al. 2021).

For the spheroid construction under rotating conditions, some considerations are important. The main reason to maintain cells rotating is to keep them in cell suspension (Baron et al. 2012), but the formation of the spheroids depends on the cell characteristics related to the gyratory velocity and time of cultivation. Besides, it is possible to use flat-bottom, treated (de Souza et al. 2021; Hultman et al. 2019) or not (Flouriot et al. 1993; Uchea et al. 2015), or round-bottom material (de Souza et al. 2021; Rodd et al. 2017). To choose the rotation velocity, it is necessary to evaluate cell

size. For bigger cells, it is necessary to have a higher velocity, which is possible to observe when comparing mammal and fish hepatocytes, cells with similar characteristics but different sizes (Baron et al. 2012); and the used material, as it is possible to form spheroids of a cell on round-bottom plates, while not on treated flat-bottom on the same velocity (de Souza et al. 2021). Rotation velocity can also influence the circularity of the spheroid, as observed by de Souza et al. 2021, where, in that case, lower velocity resulted in better circularity.

The time of construction of spheroids can vary according to when it is considered a ready spheroid and the cell type. Generally, the cells start to aggregate after 24 hours (Baron et al. 2012; Jung'a et al. 2005; Langan et al. 2016), but in a loose, not well-defined model. The spheroids are considered ready or mature when they stop growing (Baron et al. 2012) or reach the maximum spheroidal shape (de Souza et al. 2021). We here highlight that it is important to understand the cell characteristics and establish its protocol, not only repeat a protocol defined to a similar cell type. This is explicit in Faber et al. 2021 study, where the authors compared spheroid construction methods for two cell lines, and the protocol could not be transposable between them because one cell line took longer to form spheroids than the other.

One parameter that is not highly discussed is temperature. Each cell has a range of optimal temperature growth, which can have a minor variation on the organism it comes from (Bols et al. 1992). Furthermore, 3D models do not necessarily have the same response as 2D models. In general, mammal cells are more heat resistant in spheroid than in monolayer, while fish is the contrary (Xing et al. 2008). This effect on mammal spheroid is associated with increased expression of heat shock proteins (Khoei et al. 2004) and was not yet studied for fish spheroids. However, it is possible that, as spheroids usually better mimic an *in vivo* situation, fish spheroids from primary cells can better adapt to heat alterations, depending on which temperatures the fish was acclimated to. For cell lines, different stressors can influence protein expression (Iwama et al. 1998). Temperature effects are significant when it is considered to construct spheroids with different cells, in co-culture, especially when the interest cells are from distinct species.

One of the previously mentioned advantages of the spheroid model is the possible increase of culture time, but to do so, the spheroids need to be maintained in a viable microenvironment. Supplementing the spheroids medium reduces the heterogeneity, as previously mentioned, and extends their culture time (Baron et al.

2012; Flouriot et al. 1993). That happens because the spheroids consume the nutrients in the medium while being formed. Another way to overcome consumption and expand the cultivation time is by frequently changing the medium, which can also help with the spontaneous degradation of components. Hanging drop is the only method that cannot exchange the medium (de Souza et al. 2021; Kelm et al. 2003). There is no indication of adequate time between medium changes, even as there are differences for various cells. We suggest that it is important to define when working on producing spheroids.

To summarize the considerations in planning the production of spheroids, it is important to mention their toxicologic application, which is relevant for the decision-making about the previous matters. For instance, toxicologic effects can be harmed for different wrong choices, primarily related to size, which was explored above, and heterogenicity. High variability of spheroids is also observed in the results, and it decreases reproducibility and the interest in utilizing the spheroids, as the results are not considered reliable (Lammel et al. 2019). As there are differences in the metabolic rate according to spheroid size, it is an important feature to be considered when extrapolating for humans and animals (Langan et al. 2018). Both situations only indicate the necessity of an increase in the development of spheroid studies, to expand the knowledge about spheroids and their application in toxicology, to improve standardization and promote their consideration for regulatory assays.

Finally, to return to the strategies to overcome the variability of spheroid sizes and behavior, we first mention that some of the methods presented promote heterogenicity with little control related to its utilization. The methods that use Petri dishes or plates with few wells (6-48) produce several spheroids of different sizes and shapes when multi-spheroids are formed, as described by Lammel et al. 2019, with the collision of smaller spheroids. This variation was already observed in the 90s, and Flouriot et al. 1993 proposed the alteration of medium supplementation as an observed standardization of spheroid size. Lammel et al. 2019, to avoid the formation of multi-spheroids, separated each spheroid after its formation, but it increased the manipulation of the spheroids, which is laborious and not available on a large scale. So, as far as the development of spheroids construction is right now, we indicate that the better way to ensure spheroid regularity is the individualized construction, which is possible with the hanging drop method or utilizing 96-well plates. However, the pattern will only be observed for both of them if cell density is homogeneous (de Souza et al. 2021; del Duca et al. 2004; Jeong et al. 2016).

4.5.2 Human Cells

According to Saglam-Metiner et al. 2019, the first spheroid was created in 1970 by Sutherland and collaborators, with rats and mice cells (Carlsson and Yuhas 1984), as a tumoral model (Timmins and Nielsen 2007), and now there are multiple models from tumoral and non-tumoral human cells. Saglam-Metiner et al. 2019 declare that, in general, there are four common methods for human spheroid construction: with ultra-low attachment plates, on hanging drop, using bioreactors (where rotatory shaker and fluidic systems are placed) and using scaffolds. Here, we will not be comparing the methods relating it to health or tumor spheroids, for, as far as we observed, all the methods that will be presented were used, at some point, for the construction of both.

Carlsson and Yuhas 1984 indicated that the most common method for human spheroid construction was the liquid-overlay. This method consists of cultivating cells on surfaces that prevent cell attachment, usually obtained by agarose coating. Later, other materials were used to coat the surface materials, like p-HEMA, although they generated loose human cell aggregates, which was solved when Matrigel was added as a scaffold (Nagelkerke et al. 2013). More recently, is growing the use of plates that can be bought with the surface preventing cell fixing, the ultra-low attachment plates, as used by Rajan et al. 2020 to construct liver (with primary hepatocytes) and heart (with cardiomyocytes) spheroids. Carlsson and Yuhas 1984 also mentioned that non-treated plates and dishes could be used to produce spheroids on the liquid-overlay method. This was observed in the study by Tostões et al. 2012, where primary hepatocytes were induced by medium supplementation to aggregate into spheroids for 72 hours. The spheroids generated had ~81 μm of diameter, which is smaller than the indicated by the authors to form a necrotic core. A549 cells also needed a supplemented medium to be induced into aggregating on ultra-low attachment plates (Rajan et al. 2020).

The first indication of the hanging drop method was by Kennedy et al. 1994, with non-human cells, but it was later highly explored for tumor spheroid formation (del Duca et al. 2004; Kelm et al. 2003; Timmins and Nielsen 2007). The reason is the advantage related to the method, which allows the formation of spheroids of similar sizes from different cells, even the cell types that could not aggregate on other methods (Kelm et al. 2003). In addition, tumor spheroids constructed with the hanging drop method had invasion increased when compared with spheroids obtained under rotation, related to the microenvironment of the drop (del Duca et al. 2004). Agastin et

al. 2011 produced tumor spheroids of Colo205 (cancer colon cell line) with an adapted hanging drop method. The spheroids were cultivated for 12 hours on the plate lid and later resuspended. Spheroids larger than 100 µm were subject to gentle pipetting to generate smaller aggregates. Then they were placed on a microbubble of poly-dimethylsiloxane (PDMS) of approximately 207 µm. The authors did not mention the final spheroid size but that it was controlled on the microbubble to avoid variability. They mentioned that the spheroids had a necrotic core and a secondary necrotic site of the cells on the bottom of the microbubble. Besides the size, a necrotic core can be related to an aggressive tumor, with exterior cells being aggressive and metastatic. However, it is also possible to produce healthy spheroids with the hanging drop method, as made by Rajan et al. 2020, with the coculture of different brain cells.

For human spheroids, methods using rotation, based on bioreactor, were not so usual, especially when compared with fish cells, which will be discussed later. One example is from Rajan et al. 2020, for testis spheroid, but the primary testicular cells were subjected to rotation (150 g) for only 30 seconds but were cultivated on ultra-low attachment plates with a supplemented medium. Another bioreactor type of cultivation is on fluidic systems, as the automated systems here addressed. To reduce spheroid manipulation, it is possible to construct an automated system device that promotes spheroid formation, as made by Ruppen et al. 2015 and Akay et al. 2018, depending on gravity force. Ruppen et al. 2015 used round-bottom microwell and chambers to trap cells and promote aggregation. Briefly, the cells were placed on the chip with eight wells, with no confirmation of how many cells per well, resulting in similar sizes of spheroids that did not change significantly from day 3 to 11. The authors obtained spheroids of ~175 µm to ~357 µm, depending on the number of cells placed. The authors did not test the presence of a necrotic core but considered the increased cell death related to it. Akay et al. 2018 placed the cells on the channel of the chips and the spheroids were formed after seven days, with constant medium changes. The authors did not indicate spheroid sizes nor if they presented necrotic core.

For the scaffold use method, there are many possibilities. A PDMS was used as a mold, by Bhise et al. 2016, to construct liver spheroids from HepG2/C3A (human hepatocarcinoma), for five days. The spheroids obtained had ~191 µm of diameter, which the authors indicated as not having a necrotic core. Magnetic nanoparticles can also act as a scaffold to aggregate cells on magnetic levitation, as made by Timm et

al. 2013. Another material that can be used as a scaffold is agarose, which coats plates in a specific format as hydrogels to hold the cells (Napolitano et al. 2007).

It was previously described the different spheroid construction methods used by Rajan et al. 2020 for different cell types. As it was not the objective, the authors did not widely discuss the necessity of method variation, but it is probably related to the differences between the used cells, as we previously mentioned. They also did not indicate how long it took to obtain the spheroids nor the resulting spheroid sizes, but the study's objective was to evaluate the health environment. However, even when using the same cell(s), it is possible to have a discrepancy, as observed for the coculture liver-equivalent spheroid constructed with HepaRG (biopotent hepatic cell) and HHSteC (hepatic stellate cell). Lin et al. 2020, Marin et al. 2019, Maschmeyer et al. 2015a and Wagner et al. 2013 used 384-well spheroids (also known as hanging drop) plates, but Lin et al. 2020 under shaking conditions for three days, Marin et al. 2019 for four days, Maschmeyer et al. 2015a was the only study to follow Wagner et al. 2013, that was the first to describe this method, and transferred after two days to ultra-low attachment 24-well plates for three more days.

As expected, spheroid studies are not unchanged, and adaptations and development of new methods are constantly emerging.

4.5.3 Fish Cells

The first recording of a fish spheroid is from 20 years after the first mammal spheroids. It was constructed unintentionally in a rainbow trout hepatocyte culture by Blair et al. 1990 and, even more than thirty years later, the variety of possibilities is not much, especially when compared with human spheroids. Here, we acknowledge that spheroids development for humans and fish will never be equalized due to the higher quantity endpoints assessed for toxicity and human health. However, we will continue to discuss the gaps related to aquatic ecotoxicology and environmental health in comparison to human studies advances.

When they obtained rainbow trout hepatocyte spheroid unintentionally, Blair et al. 1990 mentioned how easy it was to do and that it would be of great utility in the future, but only three years later, the spheroids were constructed with studying intent (Flouriot et al. 1993; Pelissero et al. 1993). Currently, most of the studies are still with primary hepatocytes. The first spheroids constructed with lineage cells were by Vo et al. 2011 in a cell characterization. The authors suggested further applications for the

cell line, but in our research, we did not find any indications that the spheroids were used with any application. The other lineages used to construct spheroids were mainly rainbow trout (Aarattuthodi et al. 2021; Lammel et al. 2019; Langan et al. 2016, 2018). Zebrafish, a model organism, was only found in one study of the construction of spheroids from the liver and embryo (de Souza et al. 2021).

In sequence, the methods chosen to construct fish spheroids will be discussed, exposing details, comparing each other, and approaching possible further uses and improvements. Studies excluded from the results will be included here, as they present differences from the included articles. Even without mentioning it in the studies, we already highlighted that all the spheroids and methods exhibited here could be utilized to assess ecotoxicological effects. All the methods presented were first used to produce mammal spheroids and adapted to construct fish spheroids according to necessity.

Besides Blair et al. 1990, other studies generate fish hepatocytes spheroids without trying to (Braunbeck and Storch 1992; Iuchi et al. 2020). Ostrander et al. 1995, in a study complementary to Blair et al. 1990, increased the time of cultivation of hepatocytes using spheroids as a secondary structure. To do so, they used positively-charged Primaria dishes and evaluated the development of the spheroids. This promoted attachment of hepatocytes at the beginning of culture and reattachment of the cells previously on the aggregate. That shows the ability of fish hepatocytes to aggregate. Primaria dishes are known to increase attachment, yet spheroids were formed. Differently, Braunbeck and Storch 1992 and Iuchi et al. 2020 discarded spheroids.

As mentioned above, some cells can aggregate without external forces, only by their characteristics. The previous examples were of spheroid construction unintentionally, without further utilization, but it is also possible to promote cell aggregation. For fish spheroids, two ways were described: using a coating material to prevent cell adhesion (Xing et al. 2008) and increasing the quantity of serum (Jung'a et al. 2005). Xing et al. 2008 used Petri dishes coated with MPC and obtained spheroids of ZEB2J (cell line from the blastula-stage embryo of zebrafish cultivated without feeders for two years) and RTS34st (rainbow trout spleen cell line), usually used as a feeder, isolated or in coculture, depending on the temperature of cultivation. The objective of Jung'a et al. 2005 was to increase the time of cultivation of tilapia hepatocytes. The author used Primaria plates, known to promote cell attachment, and

cultivated the spheroids in different serum proportions. They observed that the serum promoted cell aggregation, which extended the cultivation time and exhibited higher protein expression levels than the monolayer. Apparently, the authors did not intend to produce spheroids, but they took advantage of this result by studying the positive effects of a hybrid (monolayer-spheroid) culture. We did not include Xing et al. 2008 in our results because they did not mention the possibility of using the spheroids produced to study toxic effects. Although, we affirm that both the spheroids and the method to construct them can be used when the interest is to assess toxicologic markers. In addition to Jung'a et al. 2005 study, it would be interesting to compare the hybrid culture with spheroids, but, as far as we know, there was no other utilization of tilapia hepatocyte to produce spheroids.

The utilization of a coated material can also be used to produce spheroids with gravity force. To do so, the spheroids need to be constructed isolated, on plates reducing attachment so that the cells can aggregate. Xing et al. 2008, to compare with the previously described method, utilized round-bottom 96-well plates coated with MPC as well, to construct isolated spheroids of ZEB2J and RTS34st. The authors did not discuss the advantages and disadvantages of each type of spheroid construction, but we previously addressed them when mentioning the necessity of standardization of spheroids size. Faber et al. 2021 and Rodd et al. 2017 constructed the spheroids with a non-adherent plate coated with agarose to produce a round-bottom, a scaffold, on still cultivation. Faber et al. 2021 used RTS-11 and RTG-2 cells (from the spleen and gonad of rainbow trout, respectively), but this method only produced spheroids of RTS-11 cells, which have a migratory capacity for being macrophages, different from the fibroblastic RTG-2 cells. Rodd produced spheroids of PLHC-1 (liver of clearfin livebearer), epithelial cells that formed compacted and homogenous spheroids. Bought round-bottom ultra-low attachment plates were used by Bloch et al. 2016 to generate neuronal eel cell spheroids.

Another method utilized by Bloch et al. 2016 to obtain the spheroids was the hanging drop, which also uses gravitational force to promote cell aggregation. The authors did not discuss possible differences in the spheroids relating it to the construction method, only with the passages of culture. We believe that, as the acting force is the same, the spheroids did not differ when constructed with one or the other method. The authors were interested in comparing early- and late- passages because the study's objective was the study of the development, not even mentioning the

possibilities for toxicologic research, the reason why the article was not included in the results. Early-passage spheroids expressed stem cell markers, being described as neurospheres, which was not observed on late-passages spheroids. We claim that both types of spheroids can be applied to neurotoxicity studies to evaluate effects on different levels of development.

Gignac et al. 2014, Servili et al. 2009 and Vo et al. 2011, when establishing the culture of different cells, also used the hanging drop method to observe the cell's capacity to aggregate. Servili et al. 2009 produced neurospheres of sea bass brain cells (SBB-W1). Gignac et al. 2014 obtained KFE-5 (from a killifish embryo). Moreover, Vo et al. 2011 studied two salmon pituitary cells (ASP309 and ASP409) but obtained tightly packed spheroids only from ASP409 cells. De Souza et al. 2021 also used hanging drop to establish a protocol, but the objective was to define a protocol for established cell lines (ZFL and ZEM2S). The authors only tried to construct ZFL spheroids, with only slight success. To generate the spheroids, rotation was necessary after the hanging drop cultivation. The four studies mentioned did not evaluate toxic effects, but they all mention their possibility. The study from Gignac et al. 2014 was the only one excluded because the authors only mentioned the spheroid construction, without discussing their results.

There are patterns related to success and failure to obtain spheroids with the hanging drop method. Epithelial-like cells, like ZFL and ASP409, could not aggregate tightly, but fibroblastic-like cells, like ASP309, SBB-W1 and KFE-5 (this cell line also presents myogenic cells but in a reduced quantity), could. De Souza et al. 2021 did not try to construct ZEM2S spheroids using the hanging drop method, but it would probably be more successful than for ZFL. However, with the other gravitational method, with the ultra-low attachment material, fibroblastic-like cells (RTG-2) could not aggregate into spheroids (Faber et al. 2021), but epithelial-like cells, like PLHC-1 (Rodd et al. 2017) and ZEB2J (Xing et al. 2008) could. We do not have a mechanistic explanation for these differences but reinforce the necessity to understand cell characteristics when considering spheroid production, as the methods cannot always be extrapolated for every cell type.

For fish spheroids construction, the method using rotation was the most utilized. Baron et al. 2012, 2017, Cravedi et al. 1996, Flouriot et al. 1993, 1995b, 1995a, Hultman et al. 2019 and Uchea et al. 2013, 2015 obtained rainbow trout hepatocyte spheroids under gyratory conditions, with some differences on the applied protocol.

Baron et al. 2012, 2017, Cravedi et al. 1996 and Flouriot et al. 1993, 1995b, 1995a used the velocity of 70 rpm to generate spheroids, while Hultman et al. 2019 used 78 rpm. Both considered that the spheroids were mature after eight days of gyratory cultivation. For Uchea et al. 2013, 2015, the maturity was reached after ten days of cultivation, with a velocity of 50 rpm. The spheroids could be maintained in culture for 30 days (Baron et al. 2012; Flouriot et al. 1993; Hultman et al. 2019; Uchea et al. 2013), with a preserved metabolic response, which is a longer time than for rat hepatocytes (Cravedi et al. 1996).

Cell lines are also available for spheroid construction under rotation. Besides the previously mentioned still method, Faber et al. 2021 also tested the possibility of constructing spheroids of RTS-11 and RTG-2 on orbital shaking, with a velocity of 65 rpm. The results obtained from RTS-11 were similar but, differently from the still method, rotation promoted RTG-2 spheroid formation. The authors did not mention how long it took to obtain mature spheroids, only that the RTG-2 took longer than the RTS-11 but resulted in better-defined spheroids, probably related to the migratory characteristics of RTS-11, which increased cell detachment from the aggregates. Lammel et al. 2019 constructed spheroids of RTL-W1 (rainbow trout liver) at 45 rpm of velocity for 4-5 days, later maintained under still culture, to avoid multi-spheroid formation. The objective of Langan et al. 2016, 2018 was to assess inside the spheroids to measure the oxygen gradient related to the necrotic core (Langan et al. 2016) and the effect on toxicologic studies (Langan et al. 2018). Two cell lines were used, RTG-2 (Langan et al. 2016) and RTgutGC (Langan et al. 2018), and the same protocol of spheroid production was applied. The rotation started at 83 rpm to encase the probe (a non-invasive method to measure oxygen) for 24 hours with a reduction for 80 rpm to continue spheroid construction. For both, spheroids were considered mature after seven days, but some differences in oxygen saturation, even with the same density of cells and similar sizes, were observed, where RTG-2 was ~34% and RTgutGC ~54%.

De Souza et al. 2021 used rotation to generate ZFL and ZEM2S spheroids, analyzing different velocities (70 and 100 rpm). The authors also compared flat- and round-bottom plates. At 100 rpm, spheroids of ZFL were not obtained on flat-bottom plates, and at 70 rpm, there was a variation in their size. Differences in the velocities were not observed for round-bottom plates, which were obtained for ZEM2S, where higher velocity resulted in smaller spheroids. Velocity also affected the circularity,

where 70 rpm resulted in spheroids being more circular. Although the spheroids continued to grow until 16 days of cultivation, the protocol established was of five days at 70 rpm for both cells.

Another method to construct fish spheroids is magnetic levitation, used by Jeong et al. 2016. They plated 200 µL with 100000. The cells grew until 80% of confluence and were treated with magnetic nanoparticles. After the cells were detached, they were transferred to plated and were magnetic levitated. The spheroids generated had 350-400 µm and were uniform. The spheroids were less sensitive than monolayer culture, presenting results more similar to *in vivo*. This study is very interesting and relevant for the application of spheroids on ecotoxicological assessment but was not considered in the results because, beyond the *in vitro* tests, they also used zebrafish embryos, which are not considered alternative methods in Brazil.

De Souza et al. 2021 and Faber et al. 2021 reported more than one method to obtain fish spheroids from cell lines, one with better results than the other. For both, the most suitable method was under rotation, compared with a method depending on gravitational forces. This result would not necessarily be similar to all cells, especially considering cost-benefices and the materials used. We discussed above the necessity to understand cell characteristics. We also indicate that the hanging drop method is low-cost and can be used as a first attempt to generate spheroids. Some cells can aggregate in this method, as reported before, and, besides the low spend, a positive result for spheroid construction would save the time of protocol definition.

Additionally, fish spheroids can also be constructed with more than one type of cell, in coculture, as made by Xing et al. 2008, with ZEB2J and RTS34st cells, but this is less explored than for human spheroids. The hybrid spheroids were obtained on Petri dishes, with different proportions between the two cells. The authors also produced isolated spheroids for both on 96-well plates. The plates could also be utilized to produce better standardized hybrid spheroids, but they did not present any reason not to do it. Faber et al. 2021 studied two cell lines (RTS-11 and RTG-2) and suggested the use of both to construct a cocultured spheroid to study infection, which was the article's objective (the reason why it was not included in the results), but it could also be applied on toxicology assessments. Their method was rotation, and there is no indication of cocultured fish spheroids produced with it. Besides the two possibilities indicated here, other cells can be employed.

Toxicological effects on spheroids can be observed by morphological alterations, with cell detachment and death (Rodd et al. 2017), or with alteration of metabolism genes expression (Uchea et al. 2015). Biotransformation activity can be evaluated by studying the metabolites (Hultman et al. 2019). The concentrations assessed must be environmentally relevant and measurable in small quantities (Baron et al. 2017). Besides, bioavailability of substances needs to be considered, as it is possibly reduced, depending on its affinity with the used material and medium components. This could be a limiting factor for a metabolic response, resulting in wrong conclusions (Hultman et al. 2019). In addition, to plan experiments for fish toxicity, it is possible to use “read-across” prediction, using mammal data, as there is little or no experimental data for fish. Nevertheless, it needs to be addressed carefully, as there are metabolic differences (Baron et al. 2017).

Every technique presented here was either used in the same way or adapted from a mammal spheroid construction method

4.5.4 Automated System to Maintain Spheroids in Culture

We could not retrieve any automated system for a spheroid model with fish cells. In contrast, it was possible to achieve 33 studies for human cells that used (research) or mentioned (review) automated systems with spheroids. Interestingly, even with more filters, more studies were obtained for automated systems with spheroids for human studies than in general applications for fish spheroids. We expected a gap in environmental toxicology, but the absence of studies of automated systems with fish spheroids was a surprise. While there was no spheroid in the automated system for fish cells, only one study (Rajan et al. 2020) presented six human spheroids on a chip to assess systemic responses, using both primary and lineage and tumoral and non-tumoral cells. Here, we will discuss human spheroid automated systems as alternatives for future fish spheroid automated systems, also considering fish automated systems already developed (with other than spheroids type of cultures).

Some considerations are necessary for the success of an automated system. The main reason to maintain the spheroids on these devices is to keep them viable. However, the benefits depend on the biocompatibility of materials and low shear stress to achieve the continuous input of nutrients and oxygen and waste output in a physiologically relevant manner (Aoun et al. 2014; Moshksayan et al. 2018). For spheroid formation, before studying the application, direct on the automated system, it

is crucial to consider the shape of the well, as flat-bottom wells generate more than one spheroid per well, while round-bottom wells generate one spheroid per well, and they were more similar to each other (Ruppen et al. 2015). For toxicologic research, the primary considerations are the previously mentioned shear stress and the facility to perform the experiments and evaluate the effects. Thus, many device shapes and designs can be utilized (Moshksayan et al. 2018).

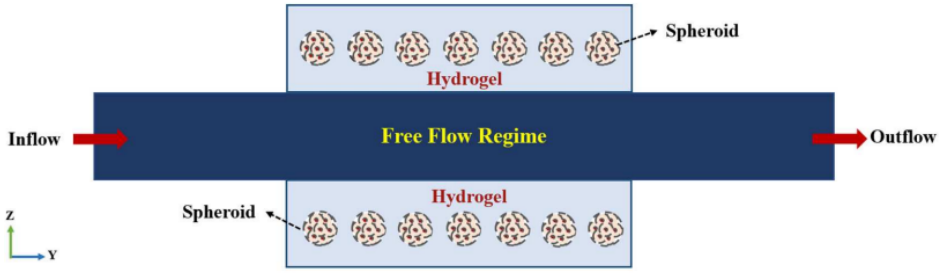
The shear stress is a mechanical stimulus present *in vivo* microphysiological environment (Ashammakhi et al. 2020b; Liu et al. 2017; Marx et al. 2016), which contributes to maintaining the gradient of nutrients and oxygen in a fluidic system (Lin et al. 2020). However, it can cause spheroid disaggregation. Each cell type has a maximum shear allowance and needs to be considered when developing fluidic systems (Moshksayan et al. 2018). On toxicologic assays, shear stress can influence the results because it can decrease cell viability (Klak et al. 2020), impact drug resistance (Moshksayan et al. 2018) and alter gene expression (Bovard et al. 2018; Kammerer 2021). It is possible to adjust shear stress on spheroids by altering the area of the channels used to deliver medium, as the widening reduces fluid flow and shear stress (Maschmeyer et al. 2015a), the flow rates (Bhatia and Ingber 2014) and by reducing the direct flow towards them, adding them to a membrane (Sharifi et al. 2019) or in a deep well (Moshksayan et al. 2018). As there is no inclusion of fish spheroids in automated systems, the effect of shear stress on them is lacking information.

The development of automated systems needs to consider analysis, as it is possible to study the toxic effects off the systems and on the systems. To evaluate off the systems, it is either after the study, by retrieving the spheroids, to analyze the final result, or the supernatant, to assess metabolic alterations, as performed by Sharifi et al. 2019, or both, as executed by Maschmeyer et al. 2015a and Tostões et al. 2012. To evaluate on the system, it is generally through microscopy to observe cell proliferation and death, as conducted by Agastin et al. 2011, Rajan et al. 2020 and Moshksayan et al. 2018. It is possible to perform both on and off-the-system analysis, as made by Bhise et al. 2016, Lin et al. 2020, Marin et al. 2019, Ruppen et al. 2015 and Wagner et al. 2013.

Because of its metabolic function, the liver is the most relevant and utilized organ to study toxicologic effects. Thinking of it, we will present liver spheroids automated systems. The spheroids (HepG2 cells) can be encapsulated in hydrogel chambers (Figure 4) to reduce sheer stress and be maintained for until four weeks,

under flow, promoted by a peristaltic pump, for posterior toxicologic research (Sharifi et al. 2019). Similarly, Bhise et al. 2016 placed HepG2/C3A spheroids on hydrogel to be used for toxicity assays in a fluidic system (but with a syringe pump) for 30 days for further high-throughput drug screening. Tostões et al. 2012 utilized a bioreactor to construct and maintain primary hepatocyte spheroids in a model without hydrogel. The difference was that the medium was supplemented with serum for the construction, for 72 hours, and without serum for four weeks of maintenance. The authors do not indicate how the medium was perfused into the system. A considerable advantage related to the maintenance of hepatic spheroids under flow conditions is the continuous nutrients and oxygen supply (Bhise et al. 2016), which is very important for hepatic models as they have higher metabolic rate and oxygen consumption (Sharifi et al. 2019). The maintenance of fish hepatocyte spheroids was reported for 30 days (Cravedi et al. 1996), with similar enzymatic activity for some markers, but differences for others, described by the authors as being related to possible spheroid maturation or medium consumption. With continuous flow, an automated system for either primary or lineage cell spheroids would help elucidate spheroid behavior and upgrade toxicity models.

Figure 4: Schematic model of bioreactor for liver spheroids cultivation. Font: Sharifi et al. 2019.



For human health studies, tumor spheroids are very relevant to assess tumor progress or drug response, but even if produced to have a necrotic core, it is important to maintain them with the proper parameters, which is challenging to perform in static models. That is observed by Agastin et al. 2011, where the medium perfusion could maintain the cells healthy, while the cells where the renewed medium could not get were necrotic. The authors used an array of PDMS microbubbles with a flow chamber gasket to place the spheroids. The chamber was connected to a syringe pump to deliver the medium, a vacuum chamber, to remove air, and a bottom to deposit the medium. The system could be maintained for five days. A similar study with a different device was performed by Ruppen et al. 2015, where lung tumor cells were maintained

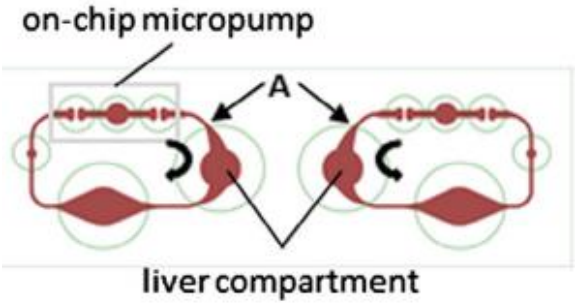
under perfusion. Their device also used a syringe pump and vacuum but could maintain the spheroids viable on the automated system for eight days. The reduced time of culture for tumor spheroids, when compared to healthy ones, is probably related to the aggressivity of the tumor cells, but we cannot affirm that other cell types could be maintained for longer, and we do not know if the whole spheroid could be kept health on these devices.

Tumor spheroids in fluidic systems were more resistant to the tested drug, maybe due to the cell-cell interaction, mass transfer limitations and limited exposure. That resembles the reality of avascular tumor growth, which is advantageous (Agastin et al. 2011). Besides, coculture spheroids are more resistant than monoculture spheroids, which is also more similar to *in vivo* cancer because of the formed barrier (Ruppen et al. 2015). The devices used for tumor research might not be the better choice to be applied to fish spheroid automated systems without an adaptation. Both models presented here favor little medium perfusion on the spheroids to reproduce a tumor better, which differs from healthy organs. As possible adaptations, we suggest the expansion of the wells, the reduction of spheroid sizes, which would allow spheroid movement, and the increase of fluid flow.

Automated systems can also be used to study interconnected effects, as observed on the 2-organs-chip (2-OC), constructed using a liver equivalent spheroid (coculture of HepaRG and HHSteC cells). The chip (Figure 5), in general, was composed of two chambers (one for the liver spheroids and the other for a barrier system), channels to connect them and a micropump to pump medium. Medium continually circulated by the chip and needed to be changed frequently, according to the specific characteristics of each model. Wagner et al. 2013 used skin biopsies as the second system. The authors produced two models of 2-OC: one where the two organs-equivalent were directly exposed to the fluid; and the other where they were inserted in a Transwell® holder, where the skin could be in an air-liquid interface, and the spheroid could be submerged in the medium. The first was cultivated for 14 days, while the second allowed 28 days of maintenance. For a 14 days study of substance *in vitro* response, Maschmeyer et al. 2015a also produced a 2-OC with skin biopsies on a Transwell® holder but studied the effects with an intestine barrier, as well, obtained with primary intestinal epithelial cells. Similarly, Marin et al. 2019 produced a liver-intestine model, but the intestine barrier, different from the previous model, was constructed with Caco-2 (colon epithelial cells) and HT-29 (colon carcinoma cells). The

model produced by Lin et al. 2020 was with a kidney barrier. The experiment was carried out for 16 days, where two of them were for adaptation. In the presented system, spheroids presented better functionality when they were maintained under flow (Marin et al. 2019), which is an example of the robustness of the model to be used as a long-term *in vitro* device (Wagner et al. 2013). Besides, the 2-OC is reliable and reproducible to study physiologic effects (Maschmeyer et al. 2015a) and can be used for drug screening and further accepted as a regulatory model (Lin et al. 2020). Multiple models of liver spheroids for fish were presented previously and could be applied in this model. An intestinal barrier was constructed with coculture of gut rainbow trout cells (RTgutF, fibroblastic, and RTgutGC, epithelial) by Drieschner et al. 2019 in a fluidic model and is an option to be included in the 2-OC model. Maybe the fish skin would not be an interesting model to be used, so we suggest that a gill system would be more toxicologically relevant for fish. The RTgill-W1, developed and characterized by BOLS et al. 1994, was recently included as a model for fish toxicity by the OECD TG 249 (OECD 2021) and previously included in a fluidic system developed for toxicity tests (Glawdel et al. 2009) and could be utilized as the barrier system.

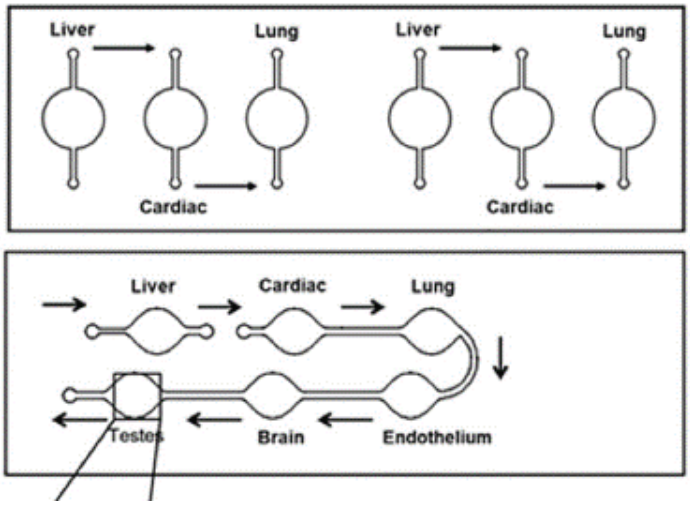
Figure 5: Representative schematic model for the 2-OC, where the smaller chamber is for the liver spheroid and the bigger chamber is for the barrier model. Font: Maschmeyer et al. 2015a.



A more complex fluidic system was constructed by Rajan et al. 2020, for three or six organs, where five of which were made as spheroid models. The system was an adhesive-based-film microfluidic chip (Figure 6), with channels to put the spheroids, with connections between them, except for the liver space so that it could be disintegrated from the system for comparative studies. To promote media flow, the authors used a peristaltic pump. Considering only one species, rainbow trout (as it is the one with more spheroid studies), to be included on the chip, it would be possible to use the spheroids previously mentioned: the liver could be from primary hepatocytes (Flouriot et al. 1993) or RTL-W1 (Lammel et al. 2019); the intestine could be from

RTgutGC (Langan et al. 2018); RTG-2 spheroids (Faber et al. 2021; Langan et al. 2016) could be used as the reproductive portion of the chip; for the brain spheroid, it is possible to follow Servili et al. 2009 or Bloch et al. 2016 protocol; lung is not an interesting model for fish toxicity evaluation so that it could be replaced by gill spheroid (still not described) or spleen (Faber et al. 2021; Xing et al. 2008); for the cardiac system, Rajan et al. 2020 produced blood vessels, that could be generated following their protocol, using red blood cells from rainbow trout, obtained, following Nombela et al. 2017 study.

Figure 6: Schematic model of the adhesive-based-film microfluidic chip for three (above) or six (below) organs. Font: Rajan et al. 2020.



A difficulty related to the maintenance of multi-organs chips is that each cell uses a specific medium, and when placed on the chips, it is necessary to be adapted, so all the cells placed there can keep developing. This is a matter not always discussed in multi-organ systems, but it was addressed by Marin et al. 2019, with the utilization of a medium composed of the two necessary media, with a proportion related to organ necessity. In the complex system proposed by Rajan et al. 2020, the circulating media was composed of testis media and endothelial cell growth medium (without serum) in 1:1 proportion. The system needed to be exposed to the shared media for some time (14 days) to stabilize the organs through autocrine and paracrine signaling, similarly to what the blood does, before starting the toxicity assays. For fish spheroids maintenance, it would be possible, as some of the media used in fish cultures were adapted from mammal culture. Besides, many fish cells use Leibovitz L-15 medium, and only some primary cultures have a specific medium (Lakra et al. 2011), but this could be considered when developing a multi-organ automated system.

As it is for spheroid construction and application, the development of new automated systems needs to continue evolving for human toxicology and must be applied for fish toxicology.

4.5.5 Study Limitations

There were difficulties related to finding studies using fish cells because using the keywords “fish” and “cells”, even as the sentence “fish cells” brought studies using the method FISH (Fluorescence *in situ* Hybridization), using other types of cells, so, even with a large number of articles, many of them were not even accessed for eligibility.

During the research about fish cell spheroids, it was observed that most of the results from Google were either repetitions of the other databases or did not meet the eligibility criteria, so it was decided not to use this database for the human cell spheroids.

Reviews were considered in the present study because most of the papers mentioned as examples of interesting were not retrieved in the search. It was observed that most of the articles retrieved were reviews for the human cell(s), which is possible due to the keywords used. Besides, it was relevant to approach the reviews, especially for human studies, because even using the same example, there were different discussions.

It is essential to highlight that possibly many studies were left out, especially from the human cells research. Most studies with cells do not use the sentence “human cell(s)”, only the name of the used cell. That situation is also observed for fish cells, but the research was more thorough by analyzing articles mentioned in the found papers. It was not a problem for the present study because the focus was the environmental studies and their delay compared to human health, and it was possible to observe the difference.

In addition, for the discussion about human cell spheroids, it was considered only the studies that constructed spheroids to be placed on automated systems and the methods already utilized in fish cell spheroid construction. The present review could be considered aid and not complete research for the in-depth research on human spheroids. That is different for fish spheroids, which were thoroughly analyzed and discussed, including possible further inclusion in automated systems.

4.5.6 Conclusions and Future Perspective

We appreciate the current pressures related to developing new and improved methods to study toxicology, but we also point out that increasing the pressure for environmental health research will not be detrimental to human health. There are many gaps in both areas, but there is a considerable delay in ecotoxicology. We also highlight that developing new approaches to evaluate ecotoxicological effects will only add to the improvement of human health, as humans are inserted into the environment, and it is or should be everyone's interest to ameliorate it.

The advances in human studies also have to continue, especially considering that, when well-established, automated system devices can be less expansive and quickly produced. Moreover, with that the application will not be only for drug screening but also for personalized, individualized medicine (Ruppen et al. 2015; Zuchowska et al. 2017), increasing the success rates and reducing collateral effects of inappropriate treatments, as it can predict better chemotherapy treatment for each patient, considering specificity and efficacy (Ruppen et al. 2015).

For human studies, the development of automated systems is advanced, including multi-organ systems, which are more robust and physiologically relevant, exposing an integrated response. Although it is not always true, in fluidic systems, 3D models present better results than 2D, and the integration of both (3D and fluidics) can be a better option for drug effects studies as they are more accurate (Rajan et al. 2020). Since various fish spheroids are available, it is possible to develop multi-organ automated systems simultaneously with one-organ systems and new spheroid construction protocols for different cells.

In conclusion, we agree with Cho and Yoon 2017 that current methods to assess human health can be used in ecotoxicology. We believe that the increasing interest in applying alternative methods on ecotoxicology assays might generate an increased quantity of studies with fish spheroids, and we hope they are in a more relevant physiological model as fluidic systems. Furthermore, methods can be developed considering both human and environmental toxicology applications. Many of the devices presented previously can be used with fish spheroid with little or no adaptation. This can be considered when creating regulatory methods for a physiologically relevant *in vitro* to assess toxic effects.

This review emphasized the considerable gap between human and fish toxicologic studies using spheroids on automated systems and the need to develop and adapt methods.

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5 CONSIDERAÇÕES FINAIS

É crescente, há anos, a pressão para o desenvolvimento de métodos alternativos ao uso de animais para avaliações (eco)toxicológicas, principalmente em dois sentidos. No sentido social, com interesse de consumidores em “produtos verdes”, que são ambientalmente amigáveis e não utilizam animais para testes. Bem como em sentido experimental, na busca de modelos de experimentos mais fáceis, rápidos, baratos e, principalmente, fisiologicamente relevantes, principalmente na abordagem da toxicologia humana, em que os testes eram principalmente realizados em animais que não apresentam respostas fisiológicas idênticas. Entretanto, apesar dos impulsos direcionados aos métodos alternativos por parte de agências regulatórias, esses métodos não são de simples desenvolvimento e, após o mesmo, precisam ser indicados como mais vantajosos que os métodos já utilizados, o que acaba atrasando e reduzindo sua aceitação.

Dentre os métodos alternativos já desenvolvidos e aceitos, são utilizadas, principalmente, culturas 2D, em sistemas estáticos, o que não condiz com a realidade biológica dos organismos. Assim sendo, o objetivo do presente trabalho foi avaliar o avanço no desenvolvimento de sistemas automatizados, fluídicos, no cultivo de esferoides células, culturas 3D fisiologicamente relevantes. Além disso, foi também comparado o progresso para a área de toxicologia e de ecotoxicologia aquática, pesquisando células humanas e de peixes, respectivamente.

Quanto aos resultados obtidos, é possível destacar que o atraso no desenvolvimento de métodos alternativos para análises ecotoxicológicas foi esperado, contudo, foi surpreendente a ausência de sistemas automatizados para cultivo de esferoides de células de peixes, principalmente ao se considerar as vantagens, amplamente abordadas acima, desses sistemas.

Para o planejamento inicial do desenvolvimento da revisão, foi considerado realizar comparações de sistemas estáticos e fluídicos para o cultivo de esferoides quanto à utilização de células de peixes e humanas, mas com o objetivo principal de destacar as realizações da área de ecotoxicologia aquática. Assim sendo, algumas adaptações foram necessárias no decorrer da pesquisa e da escrita. Com a ausência de sistemas automatizados para esferoides celulares de peixes e a grande quantidade de artigos obtidos em buscas de esferoides com células humanas, foi observado que uma abordagem aprofundada nos estudos de toxicologia poderia ofuscar os avanços na área de ecotoxicologia. A partir disso, foi decidido reduzir a quantidade de

descritores utilizados nas buscas relacionadas à saúde humana, focando somente nos estudos de sistemas automatizados, não mais considerando os sistemas estáticos no cultivo de esferoides de células humanas.

Ainda foram reduzidas as fontes de obtenção dos artigos. Para os esferoides de células de peixes, artigos utilizados como referências de outros estudos avaliados também foram considerados, o que não foi feito para os estudos com esferoides de células humanas. Além disso, foi observado que os resultados obtidos no Google Acadêmico eram repetidos de outras bases de dados previamente avaliadas e geravam uma grande quantidade de estudos que não seriam utilizados por não apresentarem as características determinadas para inclusão. Por isso, o Google Acadêmico não foi utilizado para buscar sistemas automatizados de cultivo de esferoides com células humanas.

Apesar dessas variações de pesquisas, o objetivo principal, de indicar a grande lacuna nos estudos de ecotoxicidade aquática, quando comparado à toxicologia, para o desenvolvimento de sistemas automatizados no cultivo de esferoides celulares, foi atingido. Os resultados obtidos (em menos bases de dados) de estudos com sistemas automatizados para cultivo de esferoides humanos foram em maior quantidade que os resultados obtidos para cultivo de esferoides de células de peixes em sistemas estáticos. Além da quantidade, foi observada uma grande variação dos tipos celulares utilizados na construção dos esferoides. Apesar de se observar, sim, repetições de células em diferentes estudos toxicológicos, houve uma maior diversidade quando comparado com as células utilizadas nos estudos ecotoxicológicos, que eram, principalmente, de hepatócitos primários de truta arco-íris. É necessário destacar, ainda, que essa maior variação para os esferoides humanos é observada em um menor período de tempo (a partir de 2011) quando comparado com os esferoides de peixes (a partir de 1993).

Ademais, os outros objetivos também foram cumpridos. Foi possível descrever e discutir os avanços nos estudos com esferoides construídos com células de peixes e esferoides de células humanas cultivados em sistemas automatizados. Os sistemas automatizados de cultivo de esferoides humanos apresentam melhores respostas toxicológicas, quando comparados com sistemas estáticos 2D, de modo a se assemelharem com as respostas obtidas *in vivo*. Além disso, esses sistemas permitem a integração de células, em esferoides ou em outro modelo fisiologicamente relevante, de diferentes tecidos, o que possibilita estudos que avaliem as interações

entre esses tecidos e as variações nas respostas, o que aproxima a uma realidade sistêmica. Quanto aos esferoides de peixes, mesmo em sistemas estáticos, eles já apresentam a possibilidade de um cultivo em maior tempo, em um sistema que é fisiologicamente e metabolicamente mais similar com os organismos vivos.

Quando se considera possíveis aplicações dos esferoides celulares de peixes em sistemas automatizados, não existe um motivo experimental de sua ausência. Todos os sistemas automatizados apresentados podem ser adaptados para utilização com células de peixes. Vale ressaltar que o próprio avanço na construção de esferoides não está tão adiantado. Quando se considera a possibilidade de utilização de diferentes tecidos para análises ecotóxicológicas, já é observada uma lacuna. A maioria dos estudos é com células hepáticas, que são altamente relevantes e importantes para estudos metabólicos, mas não são exclusivas. Existe a possibilidade de se utilizar células neurais, embrionárias, de brânquias, de intestino, de órgãos reprodutores, entre outras e em possíveis combinações. Além dos órgãos, existe pouca variação de espécies utilizadas para obtenção dos esferoides. Diferentes espécies de peixes podem ser encontradas em diversos ambientes, em água salgada, doce ou salobra, e em diferentes níveis tróficos. Isso faz com que haja variações entre as respostas de diferentes espécies, o que torna necessária a utilização de uma maior variedade de espécies, para melhor identificar os efeitos em diversos graus do ambiente aquático.

Considerando tudo, destacamos que o artigo científico aqui apresentado contribui com o destaque dos atrasos nos estudos ecotoxicológicos, quando comparados com os estudos toxicológicos e, consequentemente, tem potencial de favorecer o desenvolvimento de sistemas automatizados para cultivo de esferoides de células de peixes para avaliação ecotoxicológica e estudos de modo de ação.

Contudo, é importante evidenciar também a necessidade de aceitar esses sistemas como testes (eco)toxicológicos por parte das agências regulatórias. Até onde temos conhecimento, nenhum dos métodos descritos no artigo são indicados como obrigatórios para avaliação (eco)toxicológica de substâncias, mesmo com o conhecimento da maior relevância fisiológica. Entretanto, a aceitação desses sistemas pode ser pressionada pelos desenvolvimentos e resultados positivos obtidos. Assim, destacamos a necessidade de constante de desenvolvimento de métodos alternativos ao uso de animais que sejam fisiologicamente relevantes, tanto para aplicação em toxicologia, quanto para aplicação em ecotoxicologia, e ressaltamos que a utilização

de esferoides em sistemas automatizados cumpre o papel proposto e que a integração de diferentes tipos celulares nesses sistemas contribui com estudos fisiológicos e sistêmicos, se assemelhando, cada vez mais, com os animais.

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