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Methodological approaches for spermatogonia population enrichment and effect of cryopreservation on *Senegalese sole* spermatogonia quality

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Mestrado em Biologia Marinha

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Declaração de Autoria

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Maria Mafalda Almeida

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Abstract

Senegalese sole (*Solea senegalensis*) is an emerging species for aquaculture that shows reproductive issues in aquaculture conditions. The transplantation of cryopreserved spermatogonia into closely related species allows the production of surrogated broodstocks. However, the cryopreservation of spermatogonia might cause damage on the quality of spermatogonia, meaning quality assays must be performed. For these assays, it is important to perform cell enrichment protocols to achieve only pure spermatogonia population. Therefore, the aim of this project was to develop an efficient protocol for Senegalese sole spermatogonia enrichment and observe how the cryopreservation protocol affects these germ cells. For this purpose, two experiments were performed using fresh and cryopreserved using dimethyl sulphoxide (DMSO) as cryoprotectant solution. In experiment 1, cells were cultivated in several extracellular matrixes (uncoated, collagen, gelatine and laminin) to perform differential plating for cell enrichment. The results showed that gelatine and collagen plates showed higher spermatogonia presence, however due to the low percentage of spermatogonia recovery obtained this technique was considered unfit for the target species. Afterwards, serial strainers were used to observe that the appropriate size was the 5 µm strainer, which contained higher percentage of spermatogonia recovery. Then, two 5 µm strainers were used and showed slightly better and promising results. This method was validated by RT-PCR where three relevant spermatogonia markers (*gfra1*, *pou5f1/oct4* and *nanos2*) were up regulated in the samples from the strainers, yet no significance difference was observed. In experiment 2, effect of cryopreservation on the quality of spermatogonia was evaluated where the results showed that viability, DNA integrity and epigenetic were affected by cryopreservation. No difference in lipid peroxidation was observed. Future studies should focus on the limitations of the enrichment techniques to improve this method and improving the cryopreservation protocol to decrease the damaged suffered by cells during this technique.

Keywords: Senegalese sole, spermatogonia, cryopreservation, enrichment, quality assays

Resumo

O linguado senegalês (*Solea senegalensis*) é uma espécie promissora para a aquacultura europeia devido ao declínio da sua oferta no mercado de pesca, e consequentemente, o seu alto valor de mercado e também por revelar excelentes características para cultivo. Contudo, esta espécie apresenta alguns problemas reprodutivos que impedem o fecho do seu ciclo de vida em condições de aquacultura. A transplantação de espermatogónias criopreservadas em espécies intimamente relacionadas permite a produção de reprodutores substitutos. No entanto, a criopreservação de espermatogónias pode causar danos na qualidade destas como tem sido observado em espermatozóides. Para entender completamente como a criopreservação afeta as espermatogónias, devem ser realizados testes de qualidade. Para este propósito, é necessário executar protocolos de enriquecimento celular de modo a obter apenas uma população de espermatogónias puras e viáveis. Neste contexto, o objetivo deste projeto é desenvolver um protocolo eficiente para o enriquecimento de espermatogónias no linguado senegalês e observar se o protocolo de criopreservação afeta a viabilidade celular, integridade do DNA e perfil epigenético dessas células germinativas.

Neste estudo, foram usados testículos de linguado senegalês juvenil frescos e criopreservados utilizando dimetilsulfóxido (DMSO) como solução crioprotetora. Na experiência 1, as células foram cultivadas em diversas matrizes extracelulares (sem revestimento, colagénio, gelatina e laminina) durante 24h e 48h para realizar o *differential plating* para enriquecimento celular. Os resultados mostraram que o tempo ($P=0.614$) e a interação entre tempo e matrizes extracelulares ($P=0.506$) não afetou significativamente as percentagens de recuperação de espermatogónias. Contudo, existe diferença significativa entre matrizes ($P<0.001$). As placas de gelatina (5.3 ± 0.9 %) mostraram presença de espermatogónias significativamente maior quando comparado com as placas sem revestimento (1.55 ± 0.86 %; $P<0.001$) e as placas de laminina (1 ± 0.22%; $P<0.001$). As placas de colagénio também apresentaram maior presença significativa de espermatogónias em comparação com as placas sem revestimento ($P=0.011$) e placas de laminina ($P<0.001$). Não obstante, esta técnica foi considerada imprópria para a espécie alvo devido à baixa percentagem de recuperação de espermatogónias obtida (5.3%). Posteriormente, outra técnica (*strainers*) foi utilizada como método de enriquecimento celular. Uma série de tamanhos diferentes (30 µm a 1 µm) foram utilizados de modo a avaliar qual seria o tamanho mais adequado para futuras experiências. O tamanho de 5

µm demonstrou menor contaminação de outras células e maior percentagem de recuperação de espermatogónias (64.2%). Após a avaliação do tamanho, foram utilizados dois *strainers* de 5 µm. O primeiro *strainer* demonstrou uma quantidade significativamente maior de espermatogónias (26.1 ± 6.1 %) quando comparado com o *flow through* (< 5 µm) (11.6 ± 6.8 %; P=0.038) com alguma contaminação de outras células. O segundo *strainer* apresentou uma tendência semelhante à do primeiro, mas com menos quantidade de células em geral. Contudo, este não apresentou diferenças significativa nem com o primeiro *strainer* (P=0.263) nem com o *flow through* (P=0.438). No *flow through* observou-se grande quantidade de células e uma percentagem baixa de recuperação de espermatogónias. Esta técnica apresentou resultados um pouco melhores e promissores quando comparado com *differential plating* (26.1%). Esta última técnica foi validada por *real-time PCR* onde três marcadores genéticos relevantes de espermatogónias (*gfra1, pou5f1/oct4* e *nanos2*) em amostras dos *strainers* e do *flow through* (< 5 µm). O gene *gfra1* estava regulado positivamente nas amostras dos *strainers* (3.05 ± 2.45) e regulados negativamente nas amostras do *flow through* (-1.29 ± 0.29), enquanto o *pou5f1* (2.19 ± 2.35 *strainers* and 0.57 ± 2.48 *flow through*) e o *nanos2* (2.43 ± 1.66 *strainers* and 2.35 ± 0.67 *flow through*) estavam regulados positivamente para ambas as amostras. Ainda assim, não foram observadas diferenças significativas entre amostras para todos os genes. Na experiência 2, as espermatogónias criopreservadas e frescas foram enriquecidas de acordo com a metodologia selecionada na experiência 1 e a qualidade das espermatogónias foi avaliada posteriormente através de quatro técnicas (viabilidade celular, integridade do DNA, peroxidação lipídica e modificações epigenéticas). Quanto à viabilidade celular foi possível verificar que esta foi significativamente maior no grupo do fresco (78.98 ± 5.66 %) quando comparado com o grupo criopreservado (62.81 ± 3.25 %; P= 0.003). No que respeita a integridade do DNA, a fragmentação do DNA foi significativamente maior no grupo do criopreservado (37.28 ± 1.87 %) quando comparado com o grupo fresco (32.95 ± 2.28 %; P= 0.026). Em relação à peroxidação lipídica, não houve diferença significativa entre o grupo fresco (1.13 ± 0.45 µM of MDA per million spermatogonia) e o grupo criopreservado (0.91 ± 0.96 µM of MDA per million spermatogonia; P=0.701). Finalmente, em termos de modificações epigenéticas, foram observadas citosinas metiladas em três contextos diferentes, constatando-se que a maior percentagem observada, para ambos os grupos, foi no contexto CpG (81.8 ± 0.39 % fresco e 82 ± 1.05 %). Porém, nenhuma diferença significativa foi observada entre os dois grupos (P=0.733). Contudo, o grupo

criopreservado demonstrou um perfil de metilação diferente com mais DMC (*differentially metylated cytosines*) quando comparado com o fresco. Os resultados mostraram assim que apenas a viabilidade, integridade do DNA e a epigenética foram prejudicadas pela criopreservação.

Em suma, precisam de ser realizados mais estudos para abordar as limitações deste projeto e também devem ser testados mais métodos. Futuros estudos deverão se concentrar em novas soluções, tais como, avaliar a temperatura ideal e aplicar *double enrichment*, para alcançar o enriquecimento bem-sucedido das espermatogônias e otimizar as condições de cultura para apoiar a sobrevivência e a atividade mitótica das espermatogônias de Senegalese sole. Deverão igualmente ser testadas estratégias para evitar a contaminação de outras células e não perder espermatogônias durante o processo de enriquecimento, nomeadamente, mudanças na percentagem de FBS para remover células somáticas testiculares e adição de fatores de crescimento para combater a diminuição gradual do número total de espermatogônias, também devem ser testadas. Concretamente no que respeita ao efeito da criopreservação na qualidade das espermatogónia do linguado senegalês, foi possível verificar que a criopreservação causa apenas perda de viabilidade celular e danos no DNA. Futuros estudos devem focar-se em melhorar o protocolo de criopreservação para diminuir os danos sofridos pelas células durante esta técnica. Será também importante, realizar mais estudos sobre a correlação da criolesão das espermatogônias com a qualidade dos espermatozoides e oócitos após o transplante para determinar o grau de dano que pode ser aceite para fins comerciais e do GenBank. Por fim, futuros estudos devem explorar se o processo de congelamento/descongelamento ou a toxicidade dos crioprotetores é a razão que justifica a existência dos danos nas espermatogônias durante a criopreservação.

Palavras-chave: Senegalese sole, espermatogonónia, criopreservação, enriquecimento, testes de qualidade

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Abbreviations

ROS – reactive oxygen species

SSCs – spermatogonia stem cells

FACs – fluorescence- activated cell sorting

MACs – magnetic- activated cell sorting

DP – differential plating

ECMs – extracellular matrixes

PI – propidium iodide

TUNEL – terminal deoxynucleotidyle transferase mediated dUTP-biotin end-labelling

SCSA - sperm chromatic structure assay

SCGE - single cell gel electrophoresis

MDA – malondialdehyde

P/S - penicillin / streptomycin

PBS - phosphate buffered saline

FBS - fetal bovine serum

BSA - bovine serum albumin

DMSO - dimethyl sulphoxide

WGBS - whole genome bisulfite sequence

PCA - principal component analysis

DMC – differentially methylated cytosines in CpG context

DMR – differentially methylated regions

1. Introduction

1.1. Species of study: Senegalese sole

Aquaculture is one of the sectors which has been developed over the years due to the increased growth of the world's population and overexploitation of wild fisheries resources. Aquaculture has become an important economic activity in many European countries, like Portugal, France and Spain, that support intensive production systems of many species such as Senegalese sole (*Solea senegalensis*) (Morais et al., 2016).

Senegalese sole is an emerging flatfish species for aquaculture because of declining fisheries market supply and high market value (Morais et al., 2016). The rise in popularity for Senegalese sole in aquaculture is also due to having some excellent characteristics for cultivation. Its prolonged reproductive season means that embryonic and larvae development may occur under highly divergent environmental conditions causing alterations in physiological traits, which can be used to properly manage controlled captive reproduction of this species (Anguis & Cañavate, 2005). Besides, Senegalese sole has good growth rates, high larval survival, and a high capacity to adjust to intensive production (Morais et al., 2016). However, the production of this species faces some issues due to this species not being completely domesticated in captivity. The main problem is a reproductive dysfunction in cultured male breeders (F1) that do not fertilize the eggs released by females (Carazo et al., 2013; Morais et al., 2016; Fatsini et al., 2020; González-López et al., 2020). Moreover, cultured male individuals have lower sperm quality (Beirão et al., 2011), lower sperm volume (Cabrita et al., 2011a), higher variability in sperm quality (Chauvigné et al., 2016) and exhibit a dysfunctional reproductive behaviour (Carazo et al., 2011; Fatsini et al., 2020) in comparison with wild male breeders. This forces the use of wild-origin breeders, which can spawn spontaneously in captivity, making this cultivation unsustainable at a long-term period (Morais et al., 2016). For that reason, to ensure the supply for large-scale commercial production, solutions to optimize reproductive control, such as the use of hormones to induce spawning (Guzmán et al., 2011; Cabrita et al., 2011a; Chauvigné et al., 2017, 2018; Oliveira et al. 2020), the implication of social interaction during rearing (Fatsini et al., 2017; Fatsini et al., 2020; Martín et al., 2020), adjustment on broodstock nutrition (Norambuena et al., 2012a,b) and artificial fertilization (Rasines et al., 2012, 2013; Ramos-Júdez et al., 2021), are being studied. Some of these studies enhanced gamete

quality (Beirão et al., 2015) and obtained spontaneous spawning (Fatsini et al., 2020), however these results were not reproducible and without complete reproductive successful. A new practice, spermatogonia transplantation, has become more popular and studied, and might be utilised to preserve germ cell line, increase genetic gain in captivity and generate transgenic animals. Transplantation of cryopreserved spermatogonia into closely related species, without losing the ability to differentiate into gametes in the host gonads, allows the production of surrogated broodstocks. This is an important practice and a possible solution for fish that are not completely domesticated and are not able to reproduce in captivity. However, studies regarding the consequences of cryopreservation on spermatogonia quality and epigenetic profile need to be developed.

1.2. Biological characteristics of spermatogonia and spermatogenesis

Spermatogenesis is a highly coordinated and organized process where diploid spermatogonia proliferate and differentiate to form mature spermatozoa. The duration of this process in fish is influenced by water temperature and usually shorter than mammals (Schulz et al., 2010). The process can be divided into three different phases: (1) the mitotic or spermatogonial phase with the different generations of spermatogonia (undifferentiated spermatogonia and differentiated spermatogonia) (2) the meiotic phase with the primary and secondary spermatocytes and (3) the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating into motile, flagellated genome vectors called spermatozoa (Schulz et al., 2010).

Spermatogonia are early-stage male germ cells that have not yet started meiosis. These cells are large (reported to be 12 to 16 µm in diameter), self-renewing, diploid and present in the testes in varying numbers all year-round (Uribe et al., 2014; Hagedorn et al., 2018). Spermatogonia be classified in three different types: undifferentiated type-A, differentiated type-A and type-B.

Undifferentiated spermatogonia type-A are the baseline cells of spermatogenesis and are characterized by their large size, large nucleus and small amounts of heterochromatin (Uribe et al., 2014; Hagedorn et al., 2018). The largest cells in the germline, undifferentiated spermatogonia type-A, divide via mitosis into differentiated type-A spermatogonia (Hagedorn et al., 2018). These new cells share morphological

characteristics with undifferentiated spermatogonia type-A but have a lower self-renewal potential (Schulz et al., 2010). Then, differentiated type-A spermatogonia divide via mitosis into spermatogonia type-B cells (Hagedorn et al., 2018). The latter are smaller (approximately 9 to 12 µm in diameter), have a smaller nucleus containing more heterochromatin and divide rapidly for several generations (Schulz et al., 2010; Uribe et al., 2014; Hagedorn et al., 2018). Once surrounded by Sertoli cells, spermatogonia type-B differentiate in cyst-like structures - primary spermatocytes (Hagedorn et al., 2012a; Hagedorn et al., 2018). Primary spermatocytes are similar in size and shape to spermatogonia type-B cells and are also diploid (Hagedorn et al., 2018). The primary spermatocytes enter the meiotic phase where these cells will proceed to the first meiotic division, which involves DNA duplication and recombination of the genetic information, leading to the formation of secondary spermatocytes (Mañanós et al., 2008). Rapidly, the secondary spermatocytes enter the second meiotic division, where no DNA duplication occurs, leading to the formation of spermatids (Mañanós et al., 2008). Then, spermatids enter the spermiogenesis phase where the newly spermatids differentiate to ultimately become haploid sperm (1 to 3 µm in diameter) (Uribe et al., 2014; Hagedorn et al., 2018). This process does not involve cellular proliferation, only cell transformation, which is responsible for the drastic reduction in size, due to nucleus condensation and extrusion of the cytoplasmic content to the surrounding Sertoli cells (Mañanós et al., 2008).

1.3. Why cryopreservation of spermatogonia?

Spermatogonia can be used in biomedicine and biotechnological manipulation, such as cryopreservation and transplantation, in rare, endangered, or commercially cultured fish species (Xie et al., 2020). Spermatogonia transplantation is a novel technique for the field of fish farming and genetic conservation. Cryopreserved spermatogonia can be transplanted between closely related species without losing the ability to differentiate into gametes in the host gonads, granting spermatogonia xenotransplantation to become a useful tool to produce surrogated broodstocks (Pacchiarini et al., 2014).

Cryopreservation is a conservation method that allows the storage of genetic resources for an indefinite period in liquid nitrogen. To cryopreserve a cell, water is extracted and then substituted with an antifreeze material or cryoprotectant (Cabrita et al., 2008; Hagedorn et al., 2018). In a state of suspended animation, the cell can endure extreme

stress from exposure to ultra-cold temperatures (~ -196ºC) (Mazur, 1997; Hagedorn et al., 2018). Cells can remain viable for years without inducing damage to DNA if correctly frozen and stored (Mazur, 1997; Hagedorn et al., 2018). For that reason, cryopreservation technology is widely used for preserving the genetic material, which can be used for conservation or production depending on the necessities of each species (Cabrita et al., 2008; Hagedorn et al., 2018).

In fish, cryopreservation of sperm is the most established and commercialized technique. The main reason is the efficiency to which spermatozoa can be cryopreserved which stems from these cells being small, having a small surface/volume ratio, being simple structures and having high chilling resistance (Asturiano et al., 2017). However, one main disadvantage is that this implies the preservation of solely male germplasm. During the past years, cryopreservation protocols for Senegalese sole sperm have been developed and improved (Rasines et al., 2012; Valcarce & Robles, 2016; Riesco et al., 2017).

To preserve female genetic material, attempts have been made to cryopreserve eggs and embryos. Cryopreservation of fish eggs and embryos is extremely difficult due to their large size, high yolk content and low membrane permeability (Yoshizaki et al., 2011; Marinović et al., 2019), thus efficient methods have not yet been developed. Therefore, spermatogonia cells are frequently used as an alternative in cryopreservation, playing an important role in biotechnology in fish reproduction. Spermatogonia cells are particularly fitting for cryopreservation due to their relatively small cell size compared with somatic cells, low fat and yolk content, high level of sexual plasticity and the capacity of reconstitution both spermatogenesis and oogenesis after transplantation (Okutsu et al., 2007; Yoshizaki et al., 2011). Besides, spermatogonia have a higher tolerance to oxidative stress compared to sperm, due to spermatogonia having higher levels of Cu/Zn that provide them resistance to reactive oxygen species (ROS) and subsequently protection against oxidative stress (Celino et al., 2011). Okutsu et al. (2007) were able to cryopreserve spermatogonia type-A from rainbow trout (*Oncorhynchus mykiss*) and obtained a 50% survival rate after freezing and thawing procedures.

Nevertheless, the process of cryopreservation has been observed to cause damage to sperm at several levels such as plasma membrane (lipid peroxidation), mitochondria function, morphology and transcript degradation, which affects cell viability and fertilization capacity (Cabrita et al., 2005a; Riesco et al., 2017). Besides, cryopreserved spermatozoa can also suffer oxidative stress (Shaliutina et al., 2013), as mentioned above,

and epigenetic modifications (DNA methylation alterations) (Depincé et al., 2019). However, there is a lack of research regarding the consequences of cryopreservation on spermatogonia. For that reason, to understand how cryopreservation affects spermatogonia quality and epigenetic profile, quality assays must be performed. In order to perform these quality assays, it is important to have as much as possible pure spermatogonia population. Therefore, protocols for cell enrichment and purification are essential to develop.

1.4. Techniques for spermatogonia purification and quality

1.4.1. Techniques for purification

Spermatogonia stem cells (SSCs) are settled on the basal lamina of the seminiferous tubule and the number has been estimated to be only 0.03% of the total testicular cells in adult rat testis (van Pelt et al., 1996). Therefore, an important step in isolation is the purification of spermatogonia stem cells from the digested pubertal testis since this allows the elimination of the somatic testicular cells that interfere with the proliferation of spermatogonia *in vitro* (van Pelt et al., 1996). In fish, enrichment and purification techniques are also required to counteract gonad cell heterogeneity and low quantities of spermatogonia (Xie et al., 2020). The conventional approach for isolation of spermatogonia includes purification/sorting followed by *in vitro* cell culture (Conrad et al., 2014; Gat et al., 2018). Several techniques have been reported for efficient purification of spermatogonia to stem cells such as differential plating with different coating extracellular matrixes, discontinuous Percoll gradient density centrifugation and fluorescence-activated/magnetic-activated cell sorting (FACs/MACs) (Xie et al., 2020).

A basic and frequently used purification method is differential plating (DP) which incubates overnight testicular cells suspension over a coating substance (extracellular matrix) (Gat et al., 2018). The separation between somatic and germ cells is relatively simple and easy, since testicular somatic cells tend to attach tightly to the bottom of the culture plate, whereas spermatogonia are suspended or weakly attached to the somatic cells or cultured plate in fish species (Shikina & Yoshizaki, 2010). A big advantage compared with other methods is that this technique is not limited by cell concentration (Dores et al., 2015). Extracellular matrixes (ECMs) attach spermatogonia stem cells to

the basal lamina of seminiferous tubules in mammals (Tiptanavattana et al., 2015), but in fish species the attachment occurs with somatic cells (Xie et al., 2019). Various types of extracellular matrixes have been used such as gelatine, laminin, fibronectin and collagen (Kanatsu-Shinohara et al., 2008; Kim et al., 2010; Lim et al., 2014). The substrate mostly frequently used to coat culture dishes is gelatine since it is cost-effective and allows optimization in various cell types (Tiptanavattana et al., 2015; Xie et al., 2020). Yet, the laminin-coated plate has been demonstrated to improve the purifying efficacy of spermatogonia isolation in mice (*Mus musculus*; Hofmann et al., 2005), rats (*Rattus norvegicus*; Orwig et al., 2002) and cats (*Felis catus;* Tiptanavattana et al., 2015), due to the presence of specific laminin receptors in these cells.

In order to access the efficiency of the purification techniques, methods have been developed for the detection and quantification of relevant spermatogonia transcripts (*gfra1, pou5f1/oct4, nanos2*), based on real-time PCR, which has been used to evaluate the presence of spermatogonia stem cells in mice (He et al., 2007; Saga, 2010; Zheng et al., 2016) and in fish (Lacerda et al., 2013, 2014; Bosseboeuf et al., 2014; Xie et al., 2020).

1.4.2. Techniques for quality

Cryopreservation is a process that can cause damage to the integrity and permeability of cell plasma membrane, promote base oxidization or strand breaks in DNA and DNA methylation alterations. This damage can be access by different techniques that focus on cell quality through viability, DNA integrity, lipid peroxidation in the membrane and epigenetics modifications.

Cell viability is one of the easiest techniques used to test sperm and spermatogonia quality after cryopreservation. The most common method for evaluation of viability is the use of selective dyes (trypan blue and eosin-nigrosin) or fluorescent probes (propidium iodide (PI) and SYBR Green). Fluorescent probes are frequently used due to their specificity, possibility to combine with other fluorescent dyes for evolution of other cell characteristics and the possibility of scoring either by fluorescent microscopy or flow cytometry (Cabrita et al., 2008). Propidium iodide is a non-permeable substance able only to penetrate damaged or dead cells, while SYBR Green is permeable to the plasma membrane staining the nucleus of viable cells (Cabrita et al., 2008). The double labelling

with PI /SYBR Green is frequently used in fish species (Flajšhans et al., 2004; Cabrita et al., 2005b) to assess quality where live cells will fluoresce green and damaged/dead cells fluoresce red.

Different techniques are used to evaluate DNA integrity based on chromatin damage through DNA fragments detection. The most commons are TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labelling), sperm chromatic structure assay (SCSA) and single cell gel electrophoresis (SCGE) or comet assay (Cabrita et al., 2008, 2010a, 2014). The most commonly method used in fish species, such as Senegalese sole, gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), is the comet assay (Beirão et al., 2008; Cabrita et al., 2005, 2011b; Riesco et al., 2017) which analyses chromatin fragmentation based on electrophoretic migration patterns of DNA fragments based on their size (Cabrita et al., 2014). After staining, disperse DNA fragmented cells can be observe forming a comet-like tail structure, preceding the non-fragmented DNA which forms the comet head (Cabrita et al., 2014). Comet tail length can be measured manually, but specific software permits the measurement of different paraments (tail moment, percentage of DNA in tail, etc) allowing for a more objective evaluation (Cabrita et al., 2008).

Lipid peroxidation is a marker used to evaluate oxidative stress based on high amount of polyunsaturated fatty acids in the plasma membrane (Cabrita et al., 2014). Lipid peroxidation is measured by the quantification of malondialdehyde (MDA) a final product of lipid oxidation (Cabrita et al., 2014). This method is more commonly used on fish sperm (Li et al., 2010; Martínez-Páramo et al., 2012; Hagedorn et al., 2012b; Shaliutina et al., 2013). The TBARS assay can react with other type of compounds besides MDA making the assay nonspecific (Cabrita et al., 2014). For that reason, commercial kits (Oxis BIOXYTECH MDA-586) have been developed which minimize interference from other lipid peroxidation products and has been successfully used in European seabass (Martínez-Páramo et al., 2012) and Senegalese sole (Riesco et al., 2017).

DNA methylation is a key epigenetic modification that provides heritable information not encoded in the nucleotide sequence and plays a critical function in many biological processes. This modification occurs on the cytosine base and exclusively when this cytosine is followed by a guanine, which leads to refer to the methylation status of the CpG dinucleotide (Labbé et al., 2017). It regulates gene expression, genomic imprinting, cell differentiation and embryogenesis (Durcova-Hills et al., 2006; Smith et al., 2012;

Yang et al., 2014; Labbé et al., 2017). This epigenetic alteration is transmitted by the spermatozoa to the offspring and may remain cryptic within the embryo development and only be expressed later (Depincé et al., 2019). These alterations, provoked by cryopreservation, might also occur on spermatogonia, however, there is a lack of research about the consequences of cryopreservation on spermatogonia related to epigenetic alterations. The four main sequencing technologies used for exploring genome-wide DNA methylation are the followings: methylated DNA binding domain sequencing (Aberg et al., 2012), methylated DNA immunoprecipitation sequencing (Taiwo et al., 2012), whole-genome bisulfite sequencing (WGBS) (Lister et al., 2009) and reduced representation bisulfite sequencing (RRBS) (Nagarajan et al., 2014). The former two use the enrichment of methylated DNA to acquire a maximum resolution of 150 bp (Harris et al., 2010), while the latter two achieve single-base resolution through the bisulfite conversion. Generally, methods using bisulfite conversion are more accurate than those using enrichment (Harris et al., 2010; Bock et al., 2010). These methods have been used to observe epigenetics alterations exclusively on fish sperm (Lister et al., 2009; Aberg et al., 2012; Taiwo et al., 2012; Nagarajan et al., 2014).

1.5. Objectives

This project aimed to develop an efficient protocol for spermatogonia enrichment to obtain a pure and viable spermatogonia population. To achieve this goal, a new technique (strainers) and several extracellular matrixes (laminin, gelatine and collagen) for differential plating were tested. Besides, this project aimed to observe if cryopreserved spermatogonia has the same quality in terms of proliferation and cell culture compared with fresh spermatogonia. To achieve this goal, different quality parameters (cell viability, DNA integrity, lipid peroxidation and epigenetic modifications) were assessed.

2. Material and Methods

All chemicals, unless otherwise stated, were purchased from Merck (Portugal) and were reagent grade or higher. Experimental procedures were conducted in accordance with the guidelines of the European Directive (2010/63/EU) and Portuguese legislation for the use

of laboratory animals and considered the ARRIVE guidelines. CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by Direção Geral de Alimentação e Veterinária—DGAV). The authorization for experimental procedures were previously approved by DGAV (ref.0421/000/000/2022).

2.1. Animal rearing conditions

Senegalese sole (*Solea senegalensis*) male juveniles of 1 year old and approximately 35 g were used in this study. The specimens (n= 1280) were obtained from DIBAQ through CTAQUA company located in El Puerto de Santa María, Cádiz (Spain) where fish were maintained in RAS system at a constant temperature (~ 20 ºC). The fish were transported to Ramalhete station (University of Algarve, Faro, Portugal) and randomly distributed in 12 different tanks (53 height, 49 width, 106 length, 155 L of water). The tanks were maintained in a RAS system under the same conditions from the origin centre with continuous aeration and 0.3 m^3/h of water exchange. Water quality parameters (temperature, oxygen saturation, nitrites and ammonium) were monitored daily. Sole were fed ad libitum using a commercial feed from SPAROS Lda (Marine sole, 3 mm pellets) 7 days per week at a daily ration of 2-3 % biomass.

2.2. Experiment 1: Testing different spermatogonia enrichment techniques

Fish were euthanatized by an anaesthetic overdose (1000 ppm) using 2-phenoxyethanol and testes were dissected in aseptic conditions producing 3 and 2 pools containing 6 and 48 testes each, respectively. Once dissected, each pool of testes was placed in a petri dish (35x10 mm, Falcon, USA) containing L-15 medium (Leibovitz) prepared aseptically and supplemented with 2.5% of penicillin / streptomycin (P/S) and 0.5% of fungizone (L15-P/S). Afterwards, testes were minced down to 1- mm^3 fragments using sterile scissors and transferred to a petri dish containing 2 mL of dissociation solution (L-15 medium supplemented with 10% trypsin (3.6% trypsin in phosphate buffered saline (PBS), 5% fetal bovine serum (FBS), 1% P/S and 0.2% of fungizone) (L15-Dis). Testicular cells were dissociated for 2.3h with gentle pipetting 50 times every 30 min using a serological

pipette of 1 mL. Then, cell suspension was filtered through a sterile strainer (100 µm), which was previously washed with 1 mL of L15 medium supplemented with 20% of FBS, 1% of P/S and 0.2% of fungizone (L15-20%). The strainer was washed again with 2 mL of L15-20% and dissociated testicular cells were centrifuged for 6 min at 1000g at room temperature. Afterwards, the pellet was resuspended in 10 mL of L15 supplemented with 5% of FBS, 0.25% Senegalese sole serum, 1% of P/S and 0.2% of fungizone (L15-SSS). Cells were counted in a Neubaeur chamber (BRAND®) using a microscope (Nikon Eclipse E200) at 40x magnification to distinguish spermatogonia from other cells. All this procedure was repeated for all the pools processed. After this common procedure, several techniques were performed as detailed below.

2.2.1. Differential plating

For this technique, cells from each pool of testes (n=3) were seeded in uncoated, collagen, gelatine and laminin coated 6 well plates (2 x 10^5 cells/well). Each pool (n=3) was seeded twice to create replicates. Plates were incubated at 20 ºC for 24h and 48h, meaning 2 plates were prepared for each extracellular matrix (ECM). Cell cultures were inspected every day using an inverted microscope (Zeiss Axiovert 25 CFL), and photos were taken of 3 different random fields with a digital camera (VisiCam 16 Plus, VWR) to observe the evolution of the cells. Then, cell suspensions were collected and attached cells were recovered by trypsinisation using 2% trypsin for 10 min. Cells (suspension and attached of each ECM) were counted in a Neubaeur chamber (BRAND®) using a microscope (Nikon Eclipse E200) at 40x magnification to determine spermatogonia recovery. Spermatogonia cells were identified based on their morphology as described by (Shikina & Yoshizaki, 2010) as larger than the other cells and round with a spherical nucleus containing one to three nucleoli.

2.2.2. Strainers

For this technique, different sizes of strainers were used to test spermatogonia retention. Strainers (30 µm, 20 µm, 15 µm, 10 µm, 5 µm and 1 µm; pluriSelect Life Science, USA) were stacked above a 50 mL falcon and 10 mL of cell suspension from each pool of testes

(n=3) was passed through the column of the strainers. Then, each strainer was placed upside down on a 6-well plate and cells were recovered with a 1.5 mL wash of L15-SSS by gravity during 20 min.

After testing the right size of the strainer and observing that spermatogonia was mainly retained in the 5 µm strainer, the protocol was improved. Briefly, two 5 µm strainers were stacked above a 50 mL falcon and 10 mL cell suspension from each pool of testes (n=2) was passed through the two strainers. Then, each strainer was placed upside down on a 6-well plate to recover the cells with a 1.5 mL wash of L15-SSS by gravity.

In all cases, photos and cells counts were performed as previously described to determine spermatogonia recovery.

2.2.3. Spermatogonia markers validation

After spermatogonia enrichment using two 5 µm strainers was carried out (section 2.2.2), RNA was extracted from cell suspension from each pool of testes (n=2) using TRI Reagent RNA Isolation Reagent following manufacturer's instructions. RNA samples were loaded in a nanodrop (NanoDrop™ One/ OneC, Thermo Scientific) where the quality absorbance ratios were assessed (A280/260 > 1.8; A230/260 > 1.8). Afterwards, the complementary DNA (cDNA) was synthesised using Thermo Scientific Maxima First Strand cDNA Synthesis Kit For RT-qPCR with dsDNase kit following the manufacturer's protocol (Thermo Fisher Scientific Inc). Reverse transcription (RT-PCR) conditions were 25 °C for 10 min, 50 °C for 30 min and 85 °C for 5 min.

Target genes were chosen accordingly with their expression in undifferentiated type A spermatogonia (*gfra1, pou5f1/oct4, nanos2*) and differentiated type A spermatogonia (*gfra1, nanos2*) in other fish species (Table 2.1). Primers were validated by conventional PCR using 4 samples of cDNA synthetized from each well (2 samples from the 1st and 2nd strainer combined, and 2 samples from the flow-through <5 µm). The taq polymerase used to amplify the amplicon of each gene was JumpStart™ REDTaq® performing the conventional PCR using an Applied Biosystems 2720 thermocycler with the following conditions: initial denaturation step at 94 °C for 1 min, followed by 37 cycles - denaturation at 94 °C for 30s, annealing at Tm (54–57°C) for 30 s and extension at 72 °C for 1 min. Then, the amplified product was loaded in an agarose gel (1.5%) containing

0.5 µg/ml SYBR Safe gel stain (Alfagene, Portugal) and the electrophoresis was conducted using buffer TAE 1X (40 mM Tris-borate 20 mM Acetic acid 1 mM EDTA, pH 8.0), to observe the PCR amplification of the transcript products. After the electrophoresis, the DNA bands were visualized by UV light (UV Transilluminator, UPV (305/365 nm)).

Primer efficiency was evaluated by serial dilutions. The real-time PCR was run using a Bio-Rad CFX96^{TM} Thermocycler (Bio-Rad, Portugal) in 96-well plates in duplicate. Reactions were performed in 20 µl volume containing 10 µl of SsoFast EvaGreen Supermix (Bio-Rad, Portugal), 2 µl of the primers (0.5 mM) (Table 2.1), and 5 µl of cDNA at the validated dilution. Furthermore, amplifications were carried out with a systematic negative control (NTC; no template control) containing no cDNA. Standard amplification conditions contained an initial denaturation step at 95 °C for 30 s, followed by 40 cycles: denaturation at 95º C for 5 s, annealing at 57º C for 5 s and a melt curve with a 0.5º C increase (65ºC-95ºC) for 2-5 s.

Table 2.1: Primers used in this study for Senegalese sole (*Solea senegalensis*) spermatogonia. Gene name, primer sequence, biological function and reference are indicated.

Gene name	Primer sequence (5' 3')	Biological function	Reference
gfra1	F-TCAGACCATCGTACCCGTCT R-GAGTATGCGAGGAGGCAGTC	Potential for proliferation and ability to form colonies	Bosseboeuf et al., 2014 ; Xie et al., 2020
pou5f1	F-AGCAGGCCGTATGACTTCAG R-AACGCTGTGGGAAAGTTGGA	Maintenance of pluripotency and the germ lineage, transcriptional control and post-translational modifications including phosphorylation, sumoylation and ubiquitination	Bosseboeuf et al., 2014 ; Xie et al., 2020
nanos2	F-GAGCACCTGAGCGGAGATTC R-AGTGCCGTAAACTTCCTCCG	Promotes male cell differentiation, functions as an intrinsic factor to maintain the stem cell population during spermatogenesis	Lacerda et al., 2014 ; Xie et al., 2020

2.3. Experiment 2: Effect of cryopreservation on Senegalese sole spermatogonia quality

For this experiment two experimental groups (fresh and cryopreserved) were made with 4 pools of testes from different males for each group (n=4). For the first three techniques (cell viability, DNA integrity and lipid peroxidation) pools were created containing 10 and 8 testes each for the fresh and cryopreserved group, respectively. For the epigenetic modifications, pools were created containing 30 and 26 testes each for the fresh and cryopreserved group, respectively.

In case of the fresh group, fish were euthanatized by an anaesthetic overdose and testes were dissected in aseptic conditions. The testes were minced and dissociated as previously described (section 2.2) and cells were counted in a Neubaeur chamber (BRAND®).

For the cryopreserved group, fish were euthanatized by an anaesthetic overdose and testes were dissected in aseptic conditions and placed on L15-P/S. Afterwards, each testis was cut into pieces and fragments (7 to 9 per vial) were cryopreserved according to Cabrita et al. (2010b). Briefly, the fragments were transferred into a 1.5 mL cryovial containing 500 µl of freezing media (L-15 based medium supplemented with 0.5% bovine serum albumin (BSA) and 5.5 mM glucose with 1.5 M DMSO). Before freezing, each cryovial was left to equilibrate for 15 min at 4 ºC on ice. Samples were loaded into a portable free nitrogen programmed biofreezer (Asymptote EF600, Grant Instruments, Cambridge, UK) and the freezing curve was run (Figure 2.1). A total of 13 cryovials per pool were cryopreserved. Afterwards, cryovials were introduced directly in liquid nitrogen and stored in a liquid nitrogen container until further procedures. Later, cryopreserved cryovials were thawed in a water bath at 40°C for 140 s and washed in L-15 to eliminate the cryoprotectant. Once thawed, testes were dissociated as previously described (section 2.2) and cells were counted in a Neubaeur chamber (BRAND®).

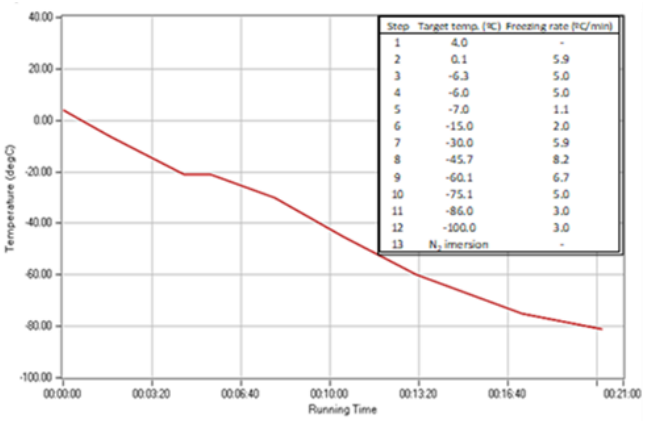

Figure 2.1: Freezing curve used for the cryopreservation protocol. The temperature and time used in each step of the freezing curve are displayed in a square in the upper right corner.

After these common procedures, several techniques were performed as detailed below.

2.3.1. Cell viability

Cell viability analysis was performed using PI/SYBR staining. Briefly, 20 µL of the fresh and cryopreserved cell suspension from each pool of testes (n=4) was mixed with 0.1 µL SYBR Green working solution (10X diluted in PBS) and 0.5 µL propidium iodide (PI stock solution). It was incubated for 5 min, and then cells were observed in a fluorescence microscope (Nikon Eclipse E200) with an excitation filter of 450 nm at 20x magnification and images were captured and recorded with a digital camera (VisiCam 5 Plus, VWR). At least 100 cells per sample were scored, and the percentage of viable cells was determined.

2.3.2. DNA integrity

DNA integrity was determined by comet assay technique following the protocol described by Cabrita et al. (2005) with some modifications. Briefly, fresh and cryopreserved cell suspension from each pool of testes (n=4) was diluted in L15 to attain a final concentration of approximately $1x10^6$ cells per 50 µL. Slides were previously prepared by applying and spreading 100 µL of 0.5 % normal melting point agarose (Invitrogen, USA) prepared in 0.1 M PBS. The slides were stored at 4 °C protected from

dust and light. After dilution, 150 µL of 0.5 % low melting point agarose (Invitrogen, USA) was added to the samples and 50 µL of this agarose mixture was added to the pre-coated slides and covered with a coverslip (24 x 24 mm) for 20 min at 4 °C to solidify. For each experimental group and pool, one slide was prepared in duplicate. After this period, the coverslip was removed and the slides were placed into a coplin jar containing lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100 and 1% lauryl sarcosine) for 1h at 4 °C. To decondense the DNA, dithiothreitol was added to the lysis buffer, at the final concentration of 10 mM, and the slides were immersed for 30 min at 4 °C. After lysis, the slides were placed horizontally in an electrophoresis cube (Sub-Cell GT, BioRad, Portugal) filled with electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) for 30 min at 4 °C to allow DNA to unwind. Electrophoresis was conducted for 10 min at 25 V and 300 mA at 4 °C. Afterwards, the slides were dried and placed into a coplin jar with neutralizing solution (0.4 M Tris, pH 7.5) for 5 min to wash and repeated twice. The slides were drained, fixed in pure ethanol and stored at 4 °C until further observation.

Visualization of the comets was carried out by pipetting 10 µL of diluted PI (50X diluted in PBS) in each sample and covered with a coverslip. Observation was performed using a fluorescence microscope (Nikon Eclipse E200) with an excitation filter of 450 nm at 40x magnification. Approximately 100 cells from each slide were captured with a digital camera (VisiCam 5 Plus, VWR). Comet analysis was performed with the Kinetic Imaging Komet v6.0 software (Andor Technology, UK). For each analysed cell, the pixels observed in the tail of the comet represent DNA fragments (damaged DNA) and the nucleus represents the head of the comet in which the undamaged DNA is located. The percentage of tail DNA (% DNA_t) was the parameter used to determine the amount of DNA fragmentation.

2.3.3. Lipid peroxidation

The lipid peroxidation level was determined by quantifying the concentration of malondialdehyde (MDA) using a colorimetric assay (kit BIOXYTECH LPO-586 ™, OxisResearch), following the protocol described by Martínez-Páramo et al. (2012). Fresh and cryopreserved cell suspension from each pool of testes (n=4) was incubated in 10 µL of 200 µM sodium ascorbate containing 40 µM ferrous sulphate for 30 min at 37 ºC in

the dark. An MDA calibration curve was prepared by diluting MDA standard solution (20 µM) in MiliQ water (Table 2.2).

Table 2.2: Curve points used in MDA protocol.

Curve Points	**1**	**2**	**3**	**4**	**5**	**6**
MDA (µM)	10	8	4	2	1	0

Subsequently, reagents provided by the kit were added to 100 µL of the cell suspension (following the manufacturer's instructions) and samples were incubated for 1 hour at 45ºC in the dark. After the incubation time, samples were centrifuged at 10000g for 10 min at 4 ºC and 200 µL of each supernatant was transferred to a 96-well flat-bottom transparent plate (Nunc.). The absorbance was read in a microplate reader (Synergy 4, Biotek Instruments. Inc.) at 586 nm. MDA concentrations were calculated from a standard curve and presented as µM of MDA per million spermatogonia.

2.3.4. Epigenetics modifications

Fresh and cryopreserved cell suspensions from each pool of testes (n=4) were used for spermatogonia enrichment with 5 µm strainers as previously described (section 2.2.2). Afterwards, the DNA was extracted using QIAamp genomic DNA kit (Qiagen, Germany). Samples were load in a nanodrop (NanoDrop™ One/ One^{C}, Thermo Scientific) where the quality absorbance ratios were assessed (A280/260 > 1.8; A230/260 > 1.8). After extraction, isolated DNA was used to perform whole genome bisulfite sequence (WGBS) by NOVOGENE company (United Kingdom). Bisulfite-converted DNA was sequenced in paired ends of 150 bp, with an expected sequencing depth of 70X (calculated from the 612.3 Mb genome size in Solea). Bisulfite conversion involves the deamination of unmodified cytosines to uracil, leaving the modified bases 5-mC and 5-hmC.

Bioinformatics was run following Nilsson et al. (2021) to observe whole genome modifications in terms of methylated region in the spermatogonia of Senegalese sole genome affected by the cryopreservation process. Briefly, quality control of the data (FASTA/FASTQ format) was undertaken using FastQC program (Krueger & Andrews, 2012). Then, low quality reads and bases were trimmed and filtered using Trim Galore

(Krueger & Andrews, 2012). This software allowed the removal of low quality bases (Phred quality score < 30), the removal of adapters and of inserts shorter than 20 bp. The bisulfite sequencing reads for each sample were mapped to Senegalese sole reference genome (Solea_v4.1) using default parameters of Bismark software (Krueger & Andrews, 2011). This step relied on read 1 and read 2 to ensure proper alignment of both sequences. All strict duplicates originating from PCR bias were removed after alignment. For CpG sites, reads from both strands were combined to calculate the methylation levels. After alignment, the cytosine methylation level was calculated as the number of C bases (methylated reads) divided by the total number of C bases (methylated reads) and T bases (unmethylated reads) at the same position of each individual cytosine. The count files generated by the Bismark program for each condition was then processed for characterisation of differentially methylated cytosine (DMCs) between treatments, using DSS program (Feng et al., 2014; Wu et al., 2015). This DMC characterization was performed without preliminary smoothing option of DSS, because the smoothing could hide some isolated CpGs which were modified by cryopreservation (smoothing is more relevant when studying biological processes, where the methylation changes always affect a broad span of CpGs). From DMC characterization, a search for regions enriched in DMC (DMRs) was then performed with DSS as well. It was based on the search for 5 consecutive CpGs (in a sliding frame of 50 bp) which had at least 75 % of significant DMCs (false discovery rate – FDR- < 5 %).

2.4. Statistical analysis

The statistical analysis was performed with SPSS Statistics 28.0 software (IBM Co., Hong Kong). All results were presented with means ± standard error (mean ± S.E.M). Shapiro-Wilks test was used to analyse data normality and data transformation was performed when this was not normal distributed.

For experiment 1, a two-way ANOVA followed by a Tukey's HSD *post-hoc* was used to assess differences between different coatings in differential plating (uncoated, collagen, gelatine and laminin) and time (24h and 48h). One-way ANOVA followed by a Tukey's HSD *post-hoc* was used to assess the differences among strainers. For quantitative real time PCR, results were normalised using two house-keeping genes, ubiquitin (ubq) and

beta-actin (β-actin), calculating geometric average (Vandesompele et al., 2002). Pfaffl method (Pfaffl, 2001) was used to obtain the mRNA abundance for each gene. Student's t-test was used to assess the differences in gene expression between the two 5 µm strainers combined and the flow through (<5 µm). Level of significance was considered with a *P-value* <0.05.

For experiment 2, Student's t-test was used to assess the differences between the experimental groups (fresh and cryopreserved). Level of significance was considered with a *P-value* <0.05.

3. Results

3.1. Experiment 1: Testing different spermatogonia enrichment techniques

3.1.1. Differential plating

To assess the effect of extracellular matrixes (ECMs) on cellular enrichment, testicular cell suspension (8.62 x 10^6 cells in total containing 3.7 ± 1.2 % of spermatogonia) was seeded at 2 x 10^5 cells/well in uncoated, collagen, gelatine and laminin coated 6 well plates for 24 h and 48 h at 20ºC. Each well contained approximately 0.085 % of spermatogonia.

After 24 hours, it was observed that all ECMs showed poor enrichment, since there were still other cells (somatic, etc) apart from spermatogonia present in the cell suspension (Figure 3.1). Nevertheless, there was significant difference between the different ECMs (P<0.001). The percentage of spermatogonia recovery for gelatine (5.3 ± 0.9 %) was significantly higher when compared with uncoated (1.55 ± 0.86 %; P<0.001) and laminin (1 ± 0.22%; P<0.001). Collagen also presented a significantly higher percentage of spermatogonia recovery (5.3 ± 1.27 %) compared with uncoated (P=0.011) and laminin (P<0.001). Despite gelatine and collagen presenting the same spermatogonia recovery percentage, there was significant difference between the two ECMs (P=0.002). The laminin plate showed lower spermatogonia recovery percentages compared with uncoated, however there was no significant difference between the two ECMs (P= 0.816). In terms of cells attached to the coatings, no cells including spermatogonia were found (0%), meaning there was no attachment occurring in any of the ECMs.

After 48 hours, similar results from 24 hours were observed with all ECMs still showing poor enrichment (Figure 3.2). In fact, the percentages of spermatogonia recovery were lower than 24 hours (5.1 ± 2.3 % in gelatine, 3.8 ± 1.50 % in collagen, 1.4 ± 0.87 % in uncoated and 0.9 ± 1.27 % in laminin). However, there was no statistically significant effect of time (P=0.614) and the interaction between time and ECMs (P=0.506) on spermatogonia recovery (Figure 3.3). Attachment was still not reported (0%).

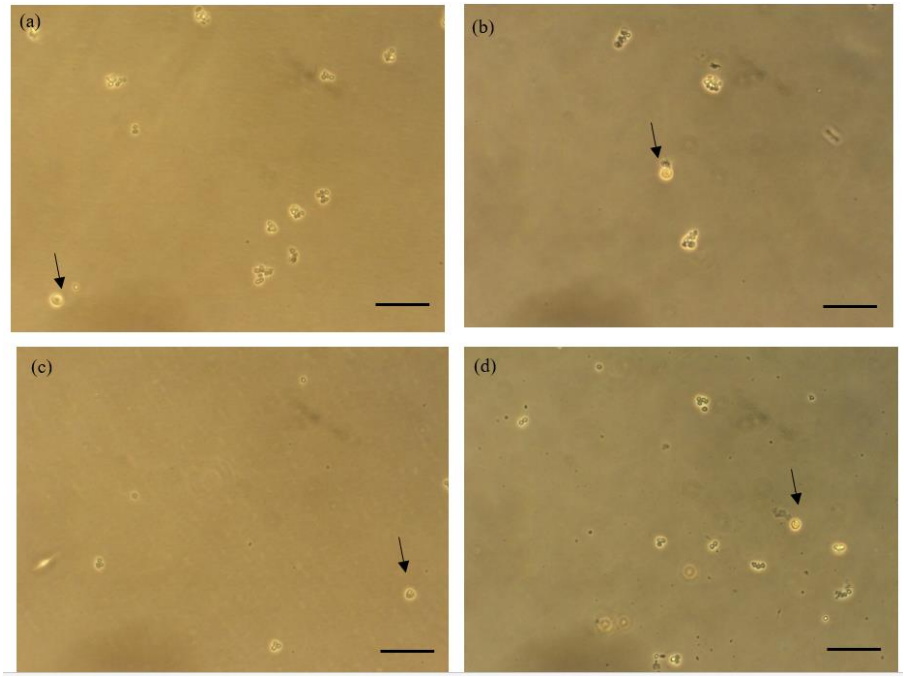

Figure 3.1: Cell suspension after 24h of culture in (a) uncoated (b) collagen (c) gelatine and (d) laminin plates. The arrows indicate the presence of spermatogonia cells. Scale bars: 50 µm.

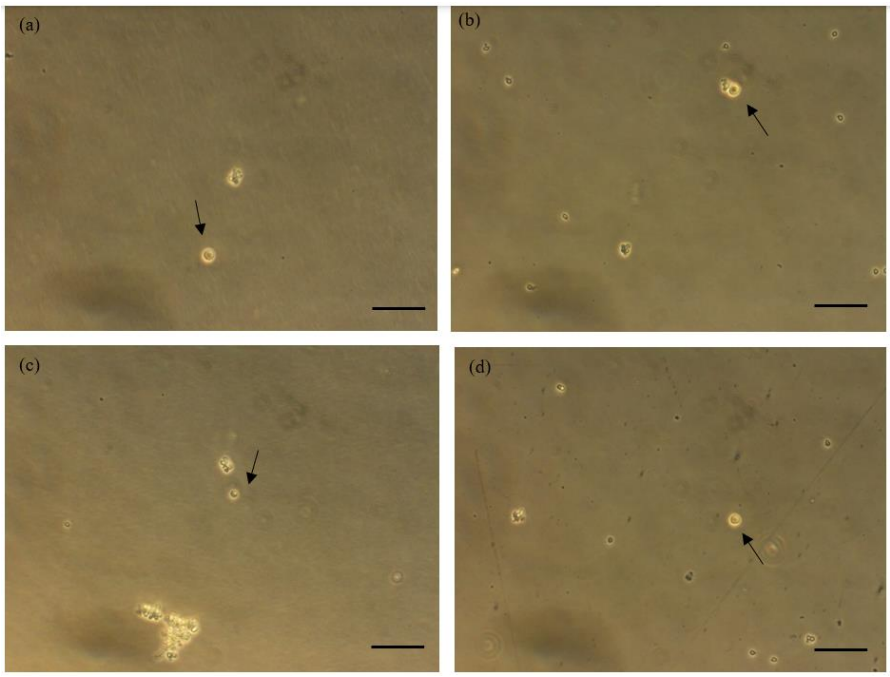

Figure 3.2: Cell suspension after 48h of culture in (a) uncoated (b) collagen (c) gelatine and (d) laminin plates. The arrows indicate the presence of spermatogonia cells. Scale bars: 50 µm.

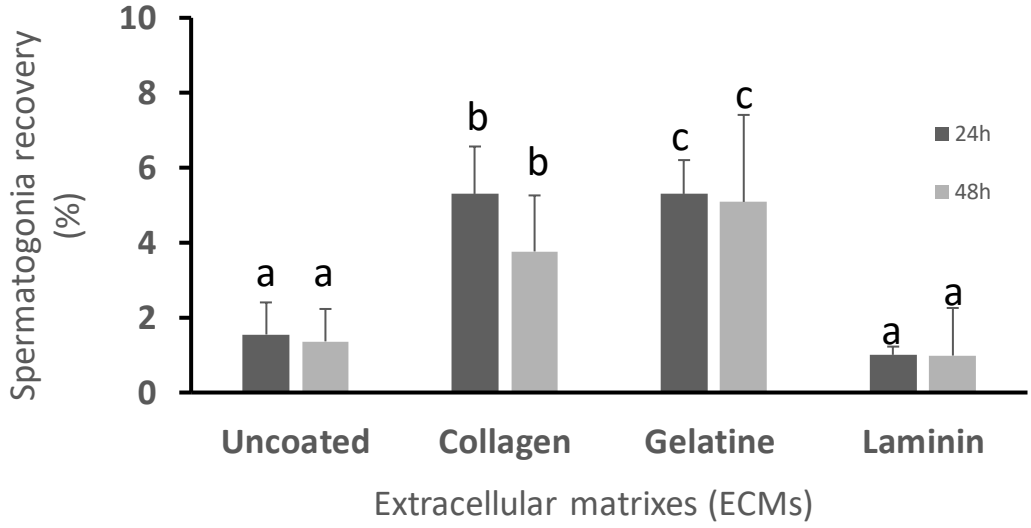

Figure 3.3: Percentage of spermatogonia recovery in different extracellular matrixes (ECMs) for 24h and 48h. Data is shown in mean ± S.E.M of 3 pools in replicates. Different letters indicate significant differences among the ECMs (Two-Way ANOVA followed by a Tukey's HSD *post-hoc*; $P < 0.05$).

3.1.2. Strainers

To test a different technique for cell sorting, testicular cell suspension (8.62 x 10^6 cells in total containing 3.7 ± 1.2 % of spermatogonia) was passed through a sequence of strainers (30 µm to 1 µm) to observe where spermatogonia retention occurred.

In the strainers of 30, 20, 15, 10 and 1 µm a high quantity of various cells with some spermatogonia was observed (Figure 3.4 and 3.5). The quantity of spermatogonia present was low for the 30, 20, 15, 10 and 1 µm strainers. There were no significant differences between these strainers (Figure 3.6). The strainer of 5 µm showed less contamination from other cells and higher amount of spermatogonia (64.2 ± 20.1 %), proving this strainer was the right size to be used in future protocols (Figure 3.5). All strainers showed significantly lower quantity of spermatogonia compared with the 5 µm strainer ($P<0.001$) (Figure 3.6).

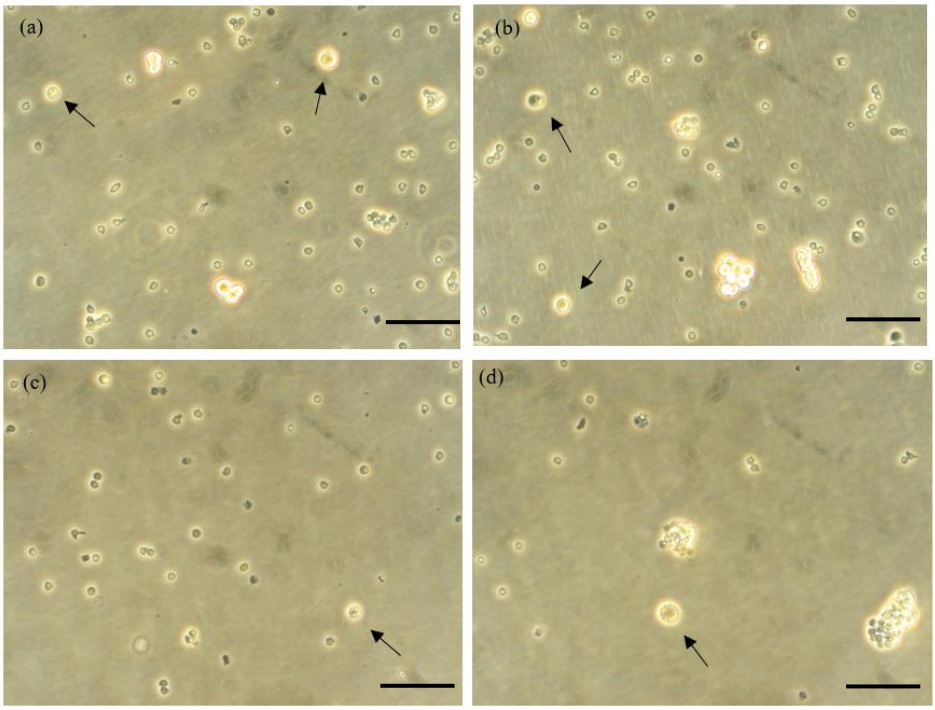

Figure 3.4: Cell suspension after passing through a (a) 30 µm (b) 20 µm (c) 15 µm and (d) 10 µm strainers. The arrows indicate the presence of spermatogonia cells. Scale bars: 50 µm.

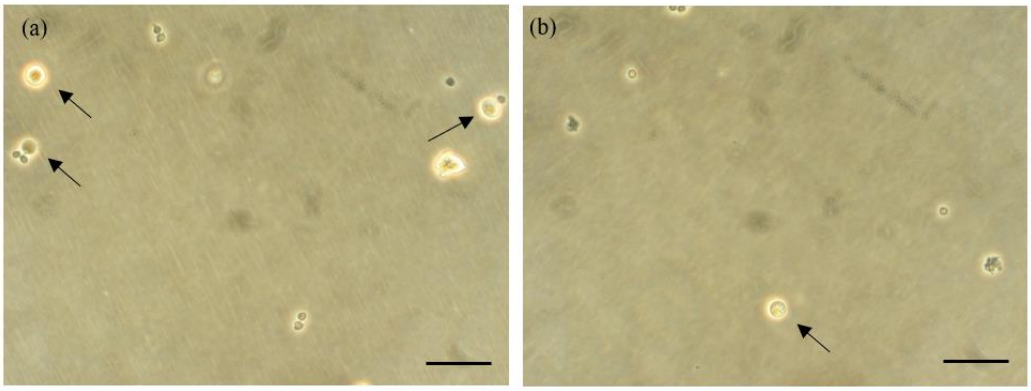

Figure 3.5: Cell suspension after passing through a (a) 5 µm and (b) 1 µm strainers. The arrows indicate the presence of spermatogonia cells. Scale bars: 50 µm.

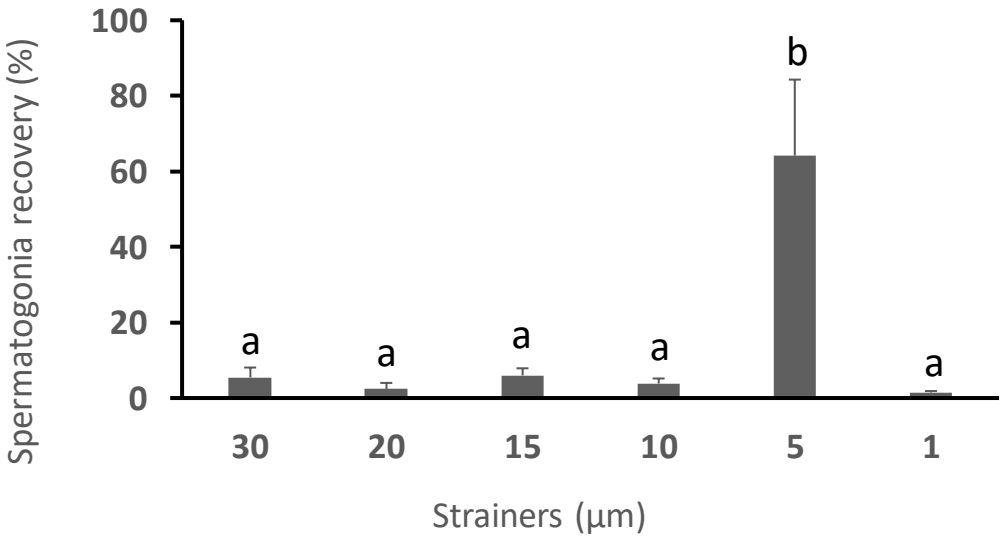

Figure 3.6: Percentage of spermatogonia recovery in different strainers (30 µm to 1 µm). Data is shown in mean ± S.E.M of 3 pools. Different letters indicate significant differences among the strainers (One-Way ANOVA followed by a Tukey's HSD *post-hoc*; P <0.05).

After observing the results from the serial strainers, the technique was improved by using two 5 µm strainer. Preliminary results obtained 59 ± 4.8 % of spermatogonia recovery in a total of 1.61 x 10^6 cells from both strainers.

Afterwards, to obtain more robust data, this method was repeated by passing testicular cell suspension (3.07 x 10^5 cells containing 4.6 ± 1.6 % spermatogonia) through both strainers. In the 1st strainer there was a significantly higher amount of spermatogonia (26.1 ± 6.1 %) compared with the flow through (<5 µm) (11.6 ± 6.8 %; P=0.038), however contamination from other cells was still present (Figure 3.7 and 3.8). The 2nd strainer showed a similar tendency as seen in the 1st strainer (Figure 3.7), with the exception that the number of cells was generally lower (17.9 ± 7.6 %), yet no significant differences were observed with the 1st strainer (P=0.263) and the flow through (P=0.438) (Figure 3.8). Finally, the flow through showed a high quantity of different cell types and lower percentage of spermatogonia recovery (Figure 3.7 and 3.8).

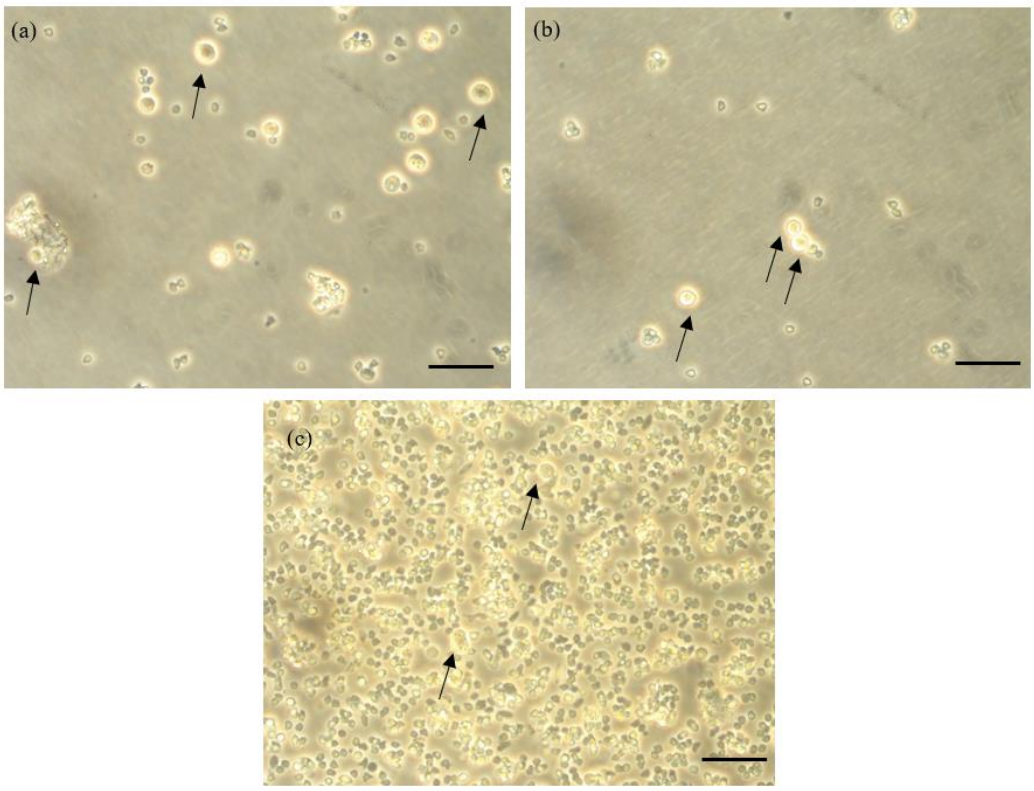

Figure 3.7: Cell suspension after passing through the (a) 1st strainer (5 µm) (b) 2nd strainer (5 µm) (c) <5 µm (flow-through). The arrows indicate the presence of spermatogonia. Scale bars: 50 µm.

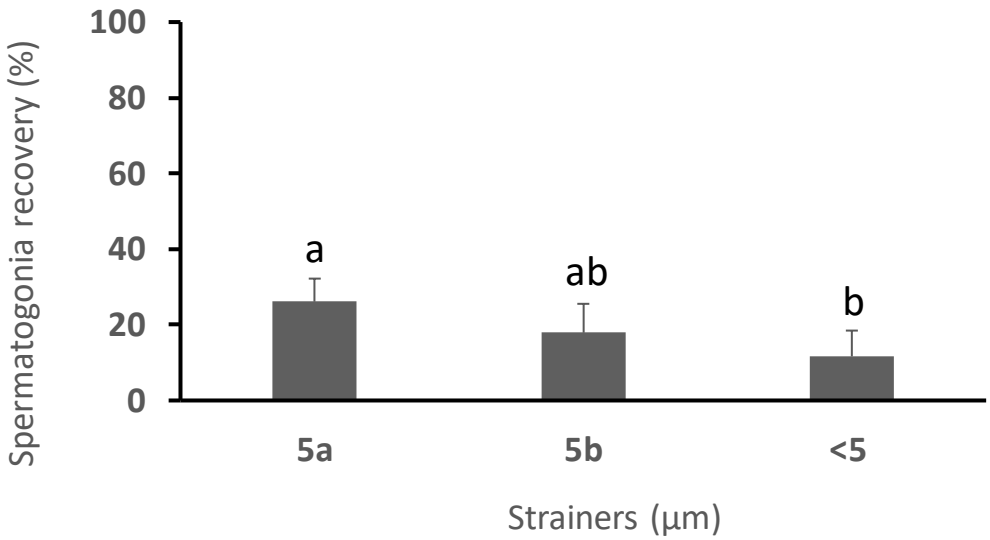

Figure 3.8: Percentage of spermatogonia recovery in 1st 5 µm strainer (5a), 2nd 5 µm strainer (5b) and flow through (<5 µm). Data is shown in mean ± S.E.M of 2 pools. Different letters indicate significant differences among the strainers (One-Way ANOVA followed by a Tukey's HSD *post-hoc*; P <0.05).

3.1.3. Spermatogonia markers validation

To assess the efficiency of the enrichment method using two strainers of 5 µm, quantitative real time PCR was run to detect relevant spermatogonia gene markers (*gfra1*, *pou5f1/oct4* and *nanos2*) in two pools of both 5 µm strainers combined and two pools of the flow through (< 5 µm). The expression of *gfra1* was up regulated in the samples from the strainers (3.05 ± 2.45) and down regulated in the samples from the flow through (-1.29 ± 0.29), while the expression of *pou5f1* (2.19 ± 2.35 strainers and 0.57 ± 2.48 flow through) and *nanos2* (2.43 ± 1.66 strainers and 2.35 ± 0.67 flow through) was up regulated for both samples. For all genes no significant difference between the two samples was observed (P=0.117 *gfra1*, P=0.392 *pou5f1* and P=0.935 *nanos2*). (Figure 3.9).

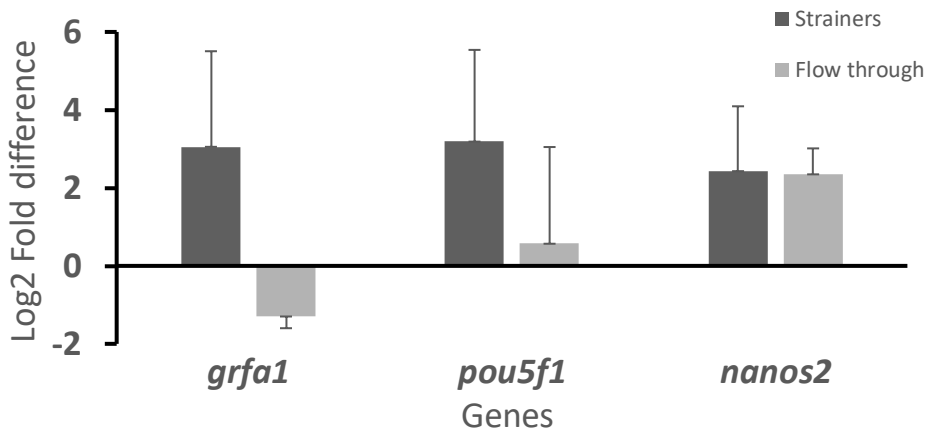

Figure 3.9: Gene expression of putative spermatogonia gene markers (*gfra1*, *pou5f1/oct4 and nanos2*) in samples of both 5 µm strainers combined and the flow through (< 5 µm). Data is shown in mean ± S.E.M of 2 pools. No letters means no differences among the strainers and the flow through (Student's t-test; P <0.05).

3.2. Experiment 2: Effect of cryopreservation on Senegalese sole spermatogonia quality

For this experiment different techniques were performed for 4 pools of testes from different males for each group (n=4) to observe the differences in quality between fresh and cryopreserved.

3.2.1. Cell viability

Cell viability was significantly higher in the fresh group (78.98 ± 5.66 %) when compared with the cryopreserved group (62.81 ± 3.25 %; P= 0.003) (Figure 3.10).

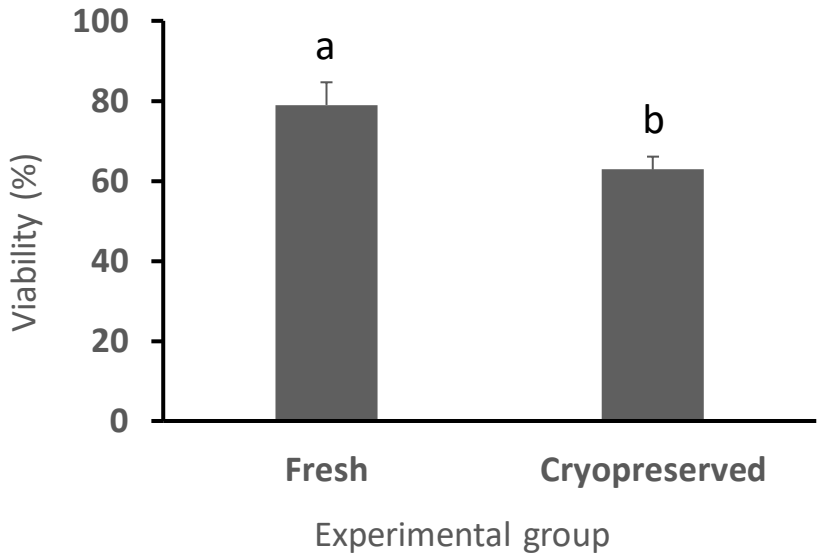

Figure 3.10: Percentage of viable cells in *S. senegalensis* fresh and post-thawed spermatogonia. Data is shown in mean ± S.E.M of 4 pools for each experimental group (fresh and cryopreserved). Different letters indicate significant differences among the experimental groups (Student's t-test; $P < 0.05$).

3.2.2. DNA integrity

The percentage of DNA fragmentation was significantly higher in the cryopreserved group (37.28 ± 1.87 %) when compared with the fresh group (32.95 ± 2.28 %; P= 0.026) (Figure 3.11).

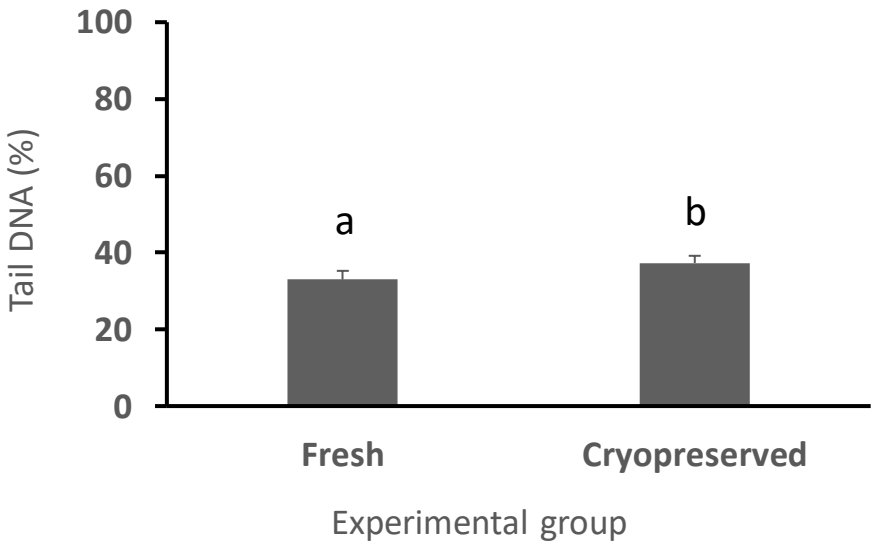

Figure 3.11: Percentage of DNA fragmentation in *S. senegalensis* fresh and post-thawed spermatogonia. Data is shown in mean ± S.E.M of 4 pools for each experimental group (fresh and cryopreserved). Different letters indicate significant differences among the experimental groups (Student's t-test; P <0.05).

3.2.3. Lipid peroxidation

There was no significant difference between the fresh (1.13 ± 0.45 µM of MDA per million spermatogonia) and cryopreserved group (0.91 ± 0.96 µM of MDA per million spermatogonia; P=0.701). Nevertheless, it can be observed that the cryopreserved group presented more variability than the fresh group (Figure 3.12).

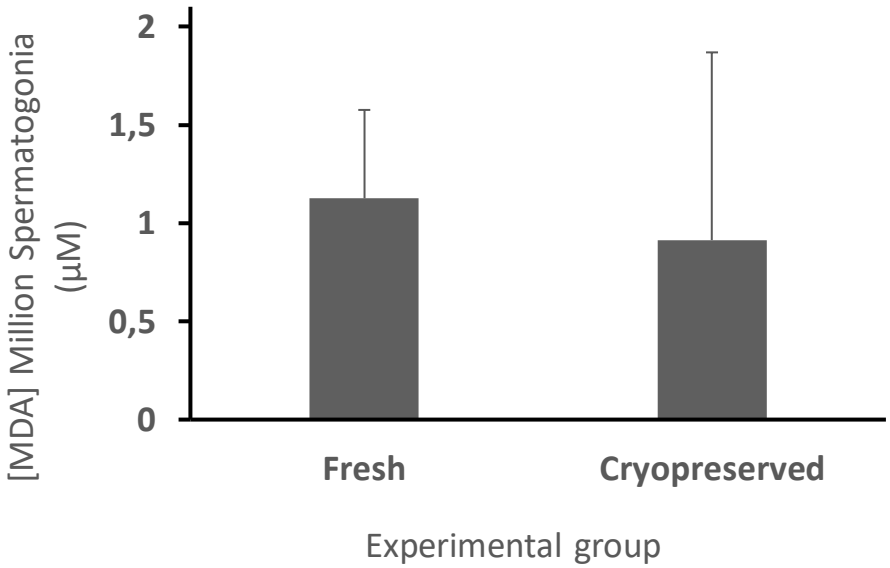

Figure 3.12: MDA concentration per million spermatogonia (µM) in *S. senegalensis* fresh and post-thawed spermatogonia. Data is shown in mean ± S.E.M of 4 pools for each experimental group (fresh and cryopreserved). No letters means no differences among the experimental groups (Student's t-test; P <0.05).

3.2.4. Epigenetics modifications

For the epigenetics modifications, samples were trimmed with success obtaining a good quality score. Then, alignment with the genome was performed and mapping efficiency was 70.8 ± 0.42% for the fresh group and 69.6 ± 1.24% for the cryopreserved group. In terms of the global overview of the cytosines methylation of the whole genome, 23,903,992 CpG dinucleotide were observed in at least one sample, which represent 96% of CpG from *Solea senegalensis* species genome (24,865,834). The bisulfite conversion efficiency was obtained after calculating the methylation percentage of the cytosine sites which were not in CpG context, and that were therefore not supposed to be methylated (number of cytosines in non-CpG context that were not converted in T, divided by the total number of cytosines in non-CpG context). This control ensured that no bias in bisulfite conversion occurred, which would lead to biased methylation assessment. Results showed that less than 0.5 % of the cytosines outside of CpG context were methylated giving a bisulfite conversion efficiency of more than 99.5 % (Table 3.1).

Methylated cytosines were observed in three contexts (CpG context, CHG context and CHH context) and the percentages of methylated cytosines were higher in CpG context for both groups (Table 3.1). However, no significant differences were observed between the fresh and cryopreserved group in the unmethylated cytosines (P=0.699), methylated cytosines in CpG context (P=0.733), methylated cytosines in CHG context (P=1) and methylated cytosines in CHH context (P=0.537). The principal component analysis (PCA) showed good separation between the two groups and higher dispersion from the cryopreserved group, meaning there was difference in the methylation profile (Figure 3.13). In fact, the comparison between fresh and cryopreserved differentially methylated cytosines in CpG context (DMC) showed that there was a total of 233 DMC of which 167 were up regulated and 66 down regulated related to 180 genes. However, no differentially methylated regions (DMR) were detected in the analysis, meaning the effect of cryopreservation was scattered all over the genome.

Table 3.1: Percentages of cytosines unmethylated and methylated in CpG, CHG and CHH context in fresh and cryopreserved samples in the whole genome. Data is shown in mean ± S.E.M of 4 pools for each experimental group (fresh and cryopreserved) (Student's t-test; P <0.05).

	Fresh	**Cryopreserved**
Unmethylated cytosines	17.28 ± 0.48%	17.05 ± 1.00%
Methylated cytosines CpG context	81.8 ± 0.39%	82 ± 1.05%
Methylated cytosines CHG context	0.48 ± 0.05%	0.48 ± 0.05%
Methylated cytosines CHH context	0.45 ± 0.06%	0.48 ± 0.05%

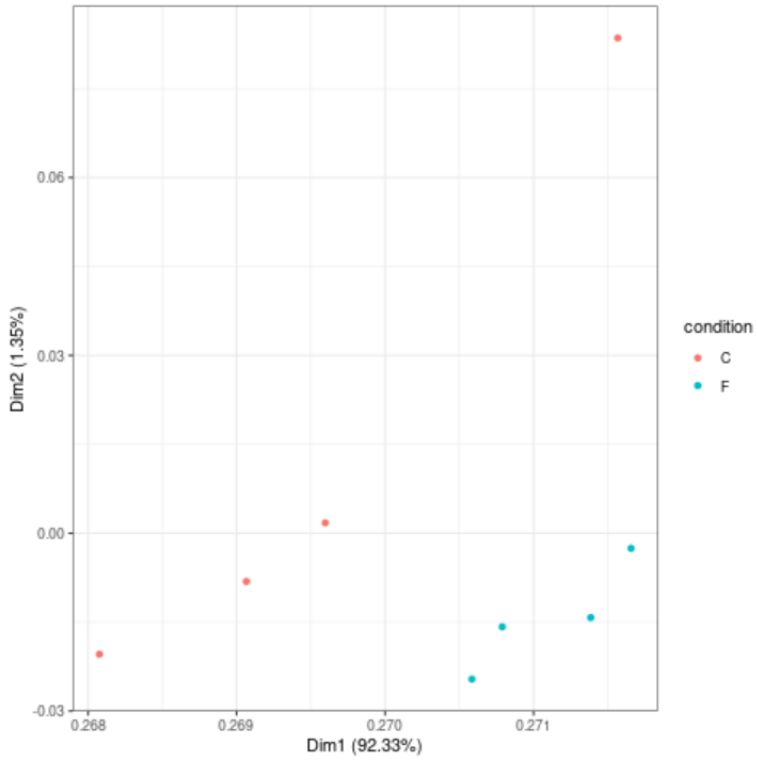

Figure 3.13: Principal component analysis (PCA) of spermatogonia for the 4 pools for each experimental group (fresh and cryopreserved).

4. Discussion

4.1. Testing different spermatogonia enrichment techniques: differential plating and strainers

Techniques for long-term *in vitro* culture of spermatogonia using differential plating have been established in several species such as humans (Gat et al., 2018), mice (Mulas et al., 2019), bulls (*Bos taurus;* Rafeeqi & Kaul, 2012), cats (Tiptanavattana et al., 2015), pigs (*Sus scrofa domesticus*; Dores et al., 2015) and in some fish species, such as zebrafish (*Danio rerio*; Kawasaki et al., 2012), rainbow trout (Shikina & Yoshizaki, 2010), Nile tilapia (*Oreochromis niloticus*; Lacerda et al., 2013), Japanese eel (*Anguilla anguilla*; Miura et al.,1991) and sturgeon (*Acipenser dabryanus*; Xie et al., 2019). In mammal species, spermatogonia stem cells tend to attach to the coating substance and somatic cells remain in suspension (Tiptanavattana et al., 2015), while in fish species spermatogonia are suspended or weakly attached to the somatic cells or cultured plate, and somatic cells attach tightly to the bottom of the culture plate (Shikina & Yoshizaki, 2010). This difference in species can cause some restraints while using this technique, however

several studies in fish species (Miura et al., 1991; Shikina & Yoshizaki, 2010; Xie et al., 2019) have shown promising results with differential plating. However, in this study, the results showed that all the cells, including somatic, were maintain in suspension, which goes against previously studies performed on different fish species. This difference in results may explain the low percentages of spermatogonia recovery using differential plating.

In the present study, we tested the effect of different extracellular matrixes (ECMs) (uncoated, collagen, gelatine and laminin) and time (24h and 48h) on spermatogonia enrichment. The results showed that only ECMs have a significant effect on spermatogonia enrichment in differential plating. The presence of spermatogonia in the collagen and gelatine plates was slightly higher compared with the other two matrixes. This is in concordance with several successful studies performed on fish (Shikina & Yoshizaki, 2010; Kawasaki et al., 2012; Lacerda et al., 2013; Xie et al., 2019), which use most frequently gelatine plates due to its cost-effective and optimization in various cell types. Collagen-coated plates have also demonstrated to improve the purification efficacy of spermatogonia isolation in mammals (Arora et al., 2003; Dhaliwal et al., 2010). Despite these two ECMs showing higher spermatogonia recovery percentage, perhaps other ECMs, such as fibronectin (Xie et al., 2020), might be more specific for Senegalese sole spermatogonia and can give better enrichment results.

Most studies in spermatogonia isolation usually combine differential plating with other separation methods (double enrichment) for a greater success. The use of a feeder cell layer has been employed in Japanese eel (Miura et al.,1991) and in zebrafish (Leal et al., 2009) showing suitable results. Lacerda et al. (2013) combined discontinuous Percoll gradient density centrifugation and differential plating to enrich tilapia spermatogonia with great success. In the present study, no combined methods were used to perform enrichment due to the low biological material recovered in Senegalese sole juvenile testes. Moreover, different studies in rainbow trout (Sato et al., 2014) and catfish (*Ictalurus punctatus*, Shang et al., 2015) have reported that Percoll has a low-resolution capacity (difficulty to distinguish spermatogonia from Sertoli cells, peritubular myoid cells and Leydig cells) which might also make this technique difficult to use in Senegalese sole.

The most important reason why DP did not produce higher enrichment/higher recovery is due to characteristics associated with the species of study. As mentioned before, this method has showed good and promising results in different fish species (Miura et

al.,1991; Shikina & Yoshizaki, 2010; Xie et al., 2019). In some fish species, such as the eel and sturgeon, immature testes present itself in the same development stage, meaning testes contain only type A and B spermatogonia in high quantities (Miura et al., 1991; Xie et al., 2020). Besides that, the development of the gonads is highly affected by temperature and can be stopped at a certain stage if changes of temperature occur (Miura et al., 1991). All of this allows the use of DP as an efficient enrichment method, since there is the possibility of obtaining a high quantity of spermatogonia from the fish gonads. In the target species, Senegalese sole, such situations do not occur, since the growth of this fish triggers maturation of the testes, making cells enter the spermatogenesis process, even at very early juvenile stages (Pauly, 2021). This means that the quantity of spermatogonia present in the gonad is very low making separation by DP challenging, due to cell differentiation during the process. Besides, it has been estimated that only 0.03% of the total testicular cells in mammals are spermatogonia (van Pelt et al., 1996), which demonstrates the importance of using animals still in puberty. All these constraints were possible to observe in the data where most Senegalese sole testicular cells suspension only contained approximately 4% of spermatogonia in total.

Other methods, such as fluorescence-activated cell sorting (FACs) can be employed for isolation of spermatogonia. In this study, we tried using FACs as an enrichment method (*data not shown*), however results were not obtained due to low quantity of spermatogonia in Senegalese sole. For that reason, strainers were used to separate cells populations with respect to their size. This method is not usually used for spermatogonia enrichment alone, but as a technique to help prepare samples before FACs (Watarai et al., 2007). Better results were observed with the strainers, with more spermatogonia and less contamination from other cells, when compared with the use of DP. This was corroborated by other studies (Langenstroth et al., 2014; Xie et al., 2020) that showed that with DP a complete separation from somatic cells is not fully effective and that a substantial quantity of cells is lost. Nevertheless, successful enrichment of spermatogonia was still not achieved meaning that other cells were still present and the presence of spermatogonia was very low. The main reason for the low percentage of spermatogonia recovery might be associated with the low quantity of spermatogonia, as previously mentioned, characteristic to Senegalese sole and the type of gonad development. Additionally, results obtained using this method were very variable with spermatogonia recovery percentages going from 6% to 59% in preliminary tests (*data not shown*). This is mainly due to

individual variability, temperature and gonad development, which affects greatly the success of the spermatogonia enrichment.

To confirm and quantify the presence of spermatogonia, putative spermatogonia markers (*gfra1, pou5f1/oct4, nanos2*) were used in real-time PCR in the samples obtained from the enrichment method with two 5 µm strainers. These transcripts have been used to evaluate the presence of spermatogonia in mice (He et al., 2007; Saga, 2010; Zheng et al., 2016) and in fish (Lacerda et al., 2013, 2014; Bosseboeuf et al., 2014; Xie et al., 2020), yet there is a lack of information on molecular marks of SSCs in Senegalese sole. Glial cell line-derived neurotrophic factor receptor alpha 1 (GFRA1) is a co-receptor of RET for the growth factor GDNF (glial cell line-derived neurotrophic factor). GDNF, produced by Sertoli cells, can regulate the proliferation and differentiation of undifferentiated spermatogonia and SSCs (He et al., 2007). The *gfra1* transcript expression has been reported in the testis of Nile tilapia (Lacerda et al., 2013) and dogfish (*Squalus acanthias*; Bosseboeuf et al., 2014) with an expression restricted to undifferentiated spermatogonia, promoting its self-renewal and maintenance. The *pou5f1* transcription factor is strongly expressed in spermatogonia and its removal results in apoptosis of primordial germ cells (Zheng et al., 2016). The expression of *pou5f1* has been observed in Nile tilapia testis, mainly in undifferentiated spermatogonia, and it is downregulated as spermatogonia differentiate into other cell types (Lacerda et al., 2013). The *nanos2* transcript is responsible for differentiation of germ cells and maintenance of SSCs (Saga, 2010). The expression of *nanos2* has been observed in undifferentiated spermatogonia populations in Nile-tilapia testis (Lacerda et al., 2013) and in rainbow trout (Bellaiche et al., 2014). The results for all three genes showed that there was no significant difference between the samples from strainers and samples from the flow-through (<5 µm), which is mainly due to a low spermatogonia recovery percentage and the sample size per group being too small (Hackshaw, 2008). However, it is important to note that all genes were up regulated in the samples from the strainers which contained more spermatogonia. This is corroborated with other studies performed on fish species where these genes are down regulated when spermatogonia differentiate (Lacerda et al., 2013, 2014; Bosseboeuf et al., 2014; Bellaiche et al., 2014). Yet, the transcripts *pou5f1* and *nanos2* were also up regulated in the samples from the flow-through, which might indicate that these genes are not exclusively specific for Senegalese sole spermatogonia

cells. This is also observed in medaka (*Oryzias* latipes) where both these genes are weakly expressed in spermatogonia (Aoki et al., 2009; Froschaeur et al., 2013).

To sum up, more studies need to be performed to address the limitations of these techniques and more methods should be tested. Future research should focus on new solutions, such as assessing optimal temperature and apply double enrichment, to achieve successful spermatogonia enrichment and optimize the culture conditions for supporting the survival and mitotic activity of sole spermatogonia. Additionally, strategies to avoid other cells contaminations and not lose spermatogonia during the enrichment process, such as changes in FBS percentage to remove testicular somatic cells and addition of growth factors to combat the gradual decrease of total number of spermatogonia (Shikina & Yoshizaki, 2010), should also be tested.

4.2. Effect of cryopreservation on Senegalese sole spermatogonia quality

Cryopreservation exposes cells to oxidative stress during dilution in the extender media, cryoprotectant exposure and cooling events. These events cause an excessive accumulation of reactive oxygen species (ROS), such as hydroperoxyl and hydroxyl radicals, that cause cell damage, like lipid peroxidation and apoptosis, and attack DNA at a sugar level resulting in fragmentation, base loss and strand break (Cabrita et al., 2014). This damage has been reported in several fish species (Li et al., 2010; Cabrita et al., 2011b; Hagerdon et al., 2012b; Shaliutina et al., 2013; Riesco et al., 2017) but has been exclusively focused only on the effects on spermatozoa. The damage reported on fish sperm are a decrease in DNA integrity, sperm motility and viability, which are key factors in sperm quality and fertilizing ability (Bobe & Labbé, 2010). Spermatogonia are early-stage male germ cells that proliferate and differentiate to form mature spermatozoa. The objective of spermatogonia is to transmit genetic information which will contribute to embryo development. Cryopreserved spermatogonia can be transplanted between closely related species, making spermatogonia xenotransplantation a useful tool to produce surrogated broodstocks (Pacchiarini et al., 2014), since spermatogonia will differentiate into spermatozoa or oocytes depending on the sex determination of the recipient specimen. Therefore, there is a great need to further explore the effects of cryopreservation on spermatogonia quality, especially due to their use in cryobanks.

In this perspective, quality assays (cell viability, DNA integrity, lipid peroxidation and epigenetic modifications) were performed to assess the effect of cryopreservation on Senegalese sole spermatogonia. In terms of cell viability, a significant difference between fresh and cryopreserved group was observed with the former showing higher percentage of viable cells than the latter, meaning spermatogonia viability was affected by cryopreservation. These results were in concordance with studies performed on different fish species (Li et al., 2006; Cabrita et al., 2005b; Cabrita et al., 2011b; Hagedorn et al., 2012b; Valcarce & Robles, 2016), where cryopreservation injures cell membranes and causes alteration in their structure, functionality, and permeability (Cabrita et al., 2010a). These alterations may affect the ability of spermatogonia to differentiate into spermatozoa or oocytes and the quality of these two gametes, which means that might cause problems on future transplantation while using cryopreserved spermatogonia.

Comet assay is a simple and sensitive method for detection of DNA damage at single cell level. This study is one of the first using comet assay on spermatogonia and the results showed a significant difference between cryopreserved and fresh group with DNA fragmentation being higher in cryopreserved cells (37.28%). These results were slightly higher than the ones obtained by our group in a previous study (Cabrita et al., 2022), but have the same relationship. Authors obtained 12% of fragmented DNA in fresh samples and 20% in cryopreserved samples where in our case the fresh samples (32.95%) already had higher damage from the beginning. This higher value in fresh might be due to individual variability, temperature or gonad development. In general, cryopreservation increased the DNA fragmentation of spermatogonia which may affect the overall quality of the gametes used to produce progeny. For that reason, future transplantation might encounter some issues using cryopreserved spermatogonia.

Lipid peroxidation is a process caused by oxidative stress where an excessive accumulation of ROS provokes oxidative degradation of lipids in the plasma membrane (Cabrita et al., 2014). Lipid peroxidation is particularly important for aquatic animals, since they normally contain greater amounts of highly unsaturated fatty acids present in their membranes than other species (Shaliutina et al., 2013). This process triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, resistance to osmotic shock, and fertilization potential (Shiva et al., 2011). Spermatozoa is highly susceptible to oxidative damage, due to their high polyunsaturated fatty acid content, causing deleterious effects on sperm function and plasma membrane structure

(Shiva et al., 2011). The results of the present study showed that there was no significant difference in MDA concentration among the experimental group. This goes against studies performed on sperm for species such as Russian and Siberian sturgeons (*Acipenser sturio and Acipenser baerii*; Shaliutina et al., 2013), European carp (*Cyprinus carpio*; Li et al., 2010), Senegalese sole (Riesco et al., 2017) and zebrafish (Hagedorn et al., 2012b), where cryopreservation induces higher levels of lipid peroxidation causing general cell dysfunction. The lack of significant difference in the results can be explained by the fact that spermatogonia compared with sperm is more resistant to lipid peroxidation due to their antioxidant defence system. Spermatogonia needs to ensure the propagation of DNA and integrity of genes required for development and continuity of life (Celino et al., 2011). For that reason, spermatogonia have evolved with higher levels of Cu/Zn that provide them resistance to reactive oxygen species (ROS) and subsequently protection against oxidative stress (Celino et al., 2011). Despite cryopreserved spermatogonia not suffer lipid peroxidation, loss of cell viability and DNA integrity occurs, meaning other processes, such as DNA methylation, might also cause an overall decrease in the quality of the gametes used to produce progeny after transplantation and cell differentiation.

DNA methylation is a key epigenetic modification that provides heritable information not encoded in the nucleotide sequence and plays a critical function in many biological processes. When sperm samples are cryopreserved with less suitable cryoprotectants, chemical-specific effects may trigger alteration on DNA methylation in damaged cells, which may be transmitted to progeny after fertilization (Depincé et al., 2019). In this study, spermatogonia global DNA methylation levels were unaffected by cryopreservation, since there was no significant difference between the fresh and cryopreserved group. This indicates that the cellular alterations induced by cryopreservation had no effect on spermatogonia global DNA methylation, meaning the cryoprotectant dimethyl sulphoxide (DMSO) might not induce any alteration in terms of DNA methylation and protects spermatogonia from cryopreservation damage. This is in accordance with a study performed in Senegalese sole that showed that DMSO was the best cryoprotectant for cryopreservation of this species sperm (Riesco et al., 2017) and spermatogonia (Cabrita et al., 2022). However, some studies performed on fish sperm showed that there is no straightforward effect of cryoprotectants, with some reporting that the cryoprotectant DMSO caused DNA hypomethylation (Depincé et al., 2019) and others DNA hypermethylation (Herranz-Jusdado et al., 2019). Despite data showing no

thorough change in the global DNA methylation of cryopreserved samples, cryopreservation induced enough changes in the CpG (region where a cytosine is followed by a guanine) status of the samples to show some statistical segregation between fresh and cryopreserved ones. Moreover, the data from this study showed that the cryopreserved group demonstrated a different methylation profile with more differentially methylated cytosines (DMC) when compared with the fresh group, yet no differentially methylated regions (DMR) were found showing that the effect of cryopreservation was scattered all over the genome and it was not restricted to small specific areas. This has also been observed in other species in sperm such as black rockfish (*Sebastes schlegelii*; Niu et al., 2022). Overall, these results demonstrate that the cryopreservation process might not highly affect the spermatogonia epigenetics. This is essential when using cryopreserved spermatogonia in transplantations since alterations on DNA methylation can be transmitted to the progeny after spermatogonia differentiate into spermatozoa or oocytes. More in depth information about the type of genes of spermatogonia affected by cryopreservation needs to be done to fully understand the impact of DMSO as a cryoprotectant and freezing/thawing process.

To sum up, cryopreservation causes loss of cell viability and DNA damage on Senegalese sole spermatogonia. Epigenetics were also observed to be affected by cryopreservation but with less impact. Future research should focus on improving the cryopreservation protocol to decrease the damage suffered by cells during this technique. Additionally, more studies should be done on the correlation of spermatogonia cryoinjury with quality of spermatozoa and oocytes after transplantation to determine the degree of damage that can be accepted for GenBank and commercial purposes. Finally, future studies should explore if the freezing/thawing process or the toxicity of cryoprotectants is the reason behind spermatogonia damage during cryopreservation.

5. Conclusion

In conclusion, differential plating is not a suitable technique to be used for spermatogonia enrichment in Senegalese sole. Other technique (strainers) has showed promising results, but more studies need to be performed to address the limitations of this study. In terms of spermatogonia quality after cryopreservation, cell viability, DNA integrity and

epigenetics were affected. Future research needs to focus on improving the cryopreservation protocol of Senegalese sole.

6. References

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