UNIVERSITY OF SÃO PAULO
School of Pharmaceutical Sciences
Graduate Program in Food Sciences
Area of Experimental Nutrition

Selenium supplementation during puberty and young adulthood mitigates obesity-induced metabolic, cellular and epigenetic alterations in rat male physiology

Gabriela de Freitas Laiber Pascoal

São Paulo

2022

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Supervisor: Prof. Dr. Thomas Prates Ong

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supervisor

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Dedication

To Almighty God who has been my source of Strength, Grace and Wisdom. To my beloved family for their love and support.

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Toute réussite déguise une abdication (S. de BEAUVOIR.)

RESUMO

PASCOAL, G.d.F.L Selenium supplementation during puberty and young adult-hood mitigates obesity-induced metabolic, cellular and epigenetic alterations in male rat physiology. ___f. Thesis (PhD) – (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo.

O papel do selênio (Se) na obesidade não é claro. Além disso, a informação sobre o papel do Se na fisiologia reprodutiva masculina, especificamente na obesidade, é escassa. Conduzimos este estudo para avaliar a eficácia da suplementação de Se especificamente durante a puberdade até à idade adulta jovem contra a desregulação induzida pela obesidade nos parâmetros metabólicos, celulares e epigenéticos na gordura epididimal e/ou nas células espermáticas num modelo de rato. O consumo elevado de gordura por ratos machos durante a puberdade e na idade adulta jovem aumentou significativamente o peso corporal, o tamanho dos adipócitos, o stress oxidativo, a expressão desregulada de genes associados à inflamação (Adiponectina, IL-6, TNF-α), adipogênese (CEBPα), biossíntese de estrogênio (CYP19) e processos epigenéticos em tecido adiposo epididimal (Dnmt3a), bem como expressão alterada de microRNA vital para a espermatogênese em células espermáticas (miR-15b e miR-497). Por outro lado, a suplementação com Se aumentou a atividade antioxidante, diminuiu o stress oxidativo e mitigou estas alterações moleculares/epigenéticas tanto no tecido adiposo epididimal como nas células espermáticas. Os nossos resultados indicam que a suplementação com selênio durante a puberdade/ idade adulta poderia melhorar a fisiologia reprodutiva masculina num contexto de obesidade. Além disso, sugere que o Se poderia potencialmente afetar positivamente a saúde da descendência.

Palavras-chave: Selênio, Obesidade, Fisiologia reprodutiva masculina, Reprogramação epigenética.

PASCOAL, G.d.F.L Selenium supplementation during puberty and young adult-hood mitigates obesity-induced metabolic, cellular and epigenetic alterations in male rat physiology. ___f. Thesis (PhD) – (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo.

Selenium (Se) role in obesity is not clear. In addition, information on Se's role in male physiology, specifically in obesity, is scarce. We conducted this study to evaluate the efficacy of Se supplementation, specifically during puberty until young adulthood, against obesity-induced deregulation of metabolic, cellular, and epigenetic parameters in epididymal fat and/or sperm cells in a rat model. High-fat-diet consumption by male rats during puberty and young adulthood significantly increased body weight, adipocyte size, oxidative stress, deregulated expression of genes associated with inflammation (Adiponectin, IL-6, TNF-α), adipogenesis (CEBPα), estrogen biosynthesis (CYP19) and epigenetic processes in epididymal adipose tissue (Dnmt3a), as well as altered microRNA expression vital for spermatogenesis in sperm cells (miR-15b and miR-497). On the other hand, Se supplementation significantly decreased oxidative stress and mitigated these molecular/epigenetic alterations in epididymal adipose tissue or sperm cells. Our results indicate that selenium supplementation during puberty/young adulthood could improve male physiology in the context of obesity. In addition, it suggests that Se could potentially positively affect offspring health.

Keywords: Selenium, Obesity, Male reproductive physiology, Epigenetics reprogramming

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Preface

Obesity, defined as a body mass index (BMI) of >30 kg/m2, is among the most alarming health concerns, impacting public health and is associated with premature mortality, an increase of proportion of the worldwide non-communicable disease burden, including type 2 diabetes, cardiovascular disease, hypertension and certain cancers (LOOS; YEO, 2022; WATANABE; NAVARRO, 2021).

Obesity is also known to disrupt male fertility and the reproduction potential, particularly through alteration in the hyperestrogenism, hypogonadotropic hypogonadism, sexual dysfunction, elevated levels of inflammatory mediators and reactive oxygen species (ROS), increased testicular heat, which cumulatively can have substantial, detrimental effects on spermatogenesis and sperm epigenetic perturbations (LEISEGANG et al., 2021). Moreover to the immediate effects that obesity has on the father, there is evidence that negative effects could be transmitted to the offspring via epigenetic mechanisms of germ cell DNA (KAHN; BRANNIGAN, 2017).

Great attention has been given to the importance of bioactive compounds and micronutrients in the management of male physiology. Several vitamins, trace elements, and phytochemicals obtained from the diet have been shown to participate in different processes involved in male reproductive function (NASSAN; CHAVARRO; TANRIKUT, 2018) In particular, Selenium (Se), an essential micronutrient for humans by acting in many biological aspects (WATANABE; NAVARRO, 2021). From the point of view of paternal nutrition, has a critical role in male reproductive tissue development and spermatogenesis. Selenium is a constituent of selenoproteins that protect spermatozoa against ROS and simultaneously increases motility and sperm viability (SKORACKA et al., 2020).

Understanding the influence of selenium interventions on male physiology can serve as a tool for dietary interventions in obese men. This could have an impact not only for themselves but potentially for their future descendants.

This thesis was divided into two chapters. Chapter 1, (under revision in Nutrients Journal) entitled “Effect of paternal diet on spermatogenesis and offspring health: Focus on epigenetics and interventions with bioactive compounds” where was approached the paternal interventions with bioactive food compounds (BFCs) as an epigenetic strategy to improve health and prevent chronic disease in the offspring. Following in chapter 2, published in Antioxidants Journal (doi:10.3390/antiox11050895), entitled “Selenium supplementation during puberty and young adulthood mitigates obesity-induced metabolic, cellular and epigenetic alterations in rat male physiology” are presented as a compilation of data from my PhD project where I evaluated the efficacy of selenium supplementation specifically during puberty until young adulthood against obesity-induced deregulation of metabolic, cellular and epigenetic parameters in epididymal fat and/or sperm cells in a rat model.

CHAPTER 1: Effect of paternal diet on spermatogenesis and offspring health: Focus on epigenetics and interventions with bioactive food compounds

1 Introduction

Infertility is defined by the World Health Organization (WHO) as the failure to achieve a pregnancy after at least 12 months of regular unprotected intercourse (ZEGERS-HOCHSCHILD et al., 2009) and has become a growing health concern for couples in present times. It is estimated that 20–70% of fertility problems are caused by the male partner and at least 30 million men worldwide are infertile (AGARWAL et al., 2015; INHORN; PATRIZIO, 2014; MARTINS; MAJZOUB; AGAWAL, 2019).

Men experience a decrease in fertility potential during aging (HARRIS et al., 2011). Importantly, imbalanced nutrition, including excessive intake of calories and malnutrition (low intake of fibers, vitamins, and bioactive food compounds [BFCs]), together with the lack of physical activity, contributes to body fat accumulation and the development of non-communicable diseases, such as cardiovascular diseases, diabetes and cancer (WORLD HEALTH ORGANIZATION, 2021). Furthermore, lifestyle factors such as smoking, alcohol abuse and poor nutritional intake can negatively impact the quality of sperm parameters, such as semen volume, sperm motility and quality, promoting a decline in male fertility potential (BELAN et al., 2019; BISCONTI et al., 2021; DANIELEWICZ; PRZYBYŁOWICZ; PRZYBYŁOWICZ, 2018; NASSAN; CHAVARRO; TANRIKUT, 2018; SHARMA et al., 2013; TAKESHIMA et al., 2021).

BFCs can be defined as nutrients and non-nutrients present in the food matrix that can produce physiological effects beyond their classical nutritional properties (CAZARIN, 2022), and have emerged as a potential treatment for male infertility (CAZARIN, 2022).Many vitamins, trace elements, and other BFCs obtained through the diet have been shown to participate in different processes involved in male reproductive function (DIAS et al., 2018; SALAS-HUETOS et al., 2019; SALAS-HUETOS; BULLÓ; SALAS-SALVADÓ, 2017; SULIGA; GŁUSZEK, 2020). These BFCs comprise molecules with antioxidant properties that are involved in cell

protection from damage due to reactive oxygen species (ROS), mediate inflammation, and support the antioxidant defense system, which can prevent reduced sperm motility, reduced sperm count and abnormal morphology (AGARWAL; SALEH; BEDAIWY, 2003; HOMA et al., 2015).

Recently, increased interest has been directed towards understanding epigenetic regulation in male reproductive physiology (SCHAGDARSURENGIN; STEGER, 2016). Epigenetics is by definition the gene regulation process without changes in DNA sequence and includes DNA methylation, posttranslational histone modifications, and non-coding RNAs, including microRNAs (miRNA) regulation (ROTONDO et al., 2021).

Epigenetic changes occur during spermatogenesis, including significant reorganization of sperm chromatin structure, thus allowing the sperm cell to become highly specialized (CRAIG et al., 2017a). Therefore spermatogenesis is particularly vulnerable to epigenetic alterations. Pre-puberty/puberty and adulthood comprise developmental windows in which the epigenome would be especially plastic and susceptible to changes induced by environmental factors such as male diet (FONTELLES et al., 2018; SOUBRY et al., 2014).

BFCs, such as ascorbic acid, α-tocopherol, polyunsaturated fatty acids (PUFAs), trace elements, carnitines, N-acetylcysteine, and coenzyme Q10, and folate have been evaluated to improve male gametogenesis, modulate epigenetics of germ cells, and the epigenetic signature of the offspring, restoring offspring metabolic health induced by stressors during early life. Thus, this review will focus on epigenetics and interventions with food bioactive compounds in the paternal diet on spermatogenesis and offspring health based on clinical and *in vivo* studies.

2 Diet and male reproductive health

Spermatogenesis is a complex process that involves continuous production of sperm cell in the seminiferous tubule (AMANN; HOWARDS, 1980). After puberty, spermatogonial stem cells (SSCs) provide the foundation of sperm cells, a process that persists throughout the majority of a male's lifetime (STUKENBORG et al., 2014). A fertile man produces over 200 million sperm cell daily within the testis (AMANN; HOWARDS, 1980). The testis is also an endocrine organ where high levels of

testosterone are produced, supporting normal spermatogenesis and male phenotypic characteristics (OATLEY; BRINSTER, 2008). Spermatogenesis consists of four differentiation stages: 1 (mitotic): SSCs undergo mitotic proliferation resulting in primary spermatocytes; 2 (meiotic): secondary spermatocytes undergo meiosis; 3 (post-meiotic): to form haploid spermatid cells; 4 (mature sperm): spermatids undergo spermiogenesis. Spermiogenesis is the final maturation stage for elongated sperm cell formation, a mature sperm cell capable of fertilization (OATLEY; BRINSTER, 2008). These stages involve many cellular events in the testis, which are regulated by hormonal and signaling pathways (CRAIG et al., 2017a; WALKER, 2021).

The steroid testosterone is the main hormone essential to maintain spermatogenesis, as it act in the testis regulating spermatogenesis (ALVES et al., 2013). Testosterone is required for critical processes: maintenance of the blood testis barrier, adherence of elongated spermatids to Sertoli cells, spermatocytes meiosis process, and the release of mature sperm cell (SMITH; WALKER, 2014). The Leydig cells produce testosterone which diffuses into the seminiferous tubules, peritubular myoid cells, Sertoli cells and Leydig cells as well as into the blood vessels. The Sertoli cells mediate metabolic factors and signals required for the proliferation and differentiation of germ cells (ALVES et al., 2013; CHANG et al., 2013). Peritubular myoid cells also provide basement membrane in the testis for SSCs that produce germ cells which will develop into sperm cells (RICHARDSON; KLEINMAN; DYM, 1995).

2.1 Effects of BFCs on male spermatogenesis

Besides genetic background, nutritional and lifestyle factors play a key role in reproductive health and can influence fertility (BENATTA et al., 2020). Adverse environmental factors in a man's life, such as malnutrition, obesity, sedentary lifestyle, stress, alcohol intake, smoking, and drug abuse, exposure to pollution or radiation make the man more susceptible to developing reproductive pathological conditions, including subfertility or infertility (ARAB et al., 2018; BENATTA et al., 2020; SHARMA et al., 2013; WORLD HEALTH ORGANIZATION, 2020).

It is well-known that consuming a diet rich in BFCs can influence men's fertility (ARAB et al., 2018). Following a nutritious diet can help maintain a healthy weight and prevent obesity, a condition that is associated with increased oxidative stress (OS), the main causative factor of infertility (WORLD HEALTH ORGANIZATION, 2020). OS

is a condition associated with increased generation of ROS and reduced cellular antioxidant capacity. In seminal plasma in human semen this condition is characterized by increased nuclear DNA damage and lipid peroxidation and decreased levels of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide and vitamins A, C and E (TAKESHIMA et al., 2021). Sperm cell plasma membranes are constituted of phospholipids and PUFAs and their cytoplasm contains low concentrations of scavenging enzymes, which makes them particularly susceptible to oxygen-induced damage (AGARWAL; SALEH; BEDAIWY, 2003). Excessive generation of ROS results in damage to sperm cell plasma membrane and subsequent loss of sperm cell quality and function, such as deregulation of capacitation, activation, motility, counts, and sperm-egg fusion (HOMA et al., 2015).

Several BFCs such as vitamins, minerals, fatty acids, amino acids, and antioxidants have been shown to improve sperm cell physiology and function through multiple mechanisms, including reduction of ROS, restoration of the antioxidant defense system, and inhibition of inflammation. The BFCs that improve sperm cell quality and functioning are summarized in **Table 1** with their reported outcomes.

Table 1 –Bioactive food compounds (BFCs) and their major outcomes on human sperm quality and functioning.

Nutritional factor	**Major outcomes**	**References**
Vitamin A	- Normal blood-testis barrier function; - Avoid germ-cell aplasia; -Fertile men have higher serum concentrations than infertile.	(AL-AZEMI et al., 2009)
Vitamin C	- Improved sperm cell count, motility, and morphology; - Lower levels of vitamin C in seminal plasma of infertile man.	(AKMAL et al., 2006; COLAGAR; MARZONY, 2009)
Vitamin E	- Higher live-birth rate, and a trend of better results of *in vitro* fertilization parameters;	(AL-AZEMI et al., 2009; KESKES-

	- Decreases the lipid peroxidation of the sperm cell and seminal plasma; - Improves sperm cell motility; -Lower levels were found in men with oligozoospermia and asthenozoospermia.	AMMAR et al., 2003; MATORRAS et al., 2020)
Vitamin D	- The expression of vitamin D receptors and metabolizing enzymes are marked in human testis, ejaculatory tract, and mature sperm cells; -Positive association between serum levels and sperm motility;	(BLOMBERG JENSEN et al., 2010, 2011; KINUTA et al., 2000)
Vitamin B9	- Protect against DNA damage.	(BOONYARANGKUL et al., 2015; HOEK et al., 2020)
Selenium	- Protect against ROS; -Deficiency promotes sperm cell abnormalities, affects motility and fertility;	(AHSAN et al., 2014; SAFARINEJAD; SAFARINEJAD, 2009)
Zinc	- Important for spermatogenesis: cofactor of enzymes involved in DNA transcription and protein synthesis; - Lower zinc levels in the seminal plasma of infertile men; -Increased the normal sperm cell morphology, sperm motility, and semen volume.	(COLAGAR; MARZONY; CHAICHI, 2009; ZHAO et al., 2016)
N-acetylcysteine	- Improved the volume, motility, and viscosity of sperm cells; - Increased the serum total antioxidant capacity; - Reduced the serum peroxide and oxidative stress;	(CIFTCI et al., 2009; SAFARINEJAD; SAFARINEJAD, 2009)

	- Increased sperm cell concentration, motility, and percent normal morphology in infertile men.	
Coenzyme Q10	- Improved sperm cell density, motility, and percent of normal morphology in infertile men; - Increased the seminal plasma and total antioxidant capacity.	(BALERCIA et al., 2009; NADJARZADEH et al., 2011; SAFARINEJAD, 2009; SAFARINEJAD et al., 2012)
Omega-3 polyunsaturated fatty acid	-Lower levels of omega-3 and greater oxidative DNA damage were found in sperm cells of infertile than infertile men; -Improved sperm cell total count, concentration, motility, and normal morphology; -Increased seminal antioxidant status and decreased the percentage of sperm cell with damage.	(ATTAMAN et al., 2012; MARTÍNEZ-SOTO et al., 2016; SAFARINEJAD, 2011; SAFARINEJAD et al., 2010; TANG et al., 2016)
Carnitines	- Increased sperm cell motility; - Improved activity toward ROS in the semen.	(BALERCIA et al., 2005; LENZI et al., 2004)

Abbreviation: asthenozoospermia, impaired sperm cell motility; oligozoospermia, low sperm cell count; ROS, reactive oxygen species.

2.2.1 Vitamins

Vitamin A, or retinoids, comprise a group of natural antioxidants that inhibit lipid peroxidation and protect against cell damage (GUDAS; WAGNER, 2011). They are necessary for a normal spermatogenic process by stimulating the transcription of genes involved in meiosis (ROSS et al., 2000). Men with normal sperm parameters presented increased retinol serum concentrations than those with low sperm cell count

(oligozoospermia) and impaired motility (asthenozoospermia) (AL-AZEMI et al., 2009).

Vitamin C (ascorbic acid) is found in fruits and vegetables and has the functionality to reduce DNA damage directly by scavenging free radicals and decreasing lipid peroxidation (PADAYATTY et al., 2003). It comprises a key component in the antioxidant system of seminal plasma. According to Colagar et. al. (2009) men with idiopathic infertility have lower levels of vitamin C in their seminal plasma than fertile men, a condition that can be a risk factor for infertility and abnormal sperm cell morphology (COLAGAR; MARZONY, 2009). Vitamin C supplementation (2mg/day for 2 months) in infertile men improved sperm cell count, motility, and morphology, indicating that seminal vitamin C may improve infertility issues in infertile men (AKMAL et al., 2006).

Vitamin E is the generic term for a group of tocopherols and tocotrienols found in vegetable oils. It is an important antioxidant component and a major cell protector of the integrity of PUFAs in the cell's membrane against ROS thus maintain their bioactivity (TRABER; ATKINSON, 2007). Lower serum α-tocopherol levels have been found in men with oligozoospermia and asthenozoospermia (AL-AZEMI et al., 2009). Matorras et. al. (2020) showed that vitamin E supplementation (400mg α-tocopherol/day) for 3 months was positively associated with live birth rate, and a trend towards better results in *in vitro* fertilization parameters but did not significantly increase progressive sperm cell motility in a double-blind, placebo-controlled, randomized study (MATORRAS et al., 2020). In another clinical trial with infertile men, Keskes-Ammar et. al. (2003) demonstrated that daily supplementation with vitamin E (400mg) and selenium (225μg) for 3 months improved sperm cell motility and decreased lipid peroxidation in sperm cell and seminal plasma (KESKES-AMMAR et al., 2003). Ener et. al. (2016) showed that vitamin E (600mg α-tocopherol/day) for 12 months increased sperm count and motile sperm count in infertile male patients that underwent varicocelectomy; however, the increase in these parameters was not statistically significant (ENER et al., 2016).

Vitamin D is mainly synthesized by exposure to sunlight that plays an important role in calcium homeostasis, bone metabolism, and cell differentiation and proliferation (ARAB et al., 2019). The importance of vitamin D has been suggested for

spermatogenesis and maturation of human sperm cell, due to the expression of vitamin D receptors and metabolizing enzymes in the human testis, ejaculatory system, and mature sperm cell (BLOMBERG JENSEN et al., 2010). Serum levels of Vitamin D positively correlated with sperm cell motility and increased intracellular calcium concentration in men (BLOMBERG JENSEN et al., 2011). However, additional studies should better identify the role of vitamin D3 on male fertility. In a study by Amini et. al. (2020) oral vitamin D supplementation (50,000 IU weekly for 8 weeks, and 50,000 IU per month in the following 4 weeks) showed no effects on the sperm cell quality in a randomized controlled trial with infertile men (AMINI et al., 2020).

Vitamin B9 or folate, comprise nutritionally essential water-soluble compounds for optimal human health and development that mediates one-carbon transfer reactions, including the methylation of DNA (NADERI; HOUSE, 2018). The methylene tetrahydrofolate reductase (MTHFR) enzyme is the key enzyme for the conversion of folate to 5-methyltetrahydrofolate, the methyl group donor necessary for methionine (Met) production from homocysteine (Hcy) (NADERI; HOUSE, 2018; SCHISTERMAN et al., 2020; YEN-MING CHAN, REGAN BAILEY, 2013). Folates are mainly found dark green leafy vegetables, animal viscera, beans whole grains and citrus fruits (CZEIZEL et al., 2013). Low levels of serum and seminal folate can led to high levels of Hcy, which may induce oxidative stress, sperm DNA damage and apoptosis lowering sperm counts (VANDERHOUT et al., 2021). In a randomized controlled trial, Boonyarangkul et. al. (2015) showed that 5 mg/day of folic acid supplement for 3 months improved sperm cell quality and protected against DNA damage in infertile male with semen abnormality (BOONYARANGKUL et al., 2015). On the other hand, three randomized controlled trials reported no significant differences regarding sperm cell volume, motility, and morphology after folate supplementation (all 5 mg/day for 12, 16 or 26 weeks) in subfertile man (RAIGANI et al., 2014; SILVA et al., 2013; WONG et al., 2002).

2.2.2 Trace elements

The trace elements selenium, calcium, copper, manganese, magnesium, sodium, potassium, and zinc are part of the seminal composition, representing key nutritional factors for proper male reproductive physiology, normal spermatogenesis, sperm

maturation, motility and function (BENATTA et al., 2020; MIRNA et al., 2019; RODRÍGUEZ et al., 2013). Calcium is essential for sperm cell quality, hyperactivation, capitulation of sperm, and acrosome reaction, leading to sperm penetration into the oocyte (MIRNA et al., 2019). Magnesium is involved in spermatogenesis, sperm cell motility, quality, and ejaculation, while sodium and potassium are involved in sperm motility and capacitation (MIRNA et al., 2019).

Zinc and selenium are the main significant elements in human semen. Selenium is an essential antioxidant micronutrient, which is critical for male reproductive tissue development, spermatogenesis, and increases the enzymatic antioxidant activity (BOITANI; PUGLISI, 2009). Fish, meat products, dairy and plants are the main dietary sources of selenium (NAVARRO-ALARCON; CABRERA-VIQUE, 2008). Selenium role is mediated by selenoproteins, the phospholipid hydroperoxide glutathione peroxidase, which is expressed by germ cells in the testis, and Selenoprotein P, a plasma protein required for selenium supply to the testis (BOITANI; PUGLISI, 2009). Selenium protect sperm cells against ROS (AHSAN et al., 2014). Its low rates during spermatogenesis can result in abnormal sperm cells, which consequently affects semen quality, sperm cell motility, and fertility (BOITANI; PUGLISI, 2009). Findings of limited number of clinical studies support selenium supplementation as a strategy to improve male reproductive physiology (HAWKES; ALKAN; WONG, 2009; SAFARINEJAD; SAFARINEJAD, 2009; SCOTT et al., 1998). In a double-blind, placebo controlled, randomized study, Safarinejad and Safarinejad (2009) administered selenium (200 µg for 26 weeks) in infertile men with idiopathic oligo-asthenoteratospermia, demonstrating improvements in semen quality (SAFARINEJAD; SAFARINEJAD, 2009). Scott et. al. (1998) used 100 µg/day of selenium for 12 weeks in subfertile men, resulting in increased sperm cell motility (SCOTT et al., 1998). However, Hawkes et. al. (2009) reported on a randomized, controlled study that selenium supplementation of 300 µg/day for 48 weeks did not affect testicular selenium status or semen quality in men (HAWKES; ALKAN; WONG, 2009). These studies must be viewed with caution due to limitation in study design and quality (QAZI et al., 2019).

Zinc is a micronutrient found in meat, wheat, and seeds. Zinc has important function in testicular development, spermatogenesis, acrosome reaction, chemotaxis,

and antioxidant action (BENATTA et al., 2020), since it acts as a cofactor of several enzymes involved in DNA transcription, protein synthesis, and antioxidant properties (FORESTA et al., 2014). According to Colagar et. Al. (2009), the seminal zinc level was positively correlated with sperm cell count and normal morphology (COLAGAR; MARZONY; CHAICHI, 2009). A systematic review and meta-analysis showed that infertile males have lower zinc levels in the seminal plasma compared to fertile men (ZHAO et al., 2016). Moreover, the double-blind, placebo-controlled interventional study of Wong et. al. (2002), the combined ingestion of zinc (66 mg zinc sulfate) and folic acid (5 mg) for 26 weeks promoted a 74% increase in total normal sperm cell count of subfertile men (WONG et al., 2002), suggesting that the trace elemento might improve sperm cell quality and male reproductive function (ZHAO et al., 2016).

The nutritional status of the man before conception might influence semen quality and male fertility. The deficiency of these trace elements can negatively influence the man reproductive health and fertility potency (MIRNA et al., 2019).

2.2.3 Other BFCs

N-acetylcystein is a cysteine derivative and a powerful antioxidant and scavenger of ROS in the treatment of OS-associated diseases (ASKARI et al., 2020). Following treatment with N-acetylcystein, ROS activity was evaluated as an approach for male infertility treatment. Ciftci et. al. (2009) showed that N-acetylcystein supplementation (600 mg/day) for 3 months improved the volume, motility, and viscosity of semen, probably due to reduced serum ROS production (CIFTCI et al., 2009). Moreover, Safarinejad and Safarinejad (2009) study demonstrated that supplementation of N-acetylcystein (600 mg) plus selenium (200 μg) orally daily for 26 weeks improved sperm cell concentration, motility, and normal morphology percent in infertile men with idiopathic oligo-asthenoteratospermia; however, no data of pregnancy occurrence were reported by authors (SAFARINEJAD; SAFARINEJAD, 2009).

Coenzyme Q10, is a ubiquinone essential for energy production in mitochondria that also has antioxidant and membrane properties. It can be synthesized by the human body and also be obtained through salmon, tuna, beef, nuts, and seeds (LITTARRU; TIANO, 2007). Three studies have evaluated the influence of coenzyme Q10 on sperm

parameters in infertile men (BALERCIA et al., 2009; SAFARINEJAD, 2009; SAFARINEJAD et al., 2012). In these studies, 200 or 300 mg/day of coenzyme Q10 was supplemented for different durations (24 to 26 weeks). Safarinejad et. al. (2009) results showed that 300 mg/day for 26 weeks improved sperm cell density, motility and morphology (SAFARINEJAD, 2009). Balercia et. al. (2009) study showed that 200 mg/day for 6 months increased the level of coenzyme Q10 and ubiquinol in seminal plasma after treatment and was effective in improving sperm cell motility (BALERCIA et al., 2009). Ubiquinol, a reduced form of coenzyme Q10, also improved sperm cell density, motility, and morphology (SAFARINEJAD et al., 2012).

Eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3) are long-chain omega-3 PUFAs obtained from the diet (e.g. fish and nuts). PUFAs are essential sperm cell membrane constituents and can influence its fluidity and integrity (DIAS et al., 2018; GIAHI et al., 2016). The increase of the ω-3 in the sperm plasma membrane phospholipids promotes adequate antioxidant properties which reduce the risk of damage to sperm cells (BAZZANO et al., 2021; DÍAZ et al., 2016). Lower concentrations of omega-3 have been found in sperm cell of infertile men (SAFARINEJAD et al., 2010). In a cross-sectional study by Attaman et. al. (2012), the omega-3 intake was positively related to adequate sperm morphology (ATTAMAN et al., 2012). Findings of Tang et. al. (2016) suggests that omega-3 PUFAs deficiency could be associated with infertility, since the infertile man had lower levels of omega-3 PUFA, and greater oxidative DNA damage in sperm cell compared with the fertile man (TANG et al., 2016). A randomized, double blind, placebo-controlled, parallel study by Martínez-Soto et. al. (2016) demonstrated that DHA (1,500 mg/day) supplementation for 10 weeks increased seminal antioxidant status and decreased the percentage of sperm cells with damage (MARTÍNEZ-SOTO et al., 2016). Safarinejad (2010) found an association between low concentrations of omega-3 in sperm cells and poor semen quality among infertile men. These findings suggest that infertile men may benefit from omega-3 fatty acids supplementation. EPA and DHA supplementation (1.84 g/day) for 32 weeks promoted increased seminal plasma antioxidants and improved semen parameters (total sperm cell count, concentration, motility, and normal morphology) (SAFARINEJAD, 2011).

Carnitines are amines mostly provided from the diet (75%) and can also be synthesized from essential amino acids such as lysine and methionine (AGARWAL; SAID, 2004; SMITS et al., 2019). They act as co-factors in mitochondrial β-oxidation of long-chain fatty acids to enhance cellular production of energy (AGARWAL; SAID, 2004). Carnitine also protects cell membranes and DNA against ROS (MONGIOI et al., 2016). L-carnitine and L-acetyl-carnitine are the two major carnitine forms, which were found in the epididymal fluid and sperm cells (BALERCIA et al., 2005). The studies of Balercia et. al. (2005) (BALERCIA et al., 2005) and Lenzi et. al. (2004) (LENZI et al., 2004) evaluated the supplementation of L-carnitine and acetyl-L-carnitine alone or in combination for 6 months. In a placebo-controlled double-blind randomized trial, Lenzi et. al. (2004) study showed that l-carnitine (2 g/day) and l-acetyl-carnitine (1 g/day) for 6 months increased sperm cell motility in infertile males with oligo-astheno-teratozoospermia, mainly in groups with lower baseline sperm cell motility levels (LENZI et al., 2004). Balercia et. al. (2005) demonstrated in a placebo-controlled double-blind randomized trial, that the therapy with l-acetyl-carnitine (3 g/day for 6 months) alone or in combination with l-carnitine (l-carnitine 2 g/day plus l-acetyl-carnitine 1 g/day for 6 months) increased sperm cell motility; the combined therapy led to straight progressive velocity improvement after 3 months (BALERCIA et al., 2005). Protection against ROS production was also observed in the semen of men with idiopathic asthenozoospermia (BALERCIA et al., 2005).

In a recent clinical study, Kopets et al. (2020) evaluated the effect of a dietary multi-vitamin supplement on sperm cell parameters and pregnancy rates in idiopathic male infertility with oligo-, astheno-, and teratozoospermia. Males received the supplement containing L-carnitine/L-acetyl-carnitine (1990 mg), L-arginine (250 mg), glutathione (100 mg), coenzyme Q 10 (40 mg), zinc (7.5 mg), vitamin B9 (234 mcg), vitamin B12 (2 mcg), selenium (50 mcg), or placebo daily for 6 months. The percentage of spontaneous pregnancies and sperm cell quality (concentration, motility, and normal morphology) in the supplemented group was greater than in the group that received a placebo (KOPETS et al., 2020).

Importantly, there are few well-controlled clinical studies that have evaluated BFCs potential protective effects in infertile men (QAZI et al., 2019). Two recent meta-analyses investigated the effect of antioxidant oral supplementation on male fertility.

Due to the high heterogeneity of studies designs, applied dose, number of participants, compounds, and evaluated parameters, further research is needed to establish more efficient methods of treating male infertility (BUHLING et al., 2019; SMITS et al., 2019). The revision of Smits et. al. (2019) concluded that antioxidant supplementation taken by subfertile man may increase the rates of pregnancy, however, the evidence are based on low-quality and small clinical trials (SMITS et al., 2019). According to Buhling et al. (2019), the meta-analysis suggests that selenium (alone or combined with N-acetylcysteine), coenzyme Q10 and the combinations of L-carnitine + acetyl-L-carnitine, folic acid + zinc, and the EPA +DHA are promising approaches for the treatment of male infertility (BUHLING et al., 2019).

3 Diet and male reproductive epigenetics

3.1 Sperm-specific epigenetics

Coming from the Greek "Epi" meaning over/on top, epigenetics means that molecules that are on top of the DNA structure are able to respond to environmental factors and are able to modify gene expression without changing the DNA sequence. The three main epigenetic mechanisms in mammals are: (i) DNA methylation and associated modifications, (ii) the histone/chromatin code which consists mainly of histone variants and their post-translational modifications, and (iii) non-coding RNA (CHAMPROUX et al., 2018). These processes are cell-specific and dynamic and could regulate how densely specific regions of DNA are compacted, thus either inhibiting or enabling access of proteins, such as transcription factors to DNA (FERNANDEZ-TWINN et al., 2019).

Male gametogenesis involves intense epigenetic remodeling (FONTELLES et al., 2018) (Figure 1). There are sensitive periods when environmental exposures might have amplified, long-lasting effects (MARCHO; OLUWAYIOSE; PILSNER, 2020). Windows of susceptibility include pre-puberty/puberty, adulthood, and the zygote phase, that stand out as stages of development. In these phases, the epigenome is especially plastic and susceptible to disturbances induced by environmental factors such as malnutrition (FONTELLES et al., 2018).

Previously, the sperm epigenome was not of significant importance, as it was thought that after fertilization, all epigenetic marks were erased. However, with the

passing of the years and the advancement of science, studies have increasingly demonstrated that epigenetic information carried by spermatozoa can indeed be transmitted between generations (GUI; YUAN, 2021; KITAMURA et al., 2015; MCSWIGGIN; O'DOHERTY, 2018; OZKOCER; KONAC, 2021; RADFORD, 2018).

Sperm cells have a unique epigenetic signature. During spermatogenesis, the sperm epigenetic profile remodeling occurs during 3 major steps: spermatogoniogenesis, spermatocytogenesis, and spermiogenesis (ZHOU et al., 2018). A rapid expansion of the spermatogonia occurs after birth. However, after this rapid clonal expansion, the germ cells lie dormant for years until the period of puberty. From the onset of puberty through the activation of the hypothalamic–pituitary–gonadal (HPG) axis until adulthood, the process of spermatogenesis occurs in the seminiferous epithelium and is supported by mitotically inactive Sertoli cells (RADFORD, 2018; SCHAGDARSURENGIN; STEGER, 2016).

During spermatogoniogenesis, the undifferentiated spermatogonia undergo clonal expansion through mitosis to produce spermatocytes (SCHAGDARSURENGIN; STEGER, 2016). Following this stage, specific paternal imprints are reestablished mainly in the primordial germ cells and end in spermatogonia (MARCHO; OLUWAYIOSE; PILSNER, 2020). Then, in spermatocytogenesis, during meiosis, most of the somatic-type histones are exchanged for testis-specific histone variants. Complete reorganization and extensive condensation of nuclear chromatin occur during spermiogenesis, leading to major replacement of most nucleosomes by protamines with histone acetylation, insertion, and removal of transition proteins (ZHOU et al., 2018). The last stage of spermiogenesis is the maturation of the epididymis and the germ cells become motile and non-coding RNA (ncRNA) mature (LI; SHEN; HUA, 2016). Soon after fertilization occurs, both parental genomes are demethylated asymmetrically. However, regions of heterochromatin around centromeres largely escape this demethylation event and with that, demethylation is not complete (SCHAGDARSURENGIN; STEGER, 2016). The epigenetic remodeling that occurs during male gametogenesis is summarized in **Figure 1**.

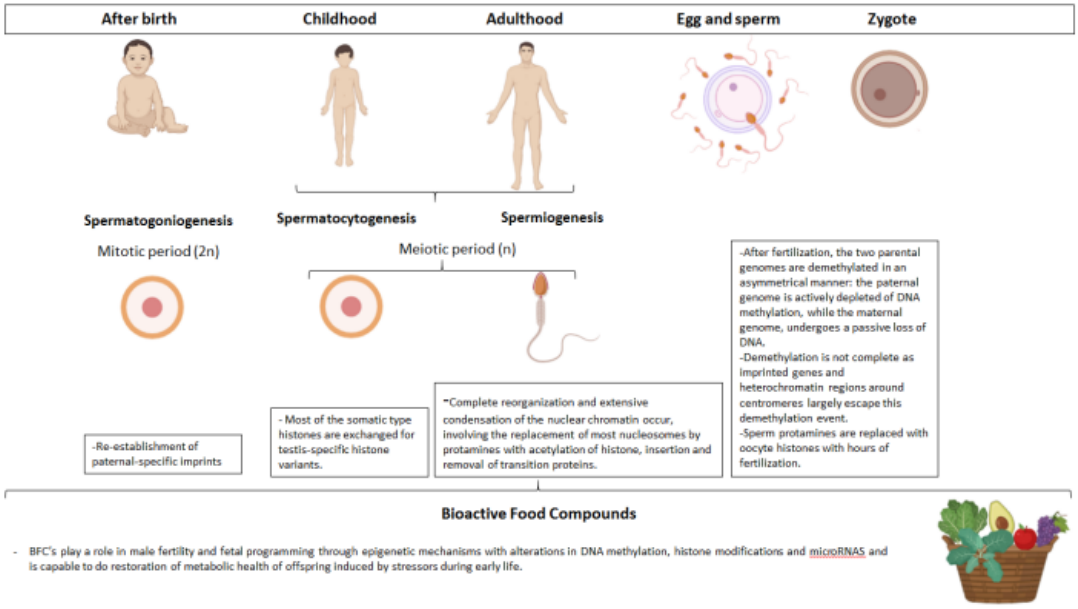

Figure 1. Consumption of Bioactive Food Compounds as a potential epigenetic strategy to improve men's reproductive heath and prevent chronic disease risk in the offspring. Created with BioRender.com

3.2 BFCs epigenetic modulation in male germ cells

Although accumulating studies show that BFCs modulate several epigenetic processes in the context of cancer prevention (ONG; MORENO; ROSS, 2012), information of such effects in the context of male reproductive physiology is scarce. These dietary compounds do not directly change DNA, but act on enzymes that add or remove epigenetic tags to or from DNA and histones that can activate or inhibit gene expression (YU et al., 2020; ZHOU et al., 2018). Regarding reproductive health, they could potentially impact the epigenetic landscape in male germ cells and sperm (SCHAGDARSURENGIN; STEGER, 2016).

Folate is a key nutrient that impacts the epigenome (DAVIS; HORD, 2005). It plays a critical role in 1-carbon metabolism. Folate metabolism generates the universal methyl donor S-adenosyl methionine, necessary DNA and histone methylation. Low paternal folate intake may alter the sperm epigenome and result in adverse pregnancy outcomes (SCHULZ et al., 2020; YU et al., 2020). Lambrot et al (2013) showed that a man's folate deficiency diet alters sperm DNA methylation at genes implicated in the development and metabolic processes (LAMBROT et al., 2013). However, there is very

limited information in humans regarding epigenetic modulation potential of BFCs in sperm cells, in the context of fertility.

A recent experimental study by Li et al (2020), examined the effect of anthocyanins on spermatogenesis during puberty in male Kunming mice contaminated by cadmium (Cd). Prevalent anthocyanin C3G found in berries effectively protected spermatogenesis in male pubertal mice from the damage elicited by Cd via normalizing histone modification, restoring the histone to protamine exchanges in spermiogenesis, and improving the antioxidative system in the testis, subsequently alleviating apoptosis. Thus, consumption of anthocyanins can be protective against Cd-induced male pubertal reproductive dysfunction (LI et al., 2020).

4 Paternal interventions with BFCs as a potential epigenetic strategy to improve health and prevent chronic disease in the offspring

4.1 Nutrition and Paternal Origins of Health and Disease (PoHAD)

According to the Developmental Origins of Health and Disease (DOHaD) paradigm, adverse environmental factors operating in early life may increase the risk of chronic noncommunicable diseases in adulthood (DAVIS; HORD, 2005; MANDY; NYIRENDA, 2018). David Barker, a British epidemiologist, was the first to propose such a link, highlighting in his pioneer study that maternal under-nutrition during gestation can increase the risk of cardiovascular and metabolic diseases in the offspring in adulthood (BARKER, 1986). Further studies by Barker and Hales (1992) (BIANCO-MIOTTO et al., 2017) on the "thrifty phenotype" hypothesis and studies on the "Dutch famine" cohort (BARKER, 1986; VAAG et al., 1992) expanded the knowledge of maternal experiences during critical windows of development (i.e. gestation and lactation) on later descendants health and disease risk.

Until recently, DoHAD research had focused mainly on the impact of maternal exposure because of the close connection between mother and fetus (FERNANDEZ-TWINN et al., 2019). However, there is growing experimental and to a lesser extent clinical evidence that paternal factors during preconception also play a significant role in the metabolic health of the offspring (SCHAGDARSURENGIN; STEGER, 2016). Studies with a paternal focus have mainly shown that psychological, metabolic and

environmental factors, such as drugs, alcohol, and diet prior to conception alter endocrine and metabolic functions and neurodevelopment in the offspring (MCGOWAN; MATTHEWS, 2018; ORNELLAS et al., 2017b; SILVA et al., 2019). Many of these studies also suggest that memory of past paternal exposures is transmitted to the progeny through the germline via epigenetic mechanisms (FONTELLES et al., 2016a; SOUBRY et al., 2013, 2014, 2015; WATKINS et al., 2018a; YESHURUN et al., 2017). Collectively, these studies can be positioned in a subfield of DoHAD termed PoHAD (SOUBRY et al., 2014).

Epigenetics has been singled out as a prominent mechanism to explain how the father´s experiences can affect offspring development (YESHURUN et al., 2017). As epigenetic marks are relatively stable and can be transmitted transgenerationally (F2 onwards, in the case of paternal exposures (ONG; OZANNE, 2015)), this may explain how such molecular changes would remain throughout offspring adult life, resulting in the altered activities of metabolic pathways and homeostatic control processes (HUR; CROPLEY; SUTER, 2017; SEGARS, 2018; ZHANG et al., 2019).

4.2 Malnutrition and PoHAD

Most of PoHAD studies have focused on the role of paternal undernutrition on their offspring health (BODDEN; HANNAN; REICHELT, 2020; MCPHERSON et al., 2014; MORGAN et al., 2020; ORNELLAS et al., 2017a; WATKINS et al., 2017; YESHURUN et al., 2017). Moderate paternal malnutrition such as protein restriction has been shown to impact the development of the offspring's organs and metabolism (DA CRUZ et al., 2018; VANHEES et al., 2014; WATKINS et al., 2017). A recent study with young male mice receiving a low-protein (LP) diet (8.9% energy from protein) showed that F1 LP female offspring presented lower birthweight, alterations in mammary gland morphology and expression of miR-451a, miR-200c, and miR-92a, and higher rates of breast cancer (DA CRUZ et al., 2018). Importantly, alterations in sperm microRNA profiles of LP fathers were also identified. With a total of 16 miRNAs differentially expressed, with eight down- and eight up- regulated (DA CRUZ et al., 2018).

F1 rat offspring of fathers that consumed a low protein diet (LPD; 9% casein), showed that displayed altered tissue angiotensin-converting enzyme (ACE) activity,

renin–angiotensin system (RAS) pathway gene expression, and vascular dysfunction (MORGAN et al., 2020). In addition, similarly to F1 offspring, juvenile F2 offspring also presented alterations in growth and tissue ACE. These alterations were further accompanied by methylation of important genes such as FTO, *Mettl3*, and *Mettl14* and modifications in histones, such as Hdac1, Hdac2 (MORGAN et al., 2020). In similar study by this same research group (WATKINS et al., 2018b), they found that sperm from LPD-fed fathers presented global hypomethylation associated with reduced testicular expression of DNA methylation enzymes Dnmt1 and Dnmt3L, and folate-cycle enzymes Dhfr, Mthfr, and Mtr expression. Offspring from LPD fathers became heavier, with increased adiposity, glucose intolerance, perturbed hepatic gene expression symptomatic of nonalcoholic fatty liver disease, and altered gut bacterial profiles. These data provide insight into programming mechanisms linking poor paternal diet with semen quality and offspring health (WATKINS et al., 2018b).

According to Hajj et al (2021) (BERNHARDT et al., 2021), paternal obesity has possible consequences on embryonic gene expression and development. *Gata6* and *Samd4b* were differentially expressed genes in embryos of high-fat diet treated fathers (BERNHARDT et al., 2021).*Gata6* and *Samd4b* are upregulated during adipocyte differentiation (BERNHARDT et al., 2021; STANESCU et al., 2015). Thus, these genes could be involved in predisposing offspring of obese fathers to diet-induced obesity in later life (BERNHARDT et al., 2021).

Paternal obesity impact on the metabolic profile of offspring was further investigated in a male mouse model of obesity (WU et al., 2021). Hyperglycemia was shown in the female offspring of obese mice fathers. Importantly, methylation of the Igf2/H19 imprinting control region (ICR) was both altered in hepatic tissue of offspring, and in the sperm of their obese fathers, suggesting that epigenetic changes in germ cells contribute to this father-offspring transmission (WU et al., 2021).

Moreover, an experimental study (ZHOU et al., 2018) demonstrated that paternal obesity influences the cognitive function of offspring via epigenetic modifications in sperm cells. Paternal obesity exerted intergenerational effects on cognition in F1 offspring by increased methylation of the BDNF gene promoter in hippocampus, which could be inherited from F0 spermatozoa. BDNF is a member of the neurotrophin family and plays a critical role in hippocampal neurogenesis and cognitive

function (ZHOU et al., 2018).

A previous study by our research group (FONTELLES et al., 2016c) showed that consumption of a high-saturated fatty acid diet (60% of calories from lard) for 9 weeks during preconception by male Sprague-Dawley rats programmed higher risk of breast cancer in the female offspring. Interestingly, high-fat diet-treated fathers presented altered miR profile in sperm (FONTELLES et al., 2016c). It is important to highlight that clinical studies in the field of Paternal Origins of Breast Cancer are lacking. Thus, an initial approach to start investigating this possibility would be to establish cohorts of daughters of obese fathers and correlate their sperm cells epigenetic marks with early indicators of breast cancer risk such as mammary density during puberty and young adulthood.

Importantly, first evidence that paternal obesity can affect descendants methylation profile on imprinted genes important in embryonic growth and cancer development came from studies from the Newborn Epigenetic Study (NEST) cohort (SOUBRY et al., 2013, 2015).

4.3 Bioactive Food Compounds and PoHAD

Folic acid and vitamins B2, B6, and B12 are essential for one-carbon metabolism and are involved in DNA methylation. Thus, they can impact the offspring's epigenome profile (STELUTI et al., 2020). One carbon metabolism consists of the interconnected folate and methionine cycles essential for transfer of 1C moieties required for cellular processes. (CLARE et al., 2019).

A recent study (CHLEILAT et al., 2021) in rats assessed whether adding a methyl donor cocktail [betaine (5 g/kg diet), choline (5.37 g/kg diet), folic acid (5.5 mg/kg diet), vitamin B12 (0.5 mg g/kg diet)] to the paternal high fat/sucrose diet improves health status in fathers and offspring. Such paternal intervention before conception reduced energy intake and increased levels of GLP-1 and PYY hormones, known to reduce food intake. In addition, it improved fertility, physiological outcomes, epigenetic and gut microbial signatures. More specifically, the methyl-donor intervention decreased in the offspring hepatic expression of miR-34a and increased miR-103, miR-107, and miR-33, which are involved in lipid metabolism and regulation of insulin sensitivity. It further elevated retroperitoneal adipose tissue expression of

DNMT1, DNMT3a, and DNMT3 in the adult offspring (CHLEILAT et al., 2021). However, when a similar dietary approach (5 g/kg diet choline chloride, 15 g/kg diet betaine, 7.5 g/kg diet methionine, 15 mg/kg diet folic acid, 1.5 mg/kg diet vitamin B12) was used on male animals on LPD, no protection was observed regarding placental physiology and epigenetic regulation (MORGAN et al., 2021). Authors of this study noted noted that such supplementation is not a 'quick-fix' (MORGAN et al., 2021).

An experimental study (MCPHERSON et al., 2016) examined whether detrimental health outcomes in offspring could be prevented by paternal micronutrient supplementation (vitamins and antioxidants). Consumption of a hypocaloric diet by male rats promoted a reduction in body weight, fertility, increased oxidative damage to sperm cell DNA and reduced overall sperm methylation. In addition, their offspring presented reduced postnatal weight and growth but somewhat paradoxically increased adiposity and dyslipidemia. Interestingly, supplementing these fathers on a restricted diet with antioxidant mix (vitamin C, vitamin E, folate, lycopene, zinc, selenium and green tea extract) normalized founder oxidative sperm DNA lesions and prevented early growth restriction, fat accumulation, and dyslipidemia in offspring. This demonstrates that paternal micronutrient supplementation during undernutrition is capable of restoring offspring metabolic health (MCPHERSON et al., 2016).

The functional analysis of the altered sperm methylome suggests that the offspring may be at increased risk for later chronic diseases such as diabetes and cancer (BARATI; NIKZAD; KARIMIAN, 2020). Another study has shown that a lifetime exposure of male mice to both folic acid deficient (0.3 mg/kg) and highly supplemented (40 mg/kg) diets result in decreased sperm counts, adverse outcomes in their offspring, and evidence of epigenetic alterations as altered imprinted gene methylation (SCHULZ et al., 2020). This U-shaped pattern is a key aspect when considering supplementing future fathers with micronutrients, as both dietary deficiencies and excess may led to same deleterious outcomes. In a previous study by our group (GUIDO et al., 2016), we evaluated the potential breast cancer programming effects of selenium deficiency during preconception in male rats. Interestingly this malnutrition condition altered mammary gland development and increased breast cancer risk in adult female offspring (GUIDO et al., 2016). In addition, supplementation of male rats with selenium during this same developmental stage did not alter breast cancer susceptibility in offspring. One

explanation would be the fact that animals were lean, not submitted to any metabolic challenge. It has been highlighted that oxidative stress background could be a key interfering factor in Se efficacy (PASCOAL, NOVAES et al., 2022). Thus, we (PASCOAL, NOVAES et al., 2022) recently found that when selenium was supplemented to obese fathers (a condition previously shown to program increased breast cancer risk (FONTELLES et al., 2016a)), during puberty and young adulthood, it led to amelioration of epididymal fat tissue obesity-induced oxidative stress and inflammation and reprogrammed sperm microRNA vital for spermatogenesis. This suggest that selenium supplementation during puberty during these developmental stages could male physiology in the context of obesity as well as it suggests that it could potentially positively affect offspring health (PASCOAL, NOVAES et al., 2022). These results suggest that the preconception stage is an important developmental window of opportunity to initiate nutritional preventive strategies of breast cancer focusing on futures father´s diet during preconception.

Clinical and *in vivo* studies focusing on BFC's and paternal programming are still scarce. Because cohorts of adult offspring of fathers submitted to specific conditions will take long to be established, one initial approach to expand the knowledge of BFCs in this context would be to conduct nutritional intake studies in fathers to become and correlate it with fetal development and newborn parameters. In addition, small clinical studies where interventions with BFCs shown to improve fertility parameters, as shown in Table 1, could be conducted in different populations of infertile man and man with specific metabolic conditions (i.e. obese/diabetic) to verify if alterations in sperm cells epigenetics are to occur. In terms of experimental studies, one approach in this context would be to test these same BFCs in previous models of paternal programming of specific phenotypes (diabetes, obesity, breast cancer, among others). Access to both fathers sperm and offspring adult tissue would allow more in-depth epigenetic mechanistic studies.

5 Conclusion

Infertility is a growing public health problem. Consumption of antioxidant micronutrients and bioactive food compounds has been highlighted as a potential strategy to protect oxidative and inflammatory damage in male reproductive system induced by obesity, alcohol, toxicants and thus improve spermatogenesis and fertility parameters. Despite the accumulating experimental studies showing protective effects on male reproductive health by dietary compounds such as vitamins A, C, D and E, selenium and zinc, and PUFAs, carnitines, N-acetylcysteine, and coenzyme Q10, few well-controlled and designed clinical trials are available.

Studies in the new field of PoHAD show that paternal malnutrition can alter the sperm epigenome and this can alter fetal development and program increased risk of metabolic diseases and breast cancer in adulthood. Paternal consumption of such BFCs could not only benefit the fathers fertility/health but also their offspring health.

This indicates that from a father´s perspective preconception is a valuable window of opportunity to start nutritional interventions in order to maximize sperm epigenetic integrity and promote adequate fetal growth and development in order to prevent chronic disease in adulthood. Because the mentioned BFCs are known epigenetic modulators, they represent promising candidates for such paternal interventions (Figure 1).

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CHAPTER 2: Selenium supplementation during puberty and young adulthood mitigates obesity-induced metabolic, cellular and epigenetic alterations in male rat physiology

1. Introduction

Obesity is among the most alarming global public health problems (LOOS; YEO, 2022). It is associated with premature mortality an increases the burden of non-communicable diseases (WATANABE; NAVARRO, 2021). Importantly, in men obesity is also linked to deregulation of reproductive physiology (CRAIG et al., 2017b). Mechanisms whereby obesogenic conditions lead to poor sperm quality and male infertility include hyperestrogenism, elevated testicular and sperm levels of inflammatory mediators and reactive oxygen species (ROS), deregulation of spermatogenesis and sperm epigenetic perturbation (LEISEGANG et al., 2021).

Selenium (Se) is an essential micronutrient with several functions in humans, such as thyroid regulation, anti-inflammatory actions and antioxidant activities (HARIHARAN; DHARMARAJ, 2020; RAYMAN, 2012a). These functions are exerted through several selenoproteins in which Se is incorporated as the aminoacid selenocysteine (RAYMAN, 2012b). Among them, Glutathione peroxidases (GPXs) encompass a key family of antioxidant enzymes, involved with removal of hydrogen peroxide, lipid hydroperoxides, and phospholipid and cholesterol hydroperoxides (RAYMAN, 2012b). Importantly, GPX activity is dependent on Se nutritional status (HARIHARAN; DHARMARAJ, 2020). The involvement of Se in metabolic diseases is a matter of increased attention in the literature (MUTAKIN et al., 2013a), although its precise role in obesity is still not clear (WONGDOKMAI et al., 2021). A clinical study showed an association between Se nutritional status and metabolic risk factors in man with visceral obesity (MUTAKIN et al., 2013b).

Se also presents a key biological role in male reproductive physiology (WATANABE; NAVARRO, 2021). It is a constituent of selenoproteins that protect spermatozoa against ROS and simultaneously increases motility and sperm viability (SKORACKA et al., 2020). Se deficiency during spermatogenesis results in fertility disorders and abnormal semen parameters (SKORACKA et al., 2020). Spermatogenesis

comprises intense epigenetic remodeling at the level of DNA methylation, histone modifications and microRNA levels (ROTONDO et al., 2021; STEWART; VESELOVSKA; KELSEY, 2016; YAO et al., 2015). Adequate sperm epigenome patterns are fundamental for fertilization and proper embryo/fetal development (CHAMPROUX et al., 2018; MARCHO; OLUWAYIOSE; PILSNER, 2020). While some studies have shown effects by Se on these epigenetic marks in cancer cells (DE MIRANDA et al., 2014; NASIR et al., 2020), its role on epigenetic processes during obesity and/or spermatogenesis is unknown.

Se deficiency is frequently observed in infertile men in different populations(BIZEREA- MOGA et al., 2021). It has been proposed that selenium nutritional status could reflect fertility competence of the young population, and its monitoring would represent a strategy orienting dietary adjustments to attain normal reproductive function (BIZEREA- MOGA et al., 2021). In addition, some studies showed a direct correlation between obesity and plasma selenium deficiency (CAVEDON et al., 2020; MCPHERSON et al., 2019). However, although Se actions are closely associated with carbohydrate and lipid metabolism, its potential roles in obesity development and in adipocyte metabolism are not clear (TINKOV et al., 2020). Furthermore, information on Se role on male physiology specifically in obesity is scarce (MCPHERSON et al., 2019). Currently, obesity is a prevalent condition in male adolescents (JUNG; YOO, 2018). In addition, schoolchildren aged 8-13 years with excess of weight were shown to have a poor selenium status, a condition that could contribute to low antioxidant protection (ORTEGA et al., 2012). Because puberty is a key window of susceptibility to spermatogenesis deregulation induced by obesity and/or Se deficiency, supplementing obese male adolescents with Se could represent a potential clinical strategy to improve their sperm epigenetic pattern. Because epididymal fat impacts epididymis physiology and consequently sperm epigenetic maturation at the level of microRNAs (DONKIN; BARRÈS, 2018; NIXON et al., 2015; SHARMA et al., 2019), Se supplementation in this context could also ameliorate the function of that fatty tissue with beneficial effects on male physiology. This could have an impact not only for man themselves but potentially for their future descendants. Importantly, from a female´s perspective, experimental evidence already highlights possible Se dietary supplementation treatment for gestating and lactating mothers to

promote their metabolic health and prevent intrauterine growth retardation, which could affect their progeny's future health in adulthood (OJEDA et al., 2019).

Thus, we conducted this study to evaluate the efficacy of Se supplementation specifically during puberty until young adulthood against obesity-induced deregulation of metabolic, cellular and epigenetic parameters in epididymal fat and/or sperm cells in a rat model.

2. Materials and methods

2.1. Animal model

This study was approved by the Ethics Committee on the Use of Animals of the School of Pharmaceutical Sciences of the University of São Paulo (CEUA/FCF/USP, nº 571). Male Sprague Dawley rats, aged 3 weeks, were maintained in a temperature- and humidity-controlled animal facility, under a 12-hour light-dark cycle (6:00 am-6:00 pm). The animals were kept in polypropylene cages (n = 4/cage) with stainless steel lids and containing previously sterilized wood shavings, changed every other day. Forty-five male Sprague-Dawley rats at 4 weeks of age were randomly assigned to 3 groups with 15 rats in each group. For a period of 9 weeks (from the 4th week to the 13th week of age), control group (CO) received a control diet (AIN-93G (REEVES; NIELSEN; FAHEY, 1993); 0.15 ppm Se, as sodium selenate); obese group (OB) received a high-fat diet based on lard (0.15 ppm Se, as sodium selenate), with 60% of calories coming mainly from lipids; and obese group supplemented with Se (OBSe) received the same high-fat diet (0.15 ppm Se, as sodium selenate) together with drinking water containing sodium selenate (0.45 ppm Se, Merck, Germany). Diets were purchased from Prag Soluções (Brazil). Diet composition is provided in Supplementary Table S1. Animals´ weights, and diet and water consumption were recorded on alternate days. At 13 weeks of age all male rats were subjected to 3-4% of inhalatory isofluorane. Euthanasia was performed by cardiac puncture (exsanguination) and the blood was stored at -80ºC until the beginning of the analyses. After this procedure epididymal adipose tissue and liver samples were placed in liquid nitrogen and stored at -80ºC for further metabolic, cellular and epigenetic analyses.

2.2 Histopathology of epididymal adipose tissue and testicles

For the morphometrical analysis, the epididymal adipose tissue and testicles were collected and fixed in 10% buffered formaldehyde and paraffin. Sections of 5.0

μm were used for histological H&E slides. Testicles analysis was performed according to Johnsen score scale 1±10 (JOHNSEN, 1970). Mean value score for 100 seminiferous tubules was calculated for every testis in each section. For histological analysis, slides were obtained in an image capture system consisting of a trinocular microscope (Axioskop 2, Zeiss, Germany) and a digital camera (Axiocam, Zeiss, Germany) (JOHNSEN, 1970). The samples were analyzed by a pathologist.

2.3. Plasma Cholesterol and fractions

This analysis was based on the classical enzymatic colorimetric method and performed by the AFIP Medicina Diagnóstica laboratory (São Paulo, Brazil).

2.4. Oxidative stress – Malondialdehyde levels (MDA)

This analysis was performed on epididymal adipose and liver tissue. The concentration of MDA was determined by reversed-phase high performance liquid chromatography (HPLC) (HONG et al., 2000). The sample emulsion was submitted to an alkaline hydrolysis and was incubated, centrifuged and the extraction of MDA by n-butanol was analyzed in an isocratic condition (HONG et al., 2000). Samples were analyzed by Synergy HT Spectrophotometer (BioTek, Vermont, USA) using Gen5 software (BioTek).

2.5. Activity of Antioxidant enzymes

These analyses were performed on liver tissue based on the classical spectrophotometrical enzyme assays Catalase (CAT) activity (NABAVI et al., 2012), Superoxide dismutase (SOD) activity (EWING; JANERO, 1995) and Glutathione peroxidase (GPx) activity (WHEELER et al., 1990).

2.6. Infiltration of immune cells into the epididymal adipose tissue

CD11C and CD68 were evaluated by immunohistochemistry (DA CRUZ et al., 2018). Epididymal adipose tissue was collected and fixed in H&E slides in sections of 5.0 μm. The dilutions of CD11C and CD68 antibodies were 1/100.

2.7. Analysis of epididymal adipose tissue expression of genes associated with inflammation, adipogenesis, estrogen biosynthesis and epigenetic processes

Primers were custom designed using the OligoAnalyzerTMTool (IDT, Brazil). The expression of the following genes: PPARγ, CEBPa, CEBPb, Adiponectin, CYP19,

IL-6, TNF-a, DNMT3a and DNMT1 were estimated in epididymal adipose tissue of animals from all groups. Around 100 mg of liquid nitrogen-sprayed in epididymal adipose tissue was homogenized in TRIZOL reagent for total RNA extraction, as described by Chomzynski and Sacchi (CHOMCZYNSKI; SACCHI, 1987). One microliter of the solution was placed in a Nano Drop 2000 apparatus (Thermo Scientic, Brazil) for RNA quantification. When samples presented a ratio 260/280nm over 2, cDNA was synthetized with reverse transcriptase from 1µg of RNA. QuantStudio 7 Flex™ Real-Time PCR System (Life Technologies, USA) was used to determine gene expression profile as described (KUBISTA et al., 2006), using SYBER Green reagent (Invitrogen, Life Technologies) as the fluorescent marker. Primers details are provided in Supplementary Table 2. B-actin gene expression was used as control.

2.8. Histones modifications

H4k16ac and H3k4me3 marks were evaluated by immunohistochemistry (DA CRUZ et al., 2018). Epididymal adipose tissue was collected and fixed in H&E slides in sections of 5.0 µm. The dilutions of histones H4k16ac and H3k4me3 antibodies were 1/100 and 1/200, respectively (WEBSTER et al., 2010).

2.9. Analysis of sperm microRNA levels

The caudal epididymis and duct deferens were punched and moved to a culture plate containing M2 medium (M2 medium with HEPES, without penicillin and streptomycin, stereo filter, appropriate for the mouse embryo; Sigma-Aldrich, USA), where it was incubated for 1 hour at 37oC. Samples were washed with PBS and incubated with somatic cell lysis buffer (SCLB; 0.1 SDS, 0.5% Triton X-100 in diethylpyrocarbonate water) for 1h, according to Platts et al (2007) (PLATTS et al., 2007). SCLB was washed with two baths of PBS and the purified sperm sample (minimum 95% purity as assessed by microscope) was pelleted and used for miRNA analysis. For total miRNA extraction the mirVanaTM miRNA Isolation Kit (ThermoFisher, USA) was used. Then, the reverse transcriptase reaction was performed with a specific primer for miRNAs (hsa-miR-200c, hsa-miR-497, hsa-miR-15b; see in supplementary table 3). The qPCR technique was performed using the TaqMan technology (Applied Biosystems® TaqMan MicroRNA Assays kits) according to the manufacturer's instructions. For endogenous control, miRNA RNU 49 was used. The

analysis was performed with an Applied Biosystems® 7500 Real-Time PCR thermocycler and quantification was performed by calculating ΔΔCt.

2.10. Statistical Analysis

Statistical analysis was conducted with GraphPad Prism 9.0 (GraphPad software Inc, USA). All data were tested for normality. One-way ANOVA was used followed by Tukey's multiple comparisons tests. Specifically, for epididymal adipocyte and testicular morphological statistical analysis, chi-square test and Student's t-test were used. $p \leq 0.05$ was accepted as threshold of statistical significance. Data are presented as mean and standard error of the mean (SEM).

3. Results

3.1. Body weight and daily intake

No differences were observed between CO and OB groups regarding initial weight ($p > 0.05$), daily feed intake ($p > 0.05$) and daily water intake ($p > 0.05$) parameters (Supplementary Figure 1). Regarding Se daily intake, this was as follows: CO group (7.2±2.4 µg/day/animal); OB group (6.5±2.5 µg/day/animal) and OBSe group (20.8±7.6 µg/day/animal). OBSe group ingested 3.2x Se levels ($p = 0.0006$) compared to OB group. Compared to CO group, OB group presented higher ($p = 0.0089$) final weight. No differences were observed between OB and OBSe groups regarding this parameter ($p > 0.05$) (Supplementary Figure 1).

3.2. Histopathology of epididymal adipose tissue and testicles

Compared to CO group, OB group had larger ($p = 0.0072$) adipocyte size while OBSe group showed no difference ($p > 0.05$) regarding this parameter. No differences ($p > 0.05$) were observed between OB and OBSe groups regarding adipocyte size (Figure 1). No differences ($p > 0.05$) were observed among all groups regarding testicular architecture (Figure 2).

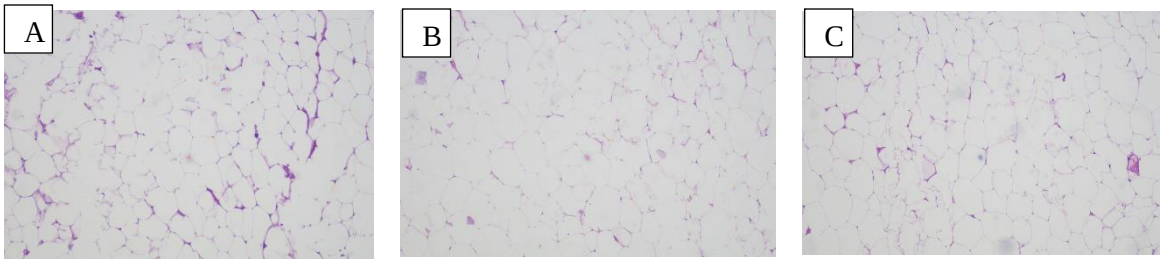


Figure 1. Representative photomicrographs of epididymal adipose tissue stained with hematoxylin-eosin. (A) CO group; (B) OB; (C) OBSe. All images are shown at ×100 magnification. Adipocyte sizes were larger ($p = 0.0072$) in OB group compared to CO group, while no differences ($p > 0.05$) were observed between OB and OBSe groups, according to chi-square test and Student's t-test.

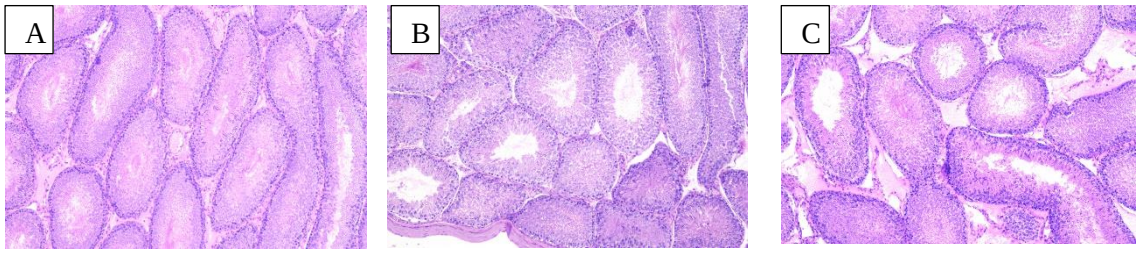


Figure 2. Testicular architecture. Representative photomicrograph of testicular tubules stained with hematoxylin-eosin. (A) CO group; (B) OB; (C) OBSe. All images are shown at ×10 magnification. No statistically significant difference ($p > 0.05$) among groups according to chi-square test and Student's t-test.

3.3. Total cholesterol and fractions

No differences were observed between OB and CO groups regarding total ($p > 0.05$), HDL ($p > 0.05$), and non-HDL plasma cholesterol levels ($p > 0.05$) (Figure 3). Compared OB group, OBSe group presented higher total ($p = 0.0032$), HDL ($p = 0.0067$) and non-HDL plasma cholesterol levels ($p = 0.0217$). Compared to CO group, OB group presented higher ($p = 0.0017$) LDL cholesterol levels, while there was no difference ($p > 0.05$), between OB and OBSe groups regarding this parameter. There was no difference between CO and OB groups regarding VLDL ($p > 0.05$) and triglycerides levels ($p > 0.05$) (Figure 3). In addition, there was no difference between CO and OB groups regarding VLDL ($p > 0.05$) and triglycerides levels ($p = 0.8302$).

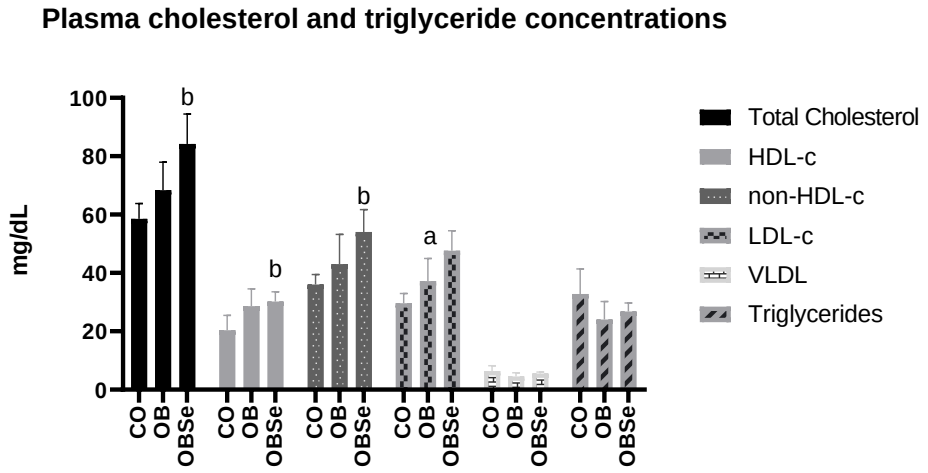


Figure 3. Effects of high-fat obesogenic diet and Se supplementation on total cholesterol and fractions and triglycerides plasmatic levels. Data represent the mean ± SEM (n = 5/group). Statistically significant difference ($p \leq 0.05$) compared to [a]CO or [b]OB group according to one-way ANOVA followed by Tukey's multiple comparisons test.

3.4. Oxidative stress – MDA levels

No differences ($p > 0.05$) were observed between CO and OB groups regarding hepatic MDA levels (Figure 4). Compared to OB group, OBSe group presented lower ($p = 0.0392$) hepatic MDA levels. Compared to CO group, OB group presented higher ($p < 0.0001$) epididymal adipose tissue MDA levels. Compared to OB group, OBSe group presented lower ($p=0.0006$) epididymal adipose tissue MDA levels (Figure 4).

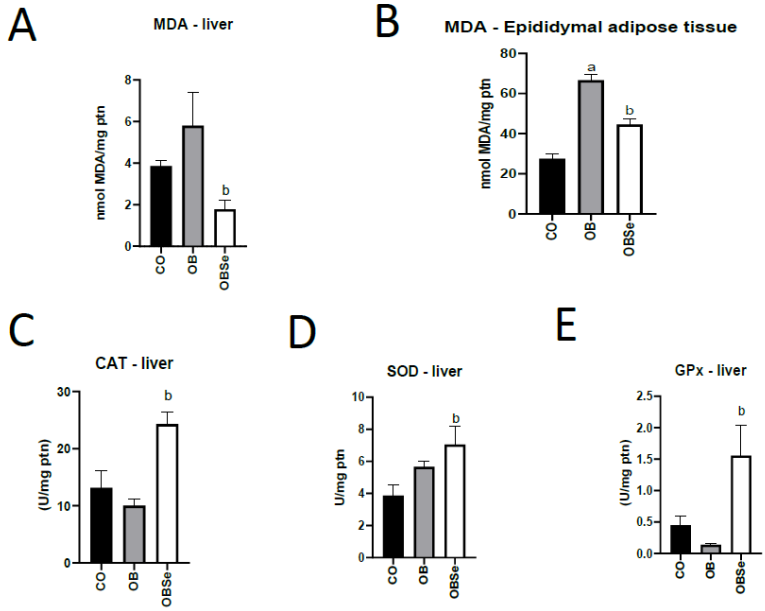


Figure 4. Effect of high-fat obesogenic diet and Se supplementation on MDA levels in liver and epididymal adipose and hepatic antioxidant enzyme activities (CAT, SOD and GPx). Data represent the mean ± SEM (n = 5/group). Statistically significant difference ($p \leq 0.05$) compared to [a]CO and [b]OB groups according to one-way ANOVA, followed by Tukey's multiple comparisons test.

3.5. Antioxidant activity

No differences ($p > 0.05$) were observed between CO and OB groups regarding CAT, SOD and GPx activity (Figure 4). Compared to OB group, OBSe group presented higher antioxidant activity of CAT ($p = 0.0002$), SOD ($p = 0.0310$) and GPx ($p<0.0001$) enzymes (Figure 4).

3.6 Infiltration of immune cells into the epididymal adipose tissue

Compared to CO group, OB group presented higher ($p = 0.0052$) amounts of positive cells per 10 fields of high magnification regarding CD68 antibody. No differences ($p > 0.05$) were observed between OB and OBSe groups regarding CD68 antibody. There was no statistical difference between CO and OB group ($p > 0.05$) and between OB and OBSe ($p > 0.05$) regarding CD11C antibody (Figure 5).

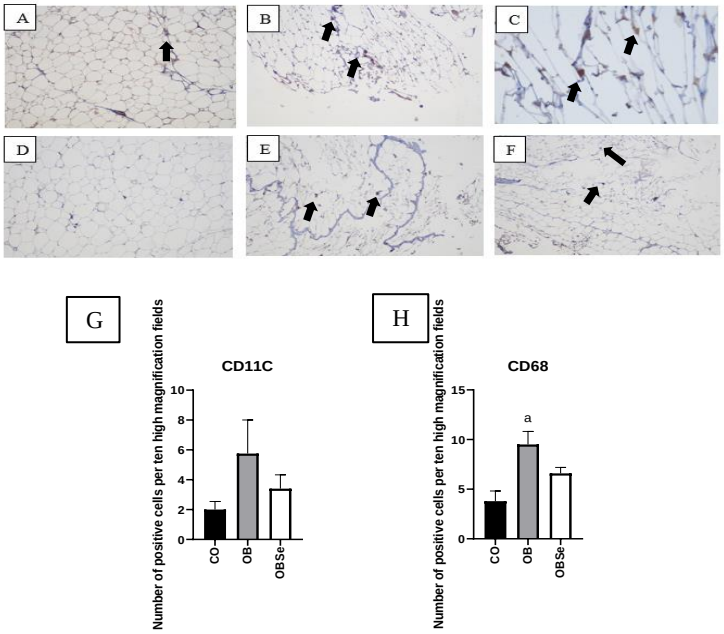
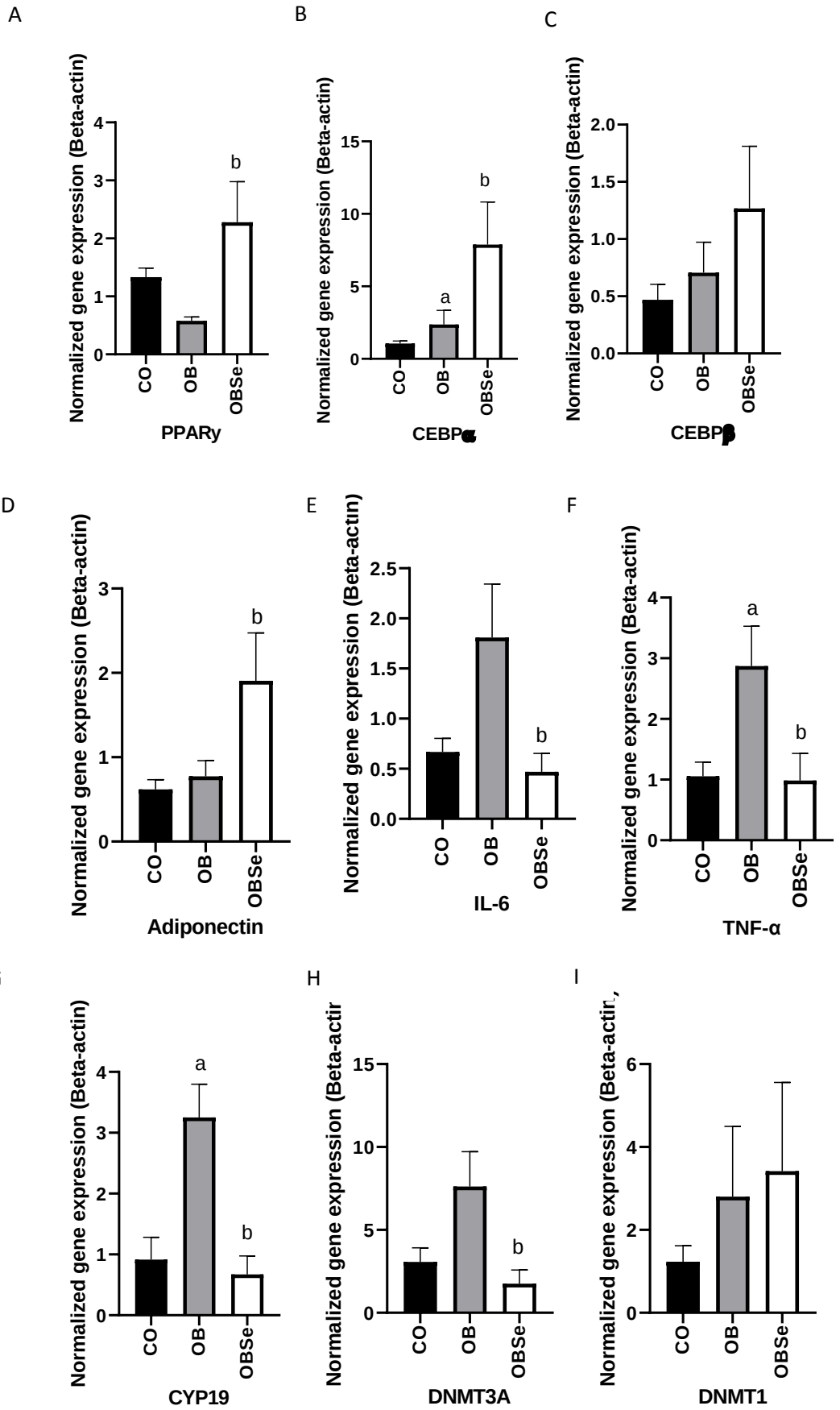


Figure 5. Representative photomicrographs of epididymal adipose tissue stained with hematoxylin-eosin. (A) CO group; (B) OB; (C) OBSe – CD11C antibody and (D) CO group; (E) OB; (F) OBSe – CD68 antibody. All images are shown at ×400 magnification. Effect of high-fat obesogenic diet and Se supplementation on infiltration of immune cells in epididymal adipose tissue, (G) – CD11C antibody and (H) – CD68 antibody. Data represent the mean ± SEM (n = 5/group). Statistically significant difference ($p \leq 0.05$) compared to [a]CO and [b]OB groups according to one-way ANOVA, followed by Tukey's multiple comparisons test.

3.7. Gene expression

Compared to CO group, no differences ($p > 0.05$) were observed between OB group regarding expression of PPARy. Compared to OB group, OBSe groups presented higher ($p = 0.0268$) expression of PPARγ. Compared to CO group, OB group presented higher ($p = 0.0317$) expression of CEBPα gene. Compared to OB group, OBSe group presented higher ($p = 0.0098$) expression of CEBPα gene. No differences ($p > 0.05$) were observed between CO and OB groups and between OB and OBSe group regarding expression of CEBPβ gene. No differences ($p > 0.05$) were observed between CO and OB groups regarding expression of adiponectin gene. Compared to OB group, OBSe group presented higher ($p = 0.0498$) expression of adiponectin gene. No differences ($p > 0.05$) were observed between CO and OB groups regarding expression of IL-6 and DNMT3A. Compared to OB, OBSe group presented lower expression of IL-6 ($p =$

0.0467) and DNMT3A genes (p = 0.0288). Compared to CO group, OB group presented higher expression of TNFα (p = 0.0382) and CYP19 (p = 0.0033). Compared OB, OBSe presented lower expression of TNFα (p = 0.0407) and CYP19 (p = 0.0015). No differences were observed between CO and OB group (p > 0.05) and between OB and OBSe group (p > 0.05) regarding expression of DNMT1 gene. See in Figure 6.


A
B
C
D
E
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G
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I
Normalized gene expression (Beta-actin)
CO
OB
OBSe
PPARγ
CEBPα
CEBPβ
Adiponectin
IL-6
TNF-α
CYP19
DNMT3A
DNMT1
a
b

Figure 6. Effect of high-fat obesogenic diet and Se supplementation on gene expression in epididymal adipose tissue. Data represent the mean ± SEM (n = 5/group). Statistically significant difference ($p \leq 0.05$) compared to [a]CO and [b]OB groups according to one-way ANOVA, followed by Tukey's multiple comparisons test.

3.8. Histones modifications

There was no statistical difference between CO and OB group ($p > 0.05$) and between OB and OBSe ($p > 0.05$) regarding the expression of H4k16ac and H3k4me3. (Supplementary figure 2).

3.9. Sperm microRNA expression

Compared to CO group, OB group presented lower ($p = 0.0441$) expression of miRNA has-miR-15b (Figure 7). Compared to OB group, OBSe group presented a tendency of increased ($p=0.0727$) expression of this miRNA. No differences ($p > 0.05$) were observed between OB and CO groups regarding expression of miRNA has-miR-200c. Compared to OB group, OBSe group presented lower ($p = 0.0357$) expression of miRNA has-miR-200c (Figure 7). Compared to CO group, OB group presented higher ($p = 0.0025$) expression of miRNA has-miR-497. Compared to OB group, OBSe group presented lower ($p = 0.0003$) expression of miRNA has-miR-497. (Figure 7)

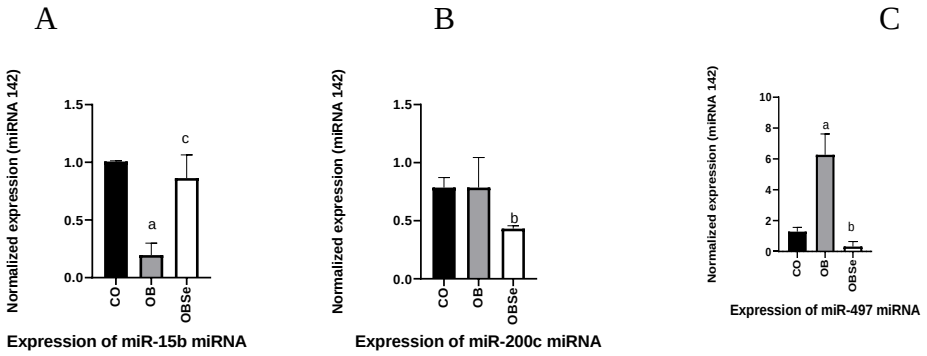

Figure 7. Effect of high-fat obesogenic diet and Se supplementation on sperm miRNA levels. Data represent the mean ± SEM (n = 7/group). Statistically significant difference ($p \leq 0.05$) compared to [a]CO and [b]OB groups according to one-way ANOVA, followed by Tukey's multiple comparisons test.

Tendency of Statistically significant difference ($p \leq 0.07$) compared to ^{c}CO according to one-way ANOVA, followed by Tukey's multiple comparisons test.

4. Discussion

We observed that Se supplementation did not alter final body weight and food intake. This suggests that at the level of intake in our study Se was not toxic. Aspects such as selenium dose and chemical form should be taken into consideration in the context of obesity as they can influence outcomes and even promote weight gain (HASANI et al., 2021). Se (0.45 ppm diet) exerted anti obesogenic effects at the level of weight gain in adult male mice treated with a high fat diet (WANG et al., 2022). Of notice, a recent clinical study showed that Se supplementation (240 µg selenomethionine/day) for three months reduced body weight of obese/overweight adult individuals. Thus, these experimental and human studies reinforce Se as a potential protective micronutrient in the context of obesity treatment.

Se effects as an hypocholesterolemic agent are still equivocal and depend on dosage and individuals cholesterol baseline levels (ZHAO et al., 2021). While in some *in vivo* studies Se supplementation did not affect total plasma cholesterol in mice (YU et al., 2021), we observed that Se increased plasma HDL-c levels. In addition, in our study Se did not affect plasma LDL-c, VLDL and triglycerides levels. These results are in accordance with a recent meta-analysis, where authors concluded that selenium supplementation did not affect triglycerides and VLDL-LDL-cholesterol levels (DJALALINIA et al., 2021).

Among obesity diverse deleterious actions, deregulation of male reproductive physiology, affecting sperm quality and fertility merits attention (LEISEGANG et al., 2021). This has been associated with obesity-induced ROS production particularly in the region close to the epididymis, where the last stage of sperm maturation occurs (MCPHERSON et al., 2019). Here we showed that high fat diet-induced obesity led to increased oxidative stress in rat epididymal adipose tissue. Importantly, Se supplementation inhibited oxidative stress in epididymal adipose tissue. Because of the close physical and biochemical connection between this adipose tissue and the epididymis (CHO; DOLES, 2020; MCPHERSON et al., 2019), Se protective effects may have occurred also in the latter tissue. This is important, since timing of the transit

of sperm through the epididymis represents the developmental window where sperm are the most susceptible to oxidative damage (MCPHERSON et al., 2019). In addition, we observed that high-fat diet-treated animals presented increased gene expression of proinflammatory cytokines TNF-α and interleukin 6 in epididymal adipose tissue. CD68 are M1-like macrophages and when activated have a pro-inflammatory action (ZELINKA-KHOBZEY et al., 2021). There are a strong relationship between adipose tissue macrophage content and indicators of adiposity provides a mechanism for the increased adipose tissue production of proinflammatory molecules associated with obesity, among these TNF-α, IL-6 (WEISBERG et al., 2003). As in our study, in a clinical study, CD68, and TNF-α expressions were significantly higher in the obese patients. These data suggest that acquired obesity is able to increase activity of macrophage and expression of inflammatory markers and (PIETILÄINEN et al., 2006). CD11c is a marker for dendritic cells (DCs), which primarily function in phagocytosis and antigen presentation. Activation of DCs is critical to the coordination of inflammatory events within fat tissue, however, no significant difference was observed between the groups in the present study (GRIFFIN et al., 2018).

On the other hand, Se presented anti-inflammatory actions in this adipose tissue by inhibiting expression of these genes and inducing expression of adiponectin, an anti-inflammatory cytokine. Similarly, selenate (0.5mg/kg b.w) presented in obese mice these oxidative stress and inflammation inhibitory effects in serum and total adipose tissue (HASANI et al., 2021). Of notice, results from our study were observed at level of selenate intake 10 times lower. McPherson et al. (2019) showed that male obese mice supplementation for 12 days, during the critical epididymal window, with the mix of antioxidants containing Se restored oxidative stress in sperm cells (MCPHERSON et al., 2019). Thus, our study expands the knowledge on Se potential in this context by showing its antioxidant and anti-inflammatory protective effects specifically on male physiology in obesity.

Se antiobesity effects could be related to modulation of adipogenesis (TINKOV et al., 2020). Se participates in key pathways involved in adipocyte differentiation and metabolism (ABO EL-MAGD et al., 2022). PPARγ is a key transcription factor for adipocyte function that presents decreased expression and activity in obesity (MOTAWI et al., 2017). In addition, Se supplementation could increase PPARγ expression in these

animals, an effect that could be related to its anti-inflammatory actions, as proposed before (DONMA; DONMA, 2016). On the other hand, in a similar mice study (KIM et al., 2015), Se antiobesity actions involved opposite effects on PPARγ. Its been highlighted that Se effects on this transcription factor would be influenced by Se characteristic and study design (TINKOV et al., 2020). In addition, these authors (KIM et al., 2015) found that selenate-administration inhibition of bodyweight gain was largely due to a decrease in adipose tissue mass, which can be attributed at least in part to decreased adipocyte hyperplasia and altered adipogenesis (including CEBPα increased expression) and lipid metabolism in adipocytes. However, in our study, Se did not alter obesity-induced adipocyte size. Thus altogether, in our study effects of Se on PPARγ and CEBPα expression could indicate a restoration of epididymal fat adipocyte metabolic function during obesity.

Literature data reports that obesity can increase DNMTs expression and activity (MAŁODOBRA-MAZUR et al., 2019; SALAH N, SALEM L, TAHA S, YOUSSEF M, ANNAKA L, HASSAN S, 2022) and that increased expression of Dnmt3a in the adipose tissue may contribute to obesity-related inflammation (KAMEI et al., 2010).Our study showed increased expression of the DNMT3a gene in epididymal adipose tissue rats treated with the high-fat diet during puberty and young adulthood. Similarly obese children and adolescents presented higher plasma DNMT3A expression (SALAH N, SALEM L, TAHA S, YOUSSEF M, ANNAKA L, HASSAN S, 2022). On the other hand, as previously reported in obese patients (MAŁODOBRA-MAZUR et al., 2019) we did not observe changes in the expression of the DNMT1 in obese animals. Information on Se epigenetic modulatory potential in metabolic disease context is limited (METES-KOSIK et al., 2012). We are unaware of such Se epigenetic studies in the context of obesity and/or male physiology. Of notice, in our study Se supplementation inhibited obesity induced DNMT3A expression in epididymal adipose tissue. This could be related, at least in part, to the previously described anti-inflammatory actions by Se in our study. Although histone deregulation has been associated with obesity (LING; RÖNN, 2019), we did not observe alterations in H3K4me3 and H4K16ac in epididymal adipose tissue after any treatment.

CYP19 is a key aromatase in estrogen biosynthesis that is responsible for converting androgens to estrogens and is associated with the inflammatory response

(GAO et al., 2012). We observed that obese animals presented increased expression of CYP19 in epididymal adipose tissue. Its high expression is associated with hormonal unbalance in the male body and damaged spermatogenesis (YUXIN et al., 2021). Importantly, we observed that Se supplementation during obesity normalized CYP19 expression, suggesting that the micronutrient protection of male physiology occurred at the level of testosterone metabolism.

According to testicular morphological analysis, we observed in all groups tubular structures with preserved architecture, accompanied by the formation of spermatids and spermatozoa within the normal limits. Similarly, Nematollahi et al. (NEMATOLLAHI et al., 2019) observed that intervention with a high fat diet did not alter in mice testicular morphological characteristics. Thus, we decided to evaluate potential alterations in sperm cells at the molecular level. MicroRNAs (miRNAs) have recently been shown to be important for spermatogenesis (CHEN et al., 2022). Obese animals presented altered epigenetic marks, including microRNA cells in spermatozoids (FONTELLES et al., 2016b). We confirm these findings and show that obesity led to decreased levels of miR-15b in sperm cells and increased levels of miR-497. miR-15b is a member of the miR-15/16 family and is primarily expressed in testis and is vital for spermatogenesis. miR-497 increased expression has been reported in sperm and seminal plasma–derived extracellular micro vesicles of men with spermatogenesis disturbances (ABU-HALIMA et al., 2013, 2020) and in plasma of men diagnosed with metabolic syndrome. Collectively our data on miRNA suggest that obesity deleterious effects on male physiology may have occurred at the epigenetic and metabolic level during spermatogenesis. Of notice, Se supplementation during obesity normalized miR-15b and miR-497 levels specifically in sperm cells. In addition, the micronutrient reduced levels of miR-200c, that is associated with metabolism and inflammation and when down regulated is important to control male germ cell development (MANTILLA-ESCALANTE et al., 2021). To the best of our knowledge, this is the first study to show Se miRNAs modulatory effects on sperm cells during obesity and to suggest that Se protective effective on male physiology could involve reprogramming of epigenetic processes factors during spermatogenesis.

More recently, increased interest has been directed towards the impact of the future father health condition and nutrition status on its offspring health, as male

gametogenesis is a highly plastic process and prone to environmental deregulation. McPherson et al. (MCPHERSON et al., 2019) showed in a seminal mice study that intervention with an antioxidant mix including Se not only mitigated obesity-induced miRNA deregulation in sperm cells but also improved fetal developmental parameters. Their data highlights the potential of supplementing future obese fathers with antioxidants including Se to improve their reproductive physiology and promote their offspring health. We previously showed that Se deficiency during preconception programmed increased risk of breast cancer in female offspring (GUIDO et al., 2016). However, Se supplementation of lean animals in this same study did not exerted protective effects (GUIDO et al., 2016). Se supplementation efficacy depends on individual metabolic context and oxidative stress context (WANG et al., 2017). Thus, based on data from the present study showing that Se supplementation on a condition of metabolic and oxidative stress improved male physiology, it would be important to investigate in further studies if Se supplementation specifically during obesity would prevent breast cancer risk in daughters, as this paternal metabolic condition was also shown to program increased disease risk (FONTELLES et al., 2016b).

5. Conclusion

Altogether, our study advances the knowledge on the role of Se on obesity and reinforce its supplementation as a potential strategy to ameliorate this condition. In addition, to best of our knowledge, this is the first study to show that Se supplementation during obesity mitigated obesity-induced deregulation of male physiology. This involved antioxidant and anti-inflammatory actions and adipogenesis and hormone-related pathways modulation in epididymal adipose tissue, as well as epigenetic reprogramming in sperm cells. Because these effects occurred during the transition between puberty and young adulthood, a developmental window were spermatogenesis is especially prone to environmental-induced disturbances, future clinical studies should focus on potential interventions with Se on this target population in order to improve male physiology. This could benefit not only themselves but also their future descendants.

Antioxidants Journal - Special Issue "Dietary Selenium and Its Antioxidant Properties Related to Growth, Lipid and Energy Metabolism"

Pascoal, G.d.F.L.; Novaes, G.M.; Sobrinho, M.d.P.; Hirayama, A.B.; Castro, I.A.; Ong, T.P. Selenium Supplementation during Puberty and Young Adulthood Mitigates Obesity-Induced Metabolic, Cellular and Epigenetic Alterations in Male Rat Physiology. Antioxidants 2022, 11, 895. https://doi.org/10.3390/ antiox11050895

Supplementary

Table S1. Diet composition

	CO group		OB group	
	g/Kg	g/500 kcal	g/Kg	g/500 kcal
Corn starch	529.486	67.81	121.667	11.69
Casein	200	25.64	264	25.38
Sucrose	100	12.82	132	12.69
Soy oil	70	8.97	92	8.84
Lard	-	-	257	24.71
Fiber	50	6.41	66	6.34
Mineral mix*	35	4.487	46	4.42
Mix of vitamins*	10	1.282	13	1.25
L-cystine	3	0.38	4	0.38
Choline bitartrate	2.5	0.32	3.3	0.32
Tert-Butylhydroquinone	0.014	0.0018	0.037	0.0035
Total	1000	128.12	1000	96.02

Control diet (AIN-93G) containing 3.9 kcal/g and hyperlipidic lard-based diet containing 5.2 kcal/g. * For mineral and vitamins mix composition, see in Reeves et al (1993). Se is present as sodium selenate in all diets. The OBSe group received the high fat diet and supplementation with sodium selenate (0.45 ppm) diluted in water.

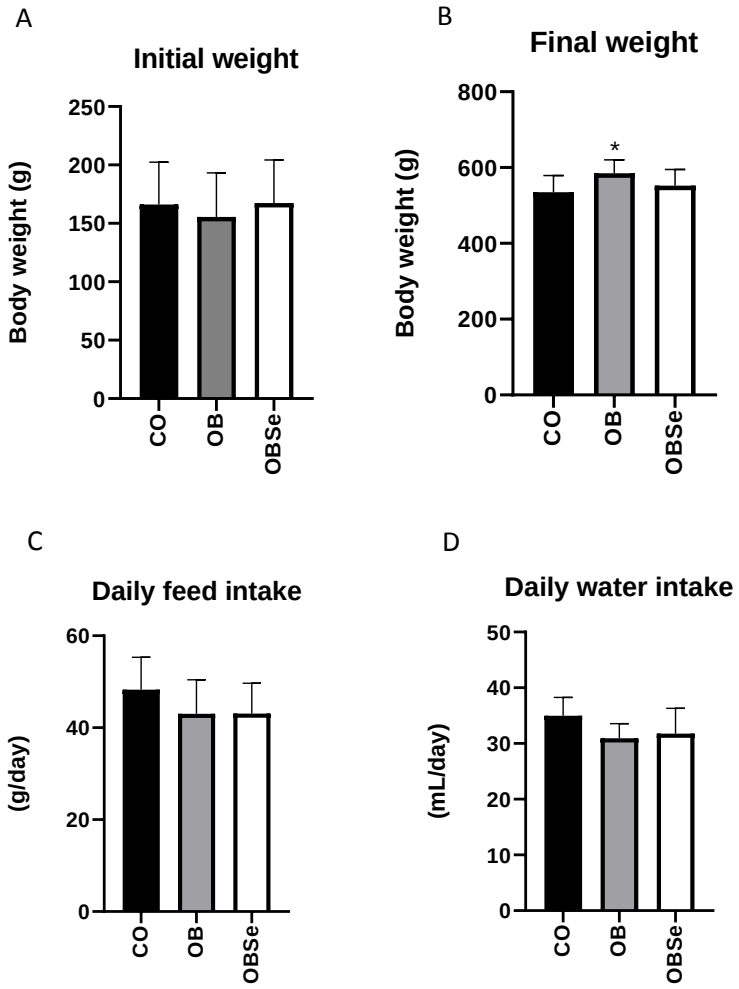

Figure S1. Initial (a) and final (b) body weight and daily feed (c) and water (d) intake. Data represent the mean ± SEM (n = 15/group). *Statistical significant difference ($p \leq 0.05$) compared to CO group according to two-way ANOVA.

Table S2. Primer sequences used in real-time PCR.

Gene	**Primer sequence (5′–3′)**
B actin (Control)	F:GACAACGGCTCCGGCATGTGCAAAG R: TTCACGGTTGGC CTTAGGGTTCAG
PPARγ	F: CTGGCCTCCCTGATGAATAAAG R: AGGCTCCATAAAGTCACCAAAG
IL-6	F: GTGGAAGACAAACCATGTTGCCGT R:TATTGCAGGTGAGCTGGACGTTCT
TNFα	F:AGAACAGCAACTCCAGAACACCCT R:TGCCAGTTCCACATCTCGGATCAT
Adiponectin	F:GCGCTCCTGTTCCTCTTAAT R:CATCCAACCTGCACAAGTTTC
C/EBPβ	F: GACTACGCAACACACGTGTAACT R: CAAAACCAAAAACATCAACAACCC
C/EBPα	F: TTACAACAGGCCAGGTTTCC R: GGCTG GCGACATACAGATCA
CYP1A1	F: CCATGACCAGGAACTATGGG R: TCTGGTGAGCATCCAGGACA
DNMT3A	F: ACGCCAAAGAAGTGTCTGCT R: CTTGGCTATTCTGCCGTGTT
DNMT1	F: GGTTCTGCGCGGGGACAGAC R: CCGGCAACATGGCCTCAGGG

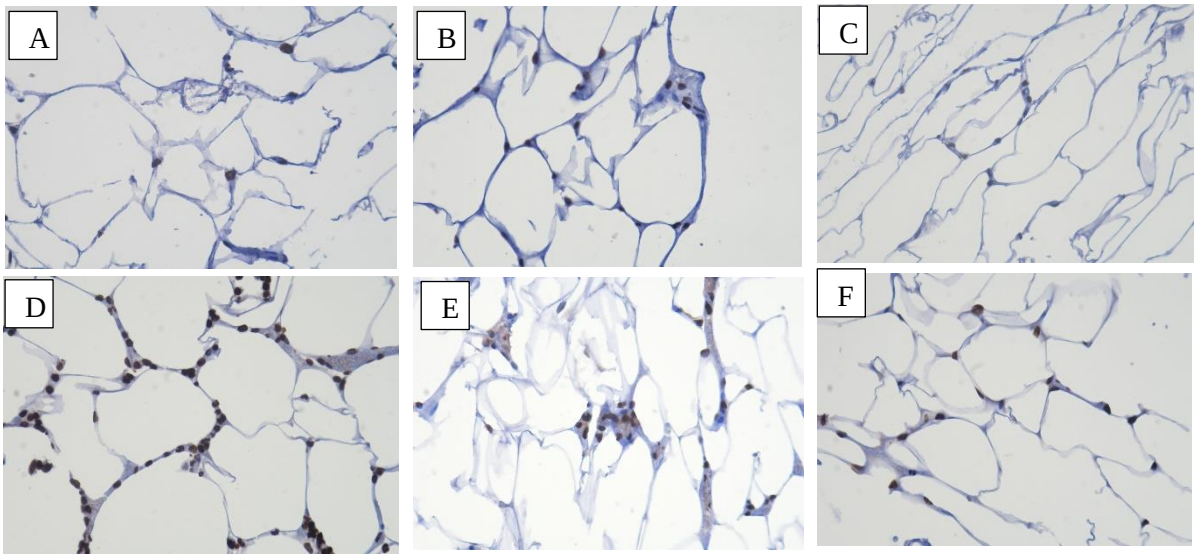

Figure S2. Effect of high fat diet and selenium supplementation on histone posttranslational modifications in epididymal adipose tissue. Photomicrograph of epididymal adipose tissue with hematoxylin-eosin stain. All images are at 10x magnification. H3k4me3 (A) Control group, (b) Obese group, and (C) Obese group supplemented with sodium selenate. H4k16ac (D) Control group, (E) Obese group, and (F) Obese group supplemented with sodium selenate.

Table S3. Taqman assays used for the analysis of miRNA expression by qPCR (Thermo Fisher Scientific, USA)

miRNA	**Assay ID**
hsa-miR-200c-3p	002300
hsa-miR-497-3p	002368
Hsa-miR-15b	000390
RNU49	000209

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