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Paternal nutrition and cancer programming: the influence of selenium deficiency or supplementation on the susceptibility of female offspring to chemically-induced mammary carcinogenesis.

Luiza Nicolosi Guido

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Thesis presented for the degree of

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Supervisor: Prof. Dr. Thomas Prates Ong

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Commission of thesis for the degree of doctor in philosophy

Prof. Dr. Thomas Prates Ong

Supervisor/President

1st examiner

2nd examiner

3rd examiner

4th examiner

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DEDICATION

To my family that have always believed in me and supported my dreams. Thank you. I love you.

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“We ourselves feel that what we are doing is just a drop in the ocean. But the ocean would be less because of that missing drop.”

Mother Teresa de Calcutá.

“Science never solves a problem without creating ten more.”

George Bernard Shaw.

“All success cloaks a surrender”.

Simone de Beauvoir

RESUMO

GUIDO Luiza N. **Nutrição paterna e programação do câncer: influência da deficiência ou suplementação com selênio na susceptibilidade da prole feminina à carcinogênese mamária quimicamente induzida.** São Paulo, 2016.

Tese de doutorado – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

O câncer de mama é um importante problema de saúde pública. O desenvolvimento da glândula mamária é um processo dinâmico que se inicia na vida intrauterina e evidências recentes mostram que a exposição do feto a fatores nutricionais maternos altera o desenvolvimento da glândula mamária e a susceptibilidade ao câncer de mama na vida adulta. Mesmo com um maior foco na nutrição materna, evidências recentes apontam que a nutrição paterna no período intrauterino e de preconcepção também afetam o desenvolvimento da glândula mamária e o risco de câncer de mama da sua prole feminina na vida adulta. Estudos apontam a modulação epigenética da expressão de genes na glândula mamária como possíveis mecanismos envolvidos na programação do câncer de mama. O selênio é um micronutriente com papel essencial em aspectos centrais da embriogênese, fertilidade masculina e que tem sido extensivamente estudado como um agente quimiopreventivo em diferentes modelos de câncer de mama. Dentre os possíveis mecanismos de ação do selênio, destacam-se a capacidade de modulação da proliferação celular, apoptose, danos do DNA e da expressão de genes e mecanismos epigenéticos. Dessa forma, foi conduzido um experimento em ratos para avaliar se a deficiência ou suplementação paterna com selênio durante o período de preconcepção poderia afetar na prole feminina o desenvolvimento da glândula mamária e o risco ao câncer de mama na vida adulta, assim como possíveis mecanismos moleculares envolvidos. Ratos machos da linhagem Sprague-Dawley com 4 semanas de vida foram submetidos à dieta experimental AIN93G contendo 0,15 (controle); 0,05 (deficiente) e 1ppm (suplementada) com selênio na forma de selenato de sódio por 9 semanas e acasalados com fêmeas controle. Com 7 semanas de vida, a carcinogênese mamária foi iniciada na prole feminina através da administração oral do carcinógeno químico 7,12 dimetilbenz[*a*] antraceno e o desenvolvimento das neoplasias mamárias foi avaliado. A deficiência paterna de selênio causou alterações no desenvolvimento da glândula mamária da prole feminina como aumento no número de terminal end buds (TEBs), aumento da elongação do epitélio mamário, aumento da proliferação celular e diminuição da apoptose que foram associados ao aumento do risco do câncer de mama (maior incidência e agressividade das lesões). Além disso, a deficiência paterna de selênio causou alterações de nível molecular na glândula mamária da prole feminina como hipometilação global, aumento dos níveis globais de H3K27me3 e alteração na expressão de genes relacionados ao desenvolvimento no início da vida e da glândula mamária, apoptose, controle de ciclo celular e reparo de danos no DNA. A suplementação paterna com selênio não foi influenciou o desenvolvimento da glândula mamária e o risco ao câncer de mama na vida adulta. Nossos resultados mostram que o risco do câncer de mama pode ser determinado no início da vida através de influências paternas por meio da modulação de mecanismos moleculares e que o período de preconcepção se caracteriza como uma importante janela de susceptibilidade para iniciar estratégias de diminuição do risco do câncer de mama. Assegurar uma ingestão adequada de selênio por homens pode ser um possível ponto de partida.

Palavras-chave: selênio, nutrição paterna, câncer de mama, desenvolvimento da glândula mamária, prole feminina, ratos.

ABSTRACT

GUIDO Luiza N. **Paternal nutrition and cancer programming: the influence of selenium deficiency or supplementation on the susceptibility of female offspring to chemically-induced mammary carcinogenesis.** São Paulo, 2016.

PhD Thesis – Faculty of Pharmaceutical Sciences, University of São Paulo.

Breast cancer is an important public health problem. As mammary gland development is a dynamic process that initiates in embryonic life, recent evidence show that in-utero life exposure to maternal nutritional factors can alter mammary gland development and program breast cancer risk in adult life. Even tough studies focus on maternal nutrition, recent evidence show that paternal nutritional factors in-utero and during preconception also affects their female offspring mammary gland development and breast cancer susceptibility in adult life. Studies highlight epigenetic modulation of gene expression in the mammary gland as possible breast cancer programming underlying mechanisms. Selenium is a micronutrient with essential role in central aspects of embryogenesis, male fertility and that has been extensively studied as a chemopreventive agent in several breast cancer models. Among selenium possible mechanisms of action, modulation of cell proliferation, apoptosis, DNA damage, gene expression and epigenetic marks are highlighted. Thus, a rat experiment was conducted to evaluate whether paternal selenium deficiency or supplementation during preconception could affect mammary gland development and breast cancer risk, as well as possible molecular mechanisms involved. Four-week old male Sprague-Dawley rats were exposed to experimental diets (AIN93G) containing 0.15 (control), 0.05 (deficient) and 1ppm (supplemented) of selenium as sodium selenate for 9 weeks and mated with control females. At 7-week old, mammary carcinogenesis was induced in their female offspring by oral administration of 7,12 dymethylbenz[a] anthracene and mammary neoplasia development was evaluated. Paternal selenium deficiency during preconception altered mammary gland development as increased terminal end buds (TEBs) number, epithelial elongation and cell proliferation and decreased apoptosis that were associated with increased breast cancer risk (higher incidence and grade tumors). In addition, paternal selenium deficiency during preconception induced molecular alterations in the mammary gland of the female offspring such as global DNA hypomethylation, increased global levels of H3K27me3 and altered expression of genes related to early life and mammary gland development, apoptosis, cell cycle control, and DNA damage repair. Paternal selenium supplementation during preconception on the other hand did not influence breast cancer programing. Our data show that breast cancer risk can be determined in early-life stages trough the male germline molecular modulation and preconception as an important window of opportunity to start breast cancer prevention strategies. Assuring and adequate selenium intake by men could be a possible starting point.

Key-words: selenium, paternal nutrition, breast cancer, mammary gland development, female offspring, rats.

List of abbreviations

Abcg2 – ATP-binding cassette, sub-family G, member 2

ABs – Alveolar buds

AIN – American Institute of Nutrition

Ang-2 – angiopoietin-2

ANOVA – Analysis of variance

Bax – BCL2 Associated X Protein

Bcl-2 – B-Cell CLL/Lymphoma 2

BORIS – Brother of the regulator of imprinted sites

BRAC1 – Breast cancer 1 gene

BRAC2 – Breast cancer 2 gene

BSA – Bovine serum albumin

Ccnd2 – Cyclin D2

cDNA – Complementary DNA

CH_3OH – Methanol

CH_3SeH – Methylselenol

CO – Control

CTCF – CCCTC-binding factor

DMBA – 7,12-dimethylbenz[a]anthracene

DNA – Deoxyribonucleic acid

DNMT1 – DNA methyl-transferase 1

DOHaD – Developmental origins of health and disease

EFSA – European Food Safety Authority

FoRC – Food research center

Gata3 – GATA binding protein 3

Gpx – Glutathione peroxidase

Gpx1 – Glutathione peroxidase 1

Gpx2 – Glutathione peroxidase 2

Gpx3 – Glutathione peroxidase 3

Gpx4 – Glutathione peroxidase 4

H_2Se – Selenide

H3K27me3 – Histone 3 lysine 27 tri-methylated

H3K9ac – Histone 3 lysine 9 acetylated

H4K16ac – Histone 4 lysine 16 acetylated

HB – Hemoglobin

HCl – Hydrochloric acid

HIF-1α – hypoxia inducible factor-1 alpha

HPLC – High-performance liquid chromatography

HPLC-DAD – High-performance liquid chromatography with diode-array detection

HPLC-FLD – High-performance liquid chromatography with fluorescence detection

Igf1 – Insulin like growth factor 1

Igf1r – Insulin like growth factor 1 receptor

Igfbp3 – Insulin-like growth factor binding protein 3

INRA – Instituit National de la Recherche Agronomique

Ki67 – Marker Of Proliferation Ki-67

LBs – Lobules

MDA – Malondialdehyde

$MgCl_2$ – Magnesium chloride

Mgmt – O-6-Methylguanine-DNA Methyltransferase

miRNAs – micro ribonucleic acid

mRNAs – messenger ribonucleic acid

MSA – methylseleninic acid

PBS – Phosphate-buffered saline

PGCs – Primordial germinative cells

pH – Potential of hydrogen

Pten – of phosphatase and tensin homolog

qPCR – Quantitative real time PCR

RDA – Recommended dietary allowances

RNA – Ribonucleic acid

RNAt-Sec – Selenocysteine RNA transporter

ROS – Reactive oxygen species

S.E.M – Standard error of mean

Se – Selenium

Se-DEF – Selenium deficient

Se-GTPs – selenium-containing tea polysaccharides

SePP – Seleno protein P

Se-SUP – Selenium supplemented

TBS-T – Tris-Buffered Saline-Tween

TDLUs – Terminal ductal lobular units

TEBs – Terminal end buds

TrxR – Thioredoxine reductase

VEGF-alpha – vascular endothelial growth factor-alpha

β-actin – Beta actin

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Summary

General Introduction

Breast cancer statistics and risk factors

Breast cancer is an important public health problem and is the most common cancer among women corresponding to the first and second cause of death in this group in developing and developed countries respectively (GLOBOCAN, 2012). Despite the increasing investments for the disease prevention and early detection breast cancer incidence is still increasing worldwide due to higher life expectancy, urbanization and adoption of western lifestyles (WHO, 2015) and 24 million cases are expected at the year 2035 (WCRF, 2015). Breast cancer mortality is higher in developing compared to developed countries in relation to the number of cases because in this less developed regions, breast cancer detection occurs in late stages, decreasing the chances of survival (GLOBOCAN, 2012).

Among the risk factors determining a woman's breast cancer susceptibility, early menarche, nulliparity, age at first gestation, late menopause, mammary density, advanced age, etiology, family history and genetic factors are described (ACS, 2016). However, breast cancer is a multifactor disease and although the genetic background and mutations such as BRAC1 and BRAC2 are important in breast cancer risk determination, they correspond to 5 – 10% of breast cancer cases, and accumulating evidence show that environmental influences contribute to the majority of cases (Anand et al., 2008; Howell et al., 2014). It has been observed that incidence rates vary across the world regions with ranges from 27/100,000 in Eastern Asia to 92/100,000 in North America (GLOBOCAN, 2012). Cohorts that have evaluated Asian immigrants in western countries show that later generations presented increased susceptibility to breast cancer and this risk was associated with the level of acculturation, suggesting that

environmental and behavioral in addition to genetic factors play important role in the breast cancer risk determination (Tseng et al., 2006). In addition to these risk factors, life style such as smoking, alcohol intake, obesity, oral contraceptive consumption, hormone therapy replacement, physical activity level, body weight and nutritional habits are also relevant (ACS, 2016) and studies suggest that about 38-50% of breast cancer cases could be avoided with the maintenance of healthy lifestyle (Anand et al., 2008; Howell et al., 2014). Most breast cancer risk factors are implicated in a woman's adult life. However, increasing interest has been directed towards early life stages such as in-utero, childhood and pre-puberty as possible windows of susceptibility for breast cancer risk determination.

Mammary gland development and differentiation

The mature mammary gland is composed of a complex network of lobes and ducts responsible for production and transportation of milk to the nipple triggered by hormones during gestation and lactation. These structures are comprised of two types of epithelial cells; the cells from the basal layer are called myoepithelial cells while cells in the surface of lobes and ducts are called luminal epithelial cells (Forsyth & Neville 2009). Mammary gland epithelium is embedded within a complex microenvironment composed by fibroblasts, adipocytes, blood vessels, nerves and diverse immune cells that present essential role for proper mammary gland development and function (Polyak & Kalluri, 2010).

Mammary gland development is a very dynamic process that initiates in embryonic life and goes through the entire woman's life, reaching full development after gestation and lactation (Gjorevski & Nelson, 2011). Experimental studies, mostly in rodents have been key to evaluate the specific developmental stages of mammary gland. In rodents, during embryonic life, there is the formation of milk lines from

overlying ectoderm followed by the formation of mammary placodes that invaginates into the mesenchyme to form rudimentary buds of the mammary gland. This mammary bud then proliferates, transforming into a rudimentary ductal structure surrounded by a fat-pad that remains dormant from birth until the onset of puberty (Watson & Khaled, 2008).

Puberty is perhaps the most remarkable stage of mammary gland development. Under the effects of increased ovarian hormones levels during this period the rudimentary mammary epithelial tree proliferates and gives rise to multilayered mammary structures called terminal end buds (TEBs) (Hinck & Silberstein, 2005). These structures are localized in the tips of mammary ducts and composed by an outer layer of cap cells and the more centrally located body cells (Hennighausen L & Robinson 2005). TEBs are the less differentiated mammary structures where cell proliferation is intense, promoting mammary ductal elongation, bifurcation and lateral branching until the complete filling of mammary fat-pad. Once the fat-pad is filled, mammary structures differentiate into alveolar buds (ABs) and lobules (LBs) and TEBs disappear (Watson & Khaled, 2008).

During pregnancy and lactation mammary gland achieves its full development into a hormone-trigged milk producer and secretor organ and after lactation mammary gland goes through an involution process with massive apoptosis (Gjorevski & Nelson, 2011). Even after this process, mammary gland does not return to the morphology before pregnancy, and presents more differentiated structures (Watson & Khaled, 2008).

Studies in breast cancer have suggested that TEBs are the site of breast cancer initiation because of the presence of stem cells and the proliferative characteristics of the epithelium. The high rate of cell proliferation combined with shorter cell cycle length increases TEBs susceptibility to malignant transformation (Russo, 2015). In

addition, in a classical model of chemically-induced breast cancer it has been shown that the cells from TEBs promote higher 7,12-dimethylbenz[*a*]anthracene (DMBA)-DNA binding, increasing the rate of cell initiation (Tay & Russo, 1981). TEBs containing transformed cells instead of differentiating into ABs, suffer hyperplasia, become progressively larger and give rise to carcinomas, while more differentiated structures either remain unmodified or give rise to hyperplasia and tubular adenomas (Russo & Russo, 1987). In humans, the terminal ductal lobular units (TDLUs) are considered the equivalent structures of TEBs found in rodents. In this regard, the increased number of TEBs/TDLUs in rodents and humans respectively is associated with increased breast cancer risk, and mammary gland differentiation driven by pregnancy and lactation is considered a protective factor (Russo, 2015).

Early life origins of breast cancer

As mammary gland development starts in embryonic life and is susceptible to environmental influences, a hypothesis has been raised that breast cancer susceptibility could be determined in early-life stages as a response to in-utero toxicants, hormones and nutrient levels exposure (Rudel et al., 2011). In 1990, based on epidemiological studies showing an association between perinatal characteristics as high birth weight and length with increased breast cancer risk in adult life, Trichopoulos (1990) suggested that fetal exposure to elevated estrogen levels during pregnancy could enhance later breast cancer susceptibility. This hypothesis has been validated in subsequent studies showing an association between dizygotic twinning or maternal diethylstilbestrol consumption with increased breast cancer risk later in life (Park et al., 2008). Among the factors postulated to affect the intra-uterine environment, maternal nutrition has attracted attention (Hilakivi-Clarke & de Assis, 2006). Experimental studies in rodents have been very important in this sense and allowed to identify possible nutritional

interventions implicated in breast cancer programming and potential mechanisms involved. In Sprague-Dawley rats, in-utero exposure to a high-fat diet based on corn oil (rich in polyunsaturated fatty-acids) increased breast cancer susceptibility (Hilakivi-Clarke et al., 1997) while the exposure a high-fat diet prevenient from lard (rich in saturated fatty-acids) decreased breast cancer incidence in the adult female offspring (de Oliveira Andrade et al., 2014). Increased breast cancer susceptibility was accompanied by increased number of TEBs, mammary epithelial elongation and cell proliferation in the mammary gland, while the opposite effect was observed in decreased breast cancer susceptibility. These data reinforce the hypothesis that the number of TEBs in the mammary gland is positively correlated with breast cancer susceptibility and that early-life exposures to maternal nutritional factors affect mammary gland development (Hilakivi-Clarke, 2007). In addition, these studies and others indicates that maternal nutrition during gestation was able to induce in the female offspring mammary gland changes in the expression of genes related to cell cycle control and apoptosis as well as some epigenetic marks, indicating possible underlying mechanisms on maternal breast cancer programming (de Oliveira Andrade et al., 2014; Govindarajah et al., 2016).

Epigenetics describes mitotically stable, heritable chromatin-based mechanisms that are capable of modulating gene expression without changes in DNA sequence (Sharma et al., 2010). Studies have shown that epigenetic mechanisms are reversible and influenced by environmental factors such as nutrient intake (Sapienza & Issa, 2016). In addition, aberrations in these mechanisms have been described as early events in cancer and have been suggested to be implicated in breast cancer programming (Dawson & Kouzarides, 2012; Eriksson, 2016). The most studied epigenetic marks are DNA methylation and post translational histone marks modification. DNA methylation is the covalent addition of methyl groups mediated by DNA-methyltransferases to the 5

position of a cytosine followed by guanine giving rise to 5-methyl-cytosines (Smith & Meissner, 2013). This modification regularly occurs in regions rich in CG known as CpG islands. Usually these regions are inserted or close to the promoter region of genes and control gene expression. DNA hypermethylation induces gene silencing by decreasing the accessibility of transcription factors to the chromatin, while the opposite occurs in DNA hypomethylation (Deaton & Bird 2011). DNA methylation is essential for diverse processes including genomic imprinting, X chromosome, DNA repeated regions and transposons silencing (Smith & Meissner, 2013). The most studied post translational histone marks are acetylation and methylation of N-terminal histone residues that are capable of altering chromatin condensation and transcription factor access to the promoter region of genes (Tessarz & Kouzarides, 2014). Histone methylation is mediated by histone methyl-transferases while acetylation is mediated my histone-acetyl-transferase and histone-deacetylase. Depending on the specific mark and the histone residue that is modified, decreased or increased accessibility and gene expression inhibition or induction is observed. The acetylation of lysine 16 in histone 4 and methylation of lysine 4 in histone 3 induces gene expression activation while methylation of lysine 27 in histone 3 induces gene silencing (Musselman et al., 2012). Recently, it was shown that miRNAs affect gene expression and are considered epigenetic mechanisms. This class of RNA is comprised by small (20-22 nucleotides) non coding RNAs that have mRNAs as targets. They are capable of binding partially or completely to mRNA and modulate gene expression by blocking translation or degrading mRNAs (Nolte-'t Hoen et al,. 2015).

Paternal programming of health and disease

Due to the strong interaction between mother and fetus/newborn, the majority of studies concerning the developmental origins of health and disease have focused on the

maternal perspective. However, accumulating evidence show that paternal experiences have also a significant impact in the offspring health (Curley et al., 2011). Initial epidemiological observations showed that fathers exposure to alcohol and drugs induced cognitive and behavior impairment (HE et al., 2006; MEEK et al., 2007) while paternal nutritional restriction increased the risk of diabetes and cardiovascular diseases in their sons and grandsons (Kaati et al., 2002). Studies in rodents have enabled to evaluate paternal nutritional interventions and the effects in their offspring health, especially in metabolic programming (Hughes, 2014).

Male consumption of a high-fat diet during preconception induced β-pancreatic cells dysfunction, insulin resistance in the absence of obesity and changes in the expression of genes implicated in cell proliferation in the pancreas and retroperitoneal adipose tissue of their female offspring (Ng et al., 2010; Ng et al., 2014). More recent studies have suggested epigenetic modulation of gene expression as a possible underlying mechanism involved in paternal programming. Male rat undernutrition promoted changes in the expression of genes related to lipid and cholesterol biosynthesis and miRNAs implicated in hepatocarcinogenesis in their offspring (Carone et al, 2010). Paternal obesity also induced miRNA expression changes in two subsequent generations of their offspring that were associated with metabolic syndrome (Fullston et al., 2013). These effects were attenuated with preconception diet or exercise intervention in the obese fathers (McPherson et al., 2015). Although convincing evidence shows the effects of paternal experiences on their offspring health, concerning breast cancer, however, few data is available in the literature. Some epidemiological studies have shown that higher paternal educational level, higher age and smoking was associated with breast cancer risk in daughters (Titus-Ernstoff et al, 2002; de Kok et al., 2008). One initial study in rats showed that daughters from fathers that were exposed

specifically in-utero to high-fat or ethinyl-oestradiol levels presented increased number of TEBs and breast cancer susceptibility in adulthood (de Assis et al., 2012).

Paternal epigenetic reprogramming

Until recently, sperm was considered as a vehicle for the delivery of only genetic information upon fertilization. However, accumulating evidence show that environmental exposures during a men's life are capable of affecting male germ cells development without altering genetic information with important outcomes in the offspring phenotype (Wu et al., 2015) and today its known that fathers transmit more than DNA but also epigenetic factors to their offspring (Ferguson-Smith & Patti, 2011).

Unlike female germ cells production, that occurs specifically during embryonic life (Hanna & Hennebold, 2014), male germ cells development starts in utero as primordial germ cells (PGCs). These PGCs suffer mitotic arrest and remain dormant as prospermatogonia until the onset of male puberty where hormone-trigged spermatogenesis begins with the production of mature, reproductive-capable male germ cells (Wu et al., 2015).

In embryonic life, after sex determination, male PGCs go through intense cell differentiation and morphological changes to prepare for genome propagation (Godmann et al, 2009). Two major epigenetic reprogramming events occur at this moment. First PGCs suffer a genome wide DNA methylation reduction as they migrate to the fetal gonad in formation. After, paternal germ cell-specific patterns of DNA methylation and histone marks are established in mitotically-arrested prospermatogonia (Carrell, 2012). As this period is characterized by intense epigenetic reprogramming, the epigenome of male germ cells are prone to in-utero environmental factors and initially the majority of studies concerning paternal programming of health and disease have focused on in-utero exposures as a risk factor and have shown induced intergenerational

and transgenerational effects through the sperm epigenome (Wu et al., 2015). However, during postnatal life, male germ cells undergo progressive development and differentiation from a prospermatogonia into diploid spermatogonia and mature haploid spermatozoa and these transitions involve stage- and test-specific gene expression, mitotic and meiotic divisions and specific chromatin remodeling that are unique to sperm (Ly et al., 2015). During this phase, male germ cells undergo more modest but significant epigenetic reprogramming until achievement of reproductive capacity (Oakes et al., 2007). During this period, DNA passive demethylation and *denovo* methylation are mediated by test-specific proteins BORIS and CTCF respectively (Loukinov et al., 2012). Histone modifications also occur in this period. During spermatozoa maturation, histones are replaced by protamine that promotes intense condensation of DNA and this is considered a sperm-specific epigenetic mechanism (Wu et al., 2015). Histone replacement in mature spermatozoa is partial and non-randomly distributed in the sperm genome and is believed to epigenetically affect development-related genes post-fertilization (Hammoud et al, 2009).

Along with in-utero exposures, accumulating evidence show that sperm epigenome during male postnatal life is prone to environmental epigenetic reprogramming, highlighting this period as an important window of susceptibility to the programming of diseases.

Selenium

Selenium (Se) is a trace element essential for human health maintenance and that exerts important role in male fertility and in central aspects of mammary carcinogenesis (Kurokawa & Berry 2013). Se was first described as a toxic element in 1817 arising as a sub product of sulfuric acid production. The element was named Se after the Greek moon goodness (Selene) because of its similar properties with Tellurium (named after

the earth) (Schomburg et al., 2004). It was only in the mid of the 20th century that Se was recognized as a micronutrient and its biological activity and importance in human health began to be studied. Experimental studies in rodents provided the first evidence of Se essentiality where feeding mice purified casein or torula-based diets promoted liver necrosis and the supplementation with Se prevented this effect (Schwarz & Foltz, 1957). Keshan disease, a congestive cardio myopathy was the first human reported disease that is associated with Se deficiency. The disease was named after the rural Se-depleted-soil region from China where it was related for the first time in 1979. The disease has also observed in New Zeeland, where Se content in soil is low (Loscalzo 2014). Se and iodine deficiency are also implicated in Kashin-Beck disease, an osteoarthropathy characterized by bone, joints and cartilage deformities (Yang et al, 2016).

Currently it is well established that Se plays important role in human health mostly through its incorporation in a family of 25 known proteins called selenoproteins. The most know and studied selenoproteins are glutathione peroxidase (Gpx), thioredoxine reductase (TrxR) and Se protein p (SePP) (Moghadaszadeh & Beggs 2006). At the physiological level, these proteins are implicated in antioxidant defense, muscle development and function, thyroid hormone metabolism, immune function, embryogenesis and fertility. Selenium status has been suggested to play important role in diseases as cancer, cardiovascular disease, male infertility, viral infection, immune system function and more recently to diabetes type 2 (Burk & Hill 2015).

Selenium intake and metabolism

Se content in food and diet depends on its concentration and bioavailability to plant uptake in the soil. Inorganic forms of selenium (selenite and selenate) occur in soil with selenate being much more easily and rapidly taken by plants than selenite under

most soil conditions (Fairweather-Tait et al, 2010). The main food groups providing selenium in human diet are cereals, meat, fish, eggs and milk/dairy products. Some food can be categorized by their capability of accumulating Se, as is the case of broccoli, cabbage, onions and garlic (Rayman et al, 2008). In addition, Brazilian nuts are known as a particular rich source of Se and although Se content in Brazilian nut varies a lot the consumption of one unit/day usually provides the Recommended Dietary Allowance (RDA) of 55µg/day for adults (Stockler-Pinto et al, 2014). This recommendation is set in order to maximize plasma selenoproteins activity. However, in a recent study from the European Food Safety Authority (EFSA) it was observed that the current RDA was not sufficient to maximize plasma SePP activity and it was suggested that the recommendation should be set at 70 µg/day (EFSA, 2014). Selenium consumption varies a lot in different regions of the world, with European countries, some regions in China and New Zealand showing low levels and seleniferous regions in China, Canada and Venezuela showing the highest and possibly toxic intakes (Morse & Stoffaneller, 2015).

In foods Se can be found in different inorganic (selenate and selenite) and organic (Se-methionine, Se-cysteine, Se-methyl-selenocysteine and γ-glutamyl-Se-methyl-selenocysteine) forms. In humans different forms of Se are absorbed and metabolized differently (Fairweather-Tait et al, 2011). When absorbed, both inorganic and organic forms of Se (with exception of γ-glutamyl-Se-methyl-selenocysteine) are converted into selenide (H_2Se) that can be considered a central point for Se interconversions. Thus, methylation of H_2Se can originate methyl-selenol (CH_3SeH) or Se can enter the pathway of selenoproteins synthesis through the production of RNAt-Sec, the specific RNA transporter that incorporates Se into selenoproteins. In addition, Se-methionine can be incorporated into nonspecific proteins in the place of methionine

and both Se-methionine and γ-glutamyl-Se-methyl-selenocysteine can be directly converted to CH_3SeH through the activity of γ- and β-lyase respectively (Suzuki et al, 2007; Ohta & Suzuki 2008; Burk & Hill 2015). Selenium is eliminated in urine, feces and breath to minimize accumulation of Se (Krittaphol et al, 2011).

SePP is the main selenoprotein implicated in Se transport through the body and although selenoproteins are synthetized in all tissues, most part of Se metabolism and SePP production occurs in the liver. Some tissues such as brain, thyroid and tests are capable to acquire and maintain Se, facilitating its retention during periods of depletion. All other organs are capable of retaining the mineral but are proner to Se deficiency (Burk & Hill 2015).

Selenium and breast cancer

Some studies that have evaluated the potential Se effects in human health have found a correlation between Se status and cancer risk (Willett et al, 1983). The interest in Se as a chemopreventive agent was increased based on initial human studies showing that increased Se content in soil and food and elevated Se blood levels were correlated with decreased total cancer mortality (Clark et al., 1996; Vinceti et al., 2014). As most of selenoproteins exerts antioxidant activity, it was proposed that increased Se status could protect DNA against oxidative damage (Moghadaszadeh & Beggs 2006) and later other mechanisms were proposed such as alteration of DNA damage repair, modulation of angiogenesis and extracellular matrix, immune function modulation, regulation of cell cycle and apoptosis and carcinogen detoxification (Fairweather-Tait et al, 2011).

Concerning breast cancer, interest was increased because of initial studies showing that Se status in blood and nails was lower in breast cancer patients than in healthy subjects (Männistö et al., 2000; Rejali et al., 2007) and some epidemiological studies showed a correlation between Se status and breast cancer risk (Cai et al., 2016).

Most Se *in vitro* and animal studies have adopted a chemopreventive approach showing that both inorganic and organic forms elicited protective effects in different models of breast carcinogenesis modulating cell proliferation, apoptosis and DNA damage (Davis et al., 2012). In female Sprague-Dawley rats Se dietary supplementation with 3-5ppm of sodium selenite decreased 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in 50% while Se deficiency increased breast cancer risk (Ip, 1981; Ip & Daniel, 1985; Thompson et al., 1982; Thompson et al., 1989) and mice supplementation with organic/inorganic Se or selenized milk casein decreased growth of intramammary tumors (Chen et al., 2013; Warrington et al., 2013).

In vitro studies conducted with different breast cancer cell lines have proposed possible mechanisms of Se on multiple cellular and molecular effects. Treatment of ER-positive MCF-7 and triple-negative MDA-MB-231 cells with different forms of Se presented growth-inhibitory effects and induced apoptosis (Guo et al., 2015). In a canine breast cancer cell line, Se treatment decreased the expression of vascular endothelial growth factor-alpha (*VEGF-alpha*), angiopoietin-2 (*Ang-2*), and hypoxia inducible factor-1 alpha (*HIF-1α*) and increased the expression of phosphatase and tensin homolog (*Pten*); effects that were associated with decreased cell proliferation and induction of apoptosis (Liu et al., 2016). The treatment of MCF-7 cells with selenium-containing tea polysaccharides (Se-GTPs) also induced cell cycle arrest that was associated with an up-regulation of *p53* expression and induction of apoptosis by the increase in *Bax/Bcl-2* ratio and subsequent *caspase-3* and *caspase-9* activation (He et al., 2013).

Recently it has been shown that selenium gene expression modulation in breast cancer cells lines could be associated with epigenetic marks. The treatment of MCF-7 cells with methylseleninic acid (MSA) or selenite induced decreased expression of

DNA methyl-transferase 1 (DNMT1) and altered the levels of global H3K9me3 and H4K16ac marks; effects that were associated with decreased cell proliferation and increased apoptosis (de Miranda et al, 2014).

However human evidence of an association between Se consumption and levels with breast cancer risk is still not clear and mixed epidemiological reports have been described and have raised concerns of a possible dubious role of Se in cancer (Vinceti et al., 2014). Based on conflicting results from the Nutritional Prevention of Cancer and the Selenium and Vitamin E Cancer Prevention Trials it has been proposed that the initial Se status (baseline) has major influence on the response of a subject to Se supplementation, where individuals with low but not high starting Se baseline presenting protective effect (Davis et al., 2012). These data, in addition with recent evidence showing that high Se doses can be associated with elevated risk of Diabetes type 2, raises concerns about Se supplementation as a chemopreventive agent for breast cancer (Ogawa-Wong et al., 2016).

Selenium and male fertility

Selenium plays important role in female and male fertility (Mistry et al., 2012; Duntas & Benvenga, 2015). The presence of adequate levels of Se in male reproductive tract is crucial for normal development, proper spermatogenesis and spermatozoa maturation and male fertility. Both decreased and increased levels impair male reproductive tract development (Ahsan et al., 2014). In Se deficient mice, impaired test development and delayed puberty were observed. These effects were more pronounced in the following generations with tests decreased weight, bilaterally atrophied, reduced seminiferous tubules diameter, reduced spermatogenic activity and impaired reproductive performance (Behne et al., 1996). Other study showed that either Se

deficiency or supplementation promoted multiple defects on mice spermatozoa, leading to decreased sperm concentration and impaired morphology (Shalini & Bansal, 2007).

Plasma SePP is the selenoprotein responsible to transport Se from blood to tests and epididymis and other selenoproteins are produced in the male reproductive tract (Kehr et al., 2009). Sperm cells are particularly prone to oxidative stress and disrupted oxidative balance can alter spermatogenesis and affect male fertility (Fullston et al., 2013). Increased levels of lipid peroxidation, malondialdehyde and reactive species of oxygen (ROS) were observed in both Se deficient and supplementation (Kaushal & Bansal, 2009). The selenoproteins Gpx1, 3 and 4 protect male germ cells from oxidative stress during spermatogenesis and spermiogenesis. Another variant of Gpx4 (nGpx4) is produced in the spermatozoa nuclei in late spermatogenesis and acts as a structural component in mature spermatozoa and in chromatin condensation stabilizing protamine-DNA binding in late spermiogenesis (Ahsan et al., 2014).

Although Se presents important role in central aspects of breast carcinogenesis, embryogenesis and fertility, few studies in the literature have evaluated the potential effects of Se in the developmental origins of health and disease. One initial study showed that maternal supplementation with Se in late gestation and lactation reduced spontaneous hepatomas in rat male offspring (Popova, 2002). More recently, it has been shown in rodents that maternal Se deficiency during gestation and lactation metabolically programmed the offspring (Nogales et al., 2013) and altered male offspring liver and colon gene expression with more pronounced effects when compared to post-weaning Se deficiency, indicating timing of exposure as a key programming factor (Barnett et al., 2015). However, the potential effects of Se on the programming of diseases through the male germline have not been described before. Thus, we aimed to evaluate whether a paternal Se deficiency or supplementation during preconception

could affect mammary gland development and DMBA-induced mammary carcinogenesis susceptibility in the female offspring as well as possible molecular mechanisms involved.

Objectives

To evaluate whether a paternal selenium deficiency or supplementation during preconception affects female offspring mammary gland development and later breast cancer susceptibility as well as possible molecular mechanisms involved.

Specific objectives

- To evaluate in rats whether paternal consumption of selenium deficient or supplemented diet affects:

  - Sperm quality and integrity;
  - Selenium blood levels, Glutathione Peroxidase activity and malondialdehyde concentration

- To evaluate if paternal selenium deficiency or supplementation affects in the female offspring:

  - Health parameters: selenium blood concentration, Gpx activity and MDA concentration;
  - Mammary gland development;
  - Global epigenetic marks (global DNA methylation and histone levels);
  - Gene expression changes in the mammary gland at 7-week old;

Articles' description

Article 1: **Paternal selenium deficiency but not supplementation during preconception alters mammary gland development and 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in female rat offspring**

While the early life origin of breast cancer has been considered predominantly form a maternal perspective, paternal influence in this context is largely unknown. This article presents the data showing that paternal selenium deficiency but not supplementation during preconception altered mammary gland development and increased 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in female rat offspring. This highlights the importance of father´s nutrition including selenium status as relevant factors affecting daughter´s breast cancer risk and paternal preconception as a potential developmental stage to start preventive strategies. **This article was published in International Journal of Cancer: DOI: 10.1002/ijc.30223.**

Article 2: **Paternal selenium deficiency breast cancer risk programming involves epigenetic marks and gene expression alterations in the mammary gland of female rat offspring**

Breast cancer risk can be programmed in early life through male malnutrition. Selenium deficiency induced mammary gland development alterations and increased breast cancer susceptibility in the female offspring. This article presents data showing altered gene expression and epigenetic marks in the mammary gland of the female offspring from fathers fed a Se-deficient diet as possible underlying mechanisms of breast cancer programming. This highlights the importance of early-life periods on mammary gland development and introduces a new sight of possible selenium mechanisms of breast cancer programming through the male germline. **This article has been formatted to be submitted to Metallomics.**

Article 1: Paternal selenium deficiency but not supplementation during preconception alters mammary gland development and 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in female rat offspring

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Short-title: Paternal selenium and breast cancer programming. **Article category**: research article.

Key words: Selenium, paternal nutrition, breast cancer, mammary gland development, female offspring. **Abbreviation list**: AIN: American Institute of Nutrition; ANOVA: analysis of variance; CO: control; DMBA: 7,12-dimethylbenz[*a*]anthracene; DOHaD: developmental origins of health and disease; Gpx: glutathione peroxidase; HB: hemoglobin; HPLC-FLD: High-performance liquid chromatography with fluorescence detection; MDA: malondialdehyde; PBS: phosphate-buffered saline; Se: selenium; Se-DEF: selenium deficient; S.E.M: standard error of mean; Se-SUP: selenium supplemented; TEBs: Terminal End Buds.

Novelty and impact: while the early life origin of breast cancer has been considered predominantly form a maternal perspective, paternal influence in this context is largely unknown. Paternal selenium deficiency but not supplementation during preconception altered mammary gland development and increased 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in female rat offspring. This highlights the importance of father´s nutrition including selenium status as relevant factors affecting daughter´s breast cancer risk and paternal preconception as a potential developmental stage to start preventive strategies.

Abstract

Breast cancer is a global public health problem and accumulating evidence indicate early-life exposures as relevant factors in the disease risk determination. Recent studies have shown that paternal nutrition can influence offspring health including breast cancer risk. Selenium is a micronutrient with essential role in central aspects of embryogenesis, male fertility and cancer and that has been extensively studied as a chemopreventive agent in several breast cancer experimental models. Thus we designed an animal study to evaluate whether paternal selenium deficiency or supplementation during preconception could affect the female offspring mammary gland development and breast cancer susceptibility. Male Sprague-Dawley rats were fed AIN93-G diet containing 0.15ppm (control diet), 0.05ppm (deficient diet) or 1ppm (supplemented diet) of selenium for 9 weeks and mated with control female rats. Mammary carcinogenesis was induced with 7,12-dimethylbenz[*a*]anthracene (DMBA) in their female offspring. Paternal selenium deficiency increased the number of terminal end buds, epithelial elongation and cell proliferation in the mammary gland of the female rat offspring and these effects were associated with higher susceptibility to DMBA-induced mammary tumors (increased incidence and higher grade tumors). On the other hand paternal selenium supplementation did not influence any of these parameters. These results highlight the importance of father´s nutrition including selenium status as a relevant factor affecting daughter´s breast cancer risk and paternal preconception as a potential developmental stage to start disease preventive strategies.

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Introduction

Breast cancer is a global public health problem and accumulating evidence indicate early-life experiences as relevant factors in the disease risk determination [1]. Mammary gland development is a dynamic process that starts in embryonic life as a rudimentary tree [2]. During this early life stage this mammary structure would be especially prone to developmental disturbances induced by abnormal in-utero conditions such as altered hormone, toxicant and nutrient levels increasing breast cancer risk in adult life [3]. Evidence of an in-utero origin of the disease in humans is shown by the association between high birth weight, dizygotic twinning or maternal diethylstilbestrol consumption with increased breast cancer risk later in life [4]. Studies performed in rodents have been key to identify breast cancer programming factors and the underlying mechanisms. Maternal nutrition has received particular attention in this regard and consumption of high-fat diets during gestation increased breast cancer susceptibility in the adult rat female offspring [5]. This effect was associated with altered mammary gland development as shown by increased number of terminal end buds (TEBs) [6]. Epigenetic modulation has been highlighted as a potential molecular mechanism [7].

Because of the strong interaction between mother and fetus/newborn the developmental origins of health and disease has been considered predominantly from a maternal perspective [8]. However accumulating evidence indicate that paternal experiences have also a significant role in the offspring health [9]. Metabolic programming through the male germline has been shown in rodents exposed to both under or overnutrition [10, 11]. Concerning breast cancer, limited data is available in the literature. Some epidemiological studies have shown that higher paternal educational level, higher age and smoking was associated with breast cancer risk in daughters [12,

13]. One initial study in rats showed that daughters from fathers that were exposed specifically in-utero to high-fat or ethinyl-oestradiol levels presented increased number of TEBs and breast cancer susceptibility in adulthood [14].

Selenium (Se) is an essential antioxidant micronutrient required at low levels for human health maintenance that can be toxic at high levels [15]. Because Se can modulate cell proliferation, apoptosis and DNA damage it has attracted attention as a potential cancer preventive agent [16]. An inverse correlation between serum Se levels and breast cancer risk has been observed and extensive preclinical data show that supplementing Se can inhibit breast carcinogenesis [17]. However, more recent studies have raised concerns of a possible dubious role for Se on cancer [18].

Although Se is essential in embryogenesis [19], few studies have evaluated its role in the developmental origins of health and disease (DOHaD) context. In rodents, maternal Se deficiency during gestation and lactation metabolically programmed the offspring [20] and altered male offspring liver and colon gene expression with more pronounced effects when compared to post-weaning Se deficiency, indicating timing of exposure as a key programming factor [21]. Maternal Se supplementation during late gestation and lactation reduced spontaneous hepatomas in the rat male offspring [22]. Adequate levels of Se are also critical for male fertility. Severe Se deficiency or supplementation impaired spermatogenesis in rodents and can induce male infertility [23]. Nevertheless the potential programming effects by Se deficiency or supplementation during male preconception have not been evaluated.

Global variation in Se intake has been observed with European countries and New Zealand showing low levels and seleniferous regions in China and Canada showing the highest and possibly toxic intakes [24]. Thus we designed a rat experiment to evaluate whether paternal Se deficiency or supplementation during preconception

could affect mammary gland development and breast cancer susceptibility in the female offspring. Our data show that paternal Se deficiency, but not supplementation, increased epithelial elongation, number of TEBs and cell proliferation index in the mammary gland of the female offspring and these alterations were associated with increased breast cancer risk in adulthood. These novel data show that male preconception comprises an important window of opportunity to initiate breast cancer preventive strategies focusing on paternal nutrition.

Material and methods

Experimental design and animal manipulation

The Study was approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil (Protocol: CEUA/FCF/382). Three-week old male (n = 30) and female (n = 60) Sprague-Dawley rats were obtained from the Colony of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil, and maintained at 22°C ± 2°C, in an atmosphere of 55% ± 10% relative humidity in a 12 hours light/dark cycle. Male rats were randomly divided into 3 groups (n = 10/group), that received experimental diets (AIN93-G; Lab Research, USA) for 9 weeks from weaning to puberty (week 4 to 13 of age): Control group (CO; 0.15 ppm Se, as recommended by AIN93 diet); Se-Deficient group (Se-DEF; 0.05ppm Se) and Se-Supplemented group (Se-SUP; 1 ppm Se). In CO and Se-SUP diets, sodium selenate was added to the mineral mix, whereas in the Se-DEF diet Se as sodium selenate was not added to the mineral mix and the only source of the mineral was casein that contains Se in the form of selenomethionine. The levels of Se in the deficient and supplemented diets were based on previous studies in rodents showing that, at these levels and after this period, both low and high Se status can be attained

respectively without inducing male infertility [25]. Mating was performed by housing one male with two females per cage. Gestational day 0 was determined by the presence of sperm in the vaginal smears. Whenever possible, 8 pups (7 female and 1 male) were maintained with their respective dams. Commercial diet (Nuvital, Brazil) was offered to female rats during pregnancy and lactation and to offspring during the entire experiment. Body mass and food intake were recorded 3 times a week for male rats receiving the experimental diets, daily for female rats during gestational and lactational periods and once a week for female offspring.

Male fertility parameters

The right testis were collected, cleaned and maintained at -20°C until determination of the daily sperm production (n = 5/group) according to Robb et al [26]. For sperm morphology analyses the left epididymis were collected, cleaned and underwent incision and subsequently immersion in phosphate-buffered saline (PBS) to promote the dissemination of gametes into the aqueous medium. The solution was placed on slides for light microscope examination. Two hundred sperm cells per animal were morphologically analyzed at 400x magnification (n = 5/group).

Determination of Se concentration, Glutathione peroxidase (Gpx) activity and Malondialdehyde (MDA) concentration in fathers

Blood samples from male rats exposed to experimental diets were collected by cardiac puncture and kept at -80°C until use. Se concentration was determined in plasma and red blood cells of fathers (n=5/group) by atomic absorption spectrometry with hydride generation coupled to quartz cell. Results are expressed as µg/L. Gpx activity in red blood cells of fathers (n=10/group) was determined using Ransel 505 – RANDOX kit (RANDOX Laboratories, UK) adapted to the Liasys® MAS automatic biochemistry analyzer (Italy) according to the manufacturer's instructions. Results are represented as

U/g hemoglobin (HB). MDA concentration in plasma and liver homogenate of fathers (n=5/group) was quantified by reverse phase high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Thiobarbituric acid-MDA conjugate was injected into a Phenomenex (USA) reverse-phase C18 analytical column (250 mm x 4.6 mm; 5 mm) with a LC8-D8 pre-column and was quantified at 515 nm (excitation) and 553 nm (emission). Isocratic mobile phase was 60% PBS (10 mmol, pH 7.4) and 40% methanol at a flow rate of 1.0 mL/min. A standard curve was prepared with 1,1,3,3-tetraetoxipropane. The results are expressed as μmol MDA/mg protein.

Mammary tumor induction

Mammary tumors were induced in 7-week old female offspring (n=24/group) by oral gavage administration of 7,12-dimethylbenz[*a*]anthracene (DMBA; 50 mg/kg body weight; Sigma, USA) dissolved in corn oil. This is a classical model of breast carcinogenesis and is based on the high susceptibility of Sprague-Dawley female rats to initiation with a single dose of this chemical carcinogen. In addition, most of the neoplastic lesions found in Sprague-Dawley rat mammary glands have their counterparts in human pathology [27]. Animals were examined for mammary tumor development 3 times a week by palpation. Latency of tumor appearance, tumor incidence and multiplicity (number of tumors/animal) were evaluated. Those animals in which tumor weight represented more than 10% of the body weight were euthanized. The other animals were euthanized 10 weeks after carcinogen administration. All tumors were collected for histological analysis.

Histological analysis of mammary tumors

The histological grade of mammary gland tumors was assessed by the Nottingham Histologic Score (Elston grade), in order to classify them in low grade (grade 1), intermediate (grade 2) and high grade (grade 3) invasive carcinoma. Elston grade is

based on the following features: tubule formation, nuclear pleomorphism and mitotic rate, which receive scores from 1 to 3. Final score 3-5, 6 or 7 and 8 or 9 features grade 1, grade 2 and grade 3 tumors, respectively [28].

Analysis of mammary gland morphology and development, cell proliferation and apoptosis in female offspring

Whole-mount preparations of the 4th abdominal mammary gland from 3- (n = 6/group) and 7-week old (n = 12/group) female offspring were obtained and the epithelium elongation and the number of TEBs were determined as described by de Assis et al [29]. These analyses were conducted 3 times by a technician that was blinded to the treatment groups and measurements compared for consistency. Cell proliferation was evaluated in mammary gland from 3- and 7-week old female offspring (n = 6/group) by Ki67 immunohistochemistry. After harvesting, mammary tissue was directly fixed in 10% buffered formalin, embedded in paraffin and sectioned. Sections were then deparaffinized in xylene and hydrated through graded ethanol. Antigen retrieval was performed with 10 mM citrate buffer pH 6 for 20 min in pressure cooker. Peroxidase blocking was performed with 10% H_2O_2 for 10 min and nonspecific binding was blocked for 1 h with 1% skimmed milk in PBS. Sections were incubated overnight with anti-rat Ki67 primary antibody (Abcam, UK) at a 1:50 dilution. After washes, sections were incubated with the LABS + System – HRP Kit (Dako-Agilent Technologies, USA) according to the manufacturer's instructions and stained with 3,3'-diaminobenzidine in chromogen solution (Dako-Agilent Technologies, USA) for 10 min, washed and counterstained for 1.5 min with hematoxylin. Cell proliferation was quantified by assessing the number of Ki67 positive cells among 1,000 cells per structure (ducts or lobules). Apoptosis analysis was performed according to Elmore et al. [30]. The results are represented as mean number of apoptotic cells/1,000 cells per structure (ducts or

lobules). The slides were evaluated using Image J software (NIH, USA).

Statistical analysis

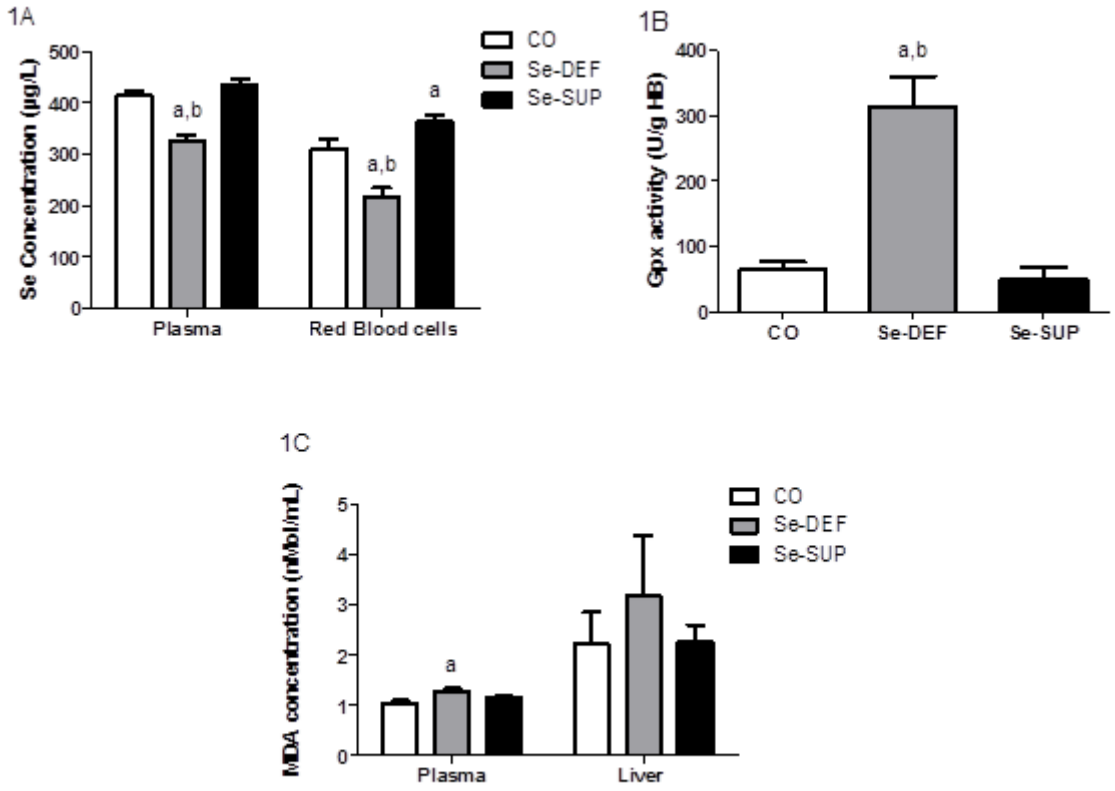
Results are expressed as mean ± standard error of mean (S.E.M.) and the statistical analysis was conducted with STATISTICA 8.0 (USA). One-way analysis of variance (ANOVA) followed by Duncan's post hoc test was used for multiple-group comparison. Repeated-measures ANOVA test was applied for caloric intake and weight gain evolution. Fisher's test was used to compare the percentage of pregnant females, number of rats with 2 or more mammary tumors and mammary tumor histological grade. Kaplan-Meier and log-rank test were performed to determine differences in tumor incidence. $P \leq 0.05$ was used as threshold for statistical significance and $p > 0.05$ and ≤ 0.08 was accepted as marginal difference, but not statiscally significant.

Results

Fathers' health parameters

There were no differences ($p > 0.05$) among the experimental groups regarding paternal caloric intake and weight gain (Data not shown). Se-DEF fathers presented lower Se concentration in plasma and red-blood cells compared to CO and Se-SUP fathers ($p \leq 0.05$) (Figure 1A). Se-SUP fathers presented increased ($p \leq 0.05$) Se concentration only in red-blood cells compared to CO fathers (Figure 1A). These data indicate that the present dietary interventions during preconception were able to induce paternal Se deficiency and increased status respectively. Se-DEF fathers showed increased ($p \leq 0.05$) GPx activity compared to CO and Se-SUP fathers, while no difference ($p > 0.05$) was observed between CO and Se-SUP fathers (Figure 1B). Compared to CO fathers, Se-DEF fathers but not Se-SUP fathers presented increased ($p \leq 0.05$) MDA plasma

concentration, while no differences (p>0.05) regarding MDA liver concentration were observed among groups (Figure 1C).



Article1 - Figure 1. Paternal Se concentration in plasma and red blood cells (A); Gpx activity in red blood cells (B) and MDA concentration in plasma and liver (C). Results are represented as mean ± S.E.M. Statistically significant differences (p≤0.05) when compared to [a]CO and [b]Se-SUP group fathers, according to ANOVA + Duncan's post hoc test. N = 5 (Se and MDA concentration) and 10 (Gpx activity).

Paternal fertility parameters

Se-DEF and Se-SUP fathers presented lighter (p≤0.05) testicles and epididymis compared do CO group fathers (Table 1). Se-SUP fathers presented heavier (p≤0.05) ventral prostates compared to CO and Se-DEF fathers. Se-DEF and Se-SUP fathers

showed decreased ($p \leq 0.05$) daily sperm production and decreased ($p \leq 0.05$) percentage of sperm with normal morphology compared to CO fathers (Table 1).

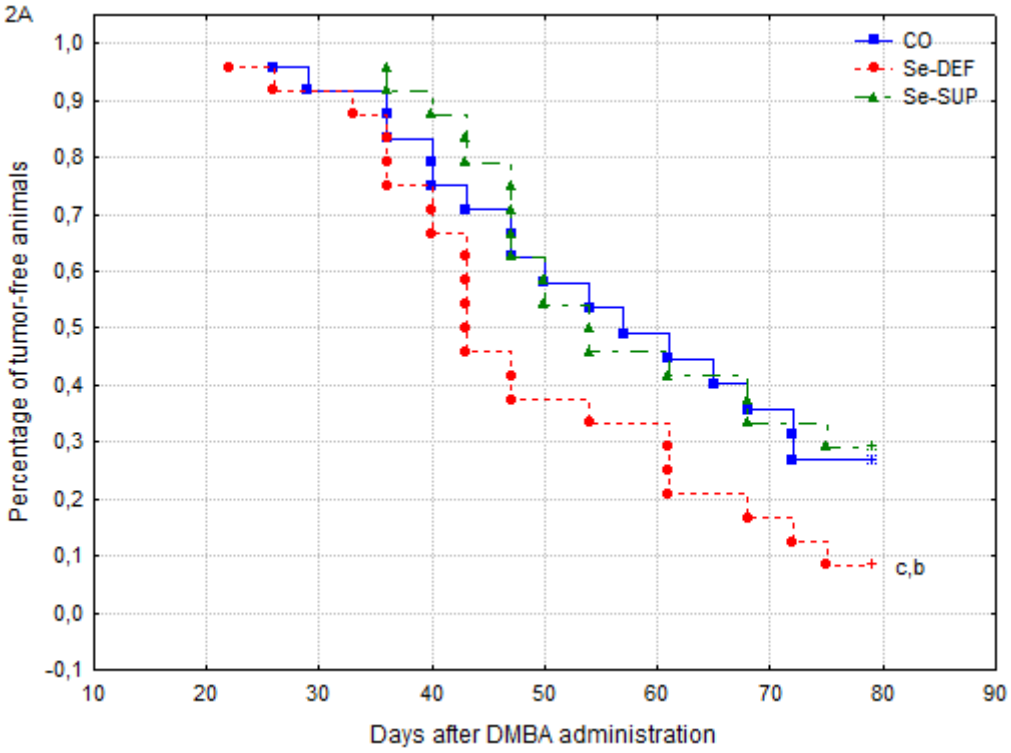
Article 1 - Table 1: Effects of paternal Se consumption on male fertility parameters

Variable	**Groups**		
	CO	**Se-DEF**	**Se-SUP**
Testicles relative weight (g/100g b.w.)	0.47 ± 0.01	0.43 ± 0.006[a]	0.43 ± 0.01[a]
Epididymis relative weight (g/100g b.w.)	0.14 ±0.003	0.12 ± 0.002[a]	0.13 ± 0.003[a]
Ventral prostate relative weight (g/100g b.w.)	0.16 ± 0.004	0.17 ± 0.004	0.18 ± 0.005[a,b]
Daily sperm production*	30 ± 1	21 ± 1[a,b]	24 ± 1[a]
Sperm morphology (%)**	64 ± 3	40 ± 5[a]	51 ± 4[a]

Results as represented as mean ± S.E.M. Statistically significant differences ($p \leq 0.05$) when compared to [a]CO and [b]Se-SUP group fathers, according to ANOVA + Duncan's post hoc test. $*10^6$ sperm/test/day; **percentage of perfect sperm. N=10 (testicles, epididymis and ventral prostate weight) and 5 (daily sperm production and sperm morphology). B.w.: Body weight.

Female offspring mammary carcinogenesis

Se-DEF group female offspring presented marginal increase but not statiscally significant ($p=0.08$) and increased ($p \leq 0.05$) mammary adenocarcinoma incidence compared to CO and Se-SUP group female offspring respectively (Figure 2A). There was no difference ($p>0.05$) regarding this parameter between CO and Se-SUP female offspring. Compared to CO group female offspring, Se-DEF group presented marginally increased but not statiscally significant ($p=0.07$) proportion of rats with 2 or more mammary adenocarcinomas (Table 2). Se-DEF group offspring presented marginally increased but not statiscally significant ($p=0.07$) and increased ($p \leq 0.05$) proportion of grade 2 mammary adenocarcinomas compared to CO group and Se-SUP group female offspring respectively (Table 2). No differences ($p>0.05$) were observed among groups regarding mammary adenocarcinoma multiplicity and 1st tumor latency of appearance.



Article 1 - Table 2: Effects of paternal Se consumption on female offspring mammary carcinogenesis

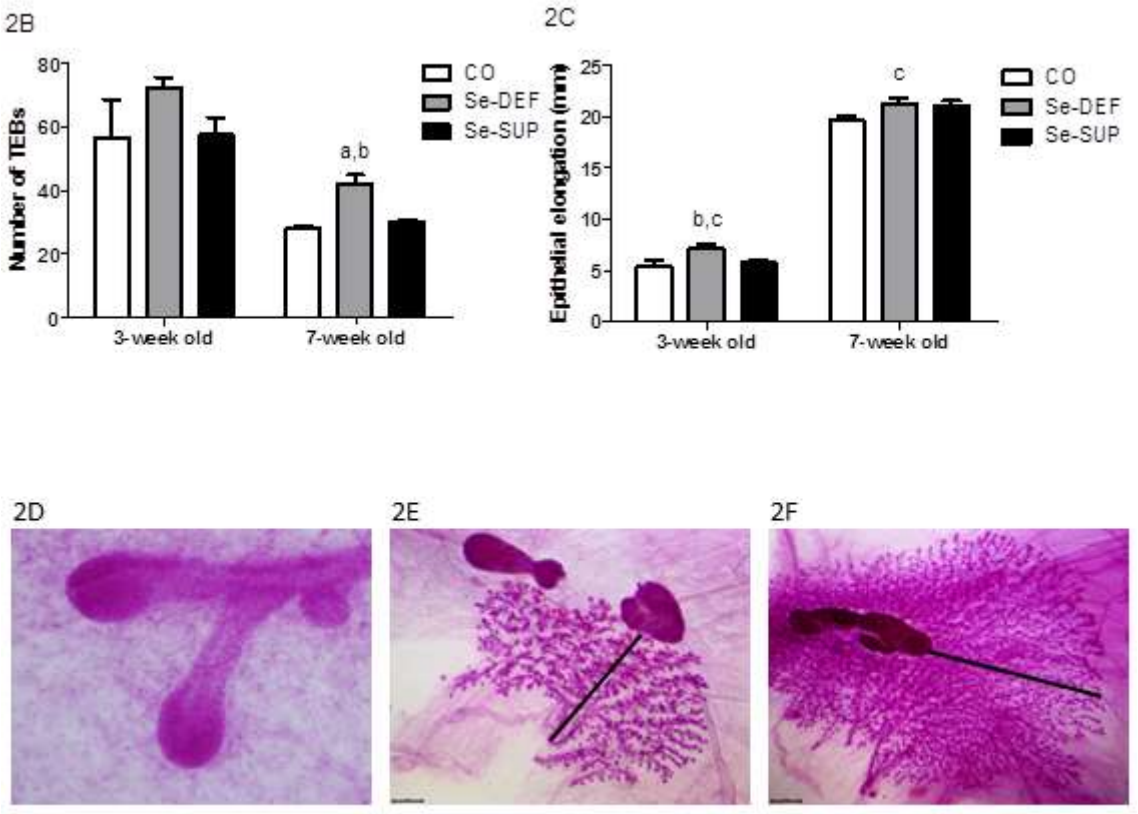
Variables	**Groups**		
	CO	**Se-DEF**	**Se-SUP**
First mammary tumor latency (days)	48 ± 3	47 ± 3	51 ± 3
Mammary tumor multiplicity	3.4 ± 0.5	3.3 ± 0.5	2.8 ± 0.4
Rats with 2 or more mammary tumors (%)	42	71[c]	50
Grade 1 mammary tumors (%)	77	61[c,b]	78
Grade 2 mammary tumors (%)	23	39[c,b]	22

Results as represented as mean ± S.E.M. Statistically significant differences ($p \leq 0.05$) when compared to [b]Se-SUP group female offspring and marginal difference but not statistically ($p > 0.05$ and ≤ 0.08) when compared to [c]CO group female offspring according to ANOVA + Duncan's post hoc test (mammary tumor multiplicity and first tumor latency) and Fisher's test (Rats with two or more mammary tumors and grade 1 and grade 2 mammary tumors). N=24 for all groups.

Female offspring mammary gland morphology

The 7-week but not 3-week old Se-DEF group female offspring presented increased ($p \leq 0.05$) number of TEBs in the mammary gland when compared to CO and Se-SUP groups female offspring (Figure 2B). The 3-week old Se-DEF group female offspring

presented marginally increased but not statiscally significant (p=0.07) and increased (p≤0.05) epithelial elongation when compared to CO and Se-SUP group female offspring respectively (Figure 2C). The 7-week old Se-DEF group female offspring presented marginally increased but not statiscally significant (p=0.06) epithelial elongation when compared to CO group female offspring (Figure 2C). There was no difference (p>0.05) regarding number of TEBs and epithelial elongation between 3- and 7-week old CO and Se-SUP female offspring groups.

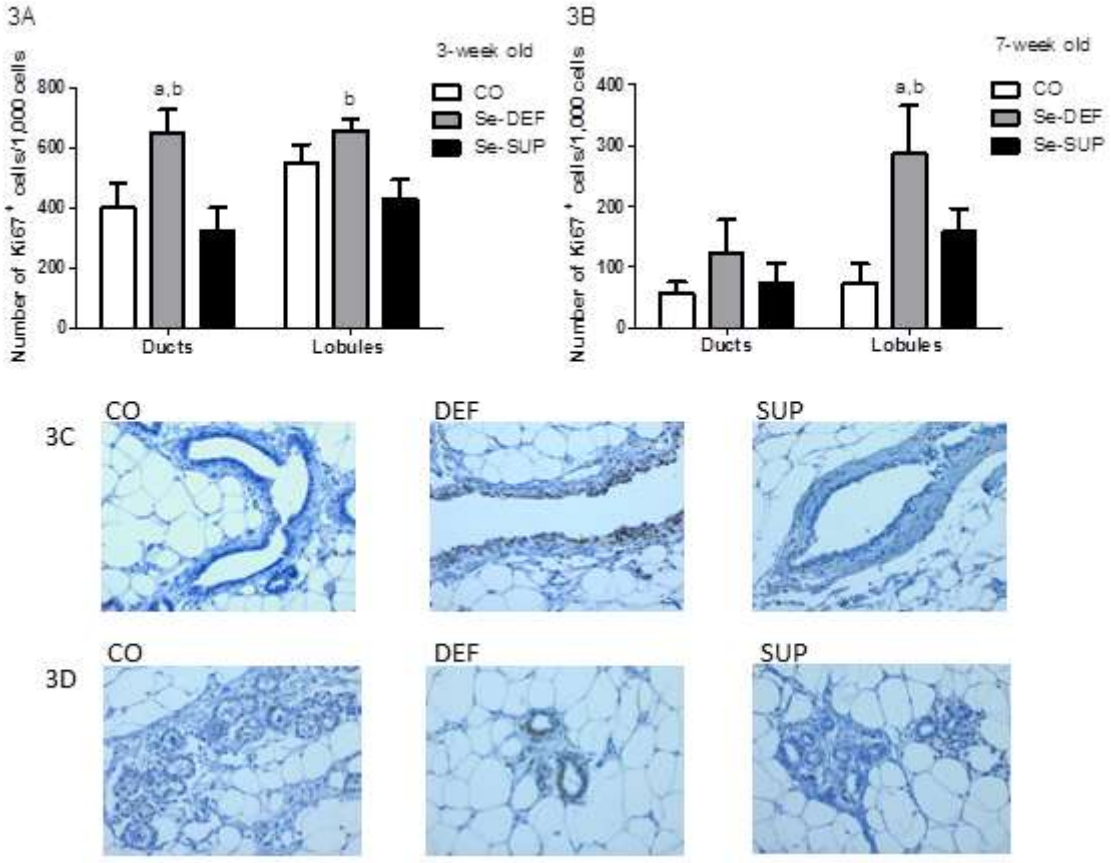


Article1 - Figure 2. Adenocarcinoma incidence (A), Number of TEBs (B) and epithelial elongation (C) in the mammary glands of CO, Se-DEF and Se-SUP groups female offspring. Photomicrography (40X) showing TEBs (D). Distance between nipple and end of the mammary epithelial tree (E) and between lymph node and end of the mammary epithelial tree (F) as shown by the bars indicates epithelial elongation in 3- and 7-week old female offspring respectively. Results are represented as mean ± S.E.M.

Statistically significant difference ($p \leq 0.05$) when compared to [a]CO and [b]Se-SUP group female offspring and marginal difference but not statistically significant ($p > 0.05$ and ≤ 0.08) when compared to [c]CO group female offspring, according to Kaplan Meyer and log-rank test (Mammary adenocarcinoma incidence; n=24) or ANOVA + Duncan's post hoc test [TEBs number and epithelial elongation; n = 6 (3-week old female offspring) and n = 12 (7-week old female offspring)].

Female offspring mammary gland cell proliferation

The 3-week old Se-DEF group female offspring presented increased ($p \leq 0.05$) cell proliferation in ducts compared to CO and Se-SUP groups female offspring and in lobules compared to Se-SUP group female offspring (Figure 3A). The 7-week old Se-DEF group female offspring presented increased ($p \leq 0.05$) cell proliferation in lobules but not in ducts compared to CO and Se-SUP groups (Figure 3B). There was no difference ($p > 0.05$) regarding cell proliferation in ducts and lobules between 3- and 7-week old CO and Se-SUP female offspring groups.

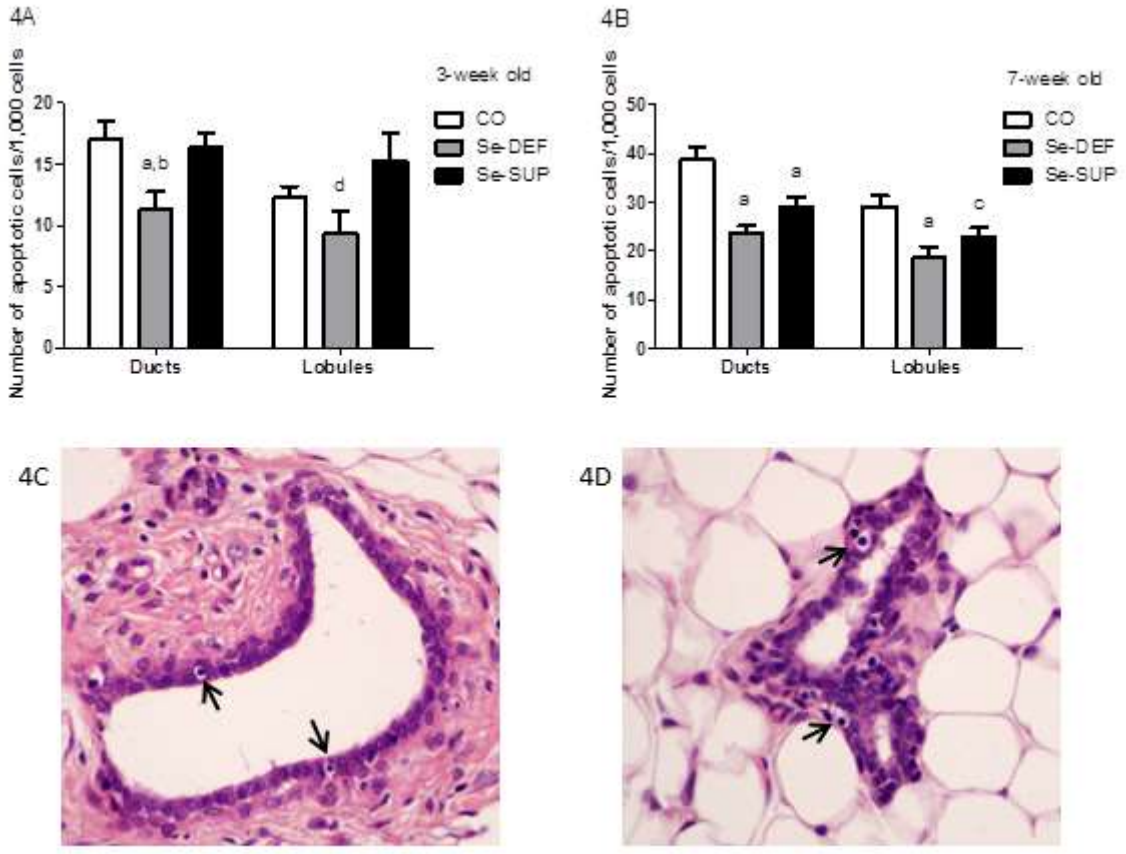


Article1 - Figure 3. Cell proliferation quantification in the mammary gland ducts and lobules of 3- (A) and 7-week old (B) female offspring. Photomicrography (40X) of Ki67 immunostaining in ducts (C) and lobules (D) of mammary glands of CO, Se-DEF and Se-SUP groups female offspring. Results are represented as mean ± S.E.M. Statistically significant difference ($p \leq 0.05$) when compared to [a]CO and [b]Se-SUP group female offspring, according to ANOVA + Duncan's post hoc test. N = 5.

Female offspring mammary gland cell apoptosis

The 3-week old Se-DEF group female offspring presented decreased ($p \leq 0.05$) apoptosis in the ducts compared to CO and Se-SUP groups female offspring and marginally decreased but not statiscally significant ($p=0.056$) apoptosis in the lobules when compared to Se-SUP but not to CO ($p>0.05$) group female offspring (Figure 4A). No differences ($p>0.05$) were observed regarding apoptosis in the ducts and lobules

between 3-week old CO and Se-SUP groups female offspring (Figure 4A). The 7-week old Se-DEF group female offspring presented decreased ($p \leq 0.05$) apoptosis in the ducts and lobules when compared to CO group female offspring (Figure 4B). The 7-week old Se-SUP group female offspring presented decreased ($p \leq 0.05$) and marginally decreased but not statiscally significant ($p=0.056$) apoptosis in the ducts and lobules respectively when compared to CO group female offspring. No differences ($p>0.05$) were observed regarding apoptosis in the ducts and lobules between 7-week old Se-DEF and Se-SUP groups female offspring.



Article1 - Figure 4. Apoptosis quantification in the mammary gland ducts and lobules of 3- (A) and 7-week old (B) of CO, Se-DEF and Se-SUP groups female offspring. Photomicrography (40X) showing apoptotic cells as indicated by arrows in ducts (C) and lobules (D) of mammary glands of female offspring. Results are represented as

mean ± S.E.M. Statistically significant difference ($p \leq 0.05$) when compared to [a]CO and [b]Se-SUP group female offspring and marginal difference but not statiscally significant ($p > 0.05$ and ≤ 0.08) when compared to [c]CO and [d]Se-SUP group female offspring, according to ANOVA + Duncan's post hoc test. N =5.

Discussion

In the present study paternal Se deficiency increased the number of TEBs, epithelial elongation and cell proliferation in the mammary gland of the female rat offspring and these effects were associated with higher susceptibility to DMBA-induced mammary tumors. On the other hand paternal Se supplementation did not influence any of these parameters. Our data suggest that breast cancer risk in daughters can be programmed through fathers' malnutrition. We are unaware of any other study showing selenium cancer programming effects through the male germline.

The participation of Se in central aspects of carcinogenesis has stimulated its development as a preventive agent for several cancers including breast cancer [18]. The majority of Se animal studies have adopted a chemopreventive approach showing that both inorganic (selenite and selenate) and organic forms (selenomethionine, selenocysteine and methylselenocysteine) elicited protective effects in different models of breast carcinogenesis [31]. In addition, in female Sprague-Dawley rats Se dietary deficiency before and after DMBA initiation enhanced mammary carcinogenesis [32]. *In vitro* studies conducted with different breast cancer cell lines have shown that Se exerts multiple cellular and molecular effects, including cell cycle control, apoptosis induction and epigenetic control of gene expression [33]. However human evidence of an association between Se consumption and levels with breast cancer risk is still not clear and mixed epidemiological reports have been described [34]. According to a

recent systematic evaluation there is no convincing evidence that Se supplementation prevents breast cancer [18]. Our results also show that paternal Se supplementation had no effects on female offspring breast cancer susceptibility. One possibility for this lack of effect could be that the Se-baseline levels were already high in the Se-supplemented fathers. Based on conflicting results from the Nutritional Prevention of Cancer and the Selenium and Vitamin E Cancer Prevention Trials, it has been proposed that starting Se status could determine the response to Se supplementation with individuals with low but not high Se starting levels showing protective effects [16]. One limitation of the present study is that we only tested supplementation with one Se form (selenate) and dose. This inorganic form was chosen because it is recommended in the AIN-93G diet [35] and because it is more stable and bioavailable than selenite [36]. However, we cannot exclude the possibility of attaining preventive effects with higher selenate doses or other Se forms. In any event paternal Se supplementation for breast cancer prevention in the daughters should be considered carefully because of recent evidence showing that high levels may increase the risk of type 2 diabetes [37]. On the other hand, in our study paternal Se deficiency programmed increased breast cancer risk in the female offspring. This suggests that rather than supplementing fathers with high Se levels, assuring an adequate paternal Se intake at the recommended dietary intake levels especially during preconception could represent a potential way of decreasing breast cancer risk in the daughters. This is of particular interest since selenium deficiency in males is commonly observed [24, 38].

Increased epithelial elongation is associated with accelerated mammary gland growth elicited by TEBs [29]. These structures are considered sites of breast cancer initiation because they present high rate of cell proliferation and lower number of differentiated cells [39]. A correlation between increased TEB number and breast cancer

susceptibility has been described in maternal intervention studies [6]. This indicates that the breast cancer programming effects by paternal Se deficiency could be associated with alterations in mammary gland development in the female offspring. Similarly male in-utero exposition to high-fat diet induced morphological alterations in the mammary gland of their female offspring that was associated with increased breast cancer risk in adult life [14]. The increased cell proliferation in the mammary gland of the Se-DEF female offspring could represent another mechanism underlying paternal programming of breast cancer risk. Se has been shown to exert cell cycle regulatory effects in breast cancer cells and breast tumor bearing animals [40, 41]. In maternal intervention studies Se deficiency altered the expression of cell cycle control genes in the offspring's colonic tissue [21]. On the other hand, the observation of decreased apoptosis in both Se-DEF and Se-SUP female offspring mammary glands suggests that this cellular process is not directly linked to increased breast cancer risk in the former group. The significance of this decrease in apoptosis remains to be elucidated.

Currently the majority of studies on paternal programming effects have focused on male in-utero environmental exposures [42]. Although male germ cells development starts in-utero as primordial germ cells, other relevant developmental phases include pre-puberty, adolescence and young adulthood [43]. During male reproductive life mature sperm cells are continuously produced indicating these phases as additional windows of susceptibility to environment-elicited alterations [44]. Our data show that in addition to fetal exposures [14], postnatal male malnutrition starting in pre-puberty also has an impact on breast cancer risk determination in the female offspring. This highlights male preconception as an important life stage when to start breast cancer prevention interventions. In previous rodents studies Se deficiency or supplementation altered testicular and epididymal development, impaired sperm production and

morphology [45], and induced male infertility [23]. Sperm cells are particularly prone to oxidative stress and disrupted oxidative balance can alter spermatogenesis [46]. On the other hand Se and selenoproteins GPx1, 3 and 4 protect male germ cells from oxidative stress during maturation and Gpx4 has also a role as a structural component in mature spermatozoa and in chromatin condensation [23]. In the present study, decreased Se blood concentration and increased oxidative stress found in Se-DEF fathers could be associated with impaired male reproductive parameters. Thus it would be important in further studies to evaluate which specific molecular male germ cells alterations could underlie the observed breast cancer programming effects.

Male gametogenesis involves profound epigenetic reprogramming [42]. Some studies conducted in rodents show that male inadequate nutrition such as low-protein or high-fat diets altered DNA methylation and microRNA expression in sperm cells [10, 46]. In addition Se deficiency or supplementation have been shown to alter epigenetic processes in different model systems including breast cancer cells [33, 38]. Thus it would be relevant in further studies to evaluate if epigenetic-based inheritance could be associated with the paternal breast cancer programming effects elicited by Se deficiency. Additionally, as family members usually share the same nutritional habits, it would be important to further analyze whether maternal Se status during pregnancy and lactation affects breast cancer risk in the daughters and to elucidate the potential interaction between maternal and paternal as well as daughters´ postnatal Se deficiency in terms of disease susceptibility.

A woman´s risk of breast cancer has been traditionally associated with her environmental exposures during adult life. Our data reinforce the hypothesis that breast cancer susceptibility can be programmed in very early life stages and through the male germline. It would be important to confirm in future studies if this phenomenon applies

to other breast cancer models. In addition, one major challenge would be to confirm in human populations the influence of paternal nutritional status on their daughters´ breast cancer incidence. Before such cohort studies data are available, possible initial strategies would be to evaluate paternal health parameters (i.e. Se status and fertility parameters) during preconception and associate them with perinatal factors postulated to affect breast cancer risk such as birth weight and length [47] and umbilical cord hormone [48] and mammary stem cell levels [49]. Furthermore, measuring breast density in girls during pubertal development and in young women using radiation-free technologies [50] could provide further insight into father´s influence on their daughter´s breast cancer susceptibility. If this possibility is confirmed in humans it is envisioned that novel breast cancer prevention strategies could be established focusing not only on a woman´s diet but also on her father´s nutrition. Assuring an adequate Se intake by men during preconception would represent a possible starting point.

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Article 2: Paternal selenium deficiency breast cancer risk programming involves epigenetic marks and gene expression alterations in the mammary gland of female rat offspring associated with altered mammary gland development

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Formatted article to be submitted to Metallomics.

Abstract

Recently we have shown that paternal selenium-deficiency during preconception alters female offspring mammary gland development and the susceptibility to 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis. Selenium is a semi-metalloid and recently it has been shown that in diverse breast cancer models selenium exerts effects on gene expression and epigenetic marks, acting as a chemopreventive agent. Thus we aimed to evaluate whether a paternal selenium deficiency during preconception alters in female offspring mammary gland the expression of genes and epigenetic marks that could be associated with altered mammary gland development and breast cancer risk previously observed by our group. Gene expression by qPCR, global DNA methylation by HPLC and global histone levels by Western blot analysis were performed in the female offspring mammary gland from fathers exposed to a selenium-deficient (0.05ppm selenium) and selenium-control (0.15ppm selenium) AIN93-G diet during preconception. Paternal selenium deficiency altered the expression of genes associated with mammary gland development, cell cycle control and DNA damage repair and induced global DNA hypomethylation and increased global

H3K27me3 levels in the female offspring mammary gland. These data suggest possible molecular mechanisms of paternal selenium-deficiency effects on breast cancer programming and highlights paternal preconception as an important window of susceptibility to start breast cancer preventive strategies.

Significance to metallomics statement: the present work contributes to the understanding of possible molecular mechanisms of a paternal selenium deficiency during preconception on breast cancer risk programming.

Introduction

Breast cancer is the most common cancer among women and its incidence and mortality has increased despite the cumulative investments for the disease prevention[1]. As mammary gland development starts in embryonic life and is susceptible to environmental disturbances[2], increasing evidence succeeded to show that breast cancer risk could be programmed trough aberrant epigenetic changes caused by early life exposures to nutrient levels[3]. Maternal nutrition during pregnancy and lactation has received particular attention and studies in rodents have shown that maternal consumption of different sources of high-fat diet altered gene expression and epigenetic marks in the mammary gland of the female offspring associated with altered breast cancer risk[4,5].

Although breast cancer risk programming through the male germline has not been extensively considered in the literature, we have recently shown that paternal selenium deficiency during preconception altered female rat offspring mammary gland development that was associated with increased susceptibility to 7,12 dimethylbenz[*a*]antracene-induced mammary carcinogenesis[6]. Recent studies concerning paternal programming of breast cancer have proposed possible epigenetic mechanisms involved as male mice exposure to a high-fat diet during preconception promoted changes in DNA methylation and miRNA expression in male sperm and female offspring mammary gland that were associated with increased later breast cancer risk[7,8]. These data reinforce the hypothesis that sperm transmit not only genetic information to the offspring but also epigenetic factors, altering the offspring health and these effects can be modulated by paternal nutritional habits[9].

Selenium (Se) is a semi-metalloid essential micronutrient for human health, including male fertility[10] and presents important role in central aspects of breast carcinogenesis[11]. In addition to cell proliferation, apoptosis and DNA damage modulation[12], recently, it has been shown that Se can modify gene expression and epigenetic marks as DNA methylation and post translational histone marks in different breast cancer models[13,14]. Selenium deficiency enhanced chemical-induced breast cancer susceptibility in a rat model[15]. However, as Se has been mostly evaluated as a chemopreventive agent in breast cancer studies[16], the possible molecular mechanisms involved in Se-deficiency effects on breast cancer risk have not been well described in the literature.

Thus, we aimed to evaluate whether paternal Se deficiency during preconception affects gene expression and epigenetic marks in the female offspring mammary gland associated with the altered mammary gland development and increased 7,12-dimethylbenz[*a*]anthracene-induced (DMBA) breast cancer susceptibility previously observed by our group[6]. Our data show that paternal Se deficiency during preconception altered female offspring mammary gland expression of genes related to cell cycle control, DNA damage repair and growth factors related to early stages of development, as well as global epigenetic marks as DNA methylation and H3K27me3 levels. These novel data suggest that epigenetic modulation and gene expression changes in the female offspring's mammary gland induced by paternal selenium deficiency during preconception could be possible mechanisms underlining breast cancer programming through the male germline and father's nutrition during this period a possible window of opportunity to decrease breast cancer risk.

Material and methods

Samples

Mammary tissue from the Control (CO) and selenium-deficient (Se-DEF) female offspring prevenient from fathers fed a Se control (0.15ppm of Se) and Se deficient (0.05 pmm of Se) for 9 weeks during preconception were collected from a previous study approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil (Protocol: CEUA/FCF/382)[6]. Tissue samples were quickly frozen in liquid nitrogen and kept at – 80C^{o} until use.

Gene expression analysis

Mammary gland (100mg) of CO and Se-DEF 7-week old female offspring RNA extraction was performed with Trizol® Reagent (Ambion-Life Technologies, France) followed by RNA clean up with RNeasy Mini-elute cleanup kit and DNAse treatment with RNase free DNase dataset (QIAGEN, France) according to manufacturer's protocols. RNA concentration and purity was determined with NanoVue Plus™ (GE Healthcare, France) and RNA integrity was accessed in 1.2% agarose gel. Purified RNA (1µg) was used for cDNA production with the SuperScript™ III Reverse Transcriptase kit (Invitrogen, France) following the manufacturer's instructions. Gene expression analysis was performed with the RT² Profiler™ PCR Array Rat Breast Cancer Kit (QIAGEN, USA) for 84 genes implicated in mammary carcinogenesis (Table 1– supplementary). Amplification was performed in Bio-Rad CFX96 Connect™ Real Time PCR Detection System (Bio-Rad, France) following RT² Profiler™ PCR Array protocol and data were analyzed with the software SABiosciences PCR Array Data Analysis, available at: www.SABiosciences.com/pcrarraydataanalysis.php. Fold change regulation was determined with the Livak method ($2^{-\Delta\Delta Ct}$) and minimal fold change

regulation accepted was 1.5. Results are represented as fold change compared to CO group.

Article 2 - Table 1 – Supplementary: RT2 Breast Cancer PCR Array Panel of analysis.

Position	Unigene	Refseq	Symbol	Description
A01	Rn.154810	NM_133401	Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A
A02	Rn.13131	NM_181381	Abcg2	ATP-binding cassette, subfamily G (WHITE), member 2
A03	Rn.24184	NM_001029899	Adam23	ADAM metallopeptidase domain 23
A04	Rn.11422	NM_033230	Akt1	V-akt murine thymoma viral oncogene homolog 1
A05	Rn.156346	NM_012499	Apc	Adenomatous polyposis coli
A06	Rn.9813	NM_012502	Ar	Androgen receptor
A07	Rn.214048	NM_001106821	Atm	Ataxia telangiectasia mutated homolog (human)
A08	Rn.36696	NM_022698	Bad	BCL2-associated agonist of cell death
A09	Rn.9996	NM_016993	Bcl2	B-cell CLL/lymphoma 2
A10	Rn.54471	NM_022274	Birc5	Baculoviral IAP repeat-containing 5
A11	Rn.217584	NM_012514	Brca1	Breast cancer 1
A12	Rn.103225	NM_031542	Brca2	Breast cancer 2
B01	Rn.102823	NM_001011949	Ccna1	Cyclin A1
B02	Rn.22279	NM_171992	Ccnd1	Cyclin D1
B03	Rn.96083	NM_022267	Ccnd2	Cyclin D2
B04	Rn.15455	NM_001100821	Ccne1	Cyclin E1
B05	Rn.1303	NM_031334	Cdh1	Cadherin 1
B06	Rn.23806	NM_138889	Cdh13	Cadherin 13
B07	Rn.104460	NM_199501	Cdk2	Cyclin dependent kinase 2
B08	Rn.10089	NM_080782	Cdkn1a	Cyclin-dependent kinase inhibitor 1A
B09	Rn.162507	NM_182735	Cdkn1c	Cyclin-dependent kinase inhibitor 1C
B10	Rn.48717	NM_031550	Cdkn2a	Cyclin-dependent kinase inhibitor 2A
B11	Rn.83632	NM_023981	Csf1	Colony stimulating factor 1 (macrophage)
B12	Rn.9609	NM_133566	Cst6	Cystatin E/M
C01	Rn.112601	NM_053357	Ctnnb1	Catenin (cadherin associated protein), beta 1
C02	Rn.11085	NM_134334	Ctsd	Cathepsin D
C03	Rn.6075	NM_012842	Egf	Epidermal growth factor
C04	Rn.37227	NM_031507	Egfr	Epidermal growth factor receptor
C05	Rn.93966	NM_017003	Erbb2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
C06	Rn.231229	NM_012689	Esr1	Estrogen receptor 1
C07	Rn.37460	NM_012754	Esr2	Estrogen receptor 2 (ER beta)
C08	Rn.10470	NM_012742	Foxa1	Forkhead box A1
C09	Rn.229174	NM_133293	Gata3	GATA binding protein 3
C10	Rn.219157	NM_001191910	Gli1	GLI family zinc finger 1

C11	Rn.28109	NM_053403	Grb7	Growth factor receptor bound protein 7
C12	Rn.87063	NM_012577	Gstp1	Glutathione S-transferase pi 1
D01	Rn.11570	NM_001107021	Hic1	Hypermethylated in cancer 1
D02	Rn.2113	NM_012797	Id1	Inhibitor of DNA binding 1
D03	Rn.201887	NM_178866	Igf1	Insulin-like growth factor 1
D04	Rn.165078	NM_052807	Igf1r	Insulin-like growth factor 1 receptor
D05	Rn.26369	NM_012588	Igfbp3	Insulin-like growth factor binding protein 3
D06	Rn.9873	NM_012589	Il6	Interleukin 6
D07	Rn.93714	NM_021835	Jun	Jun oncogene
D08	Rn.103924	NM_053976	Krt18	Keratin 18
D09	Rn.9359	NM_199498	Krt19	Keratin 19
D10	Rn.195318	NM_183333	Krt5	Keratin 5
D11	Rn.11083	NM_199370	Krt8	Keratin 8
D12	Rn.34914	NM_053842	Mapk1	Mitogen activated protein kinase 1
E01	Rn.2592	NM_017347	Mapk3	Mitogen activated protein kinase 3
E02	Rn.4090	NM_053829	Mapk8	Mitogen-activated protein kinase 8
E03	Rn.9836	NM_012861	Mgmt	O-6-methylguanine-DNA methyltransferase
E04	Rn.233802	NM_001271366	Mki67	Marker of proliferation Ki-67
E05	Rn.20391	NM_031053	Mlh1	MutL homolog 1 (E. coli)
E06	Rn.6422	NM_031054	Mmp2	Matrix metallopeptidase 2
E07	Rn.10209	NM_031055	Mmp9	Matrix metallopeptidase 9
E08	Rn.10779	NM_012602	Muc1	Mucin 1, cell surface associated
E09	Rn.12072	NM_012603	Myc	Myelocytomatosis oncogene
E10	Rn.6236	NM_138548	Nme1	Non-metastatic cells 1, protein (NM23A) expressed in
E11	Rn.25046	NM_001105721	Notch1	Notch homolog 1, translocation-associated (Drosophila)
E12	Rn.90070	NM_012576	Nr3c1	Nuclear receptor subfamily 3, group C, member 1
F01	Rn.10303	NM_022847	Pgr	Progesterone receptor
F02	Rn.6064	NM_013085	Plau	Plasminogen activator, urokinase
F03	Rn.202632	NM_001077648	Prdm2	PR domain containing 2, with ZNF domain
F04	Rn.22158	NM_031606	Pten	Phosphatase and tensin homolog
F05	Rn.217585	NM_017232	Ptgs2	Prostaglandin-endoperoxide synthase 2
F06	Rn.7817	NM_172322	Pycard	PYD and CARD domain containing
F07	Rn.220045	NM_031529	Rarb	Retinoic acid receptor, beta
F08	Rn.83042	NM_001007754	Rassf1	Ras association (RalGDS/AF-6) domain family member 1
F09	Rn.55115	NM_017045	Rb1	Retinoblastoma 1
F10	Rn.29367	NM_012620	Serpine1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
F11	N/A	XM_001065560	Sfn	Stratifin
F12	Rn.163333	NM_001276712	Sfrp1	Secreted frizzled-related protein 1
G01	Rn.99415	NM_001024745	Slc39a6	Solute carrier family 39 (zinc transporter), member 6

G02	Rn.146652	NM_022632	Slit2	Slit homolog 2 (Drosophila)
G03	Rn.43117	NM_013035	Snai2	Snail homolog 2 (Drosophila)
G04	Rn.112600	NM_031977	Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
G05	Rn.87477	NM_013042	Tff3	Trefoil factor 3, intestinal
G06	Rn.40136	NM_021578	Tgfb1	Transforming growth factor, beta 1
G07	Rn.185771	NM_001013062	Thbs1	Thrombospondin 1
G08	Rn.54443	NM_030989	Tp53	Tumor protein p53
G09	Rn.103860	NM_001108696	Tp73	Tumor protein p73
G10	Rn.161904	NM_053530	Twist1	Twist homolog 1 (Drosophila)
G11	Rn.1923	NM_031836	Vegfa	Vascular endothelial growth factor A
G12	Rn.101044	NM_001004210	Xbp1	X-box binding protein 1
H01	Rn.94978	NM_031144	Actb	Actin, beta
H02	Rn.1868	NM_012512	B2m	Beta-2 microglobulin
H03	Rn.47	NM_012583	Hprt1	Hypoxanthine phosphoribosyltransferase 1
H04	Rn.107896	NM_017025	Ldha	Lactate dehydrogenase A
H05	Rn.973	NM_001007604	Rplp1	Ribosomal protein, large, P1
H06	N/A	U26919	RGDC	Rat Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Global DNA methylation

Global DNA methylation was analyzed by high-performance liquid chromatography with diode-array detection (HPLC-DAD) in mammary tissue from CO and Se-DEF 3- and 7-week old female offspring (n = 5/group). For DNA extraction 100 mg of mammary tissue was homogenized with 3mL lysis buffer (Gentra Puregene Kit, QIAGEN - USA) containing 0.5mM deferoxamine (Sigma Aldrich, USA). Samples were incubated with proteinase K (20 mg/mL, Sigma Aldrich, USA) at 60°C overnight and treated with 15mg/mL RNase A (Sigma Aldrich, USA) in sodium acetate buffer pH 5.2 at 37°C for 2 hours. Protein was precipitated with protein precipitating solution (Gentra Puregene Kit, QIAGEN, USA). The homogenate was centrifuged at 16,000g 5

min 4°C, the supernatant replaced to a new tube and DNA was precipitated with cold isopropanol (Merk, USA). Samples were centrifuged at 16,000g 5 min 4°C and supernatant was discarded. DNA was washed with 3mL of 70% ethanol (Sigma Aldrich, USA) and centrifuged at 16,000g 5 min 4°C. Supernatant was discarded and pellet was dried at room temperature for 5 min. DNA was eluted in water containing 0.1mM deferoxamine (Sigma Aldrich, USA) and quantified by spectrophotometry (Nanodrop 1000, ThermoScientific) for concentration and purity. DNA (5µg) was hydrolyzed with 200 mM HCl/$MgCl_2$ in Tris Buffer and DNAse 1 (Sigma Aldrich, EUA) at 37°C for 1 h and incubated with phosphodiesterase (Sigma Aldrich, USA) and alkaline phosphatase at 37°C for 1 h. Enzyme excess was removed by centrifugation at 10,000g 10 min 4°C. Hydrolyzed DNA was injected into a HPLC-DAD analytical system (Shimadzu Corporation, Japan) using Luna column C18 (2), 250 mm X 4.6 mm ID, 5µm (phenomenex, Torrence, CA), with a C18 pre-column of 4.0 X 3.0 mm (Phenomenex, Torrence, CA) eluted with a gradient of formic acid (0.1% min, in water) and CH_3OH (0-25 min, 0%-18% CH_3OH; 25-27 min, 18%-0% CH_3OH; 27-37 min, 0% CH_3OH) with a flow rate of 1 mL/min at 30°C. The DAD detector was set at 260 nm for dC quantification and at 286 nm for 5-methyl-dC quantification. Calibrations curves were performed in the ranges of 0.5 nm to 8 nm for dC and 0.01 nm to 0.8 nm for 5-methyl-dC measurement. Results are represented as % 5-mdC ± S.E.M.

Global histone marks

Protein extraction from mammary tissue (100mg) from CO and Se-DEF 7-week old female offspring was performed with Trizol® Reagent (Ambion-Life Technologies, France) and quantified by DC Protein Assay (Bio-Rad, France) according to manufacturer's protocols. Protein extract (20µg) was resolved in 4-15% Precast denaturing Mini-Protean® TGX™ gel (Bio-Rad, France) (200V-35min) and transferred

to a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, France). The membrane was saturated with 5% Bovine Fetal Serum (BSA) (PAA, France) and incubated with primary anti acetyl H4K16, anti-trimethyl H3K27, anti-acetyl H3K9, 1:1,000 or with anti-β-actin (Millipore, France) 1:4,000 in 5% BSA TBS-T overnight at 4°C. Membranes were incubated with Afinity purified Dy light™ 800 anti-rabbit or Dy light™ 680 anti-mouse secondary antibody (KLP, USA) 1:15,000 in 5% BSA TBS-T for 45 min. Immunodetection was captured and quantified using Li-cor Odyssey Infrared Imaging System (Li-Cor). Data were normalized by β-actin expression and results are represented as fold change compared to CO group ± S.E.M.

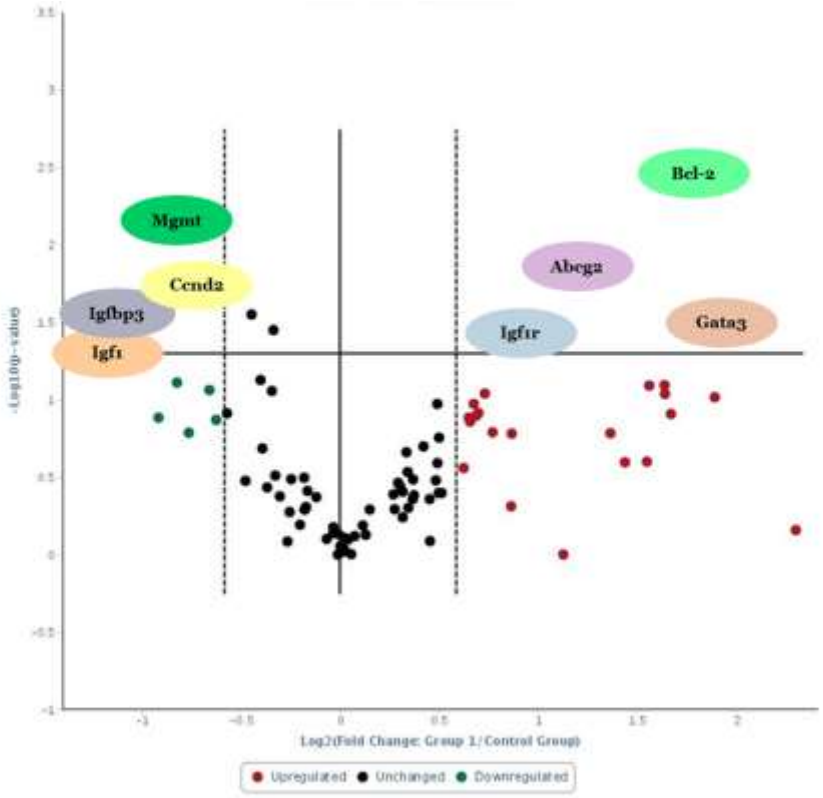
Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M.) and the statistical analysis was conducted with STATISTICA 8.0 (USA). Student's t Test was used for two-group comparison. $P \leq 0.05$ was used as threshold for statistical significance.

Results

Gene expression analysis

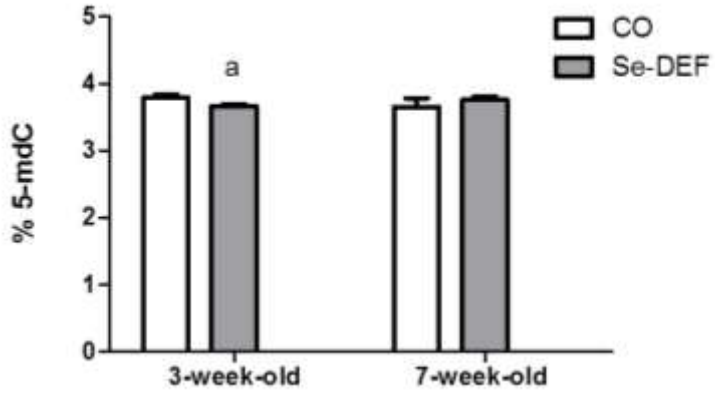
As can be seen in Figure 1, from a panel of 84 genes implicated in breast carcinogenesis the 7-week-old Se-DEF female offspring presented in the mammary gland increased ($p \leq 0.05$) expression of the genes ATP-binding cassette, sub-family G, member 2 (*Abcg2*), B-cell lymphoma 2 (Blc-2), GATA binding protein 3 (*Gata3*), insulin like growth factor 1 receptor (*Igf1r*) and decreased ($p \leq 0.05$) expression of the genes insulin like growth factor 1 (*Igf1*), Insulin-like growth factor binding protein 3 (*Igfbp3*), Cyclin D2 (*Ccnd2*) and O-6-Methylguanine-DNA Methyltransferase *(Mgmt)* in the mammary gland when compared to CO group (Figure 1).



Article 2 - Figure 1: Volcano plot with gene expression changes in the mammary gland of Se-DEF female offspring compared to CO. Fold change regulation calculated with the Livak method. Genes in the boxes are statiscally different ($p \leq 0.05$ and fold regulation ≥ 1.5) according to T Student test. N=6/group.

Global DNA methylation

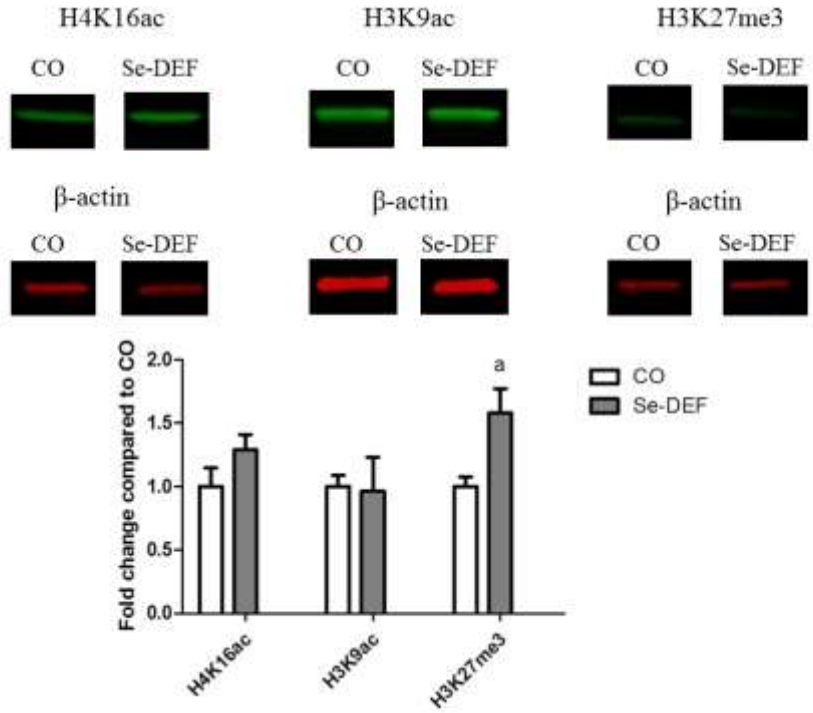
The 3-week-old Se-DEF female offspring presented global DNA hypomethylation ($p \leq 0.05$) in the mammary gland when compared to CO group female offspring. However no differences ($p > 0.05$) were observed regarding this parameter in 7-week-old female offspring between groups (Figure 2).



Article 2 - Figure 2: Global mammary gland DNA methylation analysis. Results are represented as mean ± S.E.M. Statistically significant differences ($p \leq 0.05$) when compared to aCO according to T student test. N=5/group).

Global histone marks in mammary gland of female offspring

The 7-week-old Se-DEF female offspring presented increased ($p \leq 0.05$) global levels of H3K27me3 in the mammary gland when compared to CO group. No differences ($p > 0.05$) were observed regarding H4K16ac and H3K9ac levels in the mammary gland between groups (Figure 3).



Article 2 - Figure 3: Western blot analysis for histone levels in the female offspring mammary gland. Fold change regulation compared to CO group. Results are represented as mean ± S.E.M. Statistically significant differences ($p \leq 0.05$) when compared to aCO according to T student test. N=6/group).

Discussion

In this study we show that paternal selenium deficiency during preconception altered in the female offspring mammary gland the expression of genes related to cell cycle control, DNA repair and growth factor implicated in early life and mammary gland development as well as global epigenetic marks as DNA methylation and H3K27me3 levels. These effects could be associated with altered mammary gland development and higher susceptibility to DMBA-induced mammary carcinogenesis previously observed by us[6]. Our data suggests that breast cancer risk could be determined in early life through epigenetic modulation and gene expression changes as effects of paternal malnutrition. We are unaware of other studies showing selenium-deficiency molecular modulation of breast cancer risk through the male germline.

Paternal selenium deficiency effects on *Igf1/Igf1r* expression are interesting given that this growth factor is implicated early life and mammary gland development[17]. Studies have shown that loss of *Igf1* impaired mammary gland branching and increased the number of Ki67 mammary positive cells[18]. The systemic loss of *Igf1r*, the main *Igf1* receptor in the mammary epithelium, decreases ductal branching and compromises TEBs formation, affecting mammary gland development and differentiation[19]. In addition, a study in rodents showed that mammary gland is especially prone to oncogenic transformation when *Igf1r* is up-regulated during puberty[20] and a human cohort showed that increased *Igf1r* expression in the epithelium of normal Terminal

Ductal Lobular Units (TDLUs) from benign breast biopsies was associated with increased risk of subsequent breast cancer[21]. *Igfbp3* is the most abundant *Igf* binding protein that regulates *Igf1* bioavailability and exerts independent growth inhibitory effects in the breast epithelium[22]. In *Igfbp3*$^{-/-}$ transgenic mice increased cell proliferation, earlier tumor incidence, higher tumor aggressiveness and metastasis were observed in a breast cancer model suggesting an *Igfbp3* tumor suppressor activity[23]. Thus, increased and decreased expression of *Igf1r* and *Igf1/Igfbp3* respectively in the mammary gland of Se-DEF group female offspring could be associated with altered mammary gland development and increased breast cancer risk previously observed.

Paternal Se-deficiency also affected *Ccnd2* and *Bcl-2* expression, genes that are implicated in cell cycle control and prevention[24] of apoptosis[25] respectively. Both genes expression can be affected by *Igf1* and *Igf1r* expression[26, 27]. *Ccnd2* presents dynamic expression during mammary gland development and is frequently downregulated in breast cancer through promoter hypermethylation[24]. *Bcl-2* overexpression has been reported in breast cancer, is associated with apoptosis inhibition and in previous study it has been shown that Se exerts effects on *Bcl-2* expression in breast cancer cell lines[25]. In addition to cell cycle control and apoptosis, paternal Se-deficiency affected the expression of the transcription factor *Gata3* that is also associated with mammary gland development and differentiation given that *Gata3* deletion during mice puberty resulted in failure of TEBs formation, epithelial elongation and lobule-alveolar development[28]. Our data indicate that paternal Se-deficiency during preconception affected female offspring mammary gland development in a molecular level, and the changes observed in the expression of genes implicated in cell cycle control, apoptosis and mammary gland differentiation as possible underlying mechanisms of increased breast cancer risk

in adult life. One possibility is that these effects could be orchestrated by insulin-like growth factor as a central point of paternal Se-deficiency effects.

The paternal Se-deficiency-induced down-regulation of the tumor suppressor gene *Mgmt* in the female offspring mammary gland is another interesting observation, given that *Mgmt* is implicated in DNA repair and adducts removal[29]. In our DMBA-induced breast cancer model, the chemical carcinogen is metabolized by the organism and induces malignant cell transformation trough DNA damage and adducts formation in the mammary gland[30]. Thus, the down-regulation of *Mgmt* in the specific period of DMBA-induction of breast cancer could be responsible to enhance mammary gland susceptibility to the chemical carcinogen, increasing breast cancer incidence.

The implication of the up-regulated xenobiotic transporter, *Abcg2* in the 7-week-old Se-DEF female offspring mammary gland remains to be elucidated. This is because while increased *Abcg2* expression in breast cancer is associated with chemotherapy resistance[31], in healthy mammary tissue, the *Abcg2* up-regulation could be implicated in protection against xenobiotics[32].

Paternal Se-deficiency induced DNA hypomethylation in the female offspring mammary gland is interesting given that Se is implicated in one carbon metabolism and this is an early event described in cancer[33]. Although this effect was temporary and seen in the 3- but not in the 7-week-old female offspring, we cannot exclude that it could have presented important outcome in the mammary gland development and affected later breast cancer susceptibility. Few experimental and human studies showed a correlation between Se status and DNA methylation mostly in hepatic and colonic tissue[14]. However, for breast cancer, few data is available and in one initial study in breast cancer cells, Se treatment decreased DNMT activity[13]. As Se is frequently adopted as a chemopreventive agent in breast cancer studies[16], the effects of Se-

deficiency in global DNA methylation have not been well described. In addition, paternal Se-deficiency induced increased global levels of H3K27me3 in the female offspring mammary gland. This specific histone mark has been associated with gene silencing and is constantly up-regulated in breast cancer[34]. Few studies also show a correlation between Se and histone marks, mainly as a histone deacetylase inhibitor[14]. In breast cancer cells, treatment with methylseleninic acid reduced and increased the levels of H3K9me3 and H4K16ac respectively, while sodium selenite reduced H4K16ac levels[13]. Again, as Se is adopted as a chemopreventive agent, the effects of Se-deficiency in post transcriptional histone marks have not been well described before. In this study we show that paternal Se-deficiency during preconception alters epigenetic marks in the mammary gland of their female offspring and these effects could be associated with mammary gland altered development and increased susceptibility DMBA-induced mammary carcinogenesis.

Breast cancer susceptibility is constantly associated with a woman's environmental exposures during adult life. However, recent evidence show that breast cancer risk can be determined in early life stages and trough the male germline[6-8]. Although initial studies on breast cancer programming have focused on paternal in-utero nutrition[35], we recently showed that male preconception comprises an important period on breast cancer risk determination[6]. In agreement with other recent data, we show that modulation of gene expression and epigenetic marks could be possible mechanisms involved in this programming, reinforcing the hypothesis that fathers transmit more than DNA but also epigenetic factors to their offspring, affecting health outcome. It would be interesting in further studies to evaluate if the effects observed in the present study apply to other breast cancer models as well as other epigenetic marks

as miRNA expression and gene-specific epigenetic marks as other possible mechanisms involved.

Conclusion

We conclude that paternal Se deficiency during preconception induces changes in the expression of genes implicated in cell cycle control, DNA repair and early life and mammary gland development as well as global epigenetic marks and these effects could be associated with increased DMBA-induced breast cancer risk previously observed.

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Conflict of interests: The authors declare that there are no conflicts of interest for any of the authors.

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General conclusion

This study shows that paternal selenium deficiency during preconception altered in the female offspring mammary gland development and increased breast cancer susceptibility to DMBA-induced mammary carcinogenesis. In addition results suggest that changes in mammary gland gene expression and epigenetic marks could be possible mechanisms involved in the Se effects on breast cancer programming through the male germline. This highlights the importance of father´s nutrition including selenium status as relevant factors affecting daughter´s breast cancer risk and paternal preconception as a potential developmental stage to start preventive strategies.

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