# EFFECT OF MYCOTOXINS ON SPERMATOGONIAL STEM CELLS

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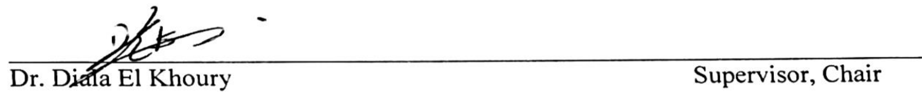

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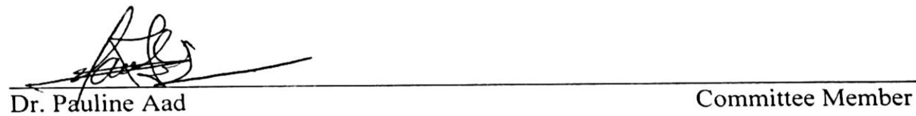

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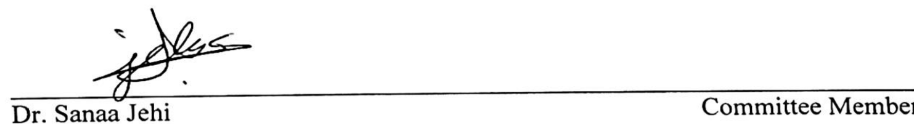

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## ABSTRACT

Fusarium mycotoxins are natural contaminants of various commodities representing significant problem worldwide. Since beauvericin (BEA), one of the fusarium toxins, represents a major concern because of its potential toxicity in humans and animal health, and its high presence in feed and food commodities, the present study was carried out to evaluate the effect of BEA on cell viability, proliferation, morphology and gene expression using spermatogonial stem cells (SSCs). First, SSCs were isolated from fresh sheep testis under aseptic technique. Isolated SSCs were cultured for 36 hours at 5% $CO_2$. Then, they were treated with different chosen BEA dose for different time points. Cell count using the hemocytometer was done to determine cell viability. BEA at 0.3, 1, and 3µM significantly decreased ($p < 0.05$) cell numbers after 12 and 24 hours of treatment. For the percentage of viable cells MTT test was performed. At concentration ≥0.3µM, BEA was found to decrease ($p < 0.05$) percentage of cell viability. Culture of SSCs on a slide treated with1 and 3 µM for 24 hours shows a morphological change after the hematoxylin and eosin staining: elongated and dispersed cells with no more clustered SSCs. RNA extraction and amplification using PCR, then the expression of Oct-4, and Smad8/9 were assessed using qRT-PCR. BEA had no effect on Oct-4 and Smad8/9 mRNA abundance ($p > 0.05$). Higher expression of Oct-4 ($p < 0.05$) was found in SSCs indicating proliferation. Whereas, a lower expression of smad8/9 ($p > 0.05$) was shown supporting the purity of SSCs isolated. Taken together these results demonstrate that BEA may impair reproductive function in sheep.

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## LIST OF ABBREVIATIONS

Aflatoxins: AFT

Aflatoxin B1: AFB1

Aflatoxin M1: AFM1

Beauvericin: BEA

Bone morphogenetic protein: BMP

Basic fibroblast growth factor: bFGF

Citrinin: CTN

Differentiated: $A_{diff}$

Daily sperm production: DSP

Deoxynivalenol: DON

Dulbecco's modified Eagle's medium: DMEM

Endocrine-disrupting chemicals: EDCs

Embryonic day: E

Epithelial cell adhesion molecule: EPCAM

Enhanced green fluorescent protein: Egfp

Ethylenediaminetetraacetic acid: EDTA

Fumonisin $B_{1}$: $FB_1$

Fetal Bovine Serum: FBS

Fluorescence-activated cell sorting: FACS

High performance liquid chromatography: HPLC

Immunocytochemistry staining: ICC

G protein-coupled receptor 125: GPR125

Glial cell line-derived neurotrophic factor: GDNF

Leukemia inhibitory factor: LIF

Nanos C2HC-Type Zinc Finger 2: NANOS2

Ochratoxins: OTA

Octamer-binding transcription factor 4: Oct-4

Primordial germ cells: PGCs

Postnatal day: P

Phosphate-buffered saline: PBS

Protein kinase B: AKT

Protein kinase: AMPK

Promyelocytic leukaemia zinc finger: PLZF

Phosphatase and tensin homolog: PTEN

POU Domain, Class 5, Transcription Factor 1: POU5F1

Stem cell growth factor receptor: SCFR

Spermatogonial stem cells: SSCs

Sex-determining region Y: SRY

Tyrosine-protein kinase Kit: c-Kit

Target genes: TG

Undifferentiated: $A_{undiff}$

Zearalenone: ZEA

Zearalenol: ZOL

Zearalanol: ZAL

Zearalanone: ZAN

# I. Introduction

Beauvericin (BEA) is a cyclic hexadepsipeptide that was first isolated from culture of the soil-borne entomopathogenic fungus Beauveria bassiana (Tang et al., 2005), and BEA is also synthesized by several Fusarium spp. parasitic to important cereal grains such as corn, wheat, rice and barley (Leslie and Summerell, 2006). Data on BEA toxicity are few (Chain, 2014). BEA was found to exert potent cytotoxicity against several mammalian cell lines (Klarić et al., 2008), but its effects on spermatogonial stem cells (SSCs) were not investigated and no data are available on its potential reproductive effects. Moreover, previous in vitro studies demonstrated that fusarium mycotoxins might affect male reproductive system (Kim et al., 2003; Sprando et al., 2005; Yang et al., 2010; Supriya et al., 2014). Zearalenone (ZEA) and deoxynivalenol (DON) induce testicular germ cell apoptosis and degeneration of mainly spermatogonia and spermatocytes (Kim et al., 2003; Sprando et al., 2005) .

Spermatogenesis is a complex process comprising proliferation, differentiation, and maturation of many different subsequent cell types (van Pelt et al., 1996) resulting in production of unlimited numbers of spermatozoa throughout the adult life of the male (Russell et al., 1993; Dobrinski, 2007). Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and comprising only 0.03% of all germ cells in testes (Tegelenbosch and de Rooij, 1993). They are defined like all other stem cells by their ability to balance self-renewing and at the same time to generate the cascade of differentiating germ cells that will eventually lead to the formation of sperm (Huckins, 1971; Clermont, 1972b; van Pelt et al., 1996; Nagano et al., 1998; Aponte et al., 2005). Moreover, Semen quality, a predictor of male fertility, has been suggested declining worldwide (Carlsen et al., 1992; Swan and Elkin, 1999; Skakkebaek et al., 2016). This continuous decline in human fertility worldwide attributed to many factors including

activities of endocrine-disrupting chemicals (EDCs) such as mycotoxins and pesticides (Ibeh et al., 1994; Martenies and Perry, 2013).

For this reason, in the present study we sought to isolate spermatogonial stem cells from sheep for the first time that could be later on treated with beauvericin (BEA) to determine this reproductive effect. First, we will perform SSCs growth curve through cell count and viability. Second, we will assess the expression of few genes in SSCs to support the purity of the cells. Then, SSCs will be treated with BEA at different times and dosage. Different test could be done such as cell count, MTT assay, staining of a cultured slide and mRNA abundance of specific gene to determine the reproductive effect of BEA.

In the present study, we isolate for the first time SSCs from sheep testis. The growth curve of sheep SSCs was examined. Moreover, the determination of BEA dose and time effect were performed on SSCs proliferation, viability, and morphology and gene expression of Oct-4, Smad8/9 and PLZF.

# II. Literature review

## 1. Mycotoxins

Mycotoxins are secondary metabolites produced by filamentous fungi, which may contaminate foods and feedstuffs worldwide (Diekman and Green, 1992; Moss, 1992; Park et al., 1996). The majority of mycotoxins are capable of causing diseases and health problems in humans and animals (Coulombe, 1993). Mycotoxins are not exhaustively studied until now, because they were subdivided into different groups. Moreover, each group of mycotoxins has a dose dependent effect on humans and animals (Coulombe, 1993). The major groups of mycotoxins are aflatoxins (AFT), ochratoxins (OTA), citrinin, patulin, ergot alkaloids and fusarium toxin (Hussein and Brasel, 2001).

Aflatoxins are produced mainly by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Applegate and Chipley, 1973; Gourama and Bullerman, 1995; Wilson and King, 1995). There are four central types of Aflatoxins, namely B1, B2, G1 and G2 (Wilson and King, 1995). In addition, aflatoxins M1 and M2 were detected in milk and dairy products (Coulter et al., 1984; Ferguson-Foos and Warren, 1984; Hassan and Kassaify, 2014). Aflatoxin B1 (AFB1) is a potent carcinogen and directly associated with adverse health effects, such as liver cancer in animals and humans (Wogan and Newberne, 1967; Hamid et al., 2013). Studies have shown that high level of aflatoxin exposure can cause a condition of aflatoxicosis in both humans and animals (Krishnamachari et al., 1975; Dhanasekaran et al., 2011). Chronic effects of aflatoxin have been reported to impair normal immunity system in the body (Turner et al., 2003; Meissonnier et al., 2008) . Aflatoxins also appeared to affect the rate of growth in kids and teenagers (Chen et al., 2018). Moreover, serious acute effects on the gastrointestinal tract were detect as a result of consumption of aflatoxin-contaminated foods especially AFB1 (Gursoy et al., 2008).

On the other hand, Ochratoxin A (OTA) are produced by different *Aspergillus* and *Penicillium* species (Abarca et al., 1994). OTA was found in a large variety of food and especially predominate in barley and wheat (Trucksess et al., 1999). Damaging effects of Ochratoxin were shown to be on human's kidneys and liver (Kuiper-Goodman and Scott, 1989; Tsuda et al., 1999). There is also evidence that OTA could impair the immune system and have a carcinogenic effect (Luster et al., 1987; Marin-Kuan et al., 2008). Therefore, OTA is a hepatotoxic/-nephrotoxic agent and a potent teratogen and carcinogen (Krogh et al., 1973; Dirheimer and Creppy, 1991).

Citrinin is a toxin produced by *Penicillium* and several *Aspergillus* species (Sankawa et al., 1983; El-banna et al., 1987; Malmstrom et al., 2000; Xu et al., 2006). Some of these *Penicillium* and *Aspergillus* species were used to produce human foodstuffs such as cheese and soy sauce (Edite Bezerra da Rocha et al., 2014). The presence of Penicillium and *Aspergillus species* in foodstuffs will increase the risk of food contamination with citrinin. Moreover, *in vitro* studies have demonstrated that Citrinin is considered to be nephrotoxic, and can act synergistically with ochratoxin to produce a collective nephrotoxic effect (Krogh et al., 1973; Berndt, 1990).

Furthermore, patulin is a toxin produced by the *Aspergillus*, *Penicillium*, and *Paecilomyces* fungal species (Sommer et al., 1974; Lovett and Thompson Jr, 1978; Santos et al., 2002). The blue mold that causes soft rot of apples, pears, cherries, and other fruits, is recognized as one of the most common causes of patulin contamination. Patulin is regularly found in unfermented apple juice, although it does not survive the fermentation into cider products (Trucksess and Tang, 1999; Reddy et al., 2010). In addition, studies shows that patulin at high concentration has an immunological, gastrointestinal and nephrotoxic effect on different

animals, such as mice and rabbits (Escoula et al., 1988; Speijers et al., 1988). Nevertheless, the Joint Food and Agriculture Organization-World Health Organization Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for patulin of 0.4 mg/kg of body weight per day (Joint et al., 1995).

Ergot is as well produced as a toxic mixture of alkaloids in *Claviceps* species (Shelby and Kelley, 1992). The ingestion of flour may contain these ergot alkaloids, and may lead to ergotism in humans (King, 1979). Ergotism can interrupt the blood supply to the extremities, and disturb the central nervous system (Saito et al., 1988). However, nowadays-modern techniques are committed to cleaning grain to reduce ergotism human disease (Blaney et al., 2003). Nevertheless, the risk of ingesting ergot alkaloids still exists when consuming contaminated vegetables.

Further, fusarium toxins are subdivided into three types, fumonisins, beauvericin (BEA), zearalenone (ZEA) and trichothecenes (Nesic et al., 2014). The most prevalent member of this fumonisins is fumonisin $B_1$ ($FB_1$). Effects of fumonisins include an alteration of sphinganine levels and myelin synthesis in the central nervous system of developing rats (Kwon et al., 1997) and liver cancer in humans (Ueno et al., 1997).

Beauvericin is a famous mycotoxin produced by many fungi, such as *Beaveria bassiana* (Hamill et al., 1969; Wang and Xu, 2012). Beauvericin is a cyclic hexadepsipeptide that belongs to the enniatin antibiotic family. It contains three D-hydroxyisovaleryl and three N-methylphenylalanyl residues in an alternating sequence (Wang and Xu, 2012). However, BEA represents a major concern because of its potential toxicity in humans and animal health, and its high presence in feed and food commodities in co-occurrence with several other Fusarium mycotoxins (Bertero et al., 2018). Their toxicity is mediated by their ability to initiate cation

transport across the cell membrane, disrupting normal intracellular cation levels, leading to apoptosis, which is accompanied by DNA fragmentation (De Saeger et al., 2016). They are very resistant to heat, acidification and proteolytic enzymes.

Zearalenone (ZEA) is one of the most studied mycotoxins subtypes possibly due to its estrogenic-like function (Kuiper-Goodman et al., 1987). ZEA is converted into a-and b-zearalenol (a-and b-ZOL), a-and b-zearalanol (a-and b-ZAL) and zearalanone (ZAN) in the liver and intestine (Kleinova et al., 2002; Mukherjee et al., 2014). Furthermore, the testis, the prostate and the hypothalamus tissues contain the major enzymes (3a-and 3b-hydroxysteroid dehydrogenase) needed to metabolize ZEA products from the liver and the intestine (Kleinova et al., 2002; Marroquin-Cardona et al., 2014). ZEA is an international contaminant of cereal and wheat crops and is commonly found in the soil in moderate and warm countries(Hussein and Brasel, 2001). In addition, ZEA was shown as a naturally chemical structure resembling produced estrogens (Kuiper-Goodman et al., 1987). Besides estrogenic effects, ZEA can also cause toxicity by production of reactive oxygen species (ROS) (El Golli Bennour et al., 2009). Therefore, due to the effects of zearalenone, the World Health organization (WHO) has established provisional maximum tolerable daily intake of 0.5 µg/kg body weight (Joint, 2002).

In addition, trichothecenes are produced by *Fusarium* species classified into four major subtypes: Type A, B, C, and D based on their functional group (Ueno, 1977, 1984); where type A tricothecenes have hydroxyl group, ester function, or no oxygen substitution at the carbon 8 position (Ueno, 1984). A Common example of type A tricothecenes is T-2 toxin with an ester substitution at carbon 8. Type B tricothecenes such as Deoxynivalenol (DON)(Krska et al., 2001), DON was first isolated in 1972 Morooka et al.(Morooka et al., 1972). These type B

tricothecenes are classified according to the presence of carbonyl functional groups substituted around the carbon 8 position (Krska et al., 2001; McCormick et al., 2011).

## 2. Mycotoxins Occurrence in Lebanon

In 2008, a study was conducted to evaluate the existence of aflatoxin (AFT), Ochratoxin A (OTA) and Deoxynivalenol (DON) in some foodstuffs from the Lebanese market and estimate the probable risk to the health of kids and teenagers in Beirut from dietary exposure to these mycotoxins. The resulted shows high levels of AFT, OTA and DON in nuts, biscuits and bread. Moreover, the calculated intake for AFT exceeded the corresponding provisional tolerable daily intake by a factor ranging from 3 to 7 (Soubra et al., 2009b). Whereas, the intakes of OTA and DON were found to be below the threshold established by international expert groups. However, the effects of repeated exposures to mycotoxins at low levels are still not well studied. Repetitive monitoring of the levels of mycotoxin contamination in foodstuffs, and the amount consumed by Lebanese kids and teenagers, such as cereals plus cereal-based products is highly needed (Soubra et al., 2009b).

Aflatoxin M1 (AFM1) is the major hydroxylated metabolite of aflatoxin B1 excreted in milk, and consequently it can be found in a large variation of dairy products thus posing a potential risk to human health when consuming these products (Ferguson-Foos and Warren, 1984; Elkak et al., 2012). A study was conducted in 2011 in order to determine the occurrence of AFM1 in cow samples (raw milk, pasteurized milk and powder milk samples), obtained from either local small farms, or markets. Using competitive enzyme linked immunosorbent assay (ELISA) method, the levels of AFM1 in various milk types marketed in Lebanon were measured (Assem et al., 2011). The results showed that the concentration of AFM1 in raw and pasteurized cow milk was higher than in powder milk and the highest concentration of AFM1

was found in the milk (Assem et al., 2011). In 2012, cheese samples taken from local small dairy farms, Lebanese industries and imported cheeses were analyzed for the presence of AFM1 levels. AFM1 was detected in 67.56% out of the 111 analyzed samples analyzed. In 17.33% of cheese samples AFM1 was found to exceed the limits (250 ng/kg) allowed by European Commission (EC) (Elkak et al., 2012). Moreover, the highest hazardous revealed ranges were detected in locally processed cheeses (Elkak et al., 2012).

In 2010, the determination of the occurrence of (DON) in crushed wheat marketed in Lebanon was done using high performance liquid chromatography (HPLC). One hundred sixty five samples of cereals (wheat, burgul and forkha) were purchased from five different mouhafazats all over Lebanon from food stores (supermarket, wholesaler and craft industry) during spring 2006. The results showed that the level of DON in all samples was below 1250 $\mu g\ kg^{-1}$ as recommended by European Union Directives, except for one sample of wheat, which contained 2307 $\mu g\ kg^{-1}$ (Antonios et al., 2010). Therefore, it is preferable to perform measures routinely to avoid high levels of DON contamination in the Lebanese market (Antonios et al., 2010).

Through my search on google scholar and PubMed about the studies done on mycotoxins in Lebanon, no more than 12 related articles were recorded, as shown in table 1. Most of the studies shown were done to determine the occurrence of aflatoxin, Ochratoxin or Deoxynivalenol. However, I did not record the publication of any article related to the occurrence of ZEA or beauvericin in the Lebanese market or their effect on human heath in Lebanon (Table 1).

Table 1: Studies testing mycotoxins occurrence in Lebanon.

| Studies in Lebanon | Results | References |
|---|---|---|
| A survey on the occurrence of aflatoxin M1 in raw and processed milk samples marketed in Lebanon | AFM1 contamination were 73.6%, 68.0%, 35.7% for the raw, pasteurized and powder milk samples, respectively. | (Assem et al., 2011) |
| Occurrence of aflatoxin M1 in cheese processed and marketed in Lebanon | AFM1 was detected in 67.56% out of the 111 samples analyzed in which the levels of AFM1 in 17.33% of the samples were found to exceed the limits. | (Elkak et al., 2012) |
| Monitoring the levels of deoxynivalenol (DON) in cereals in Lebanon and validation of an HPLC/UV detection for the determination of DON in crushed wheat (bulgur) | DON in all samples was below 1250 $\mu g\ kg^{-1}$ as recommended by EUD except for one sample of wheat, which contained 2307 $\mu g\ kg^{-1}$ | (Antonios et al., 2010) |
| Dietary exposure to aflatoxins, ochratoxin A and deoxynivalenol from a total diet study in an adult urban Lebanese population | Average dietary exposure levels to OTA and DON represented 29.9%,and 156.8% of the respective TRVs | (Raad et al., 2014) |
| Analysis of Aflatoxin $M_1$ in Breast Milk and Its Association with Nutritional and Socioeconomic Status of Lactating Mothers in Lebanon | The mean $AFM_1$ estimated daily intake was found to be 0.69 ng/day/kg of body weight. | (Elaridi et al., 2017) |
| Occurrence of total aflatoxins, ochratoxin A and deoxynivalenol in foodstuffs available on the Lebanese market and their impact on dietary exposure of children and teenagers in Beirut | The results showed that mycotoxin levels in the food samples were generally below national/European maximum limits. | (Soubra et al., 2009a) |
| The risks associated with aflatoxins M1 occurrence in Lebanese dairy products | Lebanese population daily exposure to AFM1 through consumption of daily products was estimated to be 9.22ng/L per person | (Hassan and Kassaify, 2014) |
| Occurrence of aflatoxin B1 and ochratoxin A in Lebanese cultivated wheat | 23.7% were contaminated with OTA, at a concentration > 3 mug/kg and 35.2% were contaminated with AFB1 at concentration > 2 mug/kg. | (Joubrane et al., 2011) |
| Ochratoxin A levels in human plasma and foods in Lebanon | OTA was detectable in 33% of plasma samples with a mean of 0.17±0.01ng/ml. Wheat, burghul and beer were contaminated with a mean of 0.15±0.03 µg/kg, 0.21±0.04 µg/kg and 0.19±0.12ng/mL, respectively. | (Assaf et al., 2004) |
| Occurrence of ochratoxin A- and aflatoxin B1-producing fungi in Lebanese grapes and ochratoxin a content in musts and finished wines during 2004. | 42 of the tested samples (60%) were found to be positive for OTA with low levels (0.012-0.126 µg OTA L-1) | (El Khoury et al., 2006) |
| Fungal contamination and Aflatoxin B1 and Ochratoxin A in Lebanese wine-grapes and musts. | None of these samples was contaminated by OTA at a detectable limit while 40% of these same samples were found to contain AFB1 with concentrations ranging from 0.01 to 0.46 $\mu g l^{-1}$. | (El Khoury et al., 2008) |
| Quantification of Fusarium graminearum and Fusarium culmorum by real-time PCR system and zearalenone assessment in maize. | Conventional methods of ZEA quantification and mycological detection and quantification of *F. graminearum* in maize. | (Atoui et al., 2012) |

## 3. Spermatogenesis

Spermatogenesis in mammals is a complicated process involving the division and differentiation of spermatogonial stem cells into mature spermatozoa (Clermont and Huckins, 1961; Clermont, 1972a; Hamano et al., 2007; He et al., 2009; Staub and Johnson, 2018). This process is conducted in the seminiferous tubules, which end in the rete testis (Clermont and Huckins, 1961; Hamano et al., 2007). The seminiferous tubules are a complex structure composed of germ cells and supporting somatic cells called Sertoli cell (Gwatkin, 1993; Holstein, 1994). During spermatocytogenesis, the cells extend from the basement membrane of the seminiferous tubules to reach the lumen (Gwatkin, 1993; Holstein, 1994). Spermatogenesis is divided into three major phases, spermatocytogenesis, meiosis and spermiogenesis (Clermont, 1972a; Holstein, 1994). During spermatocytogenesis, germ cells are involved in a cycle of numerous mitotic divisions leading to a rise in the yield of spermatogenesis, renewal of stem cells, and production of spermatogonia and primary spermatocytes (Clermont, 1972a; He et al., 2009; Staub and Johnson, 2018). Meiosis includes duplication and exchange of genetic material with two cell divisions, reducing the chromosome number and yielding four haploid round spermatids (Clermont, 1972a; He et al., 2009; Staub and Johnson, 2018). Spermiogenesis is the process of differentiation of round spermatids into completely mature spermatozoa (Clermont, 1972a; He et al., 2009; Staub and Johnson, 2018).

### 3.1. Spermatogonial stem cells

Primordial germ cells (PGCs) are the embryonic precursors of the germ cell lineage (Saitou and Yamaji, 2012). Germ cells are the base of new organisms and the active source for genetic diversity and evolution. There are differences in spermatogenesis cycle duration between species. Nevertheless, the process is similar between rodents and humans, making

rodents commonly used animal models to study the process of spermatogenesis (Gonzalez and Dobrinski, 2015).

In mice, the progenitors of primordial germ cells (PGCs) are derived from the epiblast of blastocyst in the yolk sac in response to bone morphogenetic protein (BMP) stimulation from the nearby visceral endoderm and extraembryonic ectoderm (Ying et al., 2001). Approximately at the embryonic day (E) 6.0, just before the epiblast separates into three germ layers: ectoderm, endoderm and mesoderm, the pluripotent cells of the most proximal posterior epiblast differentiate into PGCs (Phillips et al., 2010). At around E 13.5, migratory PGCs give rise to gonocytes which become enclosed in testicular cords formed by Sertoli precursor cells and peritubular myoid cells (Vergouwen et al., 1991; Jardins, 1993). Gonocytes are a general term that can be subcategorized into mitotic (M)-prospermatogonia, T1-prospermatogonia and T2-propspermatogonia (Vergouwen et al., 1991; Jardins, 1993; McLaren, 2003).

After birth, around postnatal day (P) 1-2, prospermatogonia differentiate into spermatogonia, as they migrate to the periphery of the testis and become flanked by somatic Sertoli cells within the tubules to support and nourish them (Nagano et al., 2000; Ibtisham et al., 2017). While most proteins and signaling networks involved in this alteration still need to be revealed, it has recently been shown that suppressing NOTCH signaling in Sertoli cells is important for maintaining quiescence in prospermatogonia (Garcia and Hofmann, 2013).

This initial neonatal spermatogonial population is heterogeneous, undifferentiated ($A_{undiff}$) and differentiated ($A_{diff}$) spermatogonia are noticeable at around P 3-4 (Ibtisham et al., 2017). The $A_{undiff}$ and the $A_{diff}$ spermatogonia differ in the expression of c-Kit (H. G. J. Schrans-Stassen et al., 1999). Stem cell growth factor receptor (SCFR), also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit or CD117, is a receptor tyrosine kinase protein that in

humans is encoded by the KIT gene (Andre et al., 1997). The transition of undifferentiated spermatogonia into differentiating spermatogonia coincides with the gain of tyrosine-protein kinase Kit (Zhang et al., 2011). The presence of Kit in spermatogonia has been routinely used as a marker to identify differentiating spermatogonia (Shinohara et al., 1999; Shinohara et al., 2000). A minor fraction of the $A_{undiff}$ spermatogonia comprises the future spermatogonial stem cells (SSCs) pool, ensuring the continuous spermatogenesis during the rest of the male reproductive lifespan (Yoshida et al., 2006; Abu Elhija et al., 2012). In mice, SSCs ($A_{single}$) and committed progenitor spermatogonia ($A_{paired}$ and $A_{aligned}$) are collectively described as undifferentiated A-spermatogonia based on morphological analyses (Oakberg, 1971; Chiarini-Garcia and Russell, 2001; Chen and Liu, 2015). During the spermatogenesis process, the products of SSCs divisions either maintain the stem cell population or produce progenitor spermatogonia for further differentiation. The differentiating product of SSCs retain a relatively large tubular connection, termed as an intercellular or cytoplasmic bridge that results from incomplete cytokinesis (Weber and Russell, 1987). Single spermatogonia are termed $A_{single}$, while those connected by an intercellular bridge are termed as $A_{paired}$ (Chiarini-Garcia and Russell, 2001). The commitment to enter meiosis is made with the transition of $A_{undiff}$ into $A_{diff}$ spermatogonia. The first differentiating spermatogonia are termed type A1. A1 cells go through mitotic cell division to differentiate in A2, then A3 and generate A4 spermatogonia. Next, two mitotic divisions form Intermediate and B spermatogonia (Chiarini-Garcia and Russell, 2001; Lacham-Kaplan, 2004).

At around P 7-10, B spermatogonia enter the first meiosis, differentiate to primary spermatocytes and then divide to produce secondary spermatocytes (Nebel et al., 1961). The meiotic phase is quite long with a period of 13 days. At the earliest post-meiotic period P 20-

21 round spermatid are detected (Kanatsu-Shinohara et al., 2005). During the following 13 days, the round spermatids differentiate into elongating spermatids and first fertilizable sperm are seen around P35 (Johnson et al., 2008; Ibtisham et al., 2017). This development cycle of germ cell into spermatid with their markers is shown in (Figure 1) for male mice and in figure (2) from different sources such as human.

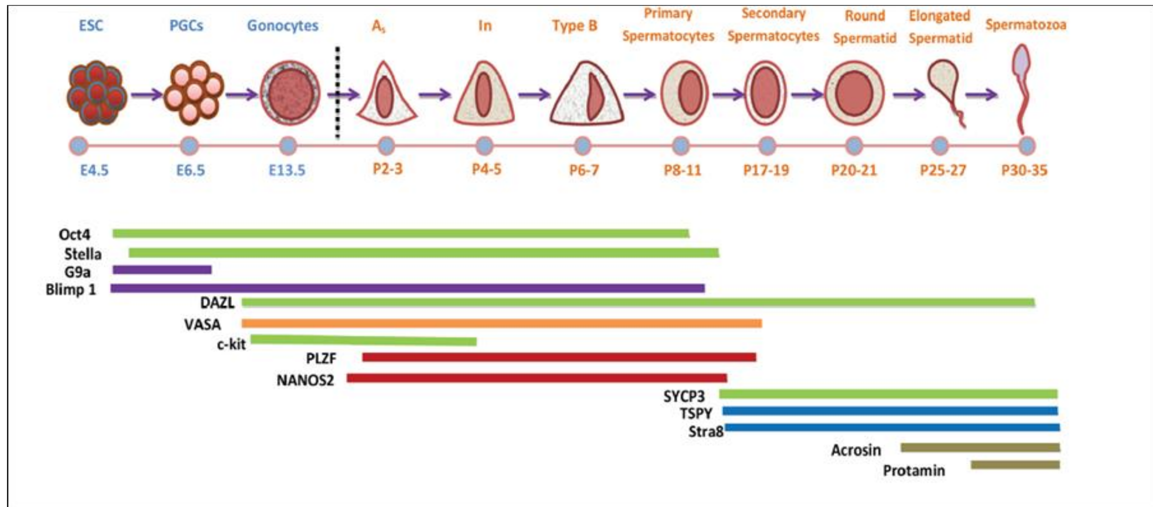


Figure 1: Sequence and timeline of male mice germ cell development and marker expression (Ibtisham et al., 2017).

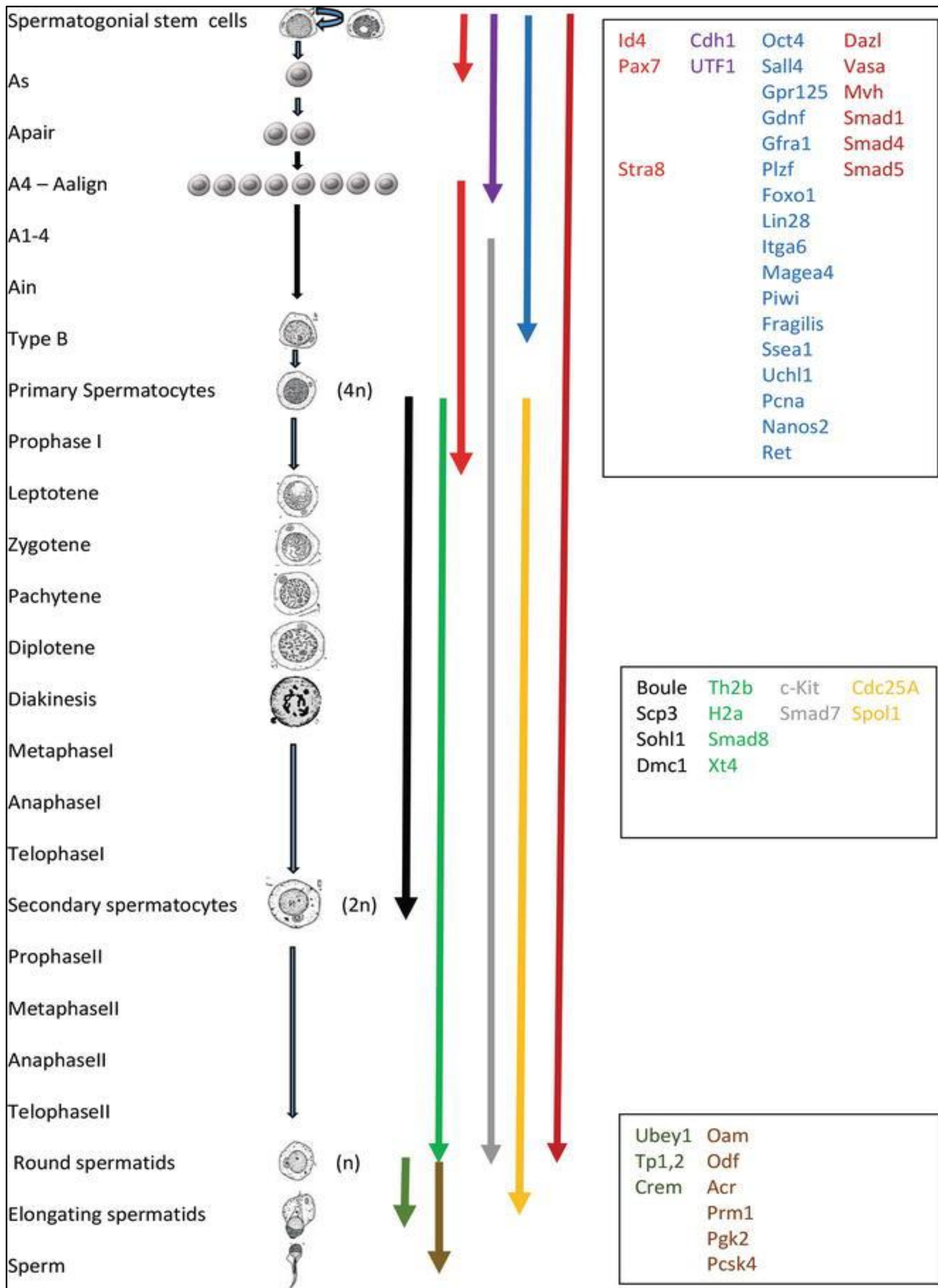


Figure 2: Stage-specific germ cell markers compiled from different sources (Dissanayake, 2018).

However, in human the male sex linked genes (SRY, SOX9) start their expression in embryonic testis from about 5-6 weeks (Hanley et al., 2000). During the second trimester, mitotically active gonocytes differentiate into pro-spermatogonia (Culty, 2009), while some of the pro-spermatogonia differentiate further into spermatogonia during embryonic development (Manku and Culty, 2015). After birth, the spermatogenic cycle initiates; it is usually longer than in mice and involves only three types of spermatogonia, $A_{dark}$, $A_{pale}$, and B (Hilscher and Engemann, 1992; Franke et al., 2004). Spermatogonia $A_{dark}$ are believed to be the reserve pool of stem cells (SSCs), whereas the proliferation of active $A_{pale}$ spermatogonia maintains spermatogenesis by balancing the production of differentiating B spermatogonia and renewing $A_{pale}$ pool (Ibtisham et al., 2017). Male spermatogenesis occurs in successive steps: mitotic, meiotic and spermiogenesis s shown in figure 3.

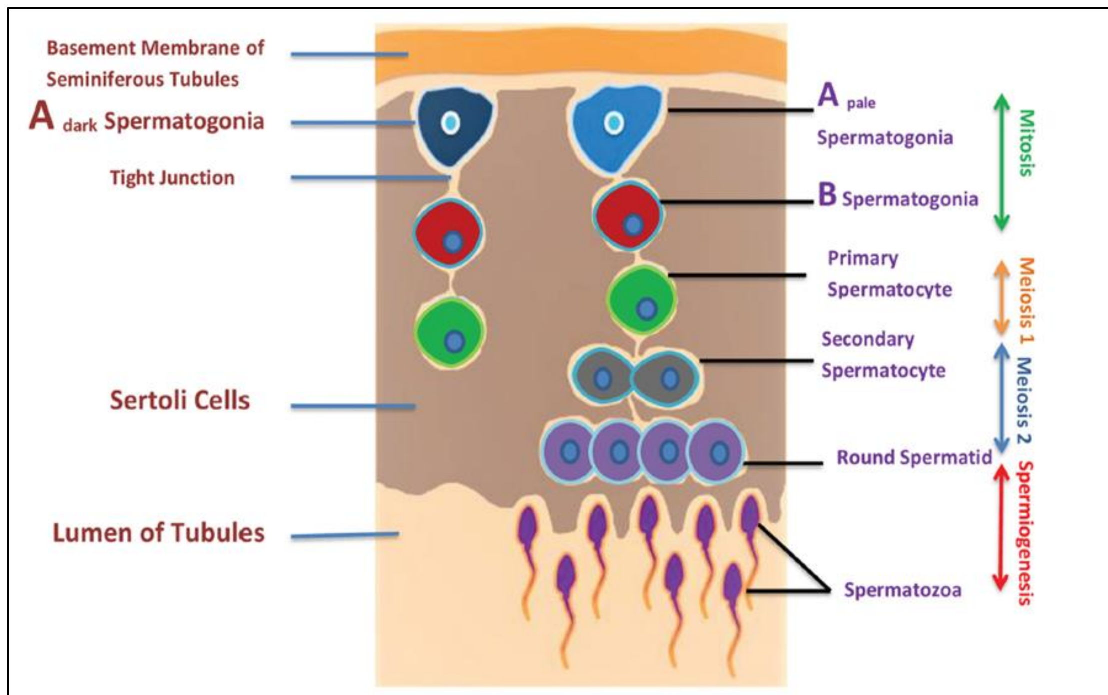


Figure 3: Illustration of spermatogenesis and approximate location of germ cells (Ibtisham et al., 2017).

Spermatogenesis starts in early puberty and is clinically recorded as an increased testicular volume. The meiotic process gives rise to haploid spermatocytes, which divide twice without additional DNA replication, producing round spermatids, which turn into spermatozoa by morphogenic process (He et al., 2010). The spermatozoa are released into the lumen of seminiferous tubules and are transported to the epididymis where they continue to mature (Ibtisham et al., 2017). Final steps of spermatogenesis occur at puberty. In humans, the whole spermatogenesis process takes 74 days including resting phases (Amann, 2008; Staub and Johnson, 2018). $A_{dark}$ and $A_{pale}$ spermatogonia are limited at the basement of seminiferous tubules. During the differentiation, spermatogonia pass to the luminal compartment. At the end of this progression, morphologically mature sperm are released into the fluid-filled lumen (Ibtisham et al., 2017). (figure 3)

SSCs are responsible for the production of $10^9$ sperm per day throughout the male mice reproductive lifespan (Kyjovska et al., 2013). A number of genes have been reported to intricate this SSCs balance, like PLZF (Buaas et al., 2004) and NANOS2 (Sada et al., 2009). Several pre-meiotic markers present on SSCs are described in the literature: Oct4 (Li et al., 2015), α6-integrin, GPR125, GFR-α1 (Azizollahi et al., 2016), Ty1, CD9 and β1-integrin, RET and CDH1 (Yaw and Mi, 2015). In this present paper, we will focus on the molecular markers that may distinguish SSCs from differentiating spermatogonia and primary spermatocyte.

In 2015, the analysis of Oct4 and c-kit expression during mice gonocytes transformation into spermatogonial stem cells (SSC) was performed using enhanced green fluorescent protein (eGFP). The results showed that gonocytes and prospermatogonia located at the tubular basement membrane were Oct4-GFP(+)/c-Kit(-), whereas through their development into SSCs (Oct4-GFP(+)/c-Kit(+)) were detected (Li et al., 2015). In addition, c-Kit has been reported as

a marker for SSCs and its expression continues until meiosis is initiated (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999). Therefore, Oct4 and c-kit expression could be used as molecular markers to differentiate between SSCs, spermatogonia and primary spermatocyte.

### 3.2. Cell culture method of spermatogonial stem cells

Spermatogonial stem cells (SSCs) culture is expected to participate in male infertility therapy, endangered species preservation, and transgenic animal technology because of their ability to differentiate into spermatozoa. A study was done in 2013, in order to isolate testicular tissues from 1-d-old male chickens and establish primary cell cultures. Testes were obtained by surgery and the dissected tissues were washed three times, with phosphate-buffered saline (PBS), supplemented with 1,000 units/mL penicillin and 1 mg/mL streptomycin to avoid microbial contamination. The tissues were then cut into small pieces and transferred into culture flasks containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 1,000 units/mL penicillin, and 1 mg/mL streptomycin. These primary cultures were incubated at 37°C in 5% $CO_2$ air, and passaged every 2–3d enzymatically with trypsin-ethylenediaminetetraacetic acid (EDTA). Then, the singled cells were seeded in 96-well culture plates with a seeding density of $1\times10^4$ cells/well and subjected to different concentrations of three growth factors of glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF). Using the colony-forming activity assay and qRT-PCR with Oct4 as primer for SSCs, the rate of success for the SSCs derivation and their maintenance *in vitro* is best in the presence of 15 ng/mL of GDNF, 20 ng/mL of bFGF, and 15 ng/mL of LIF cultures (Momeni-Moghaddam et al., 2014).

In 2016, it was shown that using a cell culture medium for mice SSCs supplemented with 15 ng/ml glial cell-derived neurotrophic factor (GDNF) the germ cells from prepuberal cat testes remained viable in culture for up to 43 days (Momeni-Moghaddam et al., 2014; Bedford-Guaus et al., 2017). Immunohistochemistry for promyelocytic leukaemia zinc finger (PLZF) protein was performed on fetal, prepuberal and adult testis sections of cat. Fluorescence-activated cell sorting (FACS) with an antibody against epithelial cell adhesion molecule (EPCAM) was used to sort live cells. Then, the gene expression profile of EPCAM-sorted cells was investigated through RT-qPCR. Notably, the results showed that EPCAM (+) cells expressed relatively high levels of c-Kit (CD117), a surface protein typically expressed in differentiating germ cells but not SSCs (Bedford-Guaus et al., 2017). On the other hand, EPCAM (-) cells expressed relatively high levels of POU domain class 5 transcription factor 1 (POU1F5 or Oct4), clearly a germ line stem cell marker. These results suggest that cat SSCs may be found within the population of EPCAM (–) cells (Bedford-Guaus et al., 2017).

In 2017, a study was performed to isolate spermatogonial cells from two months old male lambs. A biopsy was taken from the right testis of the lambs. The testicular biopsy samples were placed on ice and transferred to the laboratory within 2 h. The testes samples were minced into small pieces and suspended in DMEM, supplemented with 160.7 mM $NaHCO_3$, single-strength nonessential amino acids, 100 IU $mL^{-1}$ penicillin, 100-µg $mL^{-1}$ streptomycin, and 40-µg $mL^{-1}$ gentamicin. Then, the samples were crushed into small pieces and suspended them in DMEM containing 1 mg/ml collagenase type IV, 1 mg/ml trypsin, 1 mg/ml hyaluronidase type II and then incubated at 37°C for 40min (Qasemi-Panahi et al., 2014). After washing in DMEM, seminiferous tubules entered into secondary digestion process, with the same enzymes for 20 min at 37°C. To remove the spermatogonial cells they centrifuged the cellular suspension at 30

g for 2 min and they filtered the cells through a nylon mesh with 60 mm pore size (Qasemi-Panahi et al., 2018). After the filtration, they pelleted the cells and subjected them to co-culture of spermatogonial and sertoli cells for 10 days to reach 80% confluency in DMEM supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin (Qasemi-Panahi et al., 2018). For the identification of SSCs in sheep, immunocytochemistry staining (ICC) was used. The markers used were Anti-Promyelocytic Leukaemia Zinc-Finger (anti-PLZF) and anti-Oct4 for SSCs (Bahadorani et al., 2011) .Therefore, using anti- PLZF and anti-Oct4 in ICC can be a way in cell culture to detect the presence and quantity of spermatogonial stem cells. In addition, from this study a standard method for isolation and *in vitro* proliferation of spermatogonial stem cells in Ghezel sheep was developed (Qasemi-Panahi et al., 2018).

## 4. Effect of mycotoxins on male reproductive system

Infertility represents a growing problem in western countries affecting one out of seven couples trying to conceive an offspring (Stuppia et al., 2015). According to studies published between 1938 and 1991 about semen quality, a 1% average yearly decrease of sperm concentration was recorded (Carlsen et al., 1992). Moreover, the infertility rate increased between 1990 and 2010, from 42 million to 48.5 million couples worldwide. Studies have shown that in about 50 % of the cases, failure to conceive is due to a failure in the spermatogenesis of the male partner (Stuppia et al., 2015). Sperm morphology/motility abnormalities are also significantly increasing. In 1992, a worldwide sperm count assessment showed a decline to a mean of 71.2 million/ml in Ibadan, Nigeria, 54.6 million/ml in Lagos, Nigeria, 65.0 million/ml in Salem, Libya, 66.9 million/ml in Dar Es salaam and Tanzania (Swan et al., 1997). In 2000, Swan and colleagues confirmed that sperm concentrations in fertile males

have gradually declined overtime globally (Swan et al., 2000). This continuous decline of sperm count in human fertility worldwide was attributed to many factors including activities of endocrine-disrupting chemicals (EDCs) such as mycotoxins and pesticides (Ibeh et al., 1994; Martenies and Perry, 2013).

An extensive search of the literature revealed several studies showing the effect(s) of mycotoxins on the reproductive male system (as summarized in Table 2). Moreover, most of the studies were done using mycotoxin dose exposure on male rates *in* vivo, such as AFB1, DON, OTA, fumonisin 1 and ZEA.

## 4.1. Aflatoxin B1

Aflatoxin B1 (AFB1) is one of the most mycotoxins found in human consumed food products. AFB1 is principally hepatotoxic; however, it also affects reproduction (El Khoury et al., 2006; Joubrane et al., 2011; Supriya et al., 2014). In a study conducted in 2014, male rats were intramuscularly injected with different doses of aflatoxin B1 (10, 20, or 50-µg /kg body). The results showed that treatment with AFB1 reduces the weights of reproductive organs, daily sperm production, epididymal sperm count, viable sperm, and motile sperm (Supriya et al., 2014).

## 4.2. Ochratoxin A

Scientists have shown that Ochratoxin A, which is abundant in nature, has a negative effect on the male reproductive system (Chakraborty and Verma, 2009). Ochratoxin A established this negative effect by decreasing the sperm production and quality (Chakraborty and Verma, 2009; Zhang et al., 2018). The exposure of sperm to 10 and 100 µM OTA shows an increase in the reactive oxygen species, which will activate phophatase and tensin homolog

deleted on chromosome ten (PTEN) and activate the protein kinase (AMPK) in the exposed sperm (Zhang et al., 2018). This activation of the PTEN dephosphorylates $PIP_3$ into $PIP_2$, which results in the inhibition of the protein kinase B (AKT) signaling pathway. The AKT signaling pathway plays an important role in regulating cell growth and survival. Therefore, this inhibition of the AKT pathway is the reason behind the decrease in sperm production exposed to ochratoxin A. Moreover, results showed that the activation of the AMPK was enhanced by a decrease in the ATPase resulting in a decrease of sperm motility (Zhang et al., 2018). Consequently, Ochratoxin A works on AMPK and PTEN signaling pathway to affect the sperm production and motility.

## 4.3. Fumonisin $B_1$

In 2007, a study was done to determine the effect of fumonisin $B_1$ ($FB_1$) on sperm reserves and production of pubertal pigs. Twenty-four male pigs were assigned randomly to four diets containing different doses of fumonisin $B_1$ (0.2, 5.0, 10.0 and 15.0mg FB1/kg). The result have shown that a diet of $FB_1$ above 5mg/Kg reduce testicular and epididymal sperm reserves as well as the daily sperm production (DSP) per boar (Gbore and Egbunike, 2008). This study emphasizes that male weaning pigs for breeding should not be exposed to a diet with $FB_1$ higher than 5 mg/kg for no suppression in sperm production and reproductive performance (Gbore and Egbunike, 2008). In 2009, another study supporting this idea was done also to examine the influence of $FB_1$ on the onset of puberty, semen quality, fertility rates and testicular morphology in male rabbits. Forty male rabbits were assigned randomly to four diets containing different doses of aflatoxin $B_1$ (0.13, 5.0, 7.5 and 10.0 mg FB1/kg) for a period of 175 days. In addition, during the last week eight untreated female rabbits mated with the male rabbits treated with $FB_1$ to assess the fertility rate of the treated bucks (Ewuola and Egbunike, 2010). Results

have shown that 7.5 and 10 $FB_1$ mg/Kg delayed puberty, impaired semen quality and spermatogenesis and induced embryo mortality without a statistically adverse effect on the fertility rates of male rabbits (Ewuola and Egbunike, 2010). Therefore, the dose of fumonisin $B_1$ higher than 5 mg/Kg should be avoided in the diet to reduce this damaging effect on male reproductive system (Gbore and Egbunike, 2008; Ewuola and Egbunike, 2010).

## 4.4. T-2 Toxin

In 2010, a study was done by Yang & al. to estimate the effects of T-2 toxin on semen quality, fertility and serum testosterone concentration in mice. Adult male mice were mated with sexually mature untreated female mice after being exposed to intraperitoneal injection of T-2 toxin different doses (0, 5, 10 or 15 mg/kg body weight) daily for seven successive days. The results showed that a dose of 10 and 15 T2-toxin mg/kg leads to an increase in the number of abnormal spermatozoa and a decrease in live spermatozoa with a low pregnancy rate and high fetal resorption (Yang et al., 2010). Moreover, testicular and cauda epididymal sperm counts, efficiency of sperm production and serum testosterone concentration were reduced in mice treated with T-2 toxin at all doses in a dose-dependent manner (Yang et al., 2010). In brief, these outcomes indicated that T-2 toxin offered toxic effects on reproductive system of adult male mice.

## 4.5. Deoxynivalenol

Many studies focus to know the effect of deoxynivalenol on the male reproductive system. In 2004, male rats were exposed to different doses of DON (0.5, 1.0, 2.5, or 5.0 mg/kg DON) for 28 days via gastric intubation. The results display a dose-dependent decrease in in spermatid counts and sperm numbers in the 5.0mg/kg DON treatment group (Sprando et al.,

2005). In addition, Sperm tail abnormalities in the 5.0mg/kg dose group increased, with an increase in germ cell degeneration, sperm retention and abnormal nuclear morphology observed in the 2.5mg/kg and 5.0mg/kg dose groups (Sprando et al., 2005). This supports the idea that mycotoxins have a negative effect on male reproductive system with deoxynivalenol considered as damaging at a dose 2.5mg/kg and above.

### 4.6. Zearalenone

Studies have shown that zearalenone and its byproducts cause toxicity both by competitive binding to specific estrogen receptors and by modification of steroid metabolism (Kuiper-Goodman et al., 1987; Kim et al., 2003; Filannino et al., 2011). In 2013, Zatecka found a decrease in sperm concentration, with an increase of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V in male mice subjected *in vivo* to 0.15 µg/l zearalenone (ZEA). Consequently, this low concentration of ZEA is able to have a negative impact on the sperm parameters and testicular gene expression of CD1 mice in vivo (Zatecka et al., 2014). Zatecka suggested that one probable clarification could be that zearalenone works at the hormonal level and based on his experiments the decrease in sperm concentration has its origin at the level of spermatogonia.

## 5. Effect of mycotoxins on Spermatogonial stem cells

Spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis and male fertility. Similar to other tissue-specific stem cells, SSCs are rare, representing only 0.03 per cent of all germ cells in rodent testes (Tegelenbosch and de Rooij, 1993). In this section, we

emphasize on studies showing the effect of mycotoxin on germ cells and specifically spermatogonial stem cells.

Zearalenone (ZEA), a nonsteroidal estrogenic mycotoxin, is known to cause toxicity of testis in male rats (Kuiper-Goodman et al., 1987). To investigate whether apoptosis is involved in ZEA-induced testicular toxicity and to identify the stage and target germ cell type, male rats were treated with a single intraperitoneal dose of ZEA (5 mg/kg) and euthanized at 3, 6, 12, 24, or 48 h subsequently. By means of histopathology, germ cell degeneration was found at stages I-VI 12 h after dosing. Results (using TUNEL assay) suggested that the damaged germ cells, especially spermatogonia and spermatocytes, gradually underwent the processes leading to apoptosis. Moreover, DNA laddering on gel electrophoresis was apparent 12 h after dosing. The results demonstrated that a single dose of ZEA induces testicular germ cell apoptosis in a time-dependent and stage-specific pattern. Moreover, it was suggested that the damaged germ cells are-mostly-spermatogonia and spermatocytes (Kim et al., 2003). This study has established that apoptosis is the principal mechanism contributing to germ cell depletion and testicular atrophy following ZEA exposure (Kim et al., 2003).

Furthermore, deoxynivalenol (DON) exposure daily for 28 days via gastric incubation on male rats with a dose of 2.5mg/kg and 5.0mg/kg was found to reduce body weight, feed consumption, epididymal and seminal vesicle weights with an increase in germ cell degeneration, sperm retention and abnormal nuclear morphology at the same time (Sprando et al., 2005).

Other studies related to the effect of mycotoxin on testicular cells. Adult male mice were exposed to intraperitoneal injection of citrinin (CTN) at 0–6.25 mg/kg body weight daily for 7 days, and then mated with sexually mature untreated female mice. In CTN-exposed male, the

results showed an increase in the number of abnormal spermatozoa and decreased the number of live spermatozoa in a dose-dependent manner (Qingqing et al., 2012). Male rats were intramuscularly injected with different doses of aflatoxin B1 (10, 20, or 50-μg /kg body). The results showed that treatment with AFB1 reduces the weight of reproductive organs, daily sperm production, epididymal sperm count, viable sperm, and motile sperm (Supriya et al., 2014). A study was done to estimate the effects of T-2 toxin on semen quality, fertility and serum testosterone concentration in mice. Adult male mice were injected with T-2 toxin at different doses for 7 days successively. Then, they mated with untreated female mice. The results showed an increase in the number of abnormal spermatozoa and a decrease in spermatozoa with a low pregnancy rate and high fetal resorption after an exposure to a dose of 10 and 15 T2-toxin mg/kg (Yang et al., 2010).

Our search was conducted using the following databases: PubMed and Google Scholar. Search restriction were based on the English language, type of publication set to: journal and key words set to: "Mycotoxins AND germ cells" "Mycotoxins AND gonocytes," and "Mycotoxins AND spermatogonial stem cells." No research papers were found discussing the effect of mycotoxins on spermatogonial stem cells.

Table 2A and 2B represent a summary of all the studies testing mycotoxins effect on male reproductive system. As a conclusion from this table, a dose of 5 mg/kg of ZEA or DON can negatively affect the germ cells by increasing their degeneration process.

Table 2A: Studies testing mycotoxins effect on male reproductive system

| | Mycotoxin | Cell type | Technique | Duration | Dose | Effect | Reference |
|---|---|---|---|---|---|---|---|
| Aflatoxins | Aflatoxin B1 | Male rats 40 days old | *invivo* | 45 to 100 days of age (60 days) | 10, 20, or 50-μg aflatoxin B1/kg | **reduce** relative weights of reproductive organs, daily sperm production, viable sperm, motile sperm, and hypoosmotic swelling-tail coiled sperm were observed with **decrease** in steroidogenesis | (Supriya, Girish and Reddy, 2014) |
| | Aflatoxin B1 | Male rats 48 days old | *In vivo* | 48 days | 0.8, 1.6, 3.2 ppm/cc water /animal/day | **Decrease** developing spermatozoa in seminiferous tubules AFB1 influences the population of spermatozoa on a dose-dependent manner**.** | (Hasanzadeh and Rezazadeh, 2013) |
| Ochratoxin | Ochratoxin A | Male rats | by gastric intubation | 48h for 2, 4, 6 and 8 weeks | 289 μg/kg | **decrease** in stages I and VII whereas stages XII and XIII **increase** | (Gharbi et.al, 1993) |
| Citrinin | Citrinin | Male mice | intraperitoneal injection | Daily for 7 days | 0.0625, 0.625 and 6.25 mg/kg body weight | increase the number of abnormal spermatozoa & decrease the number of live spermatozoa reduce the sperm count and serum testosterone concentrations | (Qingqing et al., 2010) |
| Patulin | Patulin | Male rats aged 5–6 week | by gavage | 60 or 90 days | 0.1 mg/kg bw/day | sperm counts **increase** in patulin-treated rats for 60 days **decrease** in patulin-treated rats for 90 days | (Selmanolu, 2006) |

Table 2 B: Effect of Fusarium Mycotoxins on male reproductive system

| Mycotoxin | Cell type | Technique | Duration | Dose | Effect | Reference |
|---|---|---|---|---|---|---|
| Fumonisin B1 | Pigs | by diet invivo | 6 months | 0.2, 5, 10 and 15 mg FB1/kg | **reduce** testicular and epididymal sperm reserves as well as the daily sperm production | (Gobre and Egbunike, 2008) |
| Fumonisin B1 | Male rabbits | by diet invivo | 175 days | 0.13, 5, 7.5 and 10 mg FB1/kg , | **delay** puberty, impaired semen quality and spermatogenesis and induced embryo mortality | (Ewuola and Egbunike, 2010) |
| Type A T2-toxin | Male mice | Intraperitoneal injection | 7 Days | 0,5, 10 or 15 mg/kg body weight | **increase** abnormal spermatozoa, decrease in spermatozoa with integrated acrosome **Reduce** Testicular and cauda epididymal sperm counts, efficiency of sperm production and serum testosterone concentration | (Yang et al., 2010) |
| Type B DON | Male rates | via gastric intubation | 28 days | 0.5,1.0, 2.5 and 5.0mg/kg | **decrease** in homogenization resistant testicular spermatid counts, spermatid numbers **increase in germ cell degeneration,** sperm retention and abnormal nuclear-morphology | (Sprando et al., 2005) |
| Zearalenone | Male rats | intraperitoneal | 3, 6, 12, 24, or 48 hours | 5 mg/kg | Germ cells degeneration was found at stages I - VI 12 h after dosing damaged **germ cells**, especially spermatogonia and spermatocytes, leading to apoptosis | (Kim et al., 2003) |
| | Male mice | intraperitoneal | Daily for 7 days | 0, 25, 50, and 75 mg/kg body weight | Reduction of Testicular and cauda epididymal sperm counts, efficiency of sperm production and serum testosterone concentrations | (Yang et al ., 2007) |

## 6. Epigenetics and mycotoxins

Epigenetics can be defined as the study of mitotically or meiotically heritable modifications in the function of specific genes not related to modification in the DNA sequence (Russo et al., 1996). Epigenetics were simply defined as the study of biological mechanisms that will switch genes on and off (Deans and Maggert, 2015). Epigenetic mechanisms are essential for the common development and conservation of tissue-specific gene expression patterns in animals (Stuppia et al., 2015). Covalent modifications of either DNA or of histone proteins play central roles in many types of epigenetic inheritance (Zhu et al., 2017). These modifications happen through two mechanisms: DNA methylation and translational alteration (Zhu et al., 2014b). These disturbances of the epigenetic procedure may lead to altered gene function and malignant cellular conversion (Zhu et al., 2014b; Zhu et al., 2017).

Epigenetics is present everywhere. It is present in your food, live, friends, sleep, even aging. All of these can eventually cause chemical changes around the genes that will switch those genes on or off over time (Brief, 2017). Furthermore, in certain diseases such as cancer or Alzheimer's, various genes will be converted into the abnormal sequence (Deans and Maggert, 2015; Brief, 2017).

In recent years, the focus of different studies was about the epigenetic mechanisms of gene expression regulation. Scientists showed that human phenotypes are the outcome of the interaction between genes and environment. However, further studies on epigenetics determined that environmental agents modify the expression of specific genes without changing their sequence, and these changes may transmit to the offspring (Stuppia et al., 2015). In this view, epigenetic modifications are important regulators in a number of biological processes,

including spermatogenesis (Rajender et al., 2011). For example, they found that 5-aza-20-deoxycytidine, an anticancer agent, causes a decrease in global DNA methylation that leads to altered sperm morphology, decreased sperm motility, decreased fertilization capacity, and decreased embryo survival (Rajender et al., 2011). Moreover, epimutations (often hypermethylation) in several genes such as MTHFR (Kelly et al., 2005; Khazamipour et al., 2009), PAX8, NTF3, SFN, HRAS (Houshdaran et al., 2007), JHM2DA (Okada et al., 2007), IGF2, H19 (Poplinski et al., 2010), RASGRF1 (Li et al., 2004), GTL2 (Kobayashi et al., 2007), PLAG1, D1RAS3, MEST (Houshdaran et al., 2007), KCNQ1, LIT1, and SNRPN (Hammoud et al., 2010), have been reported in association with poor semen parameters or male infertility (Rajender et al., 2011). Therefore epigenetic represent a breakthrough in the field of human reproduction. In fact, since epigenetic modifications may transmit to the offspring, this will result in the increase of the infertility rate through the new generations. According to the world data atlas between 1967 and 2016, fertility rate of Lebanon was declining at a moderate rate to shrink from 5.3 births per woman in 1967 to 1.6 births per woman in 2016 due to environmental factors, such as the exposure of humans to mycotoxins with high doses. Mycotoxins, such as aflatoxin (AF), fumonisin B1, zearalenone (ZEA), and deoxynivalenol (DON), commonly found in many food commodities (Zhu et al., 2014b).

Little research worldwide has investigated the effect of zearalenone exposure on mice eggs development. The results show an increase in general DNA methylation level after Zearalenone (ZEA) treatment (Zhu et al., 2014a). Moreover, histone methylations also change by H3K4me2 as well as H3K9me3 and H4K20me1, me2, me3 levels decreased in eggs that cultured in high-dose ZEA medium (Zhu et al., 2014a). Therefore, we can conclude that ZEA might decrease the development of the egg by affecting the epigenetic modifications. Moreover,

another study was done in 2014 to discover the mechanisms of low mycotoxin dose in maize (DON at 3,875 μg/kg, ZEA at 1,897 μg/kg, and AF at 806 μg/kg). Each mycotoxin was included in diets at three different doses control, low and high (mass percentage respectively: 0, 15, and 30%) and fed to mice for 4 weeks (Zhu et al., 2014b). The results show an increase in the general DNA methylation in oocytes subjected to high dose (30%). Moreover, histone methylation of mice oocyte changed. H3K9me3 and H4K20me3 level increased in oocytes from mycotoxin-fed mice, whereas H3K27me3 and H4K20me2 level decreased in oocytes from mycotoxin-fed mice (Zhu et al., 2014b). Consequently, those results show that naturally occurring mycotoxins have effects on epigenetic changes in mice oocytes, which may be one of the explanations for reduced oocyte growing competence.

Deoxynivalenol (DON) is a prevalent trichothecene mycotoxin, which contaminates agricultural staples and produces a complex band of toxic effects on humans and animals (Pestka and Smolinski, 2005; Pestka, 2010). Studies showed that DON impairs oocyte's maturation, reproductive function and causes abnormal fetal growth in mammals (Malekinejad et al., 2007). Scientists try to explore the possible reasons behind the toxic effects of DON on porcine oocytes (Han et al., 2016b). Their results showed that DON considerably inhibited porcine oocyte maturation and disordered meiotic spindle, which delayed the cell cycle progression. Moreover, they show that DON exposure increased DNA methylation level in porcine oocytes and histone methylation levels alteration by the increase in H3K27me3 and H3K4me2 protein levels, and mRNA levels of their relative methyltransferase genes, indicating that epigenetic modifications were affected (Han et al., 2016b). Therefore, DON exposure reduced porcine oocytes maturation capability through affecting cell cycle and epigenetic modifications.

Moreover, DNA methylation has been a topic of considerable interest in the study of Ochratoxin A toxicity in recent years (Zhu et al., 2017). The changes in DNA methylation level due to OTA exposure have become the marker of some diseases such as in kidney disease (Wing et al., 2014). Therefore, each mycotoxin can have an epigenetic effect on specific organs mechanisms, which disturbing their function and possibly causing certain diseases.

Our search was conducted using the following databases: PubMed and Google Scholar. Search restriction were based on the English language, type of publication set to: journal and key words set to: “epigenetic effect” and “mycotoxins”. Only 8 research papers met our selection criteria as shown in table 3 with no paper studying the effect of beauvericin on SSCs. Through our search, beauvericin was the least studied mycotoxin worldwide. In addition, most of the mycotoxins have reproductive effect. Moreover, beauvericin effect was tested on granulosa cells but never on male reproductive system. For this reason, our Study was design to determine the effect of beauvericin on spermatogonial stem cells extracted from sheep. The reason behind choosing spermatogonial stem cells as a target of BEA effect is that fusarium group showed an increase in germ cell degeneration. Knowing that germ cells are the mother pool of the spermatogenesis pathway and any lack in those cells will affect the whole process and lead to infertility problems.

Table 3 : Studies testing the epigenetic effect of mycotoxins

| **Epigenetic Studies** | **Results** | **References** |
|---|---|---|
| Aflatoxins metabolism, effects on epigenetic mechanisms and their role in carcinogenesis | The AFB affect the epigenetic mechanisms by DNA methylation, histone modifications, maturation of miRNAs. It can lead to developing any type of cancers. | (Bbosa et al., 2013) |
| Zearalenone exposure affects epigenetic modifications of mice eggs | ZEA increased DNA methylation level and decreased egg developmental competence. | (Zhu et al., 2014a) |
| Effect of mycotoxin-containing diets on epigenetic modifications of mice oocytes by fluorescence microscopy analysis | Reduced oocyte developmental competence by increasing in DNA and histone methylation levels | (Zhu et al., 2014b) |
| Determination of the epigenetic effects of ochratoxin in a human kidney and a rat liver epithelial cell line | OTA exhibits tumors promoting properties in the liver epithelial cell, like the rat, liver epithelial cells are a primary target for carcinogens. | (Horvath et al., 2002) |
| Aflatoxin B1 induces persistent epigenomic effects in primary human hepatocytes associated with hepatocellular carcinoma | AB1 in primary human hepatocytes increase DNA methylation with a range of persistent hyper- and hypo-methylated genes identified. | (Rieswijk et al., 2016) |
| Deoxynivalenol exposure induces autophagy/apoptosis and epigenetic modification changes during porcine oocyte maturation | DON exposure reduced porcine oocytes maturation capability through affecting epigenetic modification. | (Han et al., 2016a) |
| Global DNA methylation in a population with aflatoxin B1 exposure | Association between AFB1 exposure and global DNA methylation on hepatocellular carcinoma development. | (Wu et al., 2013) |
| Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms | Protein synthesis inhibition, oxidative stress and the activation of specific cell signaling pathways, is responsible for OTA carcinogenicity. | (Marin-Kuan et al., 2008) |

# III. Materials and methods

## 1. Reagents and Consumables

Reagents used for sample isolation, treatment, RNA extraction and quantification were: Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (D8437), fetal bovine serum (F9665), collagenase from clostridium histolyticum Type I (C0130), thiazolyl blue tetrazolium bromide (M5655), trypsin from porcine pancreas (T4799), TRI Reagent (T9424) and BEA (B7510) obtained from Sigma-Aldrich Chemical Co. via Ibra Haddad (Beirut, Lebanon). Gentamicin Sulfate (L0011) and penicillin/streptomycin Solution 100X (L0022) were obtained from Biowest via Ibra Haddad (Beirut, Lebanon). From BIO-RAD the iTaq Universal Probes One-Step Kit, 100 x 20 µl rxns, 1 ml (1725140) was obtained via Bazilky (Beirut, Lebanon). As consumables 24-well plates (TPP92124), 96-well pates (TPP92196), and Clipmax 10 cm² / filter screw cap (TPP70010) were obtained from TPP Techno Plastic Products AG via Ibra Haddad (Beirut, Lebanon). The filters used during the isolation steps 40 µM (410-0001-OEM) and 70 µM (410-0002-OEM) were obtained from Foxx Life Sciences online.

## 2. Isolation of Spermatogonial stem cells

Fresh testes were collected from a local slaughterhouse. The outer layer of the testis was washed with sterile ice-cold 0.9% saline solution, 70% ethanol, and sterile ice-cold 0.9% saline solution (100 IU/L Pen/Strep) using aseptic technique. First, the tunica albuginea was removed under sterile condition and small biopsy was taken from the testis using fine scissors. Then the SSCs were isolated through two-step digestion methods. The first digestion step was using 1mg/ml collagenase type I for 60 min, while the second digestion was performed using another enzyme trypsin/EDTA (0.25 %/1 mM) for 15 min. The samples were incubated in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) containing 1mg/ml

collagenase type I in a shaking water bath at 80 oscillations (osc)/min at 37°C for 60 min. At an interval of 10 min during the digestion, the tubules were pipetted gently up and down in order to disperse them. After one hour of digestion, the seminiferous tubules fragments were separated from interstitial cells by centrifugation at 100 xg for 1 min, and the supernatant, containing mostly the interstitial cells, was discarded. The tubule fragments were washed with 10 ml of ice-cold DMEM/F12 with 5% gentamicin sulfate and 1% penicillin/streptomycin. The tube was settled on ice to allow the tubules to sediment. Washing was repeated twice in order to remove the interstitial cells. In a second digestion step, the tubules' fragments were incubated in trypsin/EDTA (0.25 %/1 mM) for 15 min at 37°C. Undigested debris were removed by centrifugation at 100 xg for 1 min. The supernatant was then processed by sequential filtration through 70 and 40 µm nylon mesh. The filtrate was centrifuged at 600 xg for 7 min, and the pellet was resuspended in DMEM/F12 supplemented with 5% gentamicin sulfate, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). Total cell number and cell viability were determined by Trypan Blue staining method. The cell suspension was mixed with 0.4 % Trypan Blue (2:1, v/v) (SIGMA T8154). The number of live (Trypan Blue excluding) and dead cells were determined using hemocytometer on an Olympus CK30, inverted microscope with phase contrast.

## 3. Validation of Cell culture system

In order to clarify that both suspended and attached cells are good SSCs for our experiment. In the first run, SSCs were plated at same concentration in 6 different wells. After 36 hours, cell count was performed for the different wells for both suspended and attached cells each one alone. Suspended cells were obtained by spinning at 1500 xg for 5 min, while attached

cells were obtained by treatment with 250µl of trypsin/EDTA (0.25 %/1 mM) for 5 min at 37°C and then scraped.

Moreover, to determine the best seeding density for the spermatogonial stem cells, the cells isolated in the second run were plated at different seeding densities of 1.5, 2.5, 3.5 and $4.5\times10^4$ cell/ ml for 36 hours. Live cells were counted via the trypan blue exclusion method as detailed below after 36 hours of culture in order to choose the best seeding density to be used in our experiment.

## 4. Cell culture

To determine the SSCs proliferation rate and RNA extraction, the cells were cultured in 24-well plates. SSCs with a concentration ($2.5x10^4$ cell/ml) were plated in 24-well plates in 500 µl of basal medium composed of a mixture of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with L-glutamine, 15 mM HEPES, and sodium bicarbonate supplemented with 5% gentamicin Sulfate, 1% penicillin/streptomycin and 10% FBS. Cultures were kept at 37°C in a humidified atmosphere with 5 % $CO_2$ for 36 hours. The same procedure was performed for SSCs in a 96 well plate for viability testing using MTT assay. The various treatments were applied using cell culture media with 10% FBS.

Each experiment was replicated 3 to 4 times and each replicate was derived from a different pool of SSCs isolated from fresh testes. For viability, test and cell count treatments were applied in duplicate culture wells. For mRNA collection, treatments were applied in duplicate culture wells with each mRNA sample being obtained from two wells.

## 5. Experimental design

Experiment 1 was carried out to evaluate the dose response of BEA on SSCs proliferation, viability, and abundance of mRNA of Oct-4, Smad8/9 and PLZF. SSCs were cultured for 36 hours in DMEM/F12 supplemented with 5% gentamicin sulfate, 1% penicillin/streptomycin and 10% FBS as described in the previous section. After 36 hours, one pool of cells was examined to determine viability and another pool of SSCs was treated for 24 hours in DMEM/F12 supplemented with 5% gentamicin sulfate, 1% penicillin/streptomycin and 10% FBS with various doses of BEA (0, 0.3, 1, 3, 10, 30 μM). After 24 hours of treatment, cells were counted, viability test was performed and RNA extraction was done. This experiment was repeated four times for four pools with the same protocol to determine the consistency of the results. In each pool, at least two replicates per treatment were done cell viability (MTT), count, and RNA extraction.

Experiment 2 was designed to evaluate the time and dose response of BEA on SSCs proliferation, viability, and the abundance of mRNA of Oct-4, Smad8/9 and PLZF. The time course was used to test if at 12 and 48 hours there is a recovery or a steroidogenic effect of BEA. SSCs were cultured for 36 hours in DMEM/F12 supplemented with 5% gentamicin sulfate, 1% penicillin-streptomycin and 10% FBS as described earlier. After 36 hours of culture without treatment, first pool of cells was examined to determine SSCs proliferation, viability and mRNA abundance. The second pool of SSCs was treated for 12 hours, 24 or 48 hours in DMEM/F12 supplemented with 5% gentamicin sulfate, 1% penicillin-streptomycin and 10% FBS with various doses of BEA (0, 0.3, 1, 3, 10, 30 μM). After the specified time, cells were counted, viability test was performed and RNA extraction was done.

## 6. Determination of SSCs numbers

The suspended cells were obtained by spinning at 1500 xg for 5 min. In the same well from which media was collected the attached cells were treated with 250µl of trypsin/EDTA (0.25 %/1 mM) for 5 min at 37°C and then scraped. The cell pellet from the media was added to the trypsinized cells with 25 µl of FBS to neutralize the effect of trypsin. Number of SSCs was determined after Trypan Blue staining. The cell suspension was mixed with 0.4 % Trypan Blue (2:1, v/v). The numbers of live cells (Trypan Blue excluding) and dead cells were determined using hemocytometer. To count cells using a hemocytometer, 10µl of trypan blue was added to 20µl of SSCs mixed gently and placed on the slide using a P-20 Pipetman. The cells were counted from the four outer squares. Finally, the cell concentration was calculated using:

The number of cells/ml =

(The number of cells counted x dilution factor x $10^4$) / Number of squares counted.

## 7. MTT test

For the viability test, 10 µL of MTT (5mg/ml) were added to the SCCs ($2.5 \times 10^4$ cell/ml) plated in 96 well plates with different doses of BEA (0, 0.3, 1, 3, 10, 30 µM) and incubated at 37ºC for 4 hours. In the next step, 100 µL of isopropanol (0.04M HCl) was added to each well. The plate was returned to the incubator for another 30 minutes with the solvent. The absorbance was measured using ELISA reader (Thermo Scientific Multiskan GO) at 570 nm. The wells which were not exposed to any cell, considered as negative control (Blank). Finally, the cell viability was calculated using:

$$\%\ viable\ cells = \frac{absorbance\ sample - absorbance\ blank}{absorbance\ control - absorbance\ blank} \text{x100}$$

## 8. Hematoxylin and eosin staining

The SSCs (2.5x$10^4$ cell/ml) were cultured for 36 hours on three separate 10 $cm^2$ clipmax microscope slide (TPP70010). One of the clipmax was treated with 1µM BEA, another clipmax with 3 µM BEA for 24 hours. Staining was performed for treated and non-treated cells to determine the morphology of the SSCs. Fixation of the cells was done using 1:1 mix of ethanol and methanol. The slide was rinsed with PBS then distilled water to remove any fixation agent. Staining of the nucleus was performed with Hematoxylin solution (SIGMA HHS16) for 2 minutes. For the next step, the slide was rinsed with water and stained with Eosin (SIGMA HT110316) for 10 seconds. Quick drying was accomplished by an increasing ethanol series (70%, 96%, and 100%). The slide was covered with a cover slip using mounting media.

## 9. RNA extraction

RNA extraction was performed using TRI Reagent for both suspended and adherent cells. Suspended cells were obtained by spinning at 1500xg for 5 min. Then, attached cells were lysed by adding 500 µl of TRI Reagent and scraped. Homogenization followed by pipetting the cell lysate solution several times. The pellet was added to the lysed cells in a 1.5 ml Eppendorf tube and left at room temperature for 5 min. Briefly, RNA was extracted following manufacturer's recommendation. A 100µl chloroform were added, vigorously shaken for 15 sec, and incubated for 5 min at room temperature. Following the centrifugation the upper aqueous layer (200-400µl), was transferred to another tube with 250 µl of isopropanol, and mixed gently. The tube was incubated for 10 min at room temperature, centrifuged, then the pellet solution was washed and left to air-dry. These pellets were then suspended in 20 µl of TE buffer and RNA quality and concentration detected on Nanodrop 2000c (ThermoScientific, DPC, Beirut, Lebanon). The samples were then stored at - 80°C until real time PCR analysis.

## 10. Primers design and validation

Primer design was performed using NCBI Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) with a preference for exon-exon junction selection and a product size between 100 and 300 maximum for Oct-4, Smad8/9 and PLZF / ZBTB16 as summarized in table 4.

Table 4: Primers used in qRT-PCR

| Gene | Primer sequence (5’–3’) | Accession Number | Product size (bp) |
|---|---|---|---|
| **Oct-4** | Forward: GGTTCTCTTTGGAAAGGTGTTC<br>Reverse: CGGTTCTCGATACTCGTCCG | Ovis XM_004018968.4 | 204 |
| **Smad8/9** | Forward: CCCCTTCCAGCACTCAGATTT<br>Reverse: TCAATCAGCACACTTCGGGA | Ovis XM_027973606.1 | 132 |
| **PLZF / ZBTB16** | Forward: GGGAGCAGTGCAGTGTATGT<br>Reverse: CAGCCATGTCTGTGCCAGTA | Capra (NM_001314273.1) homologous to predicted ovis (XM_027979222.1 | 261 |

Each primer was diluted to 100µM by adding 50µl of RNase free water to the original tube. Then, 25 µl from the diluted primer was taken and mixed with 475µl of RNase free water to get a final concentration of 5µM. The diluted primer was aliquoted into 20 separate tubes in each 15µl. At the end, the tubes containing the primers were stored at -20°C, until gene expression. The reason behind storing those primers in small volume 15 µl was to prevent freeze thaw.

## 11. Real-time PCR amplification

The differential expression of target genes (TG) in Spermatogonial stem cells was quantified using the one-step real-time qRT-PCR with SYBR green to amplify Oct-4, Smad8/9

and PLZF / ZBTB16. 18 S rRNA was used for all the runs as internal control to normalize samples for the variation in amounts of RNA loaded.

In one step qRT-PCR, the amplification of each TG and the internal control was optimized and validated separately, following which the combinations of primers for each TG and of the internal control were tested to identify the concentrations that would give optimal amplifications in the real-time PCR reaction as shown in figure 4.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Oct-4 | 200:200 MM1 | 200:200 MM1 | 200:200 MM1 | 400:400 MM2 | 400:400 MM2 | 400:400 MM2 | | NTC1 | NTC2 | | | |
| | | | | | | | | | | | | |
| Smad8/9 | 200:200 MM3 | 200:200 MM3 | 200:200 MM3 | 400:400 MM4 | 400:400 MM4 | 400:400 MM2 | | NTC3 | NTC4 | | | |
| | | | | | | | | | | | | |
| PLZF / ZBTB16 | 200:200 MM5 | 200:200 MM5 | 200:200 MM5 | 400:400 MM6 | 400:400 MM6 | 400:400 MM6 | | NTC5 | NTC6 | | | |
| | | | | | | | | | | | | |
| 18 S | 100:100 MM7 | 100:100 MM7 | 100:100 MM7 | | | | | NTC7 | | | | |
| | | | | | | | | | | | | |

Figure 4: Plate Design of the optimization

For qRT-PCR, the total reaction volume was 10 µl, including 3 µl of RNA (at 100 ng/µl), and DEPC-treated water. Thermal cycling conditions were set to 10 min at 50°C for reverse transcription, 95°C for 1 min for iTap activation, and finished with at least 50 cycles at 95°C for 10 sec for denaturing, and 60°C for 30 sec for annealing and extension. A melt-curve analysis was used to ensure that only one target was amplified according to manufacturer's instructions.

Quantification of gene expression was done by setting an arbitrary (Ct) on the SYBR green curves in the geometric portion of the qRT-PCR amplification plot after examining the

log view. Relative qualification of target gene mRNA was expressed using the comparative threshold cycle method. Briefly, the $\Delta Ct$ was determined by subtracting the internal Ct value from the target unknown value. Within each experiment, the $\Delta\Delta Ct$ was determined by subtracting the higher $\Delta Ct$ (the least expressed unknown) from all other $\Delta Ct$ values. Relative abundance in mRNA expression (fold change) was calculated as being equal to $2^{-\Delta\Delta Ct}$ .

## 12. Gel electrophoresis

To check for a successful cDNA synthesis, a 1% agarose gel was prepared by dissolving 1g of agarose in 100 ml of 1x TAE buffer. The solution was then heated in the microwave for about 42 sec until the solution was clear. Then, 3µl of ethidium bromide were added to the prepared cooled agarose gel, for band migration observation under UV, then poured in the mold to cool down. The mold was then transferred to electrophoretic chamber and 1x TAE buffer was added until the gel was soaked. 6 µl of each sample was then loaded into the wells. The chamber was placed under 70 V for 45 min. Results were observed under UV and picture was taken using the ChemiDoc Imaging System (Neumelab, Beirut, Lebanon).

## 13. Statistical analysis

The results were expressed as Means ± SEM. All experiments were repeated at least three to four times. Cell count and MTT values were analyzed using SPSS software (22), and presented as least squares means ± SEM and considered significant at $P<0.05$. Treatment effects for gene expression were analyzed as a factorial treatment design in the PROC Mixed procedure of SAS 9.0 (Statistical Analysis System INC, USA). Mean differences in mRNA abundance between treatments were determined using the Fisher's protected least significant difference (LSD) procedure and considered significantly different at $P<0.05$. Prior to all data analysis, raw

data were tested for outliers using the Grubbs outlier calculator (QuickCalcs website), and removed when significant ($P<0.05$). Moreover, the correlation between cell count, MTT assay, and gene expression for both Oct-4 and Smad8/9 was calculated using PROC CORR procedure of SAS 9.0.

## IV. Results

### 1. Validation of the cell culture system

Through our search it was shown that SSCs in culture could be attached cells and suspended cells due to their ability to sloughing off. For this reason, the first run was performed to compare the number of live attached and suspended SSCs. Figure 5 shows that the number of live attached and suspended cells was approximately the same in each well, while the number of attached SSCs was higher by 5 to 10 % as compared to suspended SSCs. These results will support the idea to use both cells attached and suspended SSCs to test the effect of BEA in our experiment.

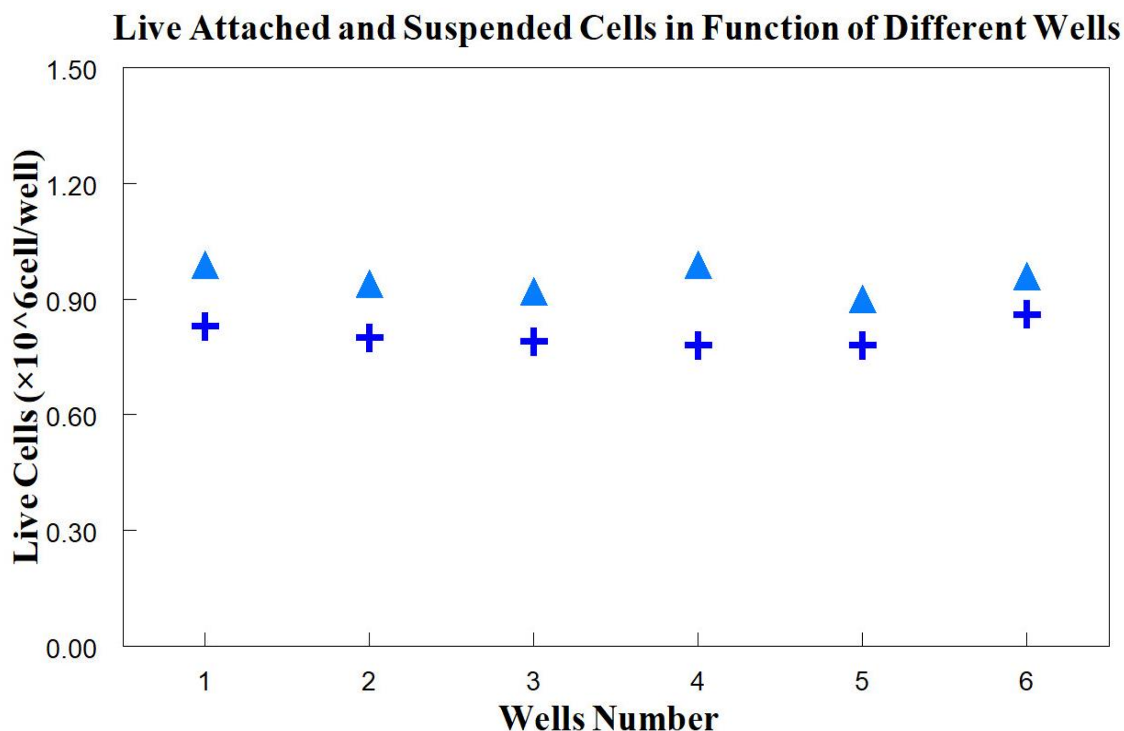


Figure 5: Live attached and suspended cells in function of different wells. The live cells were represented in ▲ for attached cells and ✚ for suspended SSCs.

In addition, in a second run SSCs were plated at different seeding densities (1.5, 2.5, 3.5 and $4.5\times10^4$cell/ml) to determine the best one for our experiment. The results shows in figure 6 that the number of live SSCs was increased after 36 hours of culture at the different seeding densities, while the maximum count of live cells $1.98\times10^6$ cell/well was recorded at a seeding density of $2.5\times10^4$cell/ml and a plateau was formed after this point.

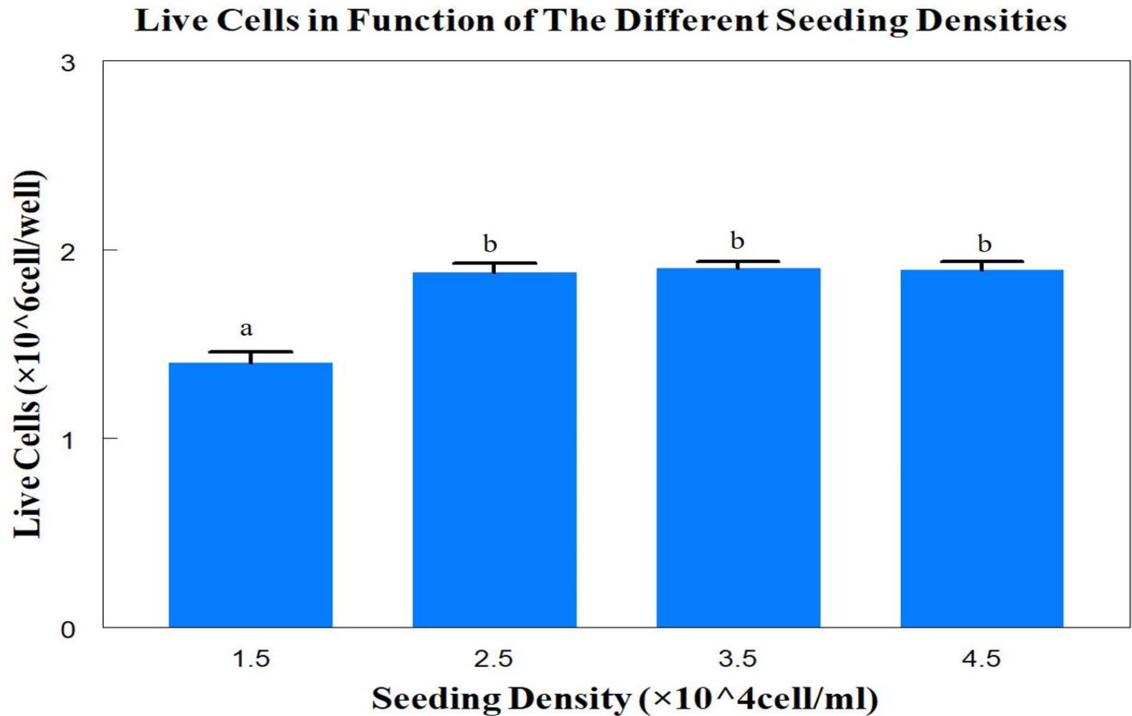


Figure 6: Live cells in function of different seeding densities. Values are means ± SEM. Means without a common letter (a-b) differ ($P < 0.05$).

## 2. Growth curve of SSCs

In order to determine the growth curve of spermatogonial stem cells, the cell count of live SSCs and the viability test (MTT assay) was performed at the different times (0, 12, 24 and 48 hours. The growth curve of SSCs based on cell count and viability test was represented in figure 7 panel A and B, respectively. Figure 7 panel A shows the cell count of spermatogonial stem cells was increased by 20% after 12 hours and by a maximum of 40% after 24 hours of plating, as compared to day 0 (control), while after 48 hours SSCs abundance starts to decrease by 15%. Similarly, in Figure 7 panel B the viability of SSCs was increased by 17% after 12 hours and by a maximum of 33% after 24 hours of plating, as compared to control (day 0), while after 48 hours SSCs abundance starts to decrease by 20%.

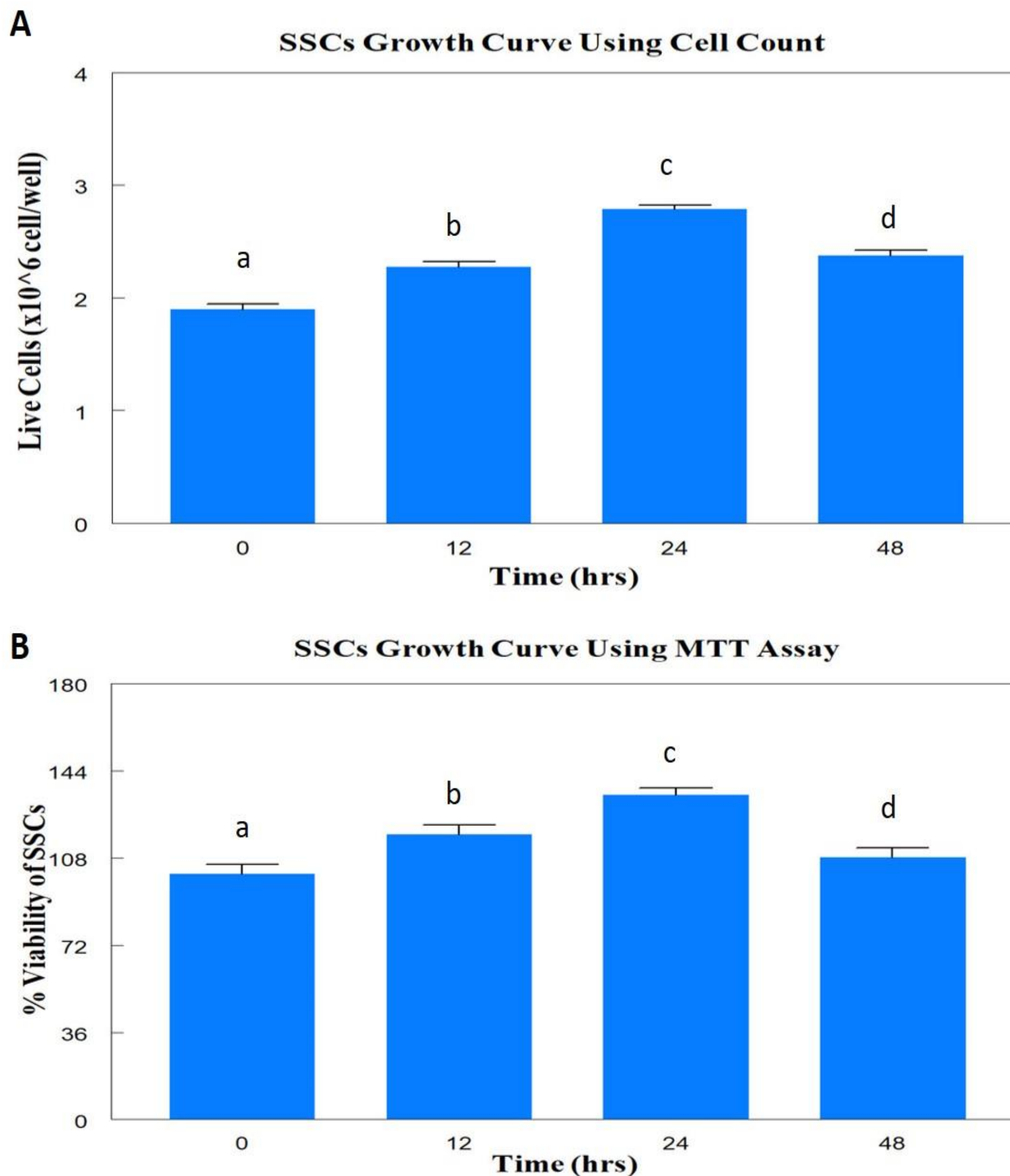


Figure 7: Spermatogonial stem cells growth curve. Panel A: cell count. Panel B: viability test (MTT assay). Cells were isolated from fresh testis and plated. At different times (0, 12, 24 and 48 hours), MTT assay and cell count test were performed. Values are means ± SEM. Means without a common letter (a-d) differ ($P < 0.05$).

## 3. Effect of BEA variable doses & times on SSCs growth and activity

First, dose effect of beauvericin was tested on SSCs after 12 hours of treatment. The cell count of live SSCs and the viability test (MTT assay) were performed for the six different doses (0, 0.3, 1, 3, 10 and 30µM) after 12 hours of treatment. Figure 8 present the dose effect of BEA on SSCs after 12 hours of treatment based on viability test and cell count panel A and B, respectively. Panel A shows that the SSCs viability was decreased by 25% after 12 hours exposure to beauvericin at a dose 0.3µM, by 35% to 38% at 1 µM and 3 µM BEA as compared to control (0 µM BEA). Similarly, in figure 8 panel B the abundance of SSCs was decreased by 15% after 12 hours exposure to BEA at a dose 0.3 µM, and by 25% at 1µM BEA as compared to control (0 µM BEA). While it slightly recovered by 20% growth for the three higher doses of 3, 10 and 30 µM BEA. These results of 12 hours treatment show that the lower percentage of viable cells was recorded at 1 and 3 µM BEA, while the lower cell count of viable SSCs was noted at 1 µM BEA.

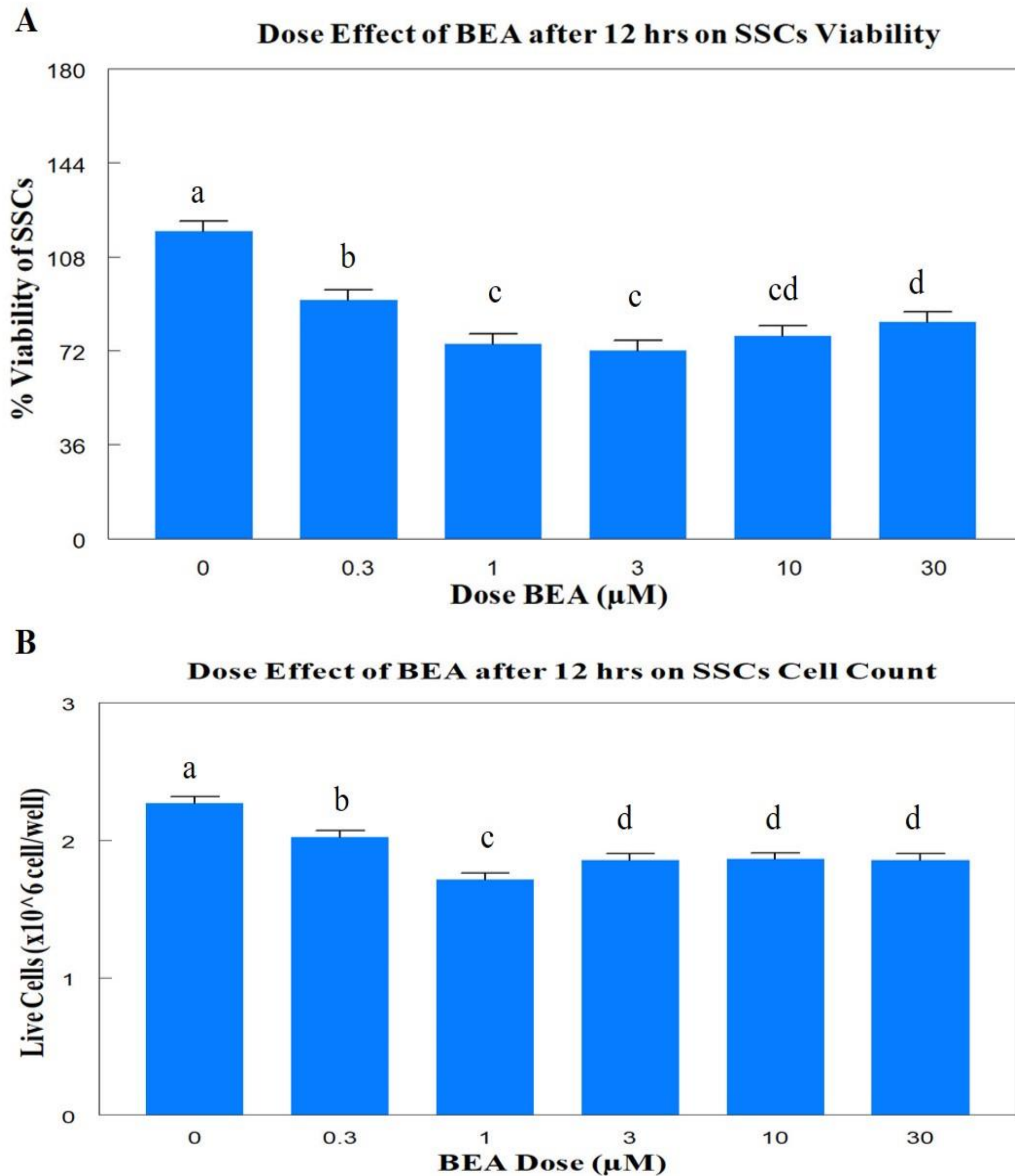


Figure 8: Dose effect of beauvericin after 12 hours on spermatogonial stem cells. Panel A: viability test (MTT Assay). Panel B: cell count. Cells were plated and treated with different doses (0, 0.3, 1, 10, and 30µM). MTT assay and cell count test were performed after 12 hours of treatment. Values are means ± SEM. Means without a common letter (a-d) differ ($P < 0.05$).

Second, dose effect of beauvericin was tested on SSCs after 24 hours of treatment. The cell count of live SSCs and the viability test (MTT assay) was performed for the six different doses (0, 0.3, 1, 3, 10 and 30µM) after 24 hours of treatment. Figure 9 present the dose effect of BEA on SSCs after 24 hours of treatment based on viability test and cell count panel A and B, respectively. Panel A shows that the SSCs viability was decreased by 25% after 24 hours exposure to BEA at a dose 0.3 µM, and by 35% to 40% at 1, 3 and 10 µM dose, compared to control (0µM BEA), while it slightly recovered when treated with 30 µM BEA. Similarly, in figure 9 panel B the abundance of SSCs was decreased after 24 hours exposure to BEA by 25% at a dose 0.3 µM, by 28% at a dose 1 µM and by a minimum 44% at 3 µM BEA as compared to control (0 µM BEA), while SSCs numbers recovered at 10 µM and 30 µM. These results of 24 hours treatment show that the lower percentage of viable cells was recorded at 1 µM BEA, while the lower cell count of viable SSCs was noted at 3 µM BEA.

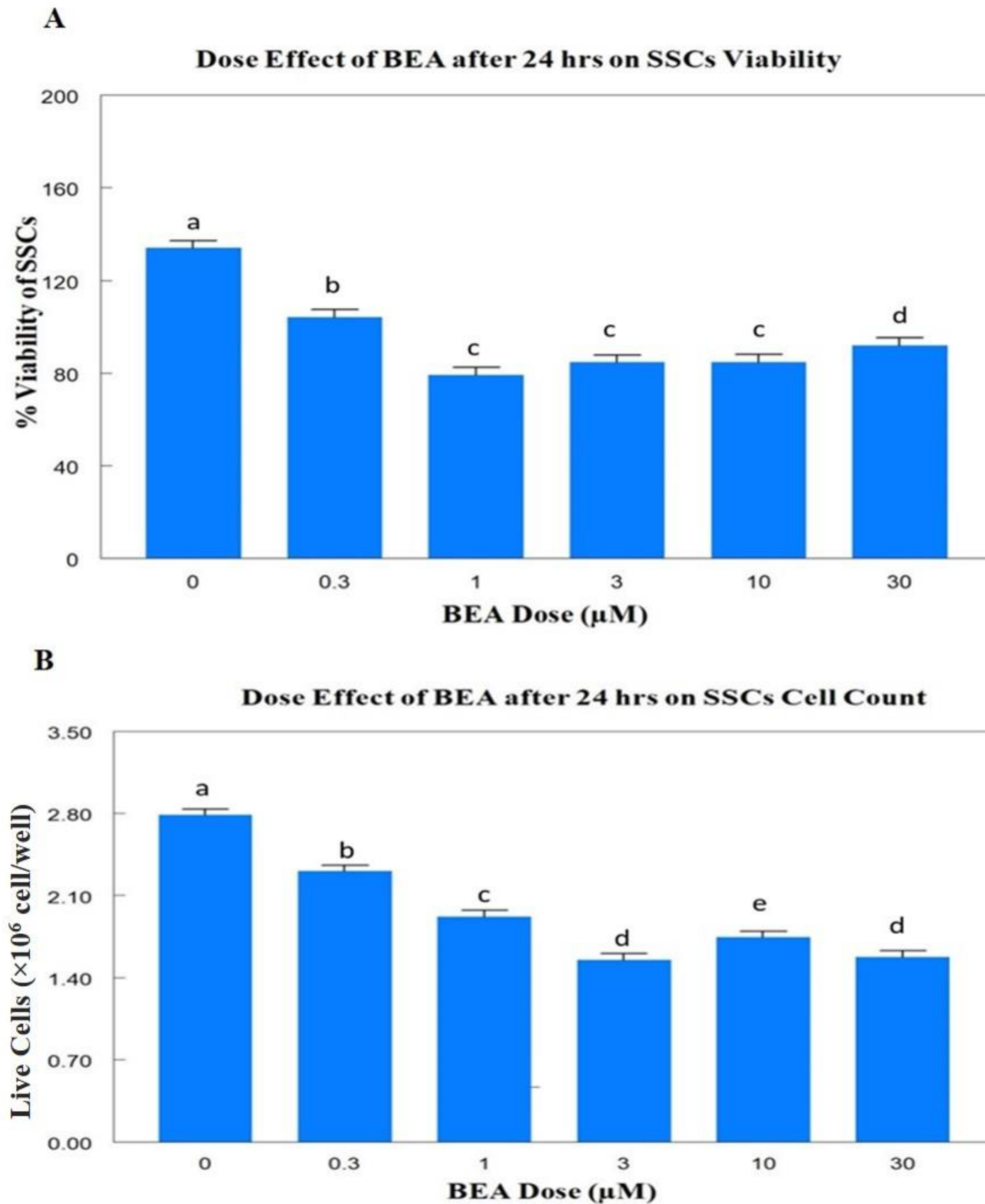


Figure 9: Dose effect of beauvericin after 24 hours on spermatogonial stem cells. Panel A: viability test (MTT Assay). Panel B: cell count. Cells were plated and treated with different doses (0, 0.3, 1, 10, and 30μM). MTT assay and cell count test were performed after 24 hours of treatment. Values are means ± SEM. Means without a common letter (a-e) differ ($P < 0.05$).

Third, dose effect of beauvericin was tested on SSCs after 48 hours of treatment. The cell count of live SSCs and the viability test (MTT assay) was performed for the six different doses (0, 0.3, 1, 3, 10 and 30µM) after 48 hours of treatment. Figure 10 present the dose effect of BEA on SSCs after 48 hours of treatment based on viability test and cell count panel A and B, respectively. Panel A the viability of SSCs was decreased by 10% after 48 hours exposure to BEA at a dose 0.3 µM, and by 20% minimum percent viability at a dose 1 µM as compared to control (0 µM BEA), while the % of viable cells recovered when treated with the three higher doses 3, 10 and 30 µM. Similarly, in figure 10 panel B shows that the SSCs number was decreased by 12% after 48 hours exposure to BEA at a dose 0.3 µM, and by 25% minimum for the four doses 1, 3, 10 and 30 µM, compared to control (0 µM BEA). These results of 48 hours treatment with a dose of 1 µM BEA show that the lower percentage viability and cell count of SSCs.

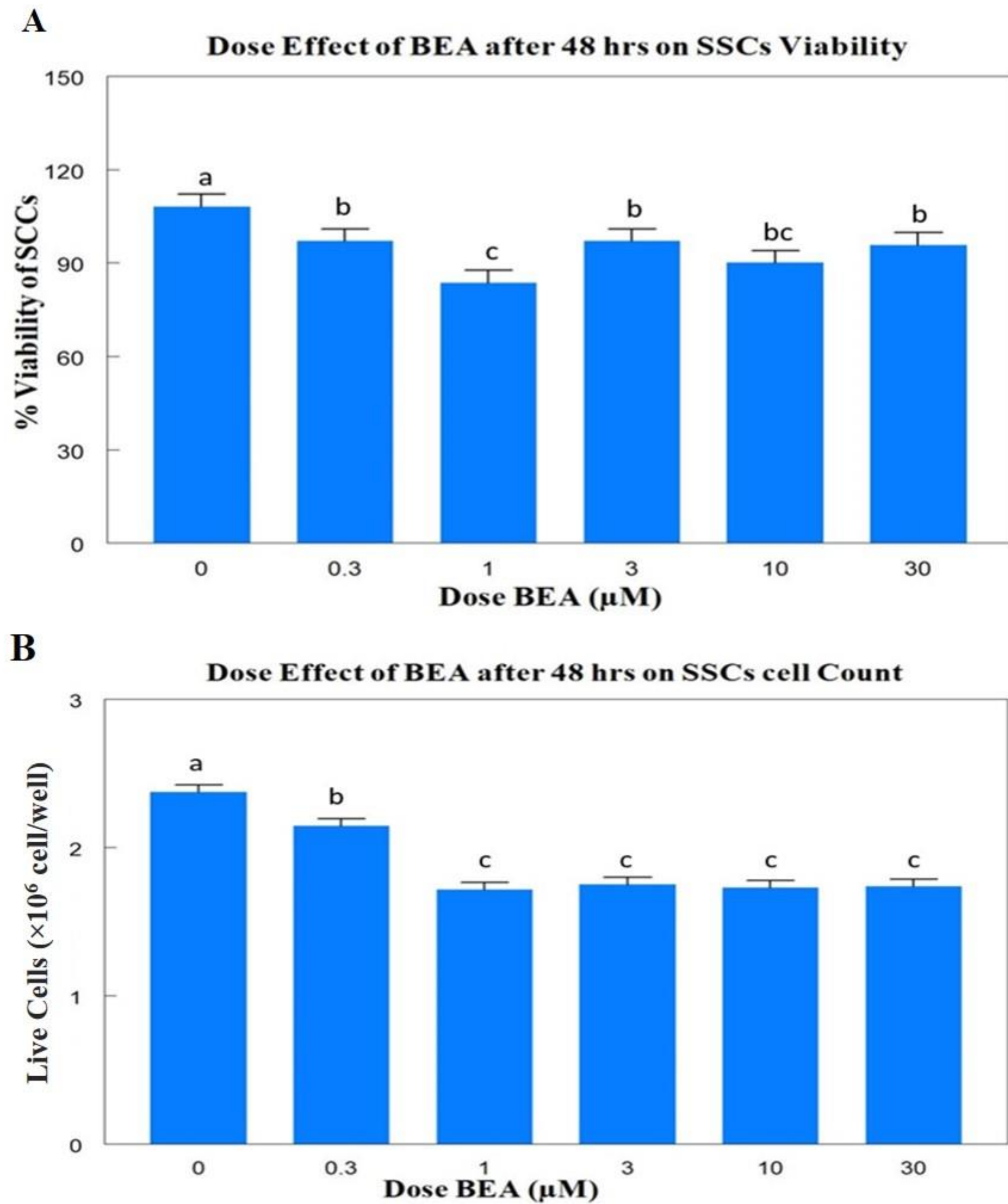


Figure 10: Dose effect of beauvericin after 48 hours on spermatogonial stem cells. Panel A: viability test (MTT assay). Panel B: cell count. Cells were plated and treated with different doses (0, 0.3, 1, 10, and 30μM). MTT assay and cell count test were performed after 48 hours of treatment. Values are means ± SEM. Means without a common letter (a-c) differ ($P < 0.05$).

Moreover, to determine if BEA has a recovery or steroidogenic effect on SSCs, the dose and time effect of beauvericin was tested on SSCs. The cell count of live SSCs and the viability test (MTT assay) was performed for the six doses (0, 0.3, 1, 3, 10 and 30µM) at the different time points (0, 12, 24 and 48 hours). Figure 11 present the dose and time effect of BEA on SSCs based on viability test and cell count panel A and B, respectively. Panel A shows a decrease in SSCs viability between 30 and 40% after the exposure to 1 and 3 µM BEA. The most decline in SSCs viability was recorded after 24 hour of BEA treatment. Similarly, figure 11 panel B shows a decrease in SSCs abundance between 20 and 30% after the exposure to 1 and 3 µM BEA. The most decline in SSCs cell count was recorded after 24 hour of BEA treatment, while after 48 hours all the concentration of treated and non-treated cell start to decline progressively. These results shows that the lower percentage viability and cell count of SSCs was recorded after 24 hours of SSCs treatment with a dose 1 and 3µM BEA.

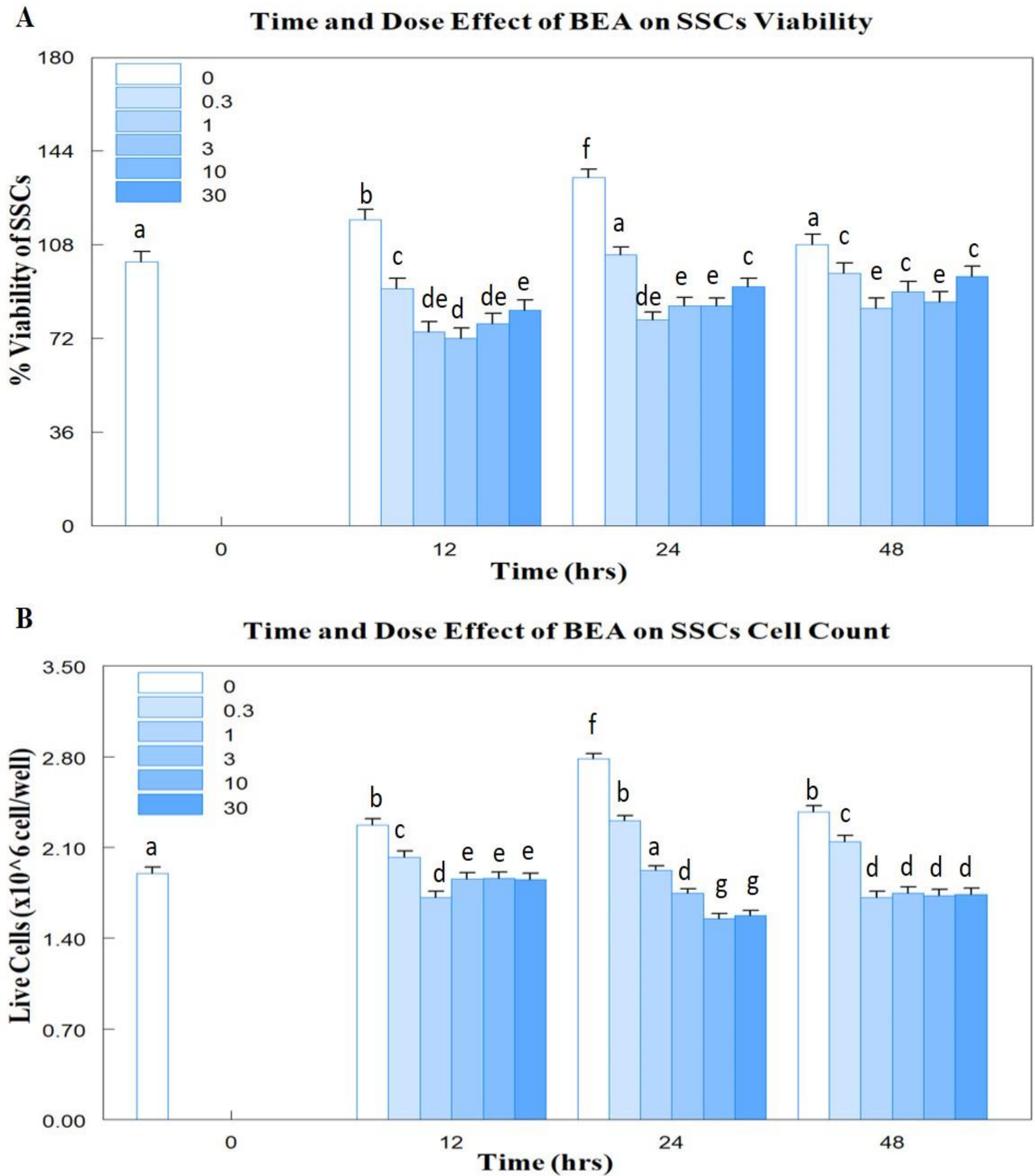


Figure 11: Time and dose effect of beauvericin on spermatogonial stem cells. Panel A: viability test (MTT Assay). Panel B: cell count. Cells were plated and treated with different doses (0, 0.3, 1, 10, and 30µM) for specific time course (0, 12, 24 and 48 hours). MTT assay and cell count test were performed for each treatment using hemocytometer. Values are means ± SEM. Means without a common letter (a-g) differ ($P < 0.05$).

## 4. Morphological analysis

Hematoxylin and eosin staining method was used to characterize the spermatogonial stem cells morphology (figure 12). Panel (A) present the morphology of non-treated spermatogonial stem cells after 24 hours of culture. It shows clusters of SSCs round or oval in shape, with a large nucleus and small cytoplasm. However, panel (B) and (C) represent the treated spermatogonial stem cells. Panel (B) treatment with 1 μM and panel (C) with 3 μM for 24 hours. In the treated SSCs panel (B&C), a morphological change was recorded by elongated and dispersed cells.

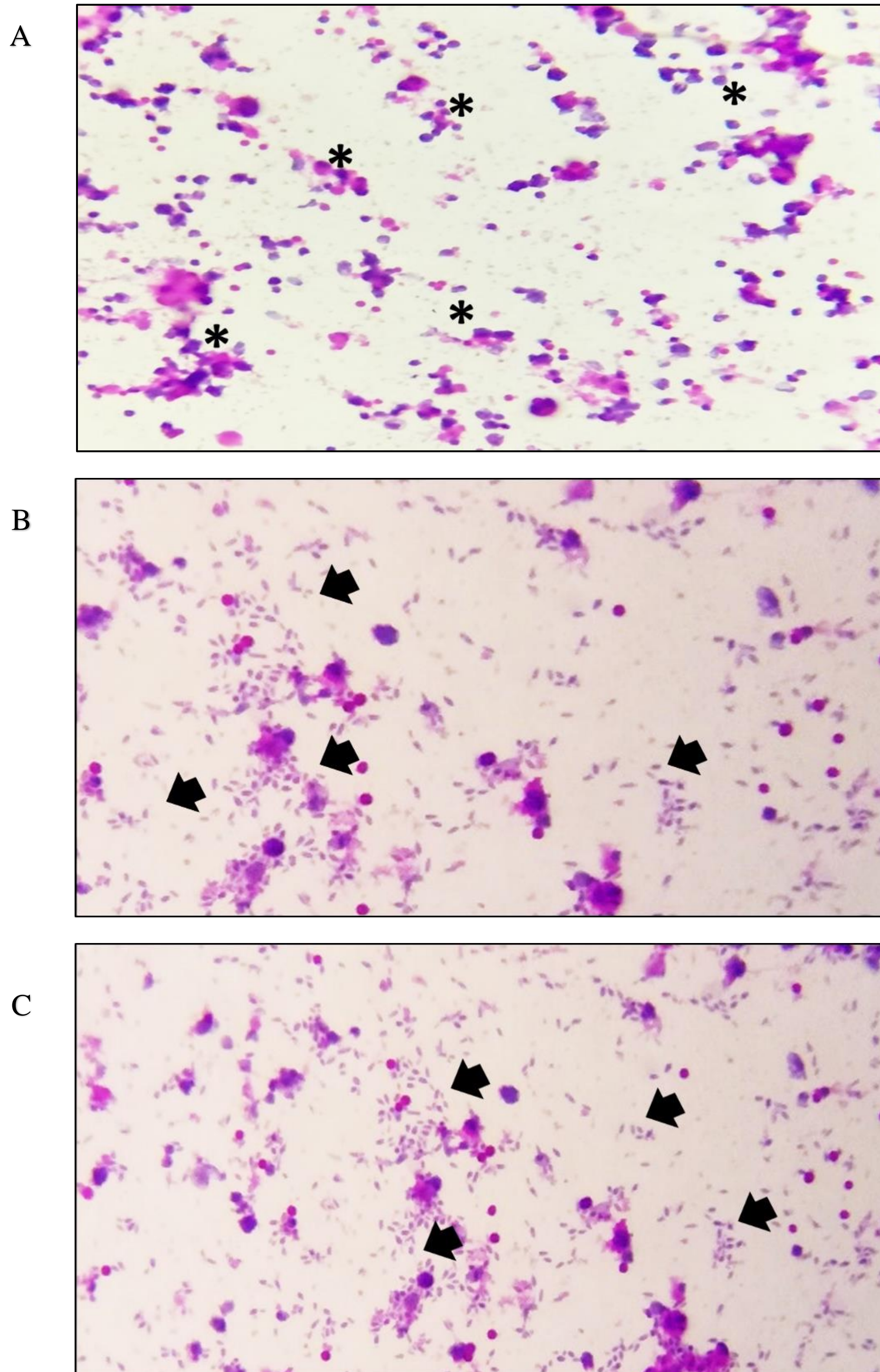


Figure 12: Characterization of SSCs using hematoxylin and eosin staining method. Non- treated SSCs (A), treated SSCs with 1 μM BEA for 24 hours (B) and SSCs treated with 3 μM BEA for 24 hours (C). Asterisk indicate normal SSCs clusters and black arrows represent SSCs morphological change.

## 5. qRT-PCR

### a- Optimization

In qRT-PCR optimization run was performed to determine the best concentration of RNA and primers for accurate results. In our experiment, different runs were performed, the first two optimization runs were with SSCs RNA, while the third run was with whole testicular RNA. Figure 13 present the amplification curves of the optimization runs based on SSCs RNA and whole testicular RNA in panel A and B, respectively. Panel A shows the amplification of the three gene, 18S after 15 cycles, Oct-4 after 28 cycles and Smad8/9 after 30 cycles, while ZBT shows no expression in SSCs. Moreover, figure 13 panel B shows the early amplification of ZBT-t in RNA extracted from whole testicular tissue after 25 cycles.

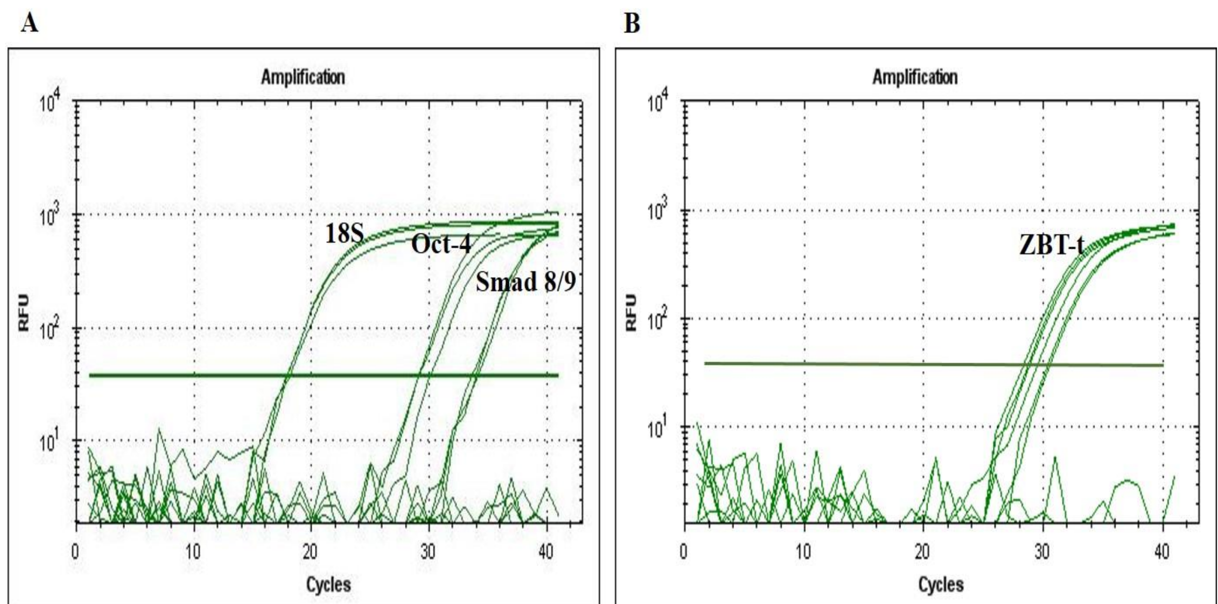


Figure 13: Amplification curves of the optimization runs based on SSCs RNA (panel A) and whole testicular RNA (panel B).

## b- Melting curve

Melting Curve analysis of all amplified gene showed the presence of one gene of interest indicating that the cycle threshold (Ct) is proportional to the amount of gene amplified by PCR as shown in figure 14.

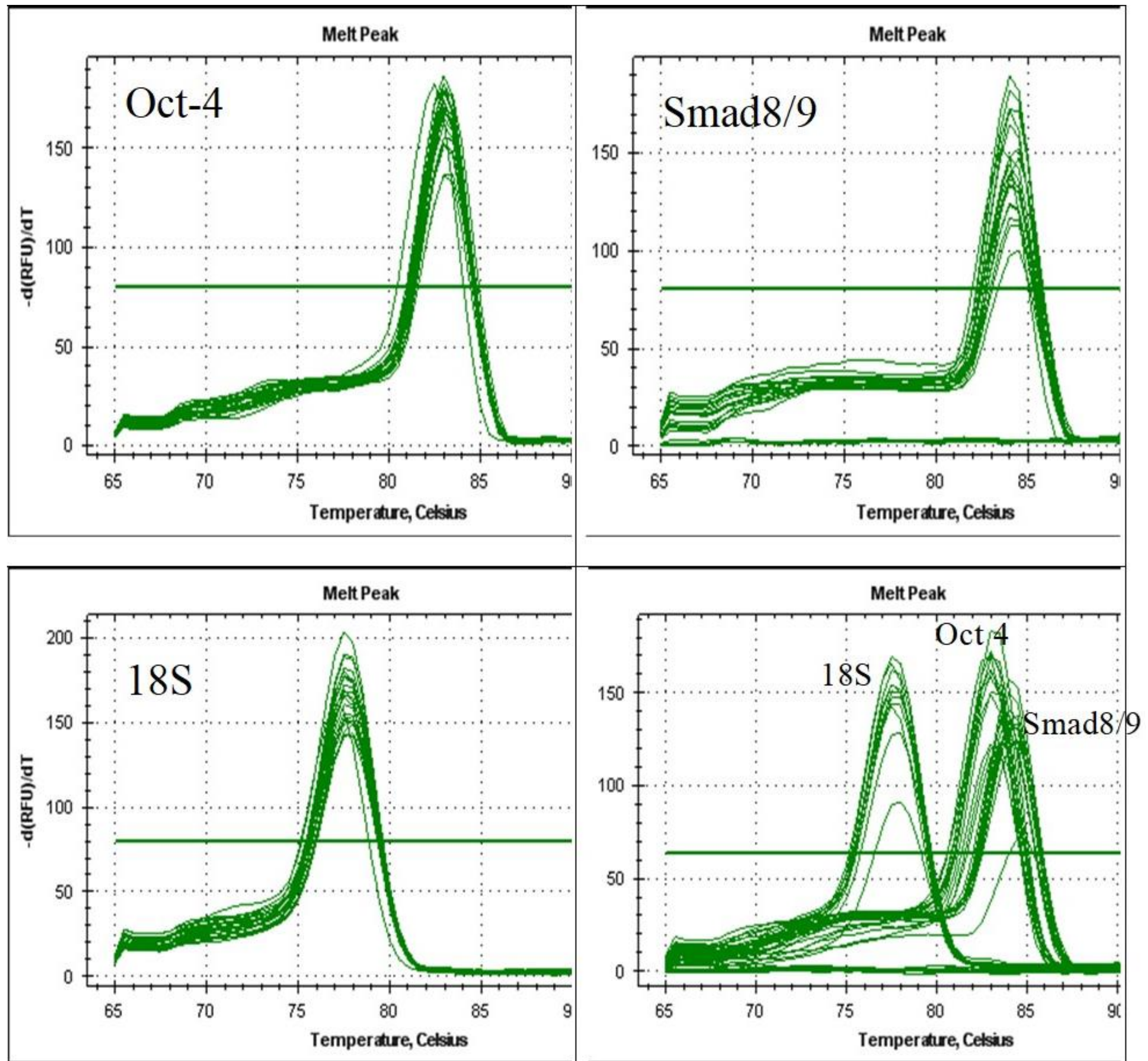


Figure 14: Melting curve analysis of Oct-4, Smad8/9 and 18S in quantitative qRT-PCR using SYBR Green. Following real time PCR quantitation, a melt curve cycle set at 65 to 95°C, increment 0.5°C 5 sec/step showed unique melting curve for Oct-4, Smad8/9, 18S and full plate.

## c- Gel electrophoresis

These results were further validated by running the PCR product on a 1% agarose gel. As shown in figure 15 were pooled amplified wells per gene showed one characteristic band as designed for Oct-4 (250 bp), Smad8/9 (200 bp), ZBT (300bp), and 18S (100 bp). Optimization of ZBT showed a lower expression in spermatogonial stem cells (as shown by later amplification signal Ct > 42 in figure 13 panel A). Doubling starting RNA or primer concentrations did not allow earlier amplification for pooled RNA extracted from SSCs. However, whole testicular RNA extract (ZBT-t) showed more ZBT RNA and signal (figure 13 panel B & 15). Highlighting the challenge in detecting ZBT signal in qRT-PCR given the adopted experimental conditions. Thus, no further analysis of ZBT in our sample was conducted.

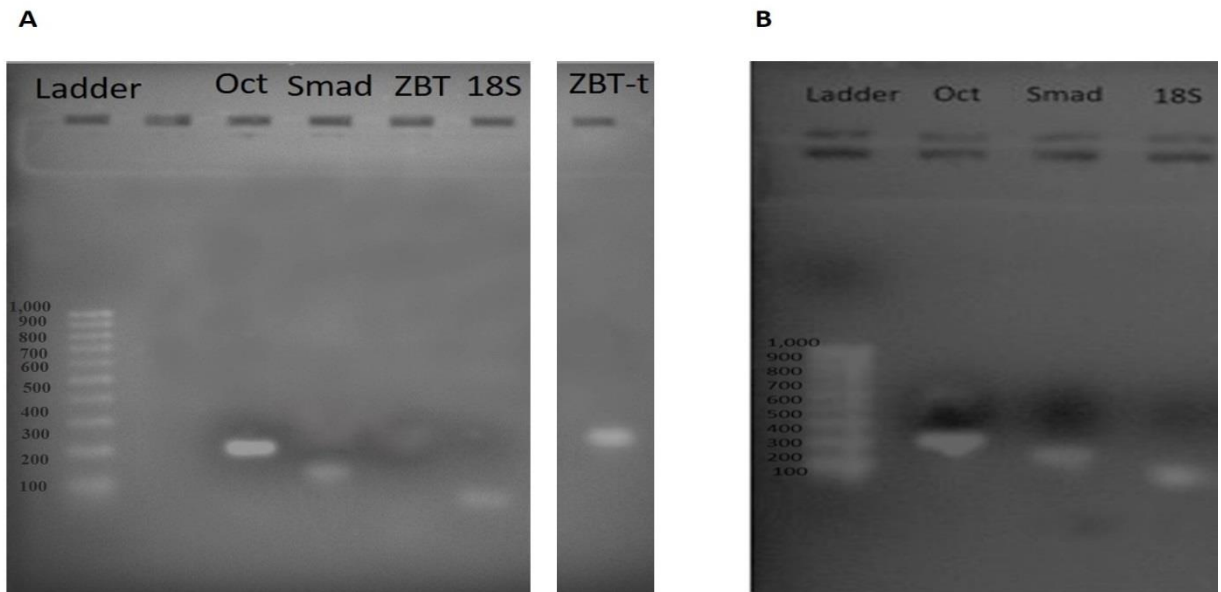


Figure 15: Gel electrophoresis showing qRT-PCR products after optimization (panel A), and after full plate run (panel B). Ladder=100bp ladder, Oct-4, Smad8/9, ZBT and 18S expressed in spermatogonial stem cells. ZBT-t expression from whole testicular RNA extract.

## 6. qRT-PCR amplification

The quantitative RT-PCR was performed for all the SSCs RNA extracted at the different time points (0, 12, 24 and 48 hours) and beauvericin doses (0, 0.3, 1, 3, 10, and 30). The results of this test qRT-PCR will determine the effect of BEA on Oct-4 and Smad8/9 expression in spermatogonial stem cell. These data of the time and dose effect of BEA on SSCs Oct-4 and Smad8/9 expression were represented in table 5 and 6, respectively.

The top two panels in figure 16 represent the fold change of Oct-4 and Smad8/9 expression in function of time in non-treated spermatogonial stem cells. Time effect showed an increase ($p < 0.05$) in Oct-4 expression after 12 hours of spermatogonial stem cell culture. Whereas, Samd8/9 expression in SSCs did not showed any significance increase or decrease with time in culture ($p > 0.05$).

Moreover, Figure 17 represent the time (0, 12, 24 and 48 hours) and beauvericin dose (0, 0.3, 1, 3, 10, and 30) effect on Oct-4 expression in spermatogonial stem cells. Top panel represents the fold change and the bottom panel is log (fold+1). It shows an increase ($p < 0.05$) in SSCs Oct-4 expression after 12, 24 and 48 hours of culture with the six different beauvericin doses, while the maximum Oct-4 expression in SSCs was recoded after 48 hours of culture. Similarly, a significance increase with a peak ($p < 0.05$) in Oct-4 expression was recorded after 12 hours of culture with a dose 10 μM.

The time (0, 12, 24 and 48 hours) and beauvericin dose (0, 0.3, 1, 3, 10, and 30) effect on Smad8/9 expression in spermatogonial stem cells were represented in figure 18. Top panel represents the fold change and the bottom panel is log (fold+1). The data showed a lower

expression of Smad8/9 in spermatogonial stem cells. Moreover, there is no significance effect of time and beauvericin dose on Smad8/9 expression in SSCs ($p > 0.05$).

Table 5: Effect of Beauvericin exposure at various dose (0, 0.3, 1, 3, 10, and 30 μM) and time points (0, 12, 24 and 48 h) on Oct-4 expression in spermatogonial stem cells. Values are presented as means ± SEM.

| Variable | Time | 0 | 12 | 24 | 48 |
|---|---|---|---|---|---|
| Ct | 0 | 32.16±1.2778 | 29.685±1.1066 | 26.75±1.565 | 26.4433±1.2778 |
| | 0.3 | | 30.63±1.1066 | 30.4133±1.2778 | 27.025±1.1066 |
| | 1 | | 29.145±1.565 | 29.0075±1.1066 | 25.7225±1.1066 |
| | 3 | | 29.7875±1.1066 | 29.9567±1.2778 | 27.3775±1.1066 |
| | 10 | | 28.53±1.565 | 28.0033±1.2778 | 28.44±1.1066 |
| | 30 | | 29.995±1.565 | 28.115±1.1066 | 26.795±1.565 |
| SCt | 0 | 10.7867±1.1404 | 13.5475±0.9876 | 10.61±1.3967 | 12.6167±1.1404 |
| | 0.3 | | 12.795±0.9876 | 12.89±1.1404 | 12.6225±0.9876 |
| | 1 | | 13.155±1.3967 | 12.3375±0.9876 | 11.8375±0.9876 |
| | 3 | | 11.95±0.9876 | 11.8267±1.1404 | 12.92±0.9876 |
| | 10 | | 15.46±1.3967 | 11.3767±1.1404 | 13.315±0.9876 |
| | 30 | | 12.17±1.3967 | 10.39±0.9876 | 10.8±1.3967 |
| DCT | 0 | 21.37±1.5835 | 16.1325±1.3714 | 16.13±1.9394 | 13.8233±1.5835 |
| | 0.3 | | 17.8375±1.3714 | 17.5233±1.5835 | 14.4±1.3714 |
| | 1 | | 15.99±1.9394 | 16.6725±1.3714 | 13.885±1.3714 |
| | 3 | | 17.84±1.3714 | 18.1333±1.5835 | 14.4575±1.3714 |
| | 10 | | 13.07±1.9394 | 16.6233±1.5835 | 15.12±1.3714 |
| | 30 | | 17.825±1.9394 | 17.725±1.3714 | 15.99±1.9394 |
| DDCT | 0 | -4.1446±1.5844 | -9.3793±1.3721 | -9.3803±1.9405 | -11.6884±1.5844 |
| | 0.3 | | -7.6764±1.3721 | -7.9918±1.5844 | -11.1132±1.3721 |
| | 1 | | -9.5285±1.9405 | -8.8439±1.3721 | -11.6314±1.3721 |
| | 3 | | -7.6757±1.3721 | -7.3809±1.5844 | -11.0559±1.3721 |
| | 10 | | -12.4423±1.9405 | -8.8895±1.5844 | -10.3938±1.3721 |
| | 30 | | -7.6884±1.9405 | -7.7911±1.3721 | -9.5198±1.9405 |
| Fold | 0 | 186.4±5134.8 | 932.48±4446.87 | 1089.96±6288.82 | 3902.42±5134.8 |
| | 0.3 | | 293.33±4446.87 | 344.87±5134.8 | 2607.32±4446.87 |
| | 1 | | 1114.31±6288.82 | 1259.42±4446.87 | 6102.76±4446.87 |
| | 3 | | 423.67±4446.87 | 185.05±5134.8 | 2170.77±4446.87 |
| | 10 | | 44107±6288.82 | 482.4±5134.8 | 2557.61±4446.87 |
| | 30 | | 235.77±6288.82 | 284.23±4446.87 | 734.74±6288.82 |
| log | 0 | 1.3633±0.4463 | 2.825±0.3865 | 2.825±0.5466 | 3.52±0.4463 |
| | 0.3 | | 2.3125±0.3865 | 2.41±0.4463 | 3.345±0.3865 |
| | 1 | | 2.87±0.5466 | 2.665±0.3865 | 3.5±0.3865 |
| | 3 | | 2.3175±0.3865 | 2.2233±0.4463 | 3.3275±0.3865 |
| | 10 | | 3.745±0.5466 | 2.6767±0.4463 | 3.1275±0.3865 |
| | 30 | | 2.32±0.5466 | 2.3475±0.3865 | 2.865±0.5466 |

Table 6: Effect of Beauvericin exposure at various dose (0, 0.3, 1, 3, 10, and 30 µM) and time points (0, 12, 24 and 48 h) on Smad8/9 expression in Spermatogonial stem cells. Values are presented as means ± SEM.

| Variable | Time | 0 | 12 | 24 | 48 |
|---|---|---|---|---|---|
| Ct | 0 | 33.3967±0.875 | 34.825±0.7578 | 34.385±1.0717 | 33.22±0.875 |
| | 0.3 | | 35.14±0.7578 | 34.1233±0.875 | 34.185±0.7578 |
| | 1 | | 34.405±1.0717 | 34.94±0.7578 | 34.29±0.7578 |
| | 3 | | 34.37±0.7578 | 35.4867±0.875 | 34.92±0.7578 |
| | 10 | | 33.73±1.5156 | 33.28±0.875 | 34.0525±0.7578 |
| | 30 | | 33.84±1.0717 | 32.6275±0.7578 | 34.785±1.0717 |
| SCT | 0 | 10.7867±1.328 | 13.5475±1.1501 | 10.61±1.6265 | 12.6167±1.328 |
| | 0.3 | | 12.795±1.1501 | 12.89±1.328 | 12.6225±1.1501 |
| | 1 | | 13.155±1.6265 | 12.3375±1.1501 | 11.8375±1.1501 |
| | 3 | | 11.95±1.1501 | 11.8267±1.328 | 12.92±1.1501 |
| | 10 | | 7.295±1.6265 | 11.3767±1.328 | 13.315±1.1501 |
| | 30 | | 12.17±1.6265 | 10.39±1.1501 | 10.8±1.6265 |
| DCT | 0 | 22.61±0.9957 | 21.2775±0.8623 | 23.77±1.2195 | 20.6±0.9957 |
| | 0.3 | | 22.345±0.8623 | 21.2333±0.9957 | 21.5625±0.8623 |
| | 1 | | 21.245±1.2195 | 22.6±0.8623 | 22.4525±0.8623 |
| | 3 | | 22.42±0.8623 | 23.66±0.9957 | 22.0025±0.8623 |
| | 10 | | 15.065±1.2195 | 21.9±0.9957 | 20.735±0.8623 |
| | 30 | | 21.675±1.2195 | 22.235±0.8623 | 23.98±1.2195 |
| DDCT | 0 | -3.0085±1.2814 | -4.338±1.1097 | -1.8439±1.5694 | -5.0139±1.2814 |
| | 0.3 | | -3.2688±1.1097 | -4.3818±1.2814 | -4.0532±1.1097 |
| | 1 | | -4.3678±1.5694 | -3.0119±1.1097 | -3.1634±1.1097 |
| | 3 | | -3.1948±1.1097 | -1.9542±1.2814 | -3.6118±1.1097 |
| | 10 | | 3.2601±1.5694 | -3.7131±1.2814 | -4.8794±1.1097 |
| | 30 | | -3.9414±1.5694 | -3.3785±1.1097 | -1.632±1.5694 |
| Fold | 0 | 8.8067±14.7211 | 30.0875±12.7489 | 6.945±18.0296 | 33.9567±14.7211 |
| | 0.3 | | 14.1075±12.7489 | 25.1967±14.7211 | 21.4475±12.7489 |
| | 1 | | 25.345±18.0296 | 13.33±12.7489 | 12.0825±12.7489 |
| | 3 | | 11.15±12.7489 | 7.79±14.7211 | 15.205±12.7489 |
| | 10 | | 22.815±18.0296 | 17.4733±14.7211 | 81.2325±12.7489 |
| | 30 | | 19.315±18.0296 | 13.7475±12.7489 | 3.45±18.0296 |
| log | 0 | 0.9633±1.6149 | 1.3325±1.3986 | 0.72±1.9779 | 1.5233±1.6149 |
| | 0.3 | | 1.0375±1.3986 | 1.3433±1.6149 | 1.25±1.3986 |
| | 1 | | 1.34±1.9779 | 0.995±1.3986 | 1.01±1.3986 |
| | 3 | | 1.015±1.3986 | 0.73±1.6149 | 1.13±1.3986 |
| | 10 | | 15.79±1.9779 | 1.1633±1.6149 | 1.5075±1.3986 |
| | 30 | | 1.22±1.9779 | 1.0675±1.3986 | 0.625±1.9779 |

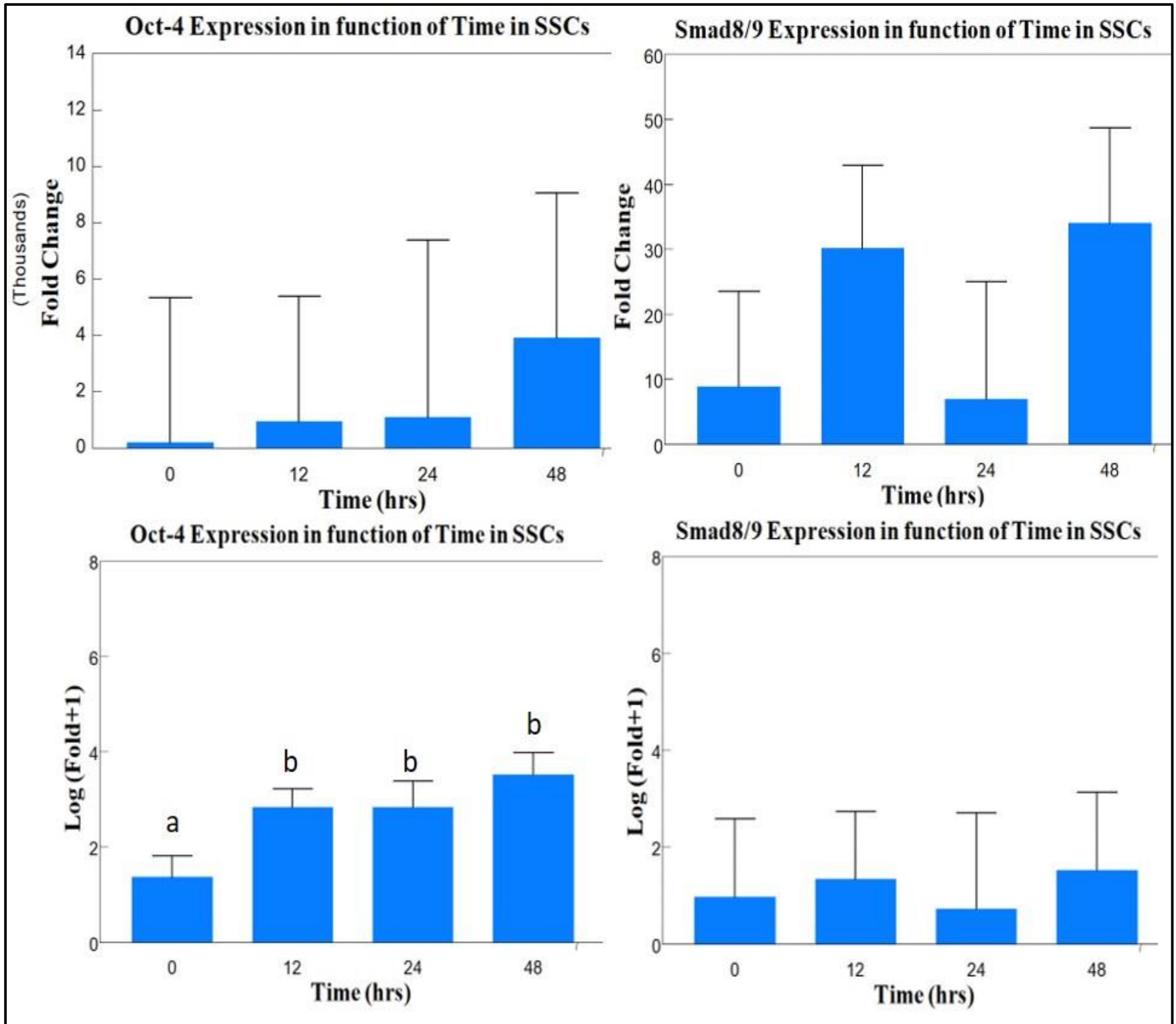


Figure 16: Oct-4 and Smad8/9 expression in non-treated spermatogonial stem cells at various durations of culture. Fold (Top Panels) and log (fold+1) (Bottom panels) of Oct-4 and Smad8/9 are presented as means ± SEM. Cells were cultured and RNA was extracted at different time points (0, 12, 24 and 48 hours). Means without a common letter (w-x) differ ($P < 0.05$).

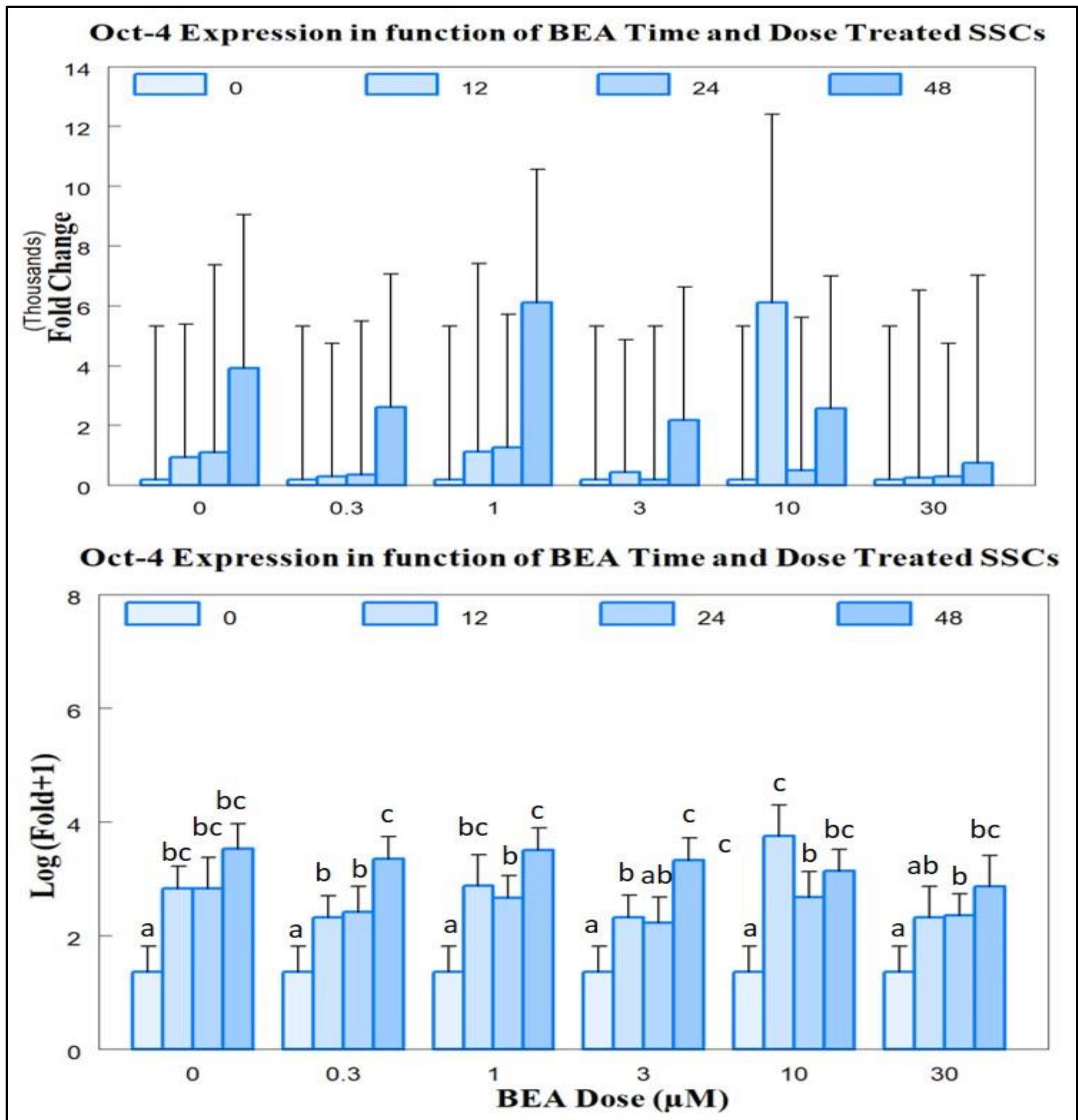


Figure 17: Time (0, 12, 24 and 48 hours) and beauvericin dose (0, 0.3, 1, 3, 10, and 30) effect on Oct-4 expression in spermatogonial stem cells. Top panel represents the fold change and the bottom panel is log (fold+1). Cells were treated with beauvericin for the specific time (0, 12, 24 and 48hours) and doses (0, 0.3, 1, 3, 10 and 30µM). The same sample for zero dose and time was used for clarity purposes. Values are means ± SEM. Statistical analysis was conducted using the log (fold+1). Means without a common letter (a-c) differ ($P < 0.05$).

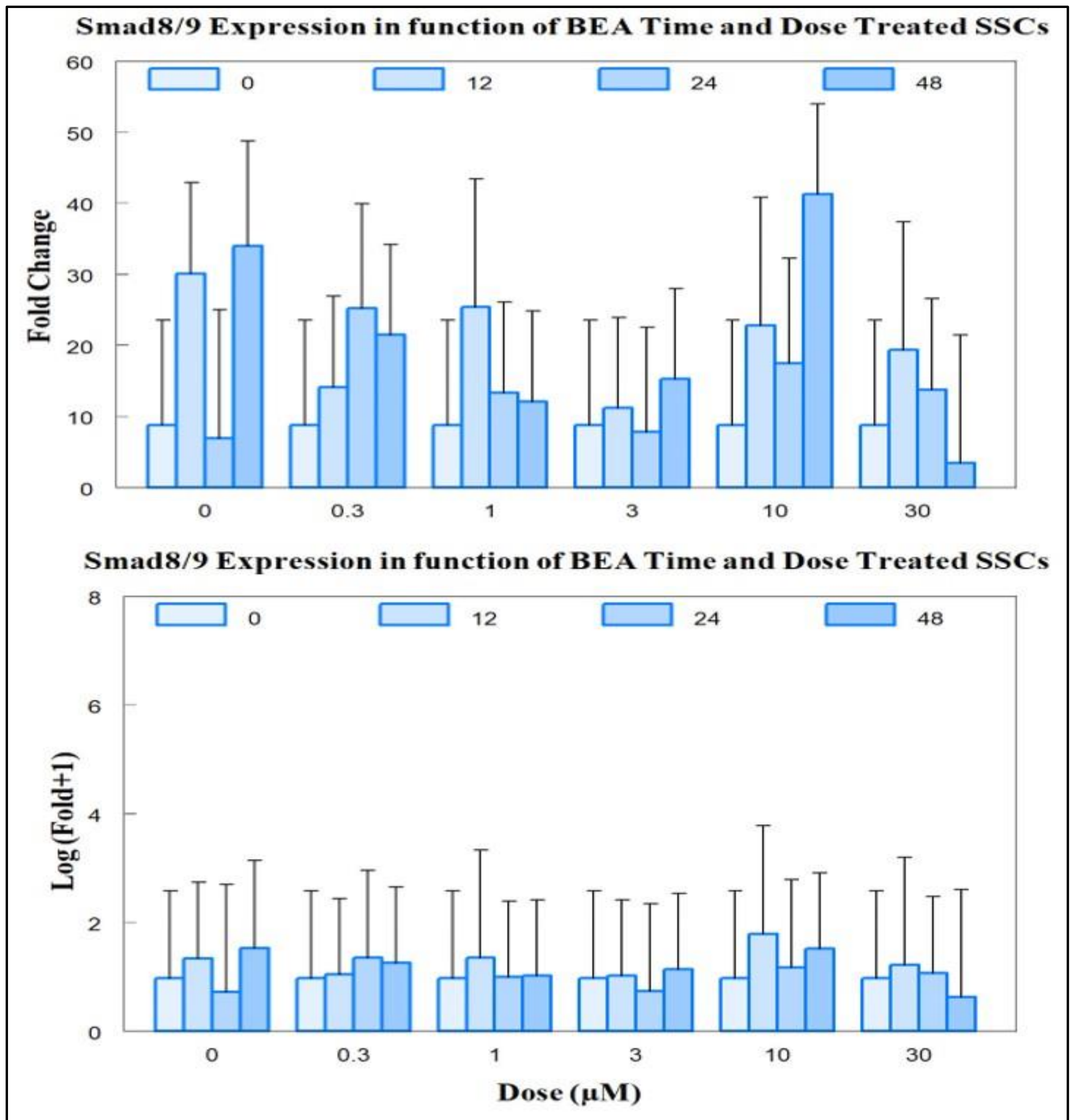


Figure 18: Time (0, 12, 24 and 48 hours) and beauvericin dose (0, 0.3, 1, 3, 10, and 30) effect on Smad8/9expression in Spermatogonial stem cells. Top panel represents the fold change and the bottom panel is log (fold+1). Cells were treated with beauvericin for the specific time (0, 12, 24 and 48hours) and doses (0, 0.3, 1, 3, 10 and 30µM). The same sample for zero dose and time was used for clarity purposes. Values are means ± SEM. Statistical analysis was conducted using the log (fold+1).

## V. Discussion and Conclusion

*In vitro* studies concerning fusariotoxins and reproductive effects have demonstrated that a key role is played by mycotoxins, either in relation to the type or amount (dosages) of these toxins (Kim et al., 2003; Sprando et al., 2005; Yang et al., 2007; Yang et al., 2010). Indeed, it was previously demonstrated that exposure to different types and doses of fusariotoxins may exert adverse effects on male germ cells viability and sperm parameters in different species, such as pigs (Gobre & Egbunike, 2008), rabbits (Ewuola & Egbunike, 2010), mice (Yang et al., 2007; Yang et al., 2010) and rats (Kim et al., 2003; Sprando et al., 2005). For this reason, choosing a new model "the sheep" was a big challenge in the present study, especially that the isolation of spermatogonial stem cells (SSCs) from sheep was not done before. In addition, BEA was tested on granulosa cells (Albonico et al., 2017) but never on sperm cells especially SSCs.

In order to optimize sheep SSCs isolation and culture, different extraction and culture runs were performed with fine-tuning measures added gradually. Some steps of the final protocol were inspired by the protocol of isolation of goat SSCs (Pramod and Mitra, 2014). Moreover, in this present study the growth curve of SSCs shows that proliferation and viability reached a maximum peak after 24 hrs of culture followed by a gradual decline, as already reported in human SSCs (Liu et al., 2011).

For the first time, we showed that BEA decreased SSCs proliferation and viability after 24 hours of treatment with a 1 or 3µM. Moreover, our results showed that cell viability was correlated with proliferation in SSCs treated with BEA. BEA modified the morphology of SSCs at a concentration of 1 or 3µM after 24 hrs of treatment, from normal SSCs, round to oval in

shape with cell clusters to elongated and dispersed cells maybe starting to differentiate. On the contrary, Oct-4 and Smad8/9 expression in SSCs was not affected by BEA dose and time effect.

Similar results were previously obtained for other mycotoxins. For example, single intraperitoneal dose of zearalenone (5mg/kg) induces testicular germ cell apoptosis in a time dependent pattern (Kim et al., 2003). Moreover, germ cell degeneration was recorded in male rats subjected to deoxynivalenol (2.5&5 mg/kg) (Sprando et al., 2005). Also, it was demonstrated that the fusarium mycotoxins BEA and FB1 may impair reproductive function in cattle by decreasing proliferation, steroid production and gene expression in bovine granulosa cells (GC) (Albonico et al., 2017). In addition, it was reported that ZEA decreased the development of the egg by affecting the epigenetic modifications (Zhu et al., 2014a). Naturally occurring DON, ZEA and AF have effects on epigenetic changes in mice oocytes, which may be one of the explanations for reduced oocyte growing competence (Zhu et al., 2014b). DON exposure reduced porcine oocytes maturation capability through affecting cell cycle and epigenetic modifications (Han et al., 2016b).

The present study shows doubling in SSCs Oct-4 expression after 12 hours of spermatogonial stem cell culture, confirming our results as already reported in previous studies about Oct-4 expression in SSCs during differentiation until primary spermatocyte stage (Ibtisham et al., 2017; Dissanayake, 2018). On the contrary, Samd8/9 expression in SSCs was lower that Oct-4 and did not showed any increase after 48 hrs of culture. Supporting the purity of SSCs isolated during the experimental protocol used.

In conclusion, results of the present study indicate that the growth of spermatogonial stem cells in culture reaches a maximum peak at 24 hrs then start to decline progressively with the high expression of Oct-4 and low expression of smad8/9. Another gene, ZBT was found

with a lower expression in SSCs RNA compared to testicular RNA. Although, one of the fusarium mycotoxin, BEA reduces SSCs viability, proliferation and alters the morphology of the cells. Further studies are needed to better elucidate the mechanism of action with the epigenetic effect of BEA on SSCs. Additional work using *in vivo* models of BEA exposure are required to determine the maximum tolerable daily intake. As well, the exact mechanism and effect of BEA on steroidogenesis remain to be determined.

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