

October 2020

EFFECT OF CERATONIA SILIQUA AND CUCURBITA PEPO SEEDS EXTRACTS ON SPERMATOGENESIS IN MALE MICE

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Recommended Citation

ElAkhdar, Nour; Bawab, Roweida; and Borjac, Jamilah (2020) "EFFECT OF CERATONIA SILIQUA AND CUCURBITA PEPO SEEDS EXTRACTS ON SPERMATOGENESIS IN MALE MICE," *BAU Journal - Health and Wellbeing*: Vol. 3 : Iss. 1 , Article 7.

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EFFECT OF CERATONIA SILIQUA AND CUCURBITA PEPO SEEDS EXTRACTS ON SPERMATOGENESIS IN MALE MICE

Abstract

Worldwide, a percentage close to 50% of infertility cases is due to male-related problems including spermatogenesis defects, the process that determines sperm cells' number and thus male fertility. Spermatogenesis is controlled by different hormones including the luteinizing hormone (LH), the follicle stimulation hormone (FSH), and testosterone. LH stimulates the Leydig cells to synthesize testosterone. FSH acts synergistically with testosterone. There is no current efficient treatment for spermatogenesis defects and people are reverting to natural remedies among which are the pumpkin and carob seeds. This study aims to evaluate the effects of pumpkin and carob seeds' aqueous extracts on spermatogenesis and different sperm parameters in Balb/c mice. Carob seed (2%) and pumpkin seed (8%) aqueous extracts were prepared then administered to six mice groups by gavage over a period of 10, 21 and 35 days. Sperm parameters were determined. The levels of LH, FSH and testosterone were measured by ELISA. The expression levels of PLC_{z1}, Rhox 5 and Ras genes were determined by RT-PCR. Histological studies of the testis seminiferous tubules were performed. Results were statistically analyzed using GraphPad Prism. The results showed that both seed extracts have no effect on the onset of puberty. In mature mice, they caused an increased in LH, FSH and testosterone levels. They also caused an increase in the expression levels of PLC_{z1}, Rhox 5 and Ras genes. The experiment proved the effectiveness of both seeds extracts as male fertility promoters improving spermatogenesis.

Keywords

Ceratoniasiliqua, Cucurbitapepo;LH; FSH; testosterone; spermatogenesis; fertility

1. INTRODUCTION

Spermatogenesis is a well-organized and coordinated process where the number of spermatozoa produced in the seminiferous tubules of the testis determines male fertility (de Rooij DG & Russel LD, 2000). The testis acts as an endocrine organ where it synthesizes the needed testosterone required for normal spermatogenesis and male characteristics (Tsai et al., 2006). Spermatogenesis relies mainly on the paracrine and autocrine communication among the Leydig, Sertoli, peritubular myoid cells, and germ cells of the testis where each type of these cells performs its own function leading to a well-coordinated process at different stages (Suede SH, Malik A & Sapra A, 2020).

Spermatogenesis depends mainly on the spermatogonial stem cells that differentiate into spermatogonia, spermatocytes, and spermatids. This process is regulated through a network of hormonal systems including Testosterone, LH and FSH. Testosterone has many important functions starting with the progression of germ cells beyond meiosis, spermiogenesis and the release of mature spermatids (Mclachlan, 2002). It aids in the maturation of spermatocytes and in the progression of round to elongated spermatids, formation of the gonads, the differentiation of the testicles (Ruwanpura, Mclachlan, & Meachem, 2010), and survival of spermatocytes and spermatids (Tapanainen, Tilly, Vihko, & Hsueh, 1993). Testosterone acts directly to change gene expression via the classical pathway, or indirectly via the non-classical pathway by activating kinases that control processes important for the maintenance of spermatogenesis (Toocheck et al., 2016). In the classical pathway, testosterone binds specific response elements to activate or repress targeted genes

(Verhoeven, Willems, Denolet, Swinnen, & Gendt, 2010). In the non-classical pathway, testosterone leads to $[Ca^{2+}]$ influx into Sertoli cells affecting gene transcription required by germ cells, the stability of the cytoskeleton and the junctions between Sertoli cells and germ cells, in addition to enabling the mobility and development of germ cells (Loss et al., 2004). It can also cause the activation of a series of kinases in Sertoli cells that in turn regulates and controls spermatogenesis (Cheng & Mruk, 2002).

In addition to testosterone, FSH and LH act as survival factors for spermatogonia where they regulate the internal apoptotic pathway and play a role in the proliferation and survival of the germ cells (Verhoeven, Willems, Denolet, Swinnen, & Gendt, 2010). LH plays an important role in stimulating Leydig cells to synthesize testosterone. FSH has more significant roles where it acts synergistically with testosterone. It acts by binding to its G protein-coupled FSH receptors (FSHRs) found on the Sertoli cells membrane (Walker, 2011) leading to the activation of protein kinase A, MAP kinase, phosphatidylinositol 3-kinase (Heckert, 2002). These in turn will activate cAMP responsive element (CRE)-binding protein (CREB), causing the transcription of genes essential for spermatogenesis. Moreover, FSH has an essential job in regulating the size and the proliferation of the Sertoli cell population which is important for the sperm output. In addition, FSH helps in the germ cells survival and proliferation, interferes in the development, maturation, survival and migration of gonocytes (Walker & Cheng, 2005).

Different genes interfere in the process of spermatogenesis including the Rhox 5 homeobox, phospholipase C zeta 1, Ras, Ink4c and Ink4d, ARID4B and many others. Rhox 5 homeobox encodes a transcription factor that is mainly expressed in Sertoli cells and is important for male germ cell survival (Krishnamurthy, Danilovich, Morales, & Sairam, 2000; Hu et al., 2010). The Ras gene that also affects spermatogenesis encodes the Ras protein that acts as molecular switches in signal transduction cascades that interfere in many processes in cell proliferation, differentiation and apoptosis. PLCz1 plays a vital role in the secretion of products required by germ cells as well as enabling their mobility and development rather than activating the testosterone-mediated $[Ca^{2+}]$ influx pathway (Maclean et al., 2005).

These factors have important effects on fertility where the lack of any of them may lead to male subfertility or infertility. Infertility is described as the failure of a couple to have a baby after one year of sexual intercourse without the use of any contraception, whereas subfertility is the reduced fertility that may need therapy for successful conception. Infertility is a worldwide problem that affects 15- 20% of couples which is about to 48.5 million couples. Males are responsible for ~ 50% of infertility cases (Nozawa, Satouh, Fujimoto, Oji, & Ikawa, 2018), (Agarwal, Mulgund, Hamada, & Chyatte, 2015). There are many causes of infertility that can be social, environmental, chromosomal or genetic. Many cases can be treated by hormonal therapy which should mainly include anti-estrogens, or estrogen receptor modulator combined with

testosterone undecanoate (TU), artificial insemination, in-vitro fertilization or spermatogonial stem cell transplantation therapy that has the potential to have wide clinical applications, e.g. in degenerative diseases of the testes, such as Klinefelter syndrome (Shiraishi & Matsuyama, 2017), (Li, 2014). These approaches are known to be expensive with low efficiency and high risk of side effects. This has raised the demand to search for natural remedies that are cheap, more efficient, and with less or no side effects (Ekor, 2014.)

Pumpkins are gourd squashes belonging to the genus *Cucurbita* and the family Cucurbitaceae. They have been used in diverse medicinal applications (Aggarwal & Kotwal, 2009), (Alarcon-Aguilar et al., 2002). They are rich in vitamins (A, B1, B2, C and E) and minerals such as potassium, phosphorus, sodium, calcium, magnesium, copper, zinc, iron, selenium, and manganese (Kikuchi, Takebayashi, Shinto, Yamada, & Tanaka, 2013), (Sedigheh, 2011), (Kikuchi, Takebayashi, Shinto, Yamada, & Tanaka, 2013). They are also a rich source of starch, phenols, polysaccharides, proteins, fibers, triterpenes, carotenes and fatty acids mainly linoleic acid, palmitic acid, stearic acid and oleic acid. They contain flavonoids, saponins, sterols, phytosterols, pectin, carotenoids, tocopherols and tocomonoenols (Nishimura, Ohkawara, Sato, Takeda, & Nishihira, 2014), (Kikuchi, Takebayashi, Shinto, Yamada, & Tanaka, 2013), (Saavedra et al., 2013), (Kikuchi et al., 2014), (Wawrzyniak et al., 2014), (Montesano, Blasi, Simonetti, Santini, & Cossignani, 2018). Due to their active ingredients, nutritional and health protective value, pumpkins, especially their seeds, have received considerable attention (Stevenson et al., 2007). Studies have shown the health properties of pumpkin seeds against many diseases, mainly diabetes, hypertension, and cancer (Bardaa et al., 2016), they aid in reducing skin damage, reducing the risk of developing cataracts, slowing the aging process, and preventing tumor growth. They also have antioxidant, antibacterial, and anti-inflammatory characteristics. Studies have shown also that pumpkins can aid in the prevention and in the treatment of prostate cancer and their lipid components are important in reducing bladder and urethral pressure and urinary tract diseases (Montesano, Blasi, Simonetti, Santini, & Cossignani, 2018.)

On the other hand, Carob, *Ceratonia siliqua* L., is an evergreen tree with a maximum height of 10 m and a thick brown trunk abundant in Lebanon and some other locations in the Middle East. Its pulp is rich in total sugars (around 50% by mass) and contains a high percentage of cellulose. It also contains minerals (sodium, potassium, iron, calcium and phosphorus), vitamins (B6, D, C, E, niacin, folic acid), tannins (16-20%), saturated and unsaturated lipids, and rich in essential and non-essential amino acids (Youssef, El-Manfaloty, & Ali, 2013), (Vafaei et al., 2018), (Battle & Tous, 1997). As for the seed, it is found to be encased with a white translucent galactomannan-rich layer that contains the endosperm that is able to produce viscous solutions when concentrations are relatively low (Zemouri, Djabeur, Frimehdi, Khelil, & Kaid-Harche, 2020.)

On a medicinal level, carob is characterized by its humongous benefits to health; it has anti-cancer, antibacterial, antidiabetic, antidiarrheal and strong antioxidant effects. Studies have also shown that carob can protect the GI tract against alcohol consumption (Vafaei et al., 2018). Its polyphenol, tannin and fiber content has allowed it to be a good prophylactic against colon cancer (Mokhtari, Sharifi & Sh, 2012). It has also been postulated that it holds several benefits to spermatogenesis witnessed mainly through the sperm parameters and their related biochemical indicators (Azab, 2017). The carob seed in particular, and mainly its peel has proven great efficiency in mood disorders therapy as well as confirmed antioxidant properties (Lakkab et al., 2019). The locust bean gum, produced from the seed endosperm has also been used as an appetite suppressant, and as a natural way to control or even treat high cholesterol levels in the plasma. It is also used to treat recurrent infant reflux due to the viscosity that it adds to food (Dakia, 2011).

As a result of the beneficial uses of pumpkins and carobs in treating many diseases and the important components they contain, this study aims to evaluate the effect of pumpkin and carob seeds on spermatogenesis in male mice.

2. MATERIALS AND METHODS

2.1 Extract Preparation

Mimicking folk medicine, a 2% carob extract and an 8% pumpkin extract were prepared by boiling 2 grams of ground carob seeds and 8 grams of ground pumpkin seeds respectively in 100 mL of water for 5 minutes. Extracts were left to simmer before filtration. Filtrate were aliquoted and stored at -20°C for weekly use.

2.2 Animals

Healthy immature (2-3 weeks old) and mature (4-6 weeks old) male albino Balb/c mice were obtained from Beirut Arab University animal house. Mice were placed in an air-conditioned room with temperature of 22-25 °C and photoperiod of 12Light:12Dark. The mice were left to acclimate for one week before initiating the experiments. All animals were carefully maintained under standard animal house conditions with free access to standard pellet diet and water. Experimental procedures were carried out in accordance with the guidelines of the Institutional Review Board at Beirut Arab University with an approval number 2019A-0037-S-R-0296.

2.3 Animal Treatment

Mice were randomly divided into experimental groups of 6 mice each. Carob and Pumpkin aqueous extracts were administered to mice by gavage (gavage needle: Popper®, 7F 05785 G). at 100 and 600 mg/kg of body weight respectively over a period of 21 and 35 days. Respective control groups received water. Mice were sacrificed after treatment. Blood samples were withdrawn through cardiac puncture and stored in Vacurate® Vacuum blood collection tubes then centrifuged at 2800 x g for 10 minutes. Serum was aliquoted and stored at -20°C. Testis were removed for histological and molecular analysis. Epididymis were removed for sperm parameters measurements.

Table 1: Description of treatment taken by each study group.

Group	Description
1	Mature mice supplied with water and considered as control for groups 2 and 3.
2	Mature mice given 100 mg/kg of body weight of carob seeds extract over a period of 21 days.
3	Mature mice supplied with 600 mg/kg of body weight of pumpkin seeds extract over a period of 21 days.
4	Mature mice supplied with water and considered as control for groups 5 and 6.
5	Mature mice supplied with 100 mg/kg of body weight of carob seeds extract over a period of 35 days.
6	Mature mice supplied with 600 mg/kg of body weight of pumpkin seeds extract over a period of 35 days.

2.4 Sperm Count

The right cauda epididymis was removed and homogenized in 1 ml of PBS buffer at 37°C. The suspension (0.5 mL) was then mixed with 0.5 mL of 10% formalin. The mixture (10 µL) was then transferred into a hemocytometer for sperm count. The number of sperm heads were observed under a light microscope at 40x magnification. The sperm number that were resistant to the homogenization was estimated by counting five quadrants and the average number was multiplied by a standard factor of 10⁶.

2.5 Sperm Motility

From the sperm suspension, 10µL were placed onto a clean glass slide whose temperature was kept at 37°C, then covered with a warm cover slip. The sperms were observed under a light microscope at 40x magnification and reduced light exposure. The progressive and the immotile sperms were counted. Later, the motile sperm percentage was calculated using the formula:

$$\text{Mobile Sperm Percentage} = \frac{\text{number of motile sperms}}{\text{total number of sperms}} \times 100$$

2.6 Sperm Viability

Sperm viability was assessed using trypan blue exclusion assay. Sperm suspension (10 μ L) were mixed with an equal volume of trypan blue solution. Following Moskovtsev, S.I., & Librach, C.L.'s method, one drop of the mixture is then transferred onto a microscope slide and smeared (Moskovtsev & Librach, 2013). Sperms were examined under a light microscope at 40x magnification. Live spermatozoa exhibit no staining, while dead ones are stained blue indicating cell death.

2.7 Histology

After sacrificing the mice, the testes were preserved in 10% formalin, sent to Hammoud Hospital Medical Centre in Saida, Lebanon for histological analysis. Samples were dehydrated in 70-90% graded alcohol, then cleared in xylene and fixed on paraffin. Sections were obtained by longitudinal cutting of 5 to 6 mm using a microtome. Slides were stained with hematoxylin and eosin (HE). All slides were observed under a light microscope and images were recorded using a visualization software under optimum brightness and contrast settings at 40x and 100x magnification.

2.8 Hormone Measurement

Determination of LH (Cat # abx154345), FSH (Cat # abx154038) and Testosterone (Cat # abx257935) levels was performed using ELISA kit based on manufacturer's recommendations. This absorbance of the final reaction was measured at 450 nm and the concentration of the different samples was determined.

2.9 Gene Expression

The expression levels of Phospholipase C, Ras, Rhox 5 genes was measured by quantitative real time polymerase chain reaction (q-RT-PCR) using QuantiFast® SYBR® Green PCR Kit (catalog # 204045, QIAGEN®, USA) according to manufacturer's instructions after RNA purification using RNeasy Plus Mini Kit (Cat #: 74134 and 74136), and cDNA synthesis using QUANTI TECT Reverse Transcription Kit (Cat #: 205311). The q-RT-PCR reaction consisted of 2x QuantiFast SYBR Green PCR Master Mix, 2 μ L of template cDNA, 2 μ L of the primers shown in table 1 (1 μ M), and 6 μ L RNase-free water were mixed in a final volume of 20 μ L PCR reaction mixture. The amplified product was visualized on 2% agarose gel electrophoresis.

Table 2: Sequence of the primers used with their melting temperature

	Sequences	Tm °C	Ref
Phospholipase C	Forward 5'-TAGACGAAGAGCCCTCTATG-3'	55.57	[14]
	Reverse 5'-GTGCGAACCTTGAACCTTCC-3'	59.41	
Ras	Forward 5'GCTGTAGAAGCTATGACAGAATAC-3'	57.00	[43]
	Reverse 5'-GCTGTGTCTAAGATGTCCAGTAG-3'	58.32	
Rhox 5	Forward 5'-GCCTGGGAGTCAAGGAA-3'	55.49	[44]
	Reverse 5' -ATTCTTCTCCTTCGCCTTC-3'	54.58	
GAPDH	Forward 5'TGGTGCTCAGGTAGCCCAG3'	64	[45]
	Reverse 5'GGACCTGACCTGCCGTCTAG3'	66	

The mean Ct values was determined from each triplicate assay. The gene expression levels were measured by normalizing each gene's threshold cycle (Ct) to the Ct of glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene. Negative controls containing RNase-free water instead of template cDNA for each primer sets primer were run to ensure that the absence of non-specific PCR products.

Δ Ct value was determined as the difference between the Ct of the gene of interest and the Ct of GAPDH. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ gene dosage ratio formula (GDR) where: $\Delta\Delta Ct = (mCt \text{ gene of interest} - mCt \text{ GAPDH}) \text{ test sample} - (mCt \text{ gene of interest} - mCt \text{ GAPDH}) \text{ control sample}$.

2.10 Statistical Analysis

All statistical analyses were performed with the standard statistical program Graph Pad Prism Ver. 7 using sample t-test. The data were expressed as mean \pm standard deviation. The statistical significance was set at $p < 0.05$

3. RESULTS

3.1 Effect of Carob and Pumpkin Seeds on Spermatogenesis

The administration of the aqueous extracts of carob and pumpkin seeds to immature mice caused no visible changes at the histological level compared to control (Figure 1A). Both groups showed low number of sex cells and undetectable mature spermatocytes.

However, in mature mice, both the pumpkin and carob extracts induced an increase in sex cells in all stages particularly in the number of mature sperms and spermatids compared to untreated ones after 21 days of treatment (Figure 1B).

After 35 days, the effect of both extracts was enhanced further with higher increase in the number of mature sex cells in treated mice versus control (Figure 1C)

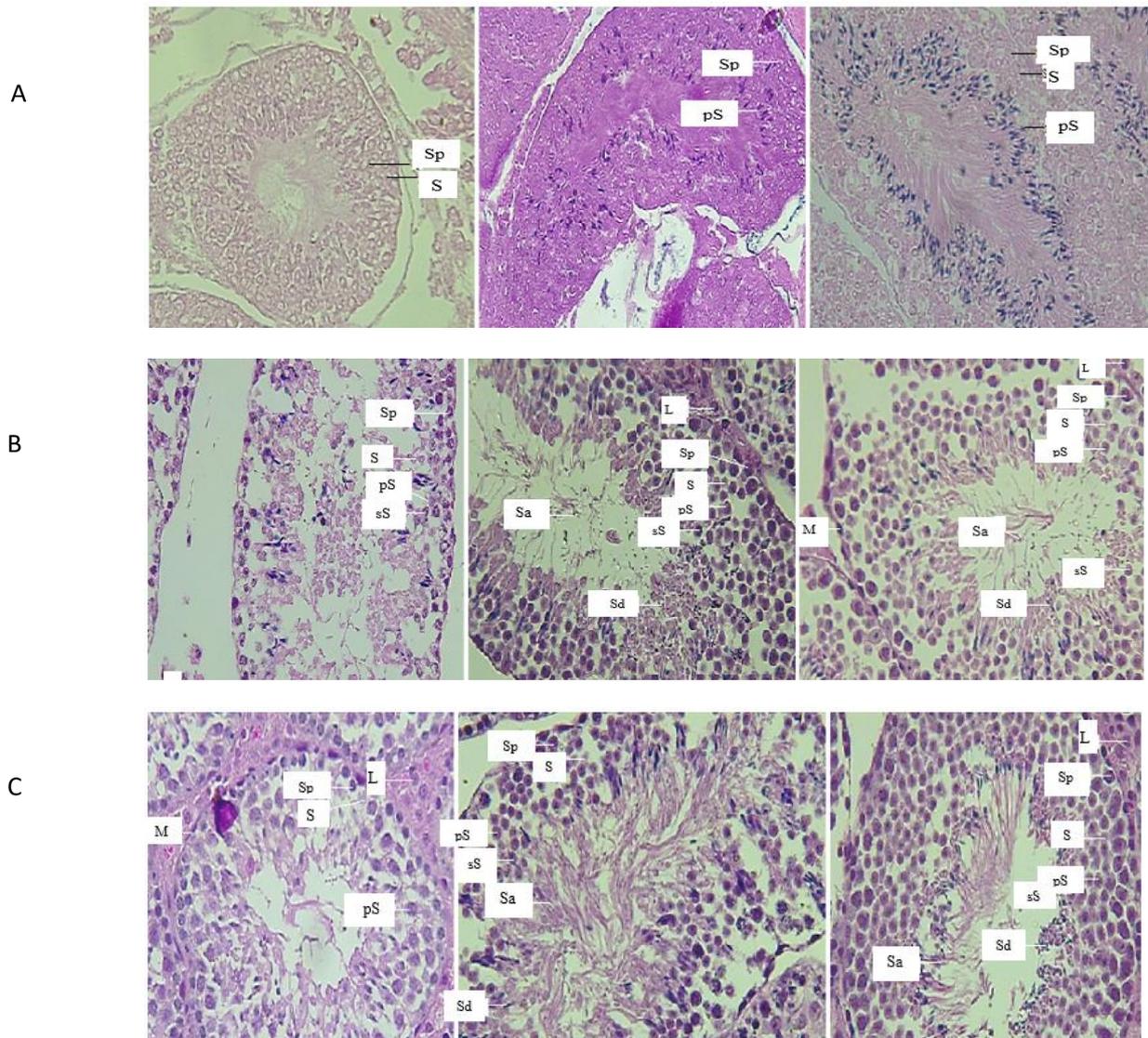


Fig.1: Histological micrographs of testes of immature mice (Panel A), mice treated for 21 days (Panel B), and mice treated for 35 days (Panel C) (H&E 400 x). Left: Control mice. Middle: Pumpkin treated mice. Right: Carob treated mice. Sp: Spermatogonium, S: Sertoli cell, pS: Primary spermatocyte, sS: Secondary spermatocyte, L: Leydig cell, Sa: Spermatozoa, Sd: Spermatids

3.2 Effect of Pumpkin and Carob Extracts on Sperm Count, Motility and Viability

The increase in the mass of the seminiferous tubules and the in the number of spermatocytes and even spermatozoa confirmed by the histological analysis imply active spermatogenesis and increase in testosterone production, two factors involved in the increase in sperm count. The effect of both extracts on sperm count was assessed. Figure 2 shows the increase in sperm count observed after 21 and 35 days of extract treatment in adult mice. A significant increase in sperm count of 40% (P value 0.0145) and 175% (P value 0.0053) was induced by pumpkin seeds extract treatment after 21 days and 35 days compared to their control groups respectively. Similarly, treatment with the carob seed extract caused a significant increase in sperm count of 136.5% (P value 0.0006) and 222.9%, (P value 0.0005) after 21 days and 35 days respectively compared to their control groups.

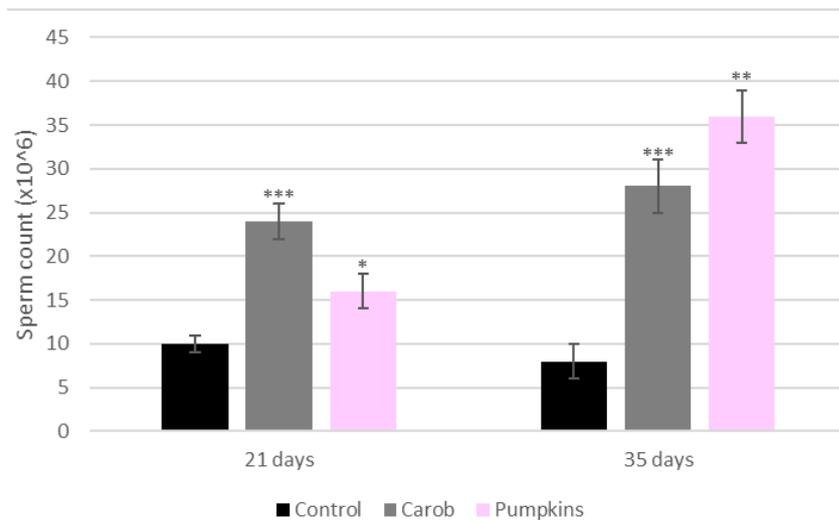


Fig.2: Effect of pumpkin seeds and carob seeds extracts on sperm count of Balb/c mice. Data represented is the average of 6 per groups with their relative controls \pm SD. Asterisks on bars represent significance relative to its respective control. (**) corresponds to $P < 0.01$ and (*) corresponds to $P < 0.05$.

However, no significant changes were observed in the sperm motility and viability.

3.3 Effect of Pumpkin and Carob Extracts on Hormonal Levels

Both extracts induced a significant increase in testosterone level. Mice treated for 21 and 35 days with pumpkin extract exhibited significant increase in testosterone levels of 45% and 67% (P values 0.0009, and 0.0014) respectively compared to their respective controls. As for those treated with carob seed extracts, significant increase in testosterone levels of 49.1% and 67.5% after 21 and 35 days respectively (P value 0.002 and 0.017) was also observed compared to their control groups as shown by Figure 3 A.

After 21 days, the effect of carob extract on the levels of the Luteinizing hormone was significant shown in Figure 3B and showed a higher increase after 35 days compared to their control groups. Whereas no significant increase was observed in pumpkin treated mice neither after 21 days nor after 35 days.

Carob seeds extract induced also a significant increase in FSH levels 4% (P value 0.024) after 21 days of treatment compared to their control groups but pumpkin seeds didn't cause significant increase. However, after 35 days of treatment, both extracts caused a significant increase compared to their control groups.

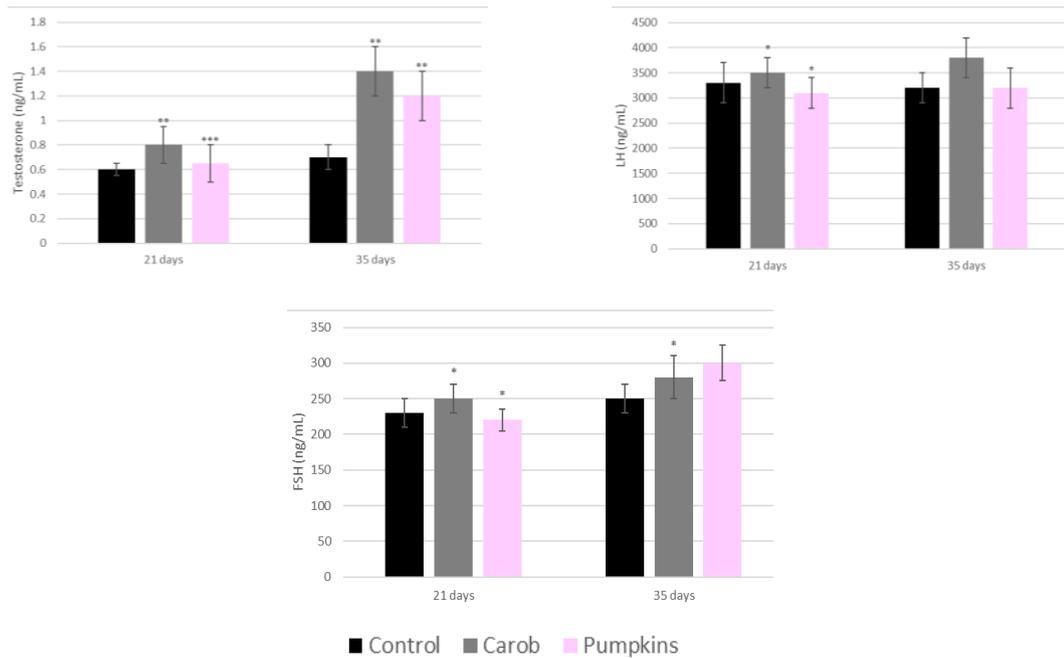


Fig.3: Effect of pumpkin and carob seeds extracts on testosterone level (A), LH levels (B) and FSH levels (C) in Balb/c mice. Data represented is the average of 3 determinations \pm SD. Asterisks on bars represent significance relative to its respective control. (***) corresponds to $P < 0.001$, (**) corresponds to $P < 0.01$ and (*) corresponds to $P < 0.05$.

3.4 Effect of Pumpkin and Carob Extracts on the expression Levels of Spermatogenesis-Related Genes

The increase in testosterone caused an increase in the expression levels of the three studied genes involved in testosterone-mediated pathways that result in the upregulation of spermatogenesis. The expression level of PLCz1 gene significantly increased after 21 and 35 days of pumpkin extract treatment by 1.8 and 3.8 folds respectively (P values 0.0026 and 0.0091) compared to their control. Pumpkin extract also induced a significant increase in Ras gene expression in mature mice treated for 21 and 35 days of 1.3 fold (P value 0.001) and 2.6 fold (P value 0.0052) respectively compared to their control groups. In addition, the expression of Rhox 5 gene significantly increased 2.1 folds (P value 0.0036) and 2.5 folds (P value 0.0049) after 21 and 35 days of pumpkin treatment compared to control.

Similarly, the expression of PLCz levels increased significantly by 0.36 and 0.33 folds (P value 0.001) following a 21-day and 35-day carob extract treatment respectively compared to their control groups. The expression levels of the Ras gene also increased by 1.9 fold (P value 0.0061) and 1.6 fold (P value 0.0052) after 21 and 35 days respectively compared to their control groups. As for the Rhox 5 expression levels, carob treatment induced a significant increase of 0.2 fold (P value 0.003) after 21 days but an insignificant one (0.14 fold, P value 0.18) after 35 days compared to their control groups.

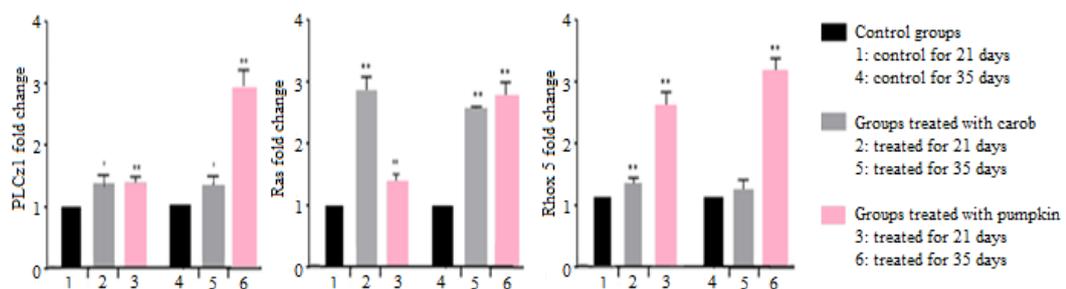


Fig.8: Effect of pumpkin and carob seeds extracts on the expression level of PLCz1, Ras and Rhox 5 genes in the testes of Balb/c mice. The data represents the average of 3 determination \pm SD. Asterisks on bars represent significance relative to its respective control. (**) corresponds to $P < 0.01$.

4. DISCUSSION

Fifteen to twenty percent of couples suffer infertility where ~50% of these cases are associated with the male rather than the female where no medical therapies are yet effective (Shiraishi & Matsuyama, 2017). However, treatment of male sub-fertility has achieved significant breakthroughs through the use of combination of hormonal therapies with receptor modulators (Newlaczyl, Coulson, & Prior, 2017) or by acting on the hypothalamo-pituitary-testis axis, stimulating Leydig and Sertoli cells to increase testosterone secretion that leads to increase in sperm production or even by using spermatogonial stem cell therapy. The aim in this study was to assess the effect of the aqueous extract of pumpkin seeds and carob seeds on spermatogenesis. The results showed that pumpkin seeds extract, and carob seeds extract induced a significant increase in sperm count, along with increase in testes' mass. Improvement in spermatogenesis was also proved by histological studies. Similar effects were obtained by Lorenzetti et al.'s using caviar homogenate that led to increase in sperm count and testis/body mass in rats (Lorenzetti et al., 2012). On the other hand, zinc oxide particles (50 or 300 mg/Kg) lead to significant decrease in sperm count and motility (Talebi, Khorsandi, & Moridian, 2013). Similarly, administration of microcystin-LR for more than 4 days (at 15 µg/kg/day) was shown to significantly decrease sperm production (Xiong, Zhong, & Xu, 2014). As for the effect of the pumpkin seeds extract and carob seeds extract on motility and viability, no changes were noticed implying their safety on viability of sperms, but this could be attributed to the cutting of the epididymis during the procedure. Moreover, both extracts did not induce spermatogenesis in immature mice showing that they do not affect the normal onset of puberty .

Furthermore, the levels of testosterone, FSH, and LH levels in mature mice significantly increased after 21 days of carob treatment and increased more significantly after 35 days of treatment with pumpkin or carob seeds extract implying that both extracts may be exerting an effect on the hypothalamo-pituitary-testes axis to stimulate the gonadotropins that in turn provoke testosterone secretion. The insignificant increase in LH after longer treatment (35 days) might be due to the negative feedback response exerted by testosterone on the level of these two hormones. Similar results were obtained by Zang et al. who showed that the herb saikokaryukotsuboreito increased testosterone concentration and elevated the quality and quantity of sperms (Zang, Ji, Zhang, Gao, & Zhang, 2016). In addition, the Malaysian ginseng that is considered as a supplement increases testosterone levels and sperm count (Michihara et al., 2013). On the other hand, testosterone and LH levels were significantly reduced in a dose- and time-dependent manner after treatment of microcystin-LR for 4 days whereas FSH secretion was not affected (Xiong, Zhong, & Xu, 2014). Moreover, androgenic, anabolic steroids led to elevation in serum androgens causing negative feedback on the pituitary gland with decrease in LH and FSH levels that in turn decreases testosterone production and consequently decreases sperm count as shown as well by de Souza & Hallak (Souza & Hallak, 2011).

At the gene level, the increase in testosterone concentration resulted in the activation of the studied key players in the three pathways through which testosterone acts. Pumpkin extract and carob extract significantly induced the expression levels of PLCz1, Ras and Rhox5 which led to proper maintenance of spermatogenesis, preventing the apoptosis of spermatocytes and spermatids and facilitating Sertoli-germ cell attachment.

In a similar study, Mokhtari et al used a hydro-alcoholic carob extract that showed similar changes in the levels of the hormones studied (Mokhtari, Sharifi & Sh, 2012). Vafaei et al proved that aqueous carob extracts increased testosterone level in infertile mice confirming its efficacy (Vafaei et al., 2018) . Our study indicates that the aqueous extract prepared, which is traditionally used as homemade infusions, is as effective on sperm parameters .

The effects of the pumpkin extract on spermatogenesis might be mediated by the presence of zinc, an essential element to the function of ~ 300 enzymes and whose deficiency can cause impairment in spermatogenesis in rats and in men (Kehinde et al., 2015). In addition, the antioxidants found in pumpkin seeds like the carotenoids, vitamins A, B, C and the different forms of E are important also for improving spermatogenesis where they have been proved to be beneficial in restoring a balance between ROS generation and scavenging activities (Adewoyin et al., 2017; (Fukuchi et al., 2004; Khaki, 2012). Similarly, the high content of pumpkin in oleic acid decreased the susceptibility of the testis to lipid peroxidation leading to a better sperm count (Khaki, 2012). This was also proved by Forouzan et al.'s who showed that the pumpkin extract

combined with ginger showed an antioxidant activity and to improve spermatogenesis (Mohammadi et al., 2013).

Similarly, Vitamin C found in carob seeds was shown to have an effect on infertile mice and a potential of improving semen quality (Akmal et al., 2006), vitamin E has shown as well to have a great potential to reverse infertility (Moslemi & Zargar, 2011). Carob seeds are also rich in aspartic acid, glutamic acid, linoleic acid, and linolenic acid (Mokhtari, Sharifi & Sh, 2012). The aspartic acid found in seeds leads to increase in testosterone release (Topo, Soricelli, D'aniello, Ronsini, & D'aniello, 2009). Exogenous treatment with Aspartic acid to humans have proven to improve spermatozoa number and motility (Fiore, Santillo, & Baccari,

As seen in the previous figures, both extracts induced an increase in sperm counts, hormonal levels and genes' levels. Carob seeds had a greater effect on the sperm count, testosterone and LH levels compared to the effect induced by pumpkin seeds. On the other hand, pumpkin seeds had a greater effect on the genes' levels and induced a more significant increase in FSH level. The carob seeds extract induced a more significant effect after 21 days compared to pumpkin seeds extract but after 35 days the effect induced by pumpkin seeds was more significant.

5. CONCLUSION

The carob and the pumpkin seeds aqueous extracts can be used to improve sperm parameters in Balb/c mice. This study was limited, in so many occasions. Longer period of treatment and studying the effect of both extract on other genes that affect spermatogenesis would be a plus. Furthermore, determining the effect of these extract on protein level would be of added value. The effect of these extracts on sterile mice, immature and on female fertility is yet to be studied.. Additional support of the results can be done with the help of checking the effect of these extract on impregnating female mice and their produced litter size. Moreover, studies to test the effect of these extract on GnRH secretion by the hypothalamus can be performed. Co-administration of pumpkin seeds extract with carob seeds extract could be studied for a faster and more amplified response mice.

6. ACKNOWLEDGEMENT

We extend our thanks to our friend lab mates, Mr. Alaa Al Mobarak, Ms. Rawan Kawach and Ms. Safaa Aridi who shared their experience and helped in the different experiments.

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