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Research Article

Effect of linseed oil on histopathology of reproductive organs of Rabbit

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Abstract

In the last decade, the fertility rate in human populations has linearly decreased due to lifestyle and low intake of essential fatty acids. The present study was designed to evaluate linseed oil's effects on the histopathology of rabbits' reproductive organs by exposing mature rabbits to low, medium, and high doses of 1%, 2%, and 3% of the diet, respectively. The research was conducted on eighty mature breeding rabbits, weighing 1-1.5kg, and classified into four groups. Each group had twenty rabbits with equal gender ratios kept in separate cages under appropriate environmental conditions. Groups A, B, and C were fed 1%, 2%, and 3% linseed oil, respectively, while group D was the control group fed basal diet only. On the 15th, 30^{th,} and 45th day of investigation, for histopathological examination, rabbits were slaughtered to remove testes and ovaries and stained tissues by H & E stain. The histopathological examination of the testes showed an improvement in the length of seminiferous tubules and the diameter of the seminiferous epithelium. In addition, the thickness of interstitial cells and Sertoli cells also increased in the testes. After histopathological findings of ovaries, it was revealed that the number and size of follicles increased in the ovaries of rabbits fed linseed oil-supplemented diets. At the same time, maximum improvement was recorded at 3% linseed oil supplementation level. Conclusively, an increase in linseed oil from 1% to 3% in the rabbit diet improved the histopathology of the reproductive gonads and can increase the overall fertility of rabbits.

Keywords: Fertility; Gonads; *Linum usitatissimum*; Microtomy; Polyunsaturated fatty acid

Introduction

Linseed (*Linum usitatissimum*) belongs to the Linaceae family and is the only species with high economic importance [1]. Linseed oil is a vital plant source with a high amount of omega 3, such as ALA (alpha-linolenic acid) [2]. However, the total amount of ALA present in linseed is 39-60.42%, which is the highest value compared to other lipids (oleic, stearic, palmitic, and linoleic acid). The amount of ALA in linseed is 5.5 times more than that of other best food sources like canola, walnut, pig, and soya bean [3].

There are three main kinds of omega-3 docosahexaenoic acid, alpha-linolenic acid, and eicosapentaenoic acid involved in the physiology of mammals. Omega 3 is a PUFA (Polyunsaturated fatty acids) that has a double carbon bond at the

position of the third carbon from the end point of the chain [4]. ALA that is present in linseed oil metabolizes into DHA (Docosahexaenoic acid) and EPA (Eicosapentaenoic acid) [5, 6]. Animals cannot produce omega 3 and omega 6 themselves. So, they consume a diet that contains enough PUFA to fulfill their body requirements [7].

Lipids play a significant role in the fertility of males because it is an essential element of the spermatozoa membrane [8]. The membrane of spermatozoa comprises about 30-50% PUFA in mammals [9]. Omega 3 increases the fertility of males by improving the blood supply to the penis and increasing the production of healthy sperm [10]. It has potential beneficial effects on the male reproduction of humans and other animals [11].

PUFA has a progressive effect on follicle development. It is also involved in the formation of different hormones like prostaglandin and sex steroids, which regulate embryo implantation, ovary activities, and other physiological processes [12]. Omega 3 improves the development of follicles and increases the ovulation rate [13]. Moreover, the EPA and DHA act as signaling factors to stimulate ovulation and improve blood flow to the ovaries to enhance the growth of follicles and the weight of the ovaries [14].

In recent times, human nutrition has become one of the significant subjects because it directly influences human health [6]. Rabbit meat is considered healthy because it contains a high amount of PUFA, i.e., omega 3, and is poor in cholesterol, sodium, and fat [15]. Research on rabbit reproduction was required to increase the success rate of rabbit development, which could have positive nutritional and economic impacts [16]. This research was designed to explore the effect of linseed oil on the fertility of male and female rabbits by examining the histopathology of reproductive organs (testes and ovaries).

Materials and Methods

Handling of animals

This research was conducted on rabbits (*Oryctolagus cuniculus*). In this investigation, female and male rabbits weighing 1-1.5kg were used as experimental animals. Rabbits were purchased from the Institute of Veterinary Research Lahore, Punjab, Pakistan. Female and male rabbits were placed in separate cages. Cages were cleaned daily to prevent diseases and stress. Rabbits were kept under controlled conditions, such as 21°C to 25°C temperature and provided a light period for twelve to fourteen hours, which is necessary for their circadian biorhythms. Rabbits were provided with clean water, fresh hay, and fresh vegetables ad libitum [17-19].

Experimental design

In the experiment, a total of eighty mature breeding rabbits were used. Rabbits were sorted randomly into four groups named A, B, C & D. Twenty rabbits were placed in each group: ten male and ten female rabbits. Rabbits in group A were treated with 1% linseed oil in the diet. Rabbits in group B were treated with 2% linseed oil in the diet. Group C rabbits were treated with 3% linseed oil in the diet. Group D rabbits were provided with a basal diet only [20]. Doses of linseed oil were orally administered to rabbits regularly at 9 am for 45 days.

Histopathological analysis

From each treatment and control group, three rabbits were randomly chosen on the 15th, 30^{th,} and 45th day of dose administration. They were slaughtered to remove testes and ovaries for

histopathology examination [21]. After removal, testes and ovaries were dipped into water to clean impurities and then transferred into 0.085% saline solution [22]. Sterilized tools of the dissection box were used to cut thin slices (3-4mm) of testes and ovaries [23].

Histopathological analysis

Microtomy was performed to examine the histopathology of the ovaries and testes of rabbits.

Tissue fixation

The first step in histopathology study is tissue fixation. It was performed to prevent tissue breakdown and to preserve morphological and chemical features of tissues. For this purpose, tissue samples were placed in Bouin's solution for seven hours before tissue processing [24].

Tissue processing

Tissue processing consists of three steps: dehydration of tissues, clearing, infiltration, and embedding [25].

Dehydration of tissues

A dehydration process was used to remove the Bouin's fixative and water from the sample tissues. For dehydration purposes, tissues were placed in several series of graded alcohol, i.e. 70%, 80%, and 90% alcohol for 90 minutes each respectively, and 100% alcohol for one hour [25].

Clearing of tissues

Xylene was used as a clearing agent because it is compatible with alcohol and replaces it before the infiltration of paraffin wax. So, tissues were placed in Xylene I and Xylene II for one hour each [25].

Infiltration and embedding

For infiltration, molten paraffin wax was poured into glass tubes. The sample tissues were transferred into these glass tubes and placed in an incubator at 56 ° C for the whole night.

For embedding, paper blocks were formed with the help of wooden chunks. Solid paraffin wax was placed into the wax dispenser and liquefied at 60 ° C. Melted wax was then poured into paper blocks, and tissue was placed in the center of the wax with the help of forceps and allowed to solidify at room temperature. After solidification, the blocks were placed in the refrigerator for ten hours [25].

Trimming

The paper mold that surrounded the wax blocks was removed. Wax blocks were trimmed from all sides to remove extra wax with the help of a sharp blade [25].

Mounting of wax block

One side of the wax block was heated with a flame and then mounted on a microtome machine holder. For proper cutting, it was required to fix the wax block properly in a parallel position to the blade of the microtome machine [22].

Water bath

The water bath was first washed to clean dust and then filled with clean water. It was set at a temperature 45^{o}C less than the melting point of paraffin wax [26].

Sectioning

A microtome (YD-202) was used to cut thin sections of tissues. For this purpose, the wheel of the microtome was held in a sealed position, and the wax block was attached with the microtome block holder. The thickness of tissue sections, about 4-5 micrometers, was adjusted on the microtome, and thin wax ribbons of tissues were obtained. After that, ribbons were transferred with the help of a camel hair brush to water bath that was used to eliminate the wrinkles of tissue sections [27].

Mounting of sections

Before mounting sections, slides were cleaned with alcohol and labeled properly. To prepare Mayer's albumin, glycerin and

egg white were mixed in a 1:1 ratio. Before tissue mounting, Mayer's albumin was spread on slides. Then, the slides were put in floating water bath and tissue ribbons were mounted carefully on the slides. Then, the slides were placed in a vertical position and allowed to dry at room temperature for fifteen minutes [27].

Staining

Staining is a significant step in the histopathology procedure. To remove wax, slides were placed in xylene for 3 minutes. Then, the slides were cleaned properly with tissue paper. Tissues were subsequently rehydrated in a series of alcohol, i.e., 100%, 90%, and 70% alcohol for 5 min each. Slides were put in tap water for 1 min and dipped in distilled water four times. After rehydration, tissues were stained with hematoxylin for two minutes. Then, the slides were kept in tap water for five min and washed in 70% and 90% alcohol for three min each. At last, the Slides were immersed in eosin solution for two min and washed in 90% alcohol and absolute alcohol for two min each [24].

Lubrication

After staining, the lubrication of tissues was done. Slides were placed in cedar wood oil for 1 minute [24].

Clearing of tissues

Pure xylene was used to clear tissues. After lubrication, slides were placed in xylene for two minutes [24].

Mounting

The last step in the histopathology procedure is the mounting of cover slip. For mounting purposes, one to two drops of Canada balsam was put on the slide and then the coverslip was placed gently on the slide to avoid bubbles formation. Slides were dried at room temperature [21].

Study of slides

Histopathology of the testes and ovaries was examined under the stereo microscope with a digital camera (FRL Lx400). Photomicrographs of each slide were then captured.

Results and Discussion

The results of the present research showed that linseed oil has positive effects on the histopathology of the reproductive organs (testes and ovaries) of rabbits.

It was indicated that linseed oil increased the diameter of seminiferous tubules and showed a regular and compact arrangement of germ cells in seminiferous epithelial in treated groups as compared to the control group. The thickness of interstitial cells and Sertoli cells noticeably increased in all treatment groups (Fig. 1, 2 & 3). Therefore, another finding showed high spermatogenesis found in avian testes when they received a diet with the addition of omega 3 because it enhanced the production of testosterone hormone [28].

The histopathological findings of ovaries indicated that the number and size of follicles increased in rabbits fed a linseed oil-supplemented diet. It produced a significant increase in primordial follicles in treated rabbits (Fig. 4, 5 & 6). Similarly, it was reported that follicle numbers in ovaries improved when cows received an omega-3 enriched diet such as linseed [29-31]. Moreover, it was observed that cows fed fish oil as a source of DHA and EPA showed a high number of follicles [32].

Histopathological changes in the testes of Rabbit

The histopathological results of testes (Fig. 1, 2 & 3) revealed that linseed oil-supplemented diets have beneficial impacts on the structure of testes. It increased the thickness of interstitial cells and Sertoli cells. Seminiferous tubules become elongated, and the thickness of germ cells increases, enhancing the diameter of the seminiferous epithelial in

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the testes of treated rabbits as compared to the control group. These findings resembled the results of [33], who demonstrated that the structural integrity of testes increased when rats consumed stable PUFA, i.e., omega 3 and omega 6 balanced ratio in their diet. It was reported that when linseed oil was supplemented to rams, it increased the diameter of the seminiferous epithelium and the length of spermatozoa [34]. In addition, a previous study found that bucks supplemented with 1% linseed oil, 2% linseed oil, and omega 3 increased the tubular diameter and thickness of interstitial cells [20].

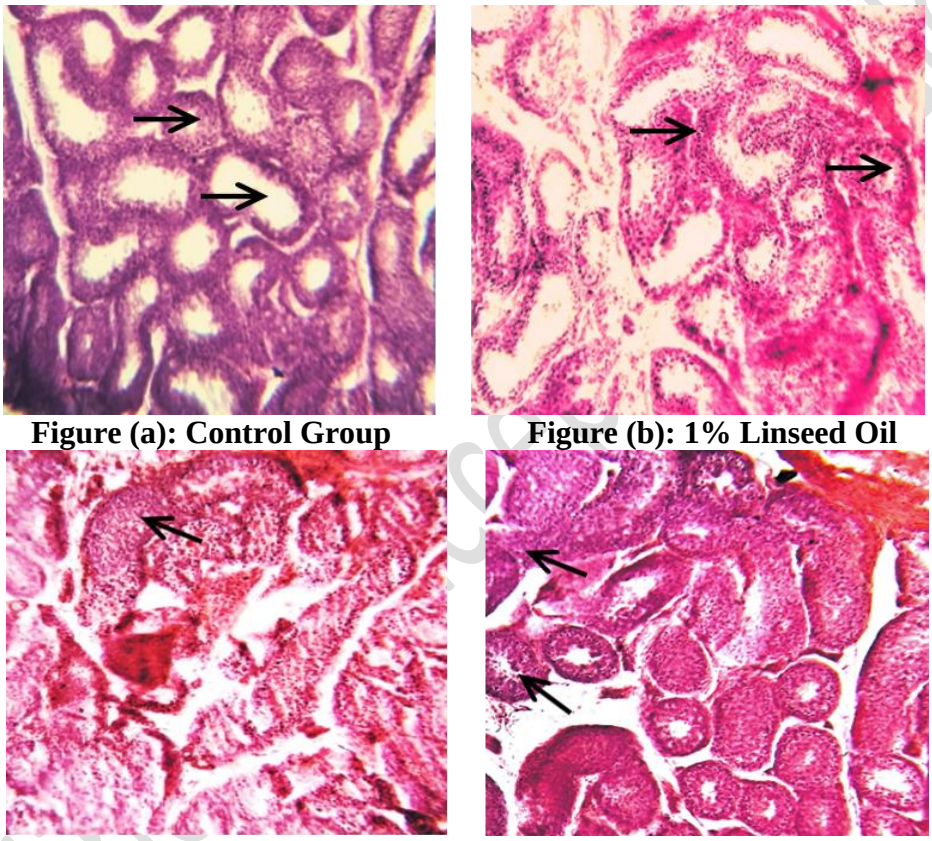

Figure (a): Control Group **Figure (b): 1% Linseed Oil**

Figure (c): 2% Linseed Oil **Figure (d): 3% Linseed Oil**

Figure 1: Comparative histopathological effects of linseed oil on testes of male Rabbits at 10X magnification power under light microscope on 15th day of dose administration

Photomicrograph of cross section of testes of male rabbits at the 15th day-Figure (a): The control group shows the normal histological structure of testes, e.g., Sertoli cells, lumen. Figure (b) shows the thickness of interstitial cells and rounded seminiferous tubules. Figure (c) shows the elongation of seminiferous tubules. Figure (d) shows an increase in the thickness of primary spermatocytes and the thickness of interstitial cells.

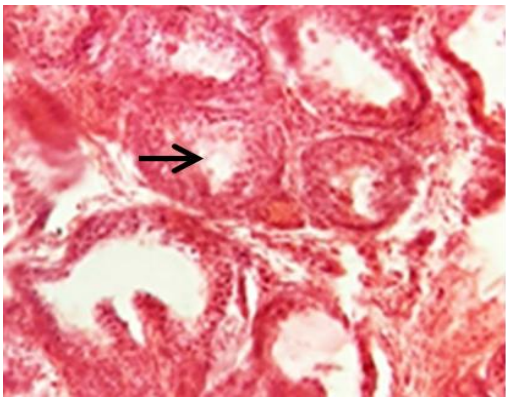

Figure (a): Control Group

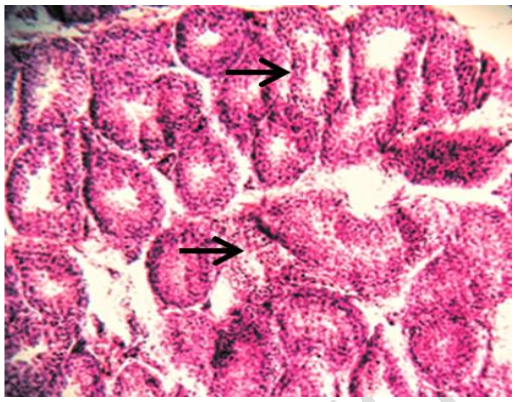

Figure (b): 1% Linseed Oil

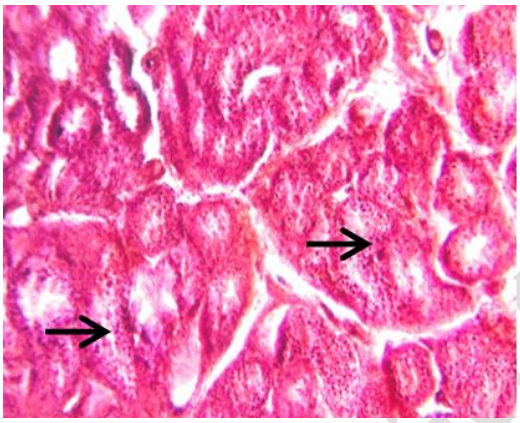

Figure (c): 2% Linseed Oil

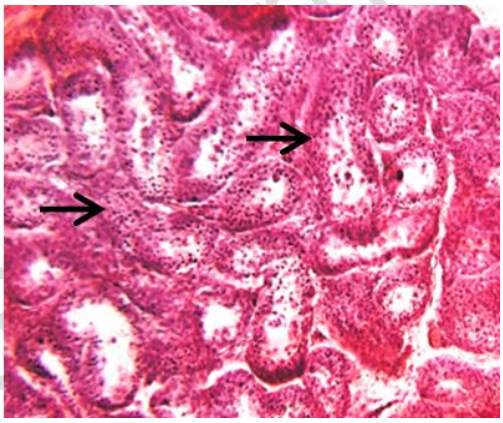

Figure (d): 3% Linseed Oil

Figure 2: Comparative histopathological effects of linseed oil on testes of male Rabbits at 10X magnification power under light microscope at 30th day of dose administration

Photomicrograph of a cross-section of testes of male rabbits on the 30th day- Figure (a) The control: The control group shows normal rounded seminiferous tubules. Figure (b) shows an increase in the diameter of seminiferous tubules and the thickness of interstitial cells. Figure (c) shows the increase in the number of primary spermatocytes. Figure (d) shows an increasing diameter of seminiferous epithelium and thickness of interstitial cells.

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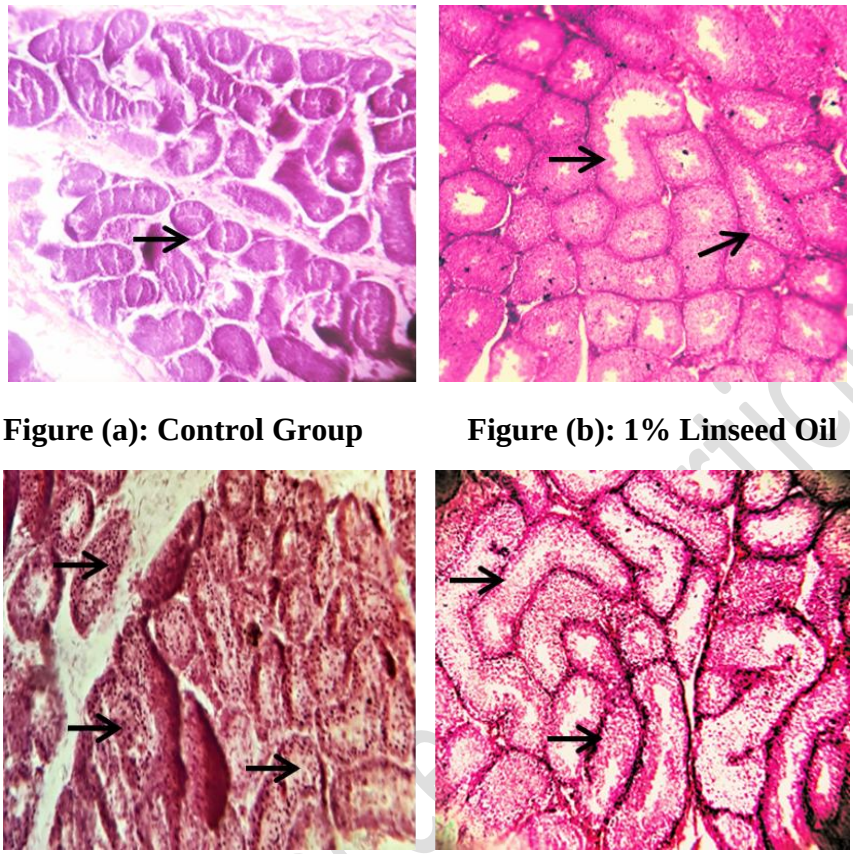

Figure (a): Control Group **Figure (b): 1% Linseed Oil**

Figure (c): 2% Linseed Oil **Figure (d): 3% Linseed Oil**

Figure 3: Comparative histopathological effects of linseed oil on testes of male Rabbits at 10X magnification power under light microscope at 45th day of dose administration

Photomicrograph of a cross-section of testes of male rabbits on 45th day-Figure (a): The control group shows the normal structure of seminiferous tubules. Figure (b): shows an increase in the diameter of seminiferous epithelium. Figure (c) shows cross-sections of elongated seminiferous tubules with high thickness of secondary spermatocytes and increased thickness of Sertoli cells. Figure (d) shows the elongation of seminiferous tubules and the increase in the thickness of primary spermatocytes in testes.

Histopathological changes in ovaries of Rabbit

Histopathological results of ovaries (Fig. 4, 5 & 6) in female rabbits showed an increase in the number of follicles, especially primordial follicles, and the growth of follicles in treatment groups as compared to the control group. These results are supported by [35], who examined ewes that received fish oil as a source of omega 3 and showed a greater number of primordial follicles. It was reported that when cows received an omega 3 and omega 6 FA-enriched diet, the number of follicles increased in ovaries [36]. Another study observed that the ovulation rate in rats was enhanced upon exposure to fish oil as a source of omega 3 [37]. In addition, it was shown that a high intake of EPA and DHA increased follicle development and ovulation in rats [38].

Similarly, it was also observed that the intake of omega 3 increased folliculogenesis in the ovaries of bovine [39] and dairy cows [40]. Moreover, the findings of [41] revealed that fish oil as a source of omega 3 improved the development of ovarian follicles in hens. Similarly, it was reported that when goats were fed fish oil, the pre-ovulatory follicle numbers and ovulation rate increased [42].

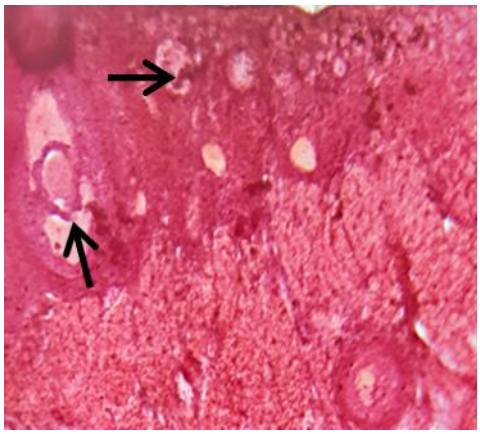

Figure (a): Control Group

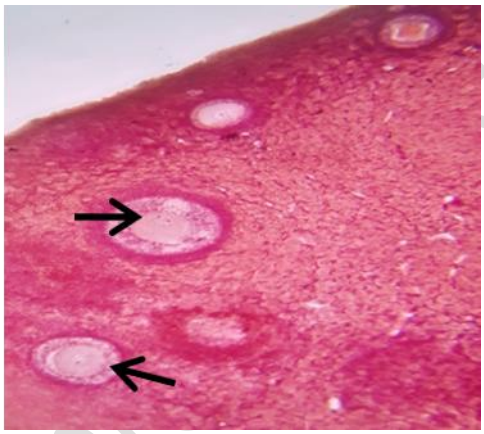

Figure (b): 1% Linseed Oil

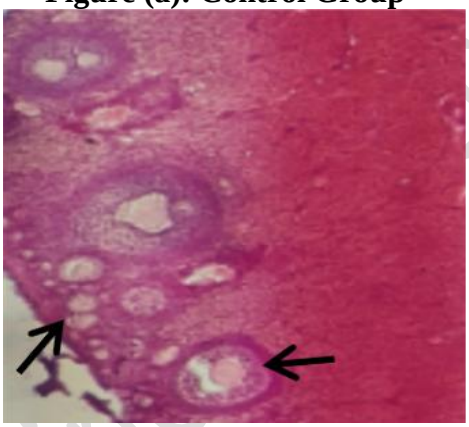

Figure (c): 2% Linseed Oil

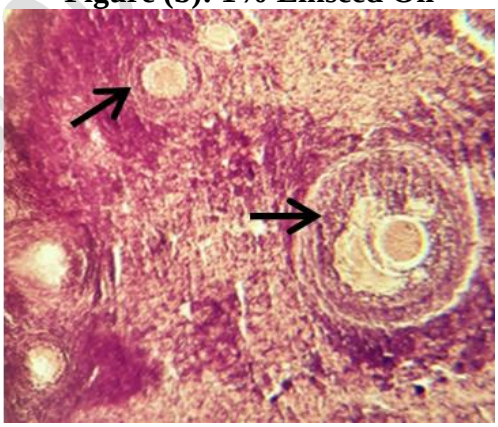

Figure (d): 3% Linseed Oil

Figure 4: Comparative histopathological effects of linseed oil on ovaries at 10X magnification power under light microscope on 15th day of dose administration

Photomicrograph of the cross section of ovaries on the 15th day. Figure (a): The control group shows the oocyte and primary follicle. Figure (b) shows an increase in primary follicle size. Figure (c): shows an increase in the size and number of Primordial and primary follicles. Figure (d) shows an increase in the number of primary follicles and the growth of the secondary follicles.

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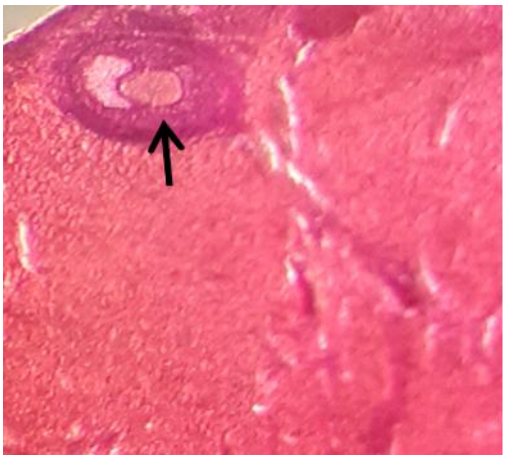

Figure (a): Control Group

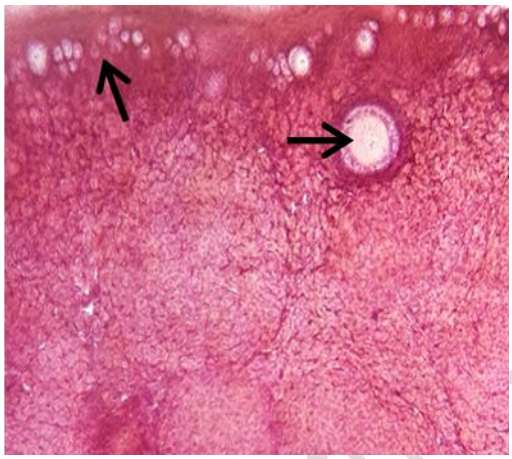

Figure (b): 1% Linseed Oil

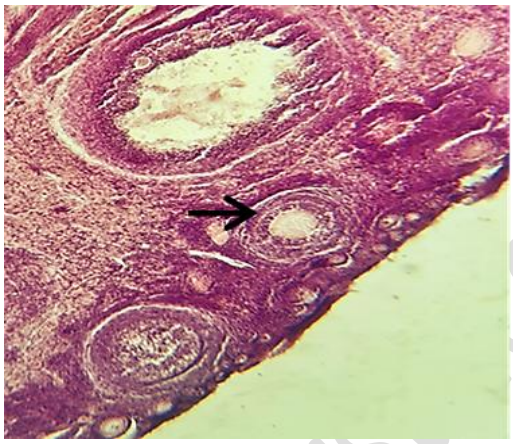

Figure (c): 2% Linseed Oil

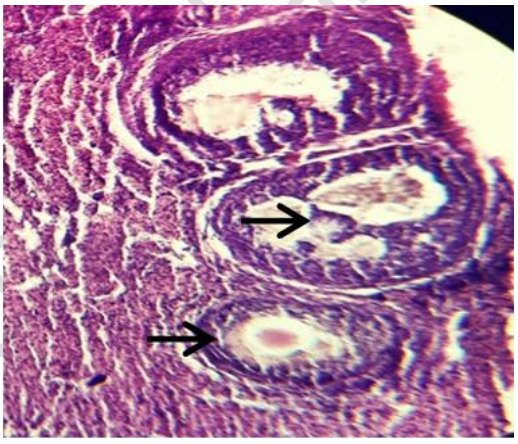

Figure (d): 3% Linseed Oil

Figure 5: Comparative histopathological effects of linseed oil on ovaries at 10X magnification power under light microscope on 30th day of dose administration

Photomicrograph of a cross section of ovaries on the 30th day-Figure (a): The control group shows the mature oocyte surrounded by a secondary follicle. Figure (b) shows an increase in the growth of many primordial follicles. Figure (c): shows an increase in growth of primary follicles in ovaries. Figure (d) shows the high growth of secondary follicles.

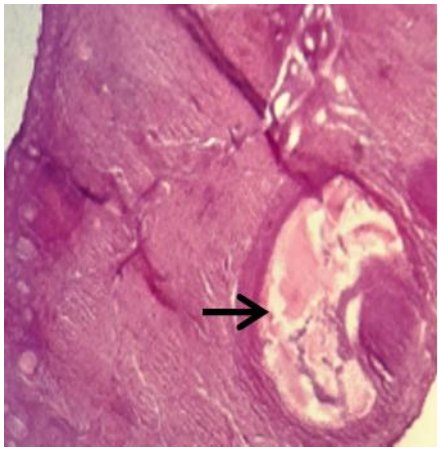

Figure (a): Control Group

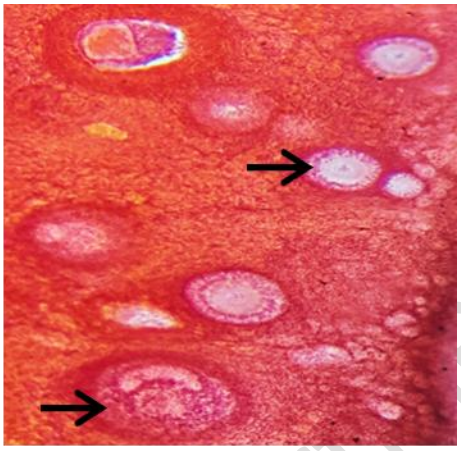

Figure (b): 1% Linseed Oil

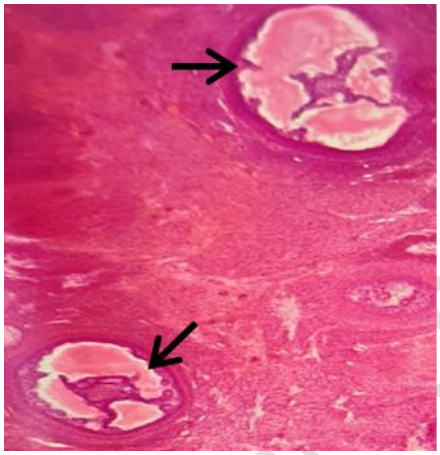

Figure (c): 2% Linseed Oil

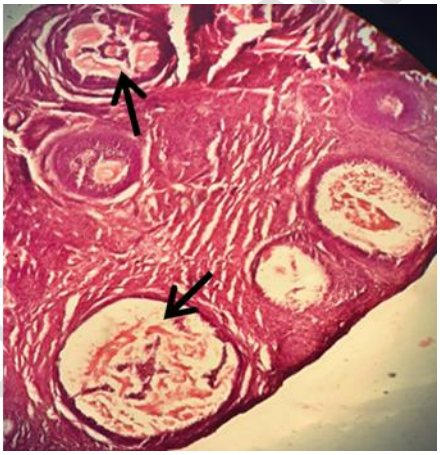

Figure (d): 3% Linseed Oil

Figure 6: Comparative histopathological effects of linseed oil on ovaries at 10X magnification power under light microscope at 45th day of dose administration

Photomicrograph of a cross section of ovaries on the 45th day Figure (a): The control group shows the normal structure of the follicle. Figure (b) shows the growth of a large number of primary follicles and secondary follicles. Figure (c) shows an increase in the number and growth of secondary follicles. Figure (d): shows improvement in the growth of secondary follicles and a large number of Graafian follicles (corpus luteum) after ovulation.

Conclusion

The present study concluded that linseed oil has beneficial impacts on the reproduction of rabbits. Histopathological examination showed that linseed oil has advantageous effects on the structure of testes and ovaries as it increases spermatogenesis in testes and the growth of follicles in ovaries. So, it is concluded that linseed oil can enhance the fertility of rabbits. These findings suggest that linseed oil can be the best natural remedy to increase fertility in mammals and other animals.

Authors' contributions

Conceived and designed the experiment: R Iqbal & M Mubasher, Performed the experiment: M Mubasher, Analyzed the data: R Iqbal, Contributed reagents/

materials/ analysis tools: R Iqbal, Wrote the paper: M Mubasher

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