

# Postnatal Exposure to Exogenous progesterone Exacerbates the Prenatally-Induced Abnormal Sperms Production and Function in Rats

## La exposición Posnatal a la Progesterona Exógena Exacerba la Producción y Función Anormales de Espermatozoides Inducidas Prenatalmente en Ratas

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**SUMMARY:** This study aimed at clarifying the impact of long-term prenatal and postnatal exposure to exogenous progesterone on sperm production and function, relative sex organs weights, and the levels of the relevant hormones in rats. Sixty male Wistar rats were included and classified into three groups (n=20 in each). A test I group had mature rats born to dams treated with progesterone prenatally. A test II group included rats exposed to progesterone during prenatal as well as postnatal periods, and a control group had rats treated with a placebo (olive oil). The test groups revealed a significant reduction in sperm count, motility, and viability with higher abnormal forms than the control group ( $P < 0.05$ ). Similarly, the test groups revealed significantly lower serum testosterone and higher FSH and LH levels ( $P < 0.001$ ). Interestingly, the test II group showed pronounced sperm abnormalities, an alarming decrease in sperm viability and motility, and a significant accretion in the relative testicular weight compared to the test I group ( $p < 0.001$ ). Long-term (prenatal and early postnatal) treatment with synthetic progesterone hurts sperm quantity and quality, adversely affecting future male fertility.

**KEY WORDS:** Wistar rats; Progesterone; Sperm; Viability; Motility.

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## INTRODUCTION

Synthetic progestins are widely used in fertility clinics during the prenatal and postnatal periods for therapeutic purposes, which entails many women worldwide being exposed to a higher dose of sex hormones. They are traditionally used as contraceptives, ovulation inducers in primary infertility cases and pregnancy supporters for threatened miscarriages and transplanted IVF embryos (Vidaeff & Belfort, 2013).

Recent studies reported that prenatal exposure to progesterone induces abnormal testicular morphometric and ultra-structure changes such as the unusual configuration of seminiferous tubules; folded or detached basal lamina; remarkable apoptosis; cellular cavitation with fatty drops; and ample interstitial space with significant interstitial cells (Leydig cells) and sustentacular cells (Sertoli cells) count reduction (Ahmed *et al.*, 2016a).

The rise in the infertility rate worldwide has become a serious matter, requiring scientific researchers to explore the underlying causes through appropriately designed

experiments. Therefore, recent studies highlighted the impact of exposure to exogenous steroids during prenatal or postnatal (lactation) periods that may contribute to testicular abnormalities and infertility (Kavlock *et al.*, 1996)

Many clinical and epidemiological studies suggested that exposure to synthetic steroids may interfere with the endogenous hormones, which may pose the highest risk during prenatal or postnatal development and be accused of eliciting permanent adverse effects on future generations' reproductive health and fertility. However, they may not be apparent until much later in life (Harini *et al.*, 2009).

Although the adverse effects of prenatal exposure to exogenous steroids on sperm reproduction and function were amply reported (Goyal *et al.*, 2003; Storgaard *et al.*, 2006; Ahmed *et al.*, 2016a) there are little data and controversy concerning the possible causal link between male reproduction abnormalities exacerbation and long-term (prenatal-postnatal) exposure to such chemicals which justifies the need for further clarification studies.

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In light of the review mentioned above, the current experimental study was designed to clarify the impact of prenatal and early postnatal exposure to synthetic progesterone on sperm production and function, relative sex organs weights, and the levels of the relevant reproductive hormones.

## MATERIAL AND METHOD

**Test chemicals:** Synthetic progesterone: hydroxyprogesterone caproate (trade name: Proluton Depo ®) and medroxyprogesterone (trade name: Depoprovera ®) (250 mg in 1.0 ml) are available in an oily solution diluted by pure Spanish olive oil (1:4ml).

**Animals and husbandry:** Wistar strain albino rats eight to ten weeks old females (n = 21) and males (n = 9) with body weight 200-250g.

After one-week acclimatization, rats were housed for mating, female rats were examined every morning for the presence of cervical plugs and/or sperm in a vaginal smear. Proved-mated female rats were kept separate in sterilized polypropylene cages (90 cm × 45 cm × 15 cm) and were divided into three groups. The rats were maintained at 23 ± 2 °C, under a regulated light/dark (12:12 h) cycle, fed with a pellet diet and water ad libitum. Group (A) act as a control and received the same olive oil injections as the test groups. Groups B and C (test groups) were subcutaneously injected with proluton depot (Hydroxyprogesterone hexanoate; Schering AG; Germany) 10 mg/kg BW on the 1st, 7th and 14th day of gestation. After delivery, mothers of the test C group were further injected with the same dose of synthetic progesterone (medroxyprogesterone; 10 mg/kg maternal body weight) every other day for the first 15 postnatal days of the lactation period. The dose was in regular clinical use and was adjusted daily to each dam's body weight (Pushpalatha *et al.*, 2003a; Harini *et al.*, 2009; Ahmed *et al.*, 2016b).

Sixty (60) male offspring born from these groups (20 puppies each) were allowed to reach maturity (90 postnatal days). Male offspring exposed and treated with synthetic progesterone during pregnancy were named (test I group), and those treated during both pregnancy and lactation were named (test II group). In contrast, those who were born to dams treated with a placebo served as a control group.

**Tissue collection and preparation:** On the 90<sup>th</sup> postnatal day, male offspring were sacrificed by cervical dislocation and blood samples were taken through the cardiac puncture

to assess the testosterone (T), luteinizing (LH) and follicle-stimulating (FSH) hormones serum levels.

Meantime, the relative weights of cleaned right testis and epididymis were calculated. The epididymis was minced in a Petri dish and oozed sperms containing fluid were diluted in 1 mL physiological saline solution (0.9 % NaCl). Then, the mixed sperms suspension was kept at 37 °C for five minutes for seminal analysis (Ahmed *et al.*, 2016b).

### Seminal analysis

**Sperm Counts:** A drop of sperm's suspension (1:20) was taken to assess the sperm's total number for each rat by using a Neubauer hemocytometer and a Carl Zeiss (Germany) Axio 2 Plus microscope at x100 magnification. Consequently, counted sperms were expressed as sperm X 10<sup>6</sup>/ml. Two samples per - epididymis were assessed and the average counts were recorded (Franca *et al.*, 2006; Ahmed *et al.*, 2020).

**Sperm Motility:** Sperm's motility was immediately evaluated within 5 minutes following their isolation at 37°C. A drop of sperm suspension was transferred to a hemocytometer and observed under a Carl Zeiss (Germany) Axio 2 Plus microscope at × 200 magnification. Each sperm with any of the different motility types was recorded as motile sperm for each rat, and 10 random fields were selected. The percentage of motile sperm in 200 counted sperms was recorded (Harini *et al.*, 2009; Ahmed *et al.*, 2020). Two separate drops for each rat were assessed to obtain the average.

**Sperm Viability:** Sperm viability was assessed by mixing 1ml of the sperm suspension and a drop of 1 % Eosin stain in an Eppendorf tube at 37 °C. Then, a 10mL sample was observed under a Carl Zeiss (Germany) Axio 2 Plus microscope. The dead spermatozoa were stained dark red while the live spermatozoa remained unstained. Sperm viability was expressed as a percentage of live sperm of the 200 sperms counted in 10 randomly selected fields for each rat (Pushpalatha *et al.*, 2003b; Harini *et al.*, 2009; Ahmed *et al.*, 2020). Two separate hanging drops for each animal were prepared, assessed by two independent observers, and obtained the average.

**Sperm Morphology:** A drop from a thoroughly mixed sperm suspension was applied to a cleaned microscopic slide and air-dried a thin film was obtained to determine sperm morphology under a Carl Zeiss (Germany) Axio 2 Plus microscope using x100 magnification. For each rat sample, 10 different optical fields were randomly selected, and the abnormal sperm relative percentages were counted. The

counted Abnormalities are headless, tailless, and coiled tails (Pizzi *et al.*, 1977; Ahmed *et al.*, 2020). Two separate thin films were performed on each rat and evaluated by different observers to obtain the average.

**Serum Collection & Hormonal Assay:** The blood sample for each rat was collected, centrifuged at 4,000 rpm for 5 minutes after overnight storage at 4 °C and then stored at 20 °C (Pushpalatha *et al.*, 2003a; Harini *et al.*, 2009; Ahmed *et al.*, 2020) (Table V). ELISA kits, purchased from Elabscience Biotechnology Co., Ltd was used to measure the serum levels of the luteinizing hormone (LH) (Catalog No: E-ELR0026, testosterone (T) (Catalog No: E-EL-R0155) and follicles stimulation hormone (FSH) (Catalog No: E-EL-R0391) according to their manufacturer's method instructions listed.

**Statistical analysis.** The data were analyzed using the SPSS version 16 (Chicago, USA) software program. Data were presented as mean ±SD. One-way analysis of variance (ANOVA) was used to test the significant difference between different groups, with the level of significance set at p<0.05

**Ethical approval.** This study was committed to national and international ethical experimental protocols and approved by a college of medicine ethical approval committee – at Najran University- KSA.

## RESULTS

**Sperm count:** The mean percentage of the sperm count of the test groups was significantly lower than that of the con-

trol (p<0.001) (Table I), which reflected a clear difference in spermatozoa density between the test and control groups. Moreover, no statistically significant difference in the sperm count between the test groups.

**Sperm motility and viability:** As shown in (Table I, Fig. 1), the mean percentage of sperm motility and viable spermatozoa showed a significant reduction in the test groups compared to the control (p<0.001). At the same time, this reduction was more pronounced in test group II.

**Sperm morphology:** The means percentages of the total and specific sperms abnormalities were significantly increased among the test groups compared to the control. Meantime, the abnormalities were significantly higher in the test II group (p<0.05). Furthermore, coiled tail abnormality showed a tremendous and horrible increase in the test II group compared to the test I group (p<0.001) (Table II, Fig. 2).

**Hormonal Assay:** Compared to the control rats, serum testosterone levels showed a startling reduction (p<0.001), whereas serum FSH and LH levels were both significantly increased in the test groups (p<0.001). Although there was a mild change in the recorded serum hormone levels between the test groups (Table III), these changes were statistically insignificant.

**Testicular and epididymal relative weights:** Compared to the control group, the mean percentage of the relative testicular and epididymal weight of the test I group showed a significant reduction (p<0.001) while, the test II group showed a remarkable increase in the mean relative testicular and epididymal weight (p < 0.001) (Table IV).

Table I. Effects of synthetic progesterone on sperm count, motility and viability.

| Parameter                                | Control                      | Test I Group                | Test II Group                    |
|--|------------------------------|-----------------------------|----------------------------------|
| Sperm count X (10 <sup>6</sup> sperm/ml) | (113.55 ±10.46) <sup>a</sup> | (81.72 ± 5.61) <sup>b</sup> | (79.17 ±5.03) <sup>c&amp;b</sup> |
| Motility ( %)                            | (82.42± 6.62) <sup>a</sup>   | (63.65 ± 6.49) <sup>b</sup> | (55.33± 6.10) <sup>c</sup>       |
| Viability ( %)                           | (81.82± 6.54) <sup>a</sup>   | (64.90 ± 6.10) <sup>b</sup> | (56.60 ± 6.04) <sup>c</sup>      |

Data are presented as mean ± SD. a, b, c. Values with different superscripts in the same row are significantly different (p<0.001).c&b Value with the same superscripts in the same row is not significantly different.

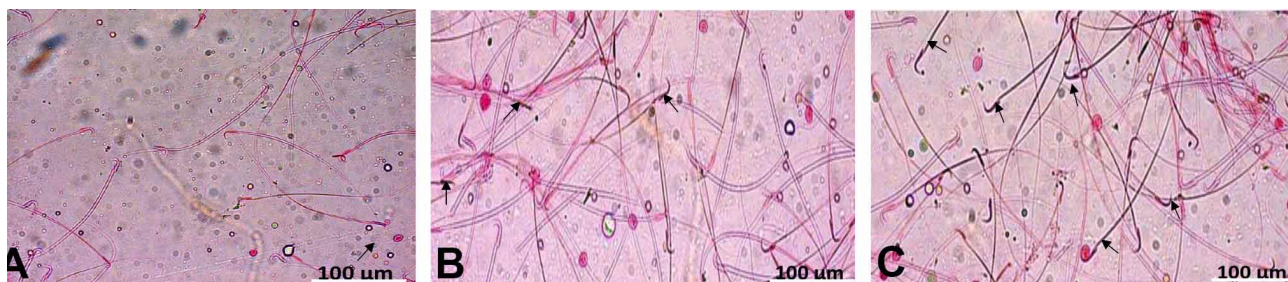


Fig. 1. Micrographs illustrating sperm viability: control (A), test I (B) and test II (C). Note the difference in the number of dark-stained dead sperms (black arrows) (X200).

Table II. Effects of synthetic progesterone on sperm abnormalities (%).

| Parameter                   | Control                     | Test I                      | Test II                     |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Total sperm abnormality (%) | (15.10 ± 1.42) <sup>a</sup> | (41.16 ± 3.93) <sup>b</sup> | (58.28 ± 5.10) <sup>c</sup> |
| Headless (%)                | (1.42±.41)                  | (9.78±1.05) <sup>b</sup>    | (11.88±1.56) <sup>c</sup>   |
| Tailless (%)                | (1.91±.48) <sup>a</sup>     | (16.42±2.78) <sup>b</sup>   | (19.44±2.45) <sup>c</sup>   |
| Coiled Tail (%)             | (2.86±.52) <sup>a</sup>     | (16.38±3.89) <sup>b</sup>   | (22.26±4.52) <sup>c</sup>   |

Table III. Effects of synthetic progesterone on the serum hormones level (ng/ml).

| Parameter    | Control                    | Test I                     | Test II                          |
|--------------|----------------------------|----------------------------|----------------------------------|
| LH           | (3.13 ± 0.10) <sup>a</sup> | (3.9 ± 0.10) <sup>b</sup>  | (4.20 ± 0.14) <sup>c&amp;b</sup> |
| FSH          | (3.90 ± 0.14) <sup>a</sup> | (5.0 ± 0.10) <sup>b</sup>  | (5.30 ± 0.10) <sup>c&amp;b</sup> |
| Testosterone | (2.33 ± 0.09) <sup>a</sup> | (1.04 ± 0.06) <sup>b</sup> | (0.81 ± 0.05) <sup>c&amp;b</sup> |

Data are presented as mean ± SD. a, b, c. Values with different superscripts in the same row are significantly different (p<0.001). c&b Value with the same superscripts in the same row is not significantly different.

Table IV. Effects of synthetic progesterone on the testicular & Epididymal Relative's weight (g).

| Parameter                  | Control                       | Test I                        | Test II                        |
|----------------------------|-------------------------------|-------------------------------|--------------------------------|
| Relative Testicular Weight | (0.011 ± 0.0002) <sup>a</sup> | (0.0062± 0.0002) <sup>b</sup> | (0.018 ± 0.0003) <sup>c</sup>  |
| Relative Epididymal Weight | (0.004 ± 0.001) <sup>a</sup>  | (0.002 ± 0.0002) <sup>b</sup> | (0.0046 ± 0.0001) <sup>c</sup> |

Data are presented as mean ± SD. a, b, c. Values with different superscripts in the same row are significantly different (P<0.001). c & b Value with the same superscripts in the same row is not significantly different.

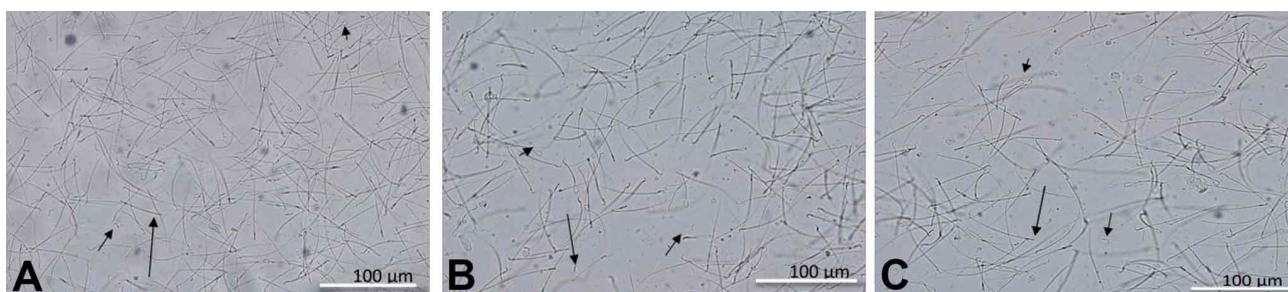


Fig. 2. Micrographs showing sperms with abnormal morphologies including headless (long arrow), tailless (short arrow), and coiled tail (arrowhead) in the control (A), test I group (B) and test II group (C) (X100). Note that the number of abnormal sperms is higher in the test II group, precisely the coiled tail compared to the control.

## DISCUSSION

Exogenous sex hormones are commonly prescribed drugs by obstetricians, particularly for pregnancy support and contraception. Therefore, increased awareness has been directed to the emerging, very worrisome potential effects of prenatal or postnatal exposure to such female sex hormones on future male reproduction. The present study findings supported and further clarified the previously reported results, including recent studies (Goyal *et al.*, 2003; Harini *et al.*, 2009; Pushpalatha *et al.*, 2003b). The significant testicular changes among the hormone-treated groups compared to the control are most likely progesterone-induced adverse effects during the in-utero critical time point of development, i.e., the embryonic period, which may persist to adulthood.

In the present study, the significant reduction in the viability and motility observed in the test II group interprets that exposure to progesterone in prenatal and postnatal periods negatively affects sperm fertilizing ability due to deleterious influence on the sperm maturation and epididymal functions (Ahmed *et al.*, 2020; Chioccarelli *et al.*, 2020). Interestingly, the significant increments in the percentages of the total abnormal spermatozoa (specifically the coiled tail abnormality) in the test II group compared to the test I and control groups signify that such exposure has more adverse effects that interfere with spermatogenic cells production and reproductive hormones levels (Fielden *et al.*, 2002; Chioccarelli *et al.*, 2020).



The alarming reduction in serum testosterone levels in the present study's test groups underpins the previously reported results, including very recent ones (Atanassova *et al.*, 1999; Ahmed *et al.*, 2020). The low testosterone level could be explained by the reduction in the hormone-secreting (interstitial cells) count, unresponsiveness of these cells to the tropic (LH) hormone, or the inhibition of testicular ability to form sex steroids (steroidogenesis) (Harini *et al.*, 2009; Fielden *et al.*, 2002). Harmoniously, a significant reduction in the 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) and 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) activities and steroidogenesis was reported in prenatally progesterone-treated mice, (T (Pushpalatha *et al.*, 2003a). As spermatogenesis stimulates hormones, low testosterone levels contribute to reduced sperm count. The increased serum FSH levels in the present study could be explained by the interruption of the negative feedback loop on FSH secretion. The damage in testicular sustentacular cells decreases inhibin hormone secretion, which is responsible for the negative feedback. Likewise, the impairment of the interstitial cells structure or function would reduce serum testosterone serum levels. As a result, testosterone's negative feedback on LH secretion would be interrupted, explaining the high LH levels (Ye *et al.*, 2011; Carreau *et al.*, 2011).

Moreover, the present study reported a significant decrease in the test I group's relative testicular and epididymal weights compared to the control rats. This result could be explained by germinal and somatic cell loss or hypotrophy, seminiferous tubules shrinkage, decreased sperm count, and increased tailless sperms (Pushpalatha *et al.*, 2003b; Ahmed *et al.*, 2016a,b; Ahmed *et al.*, 2020). More recently, Chioccarelli *et al.* (2020) reported that endocrine disruption with estrogenic activity interferes with the epididymal role of sperm maturation.

Despite the presence of insignificant sperm count reduction and the significant increase in the total abnormal sperm in the test II group compared to the test, I group of the present study, the test II group showed unforeseen increments in the relative testicular and epididymal weights. These results have pointed out that long-term (prenatal-postnatal) exposure to progesterone may upset the testicular progesterone-estrogen balance or estrogen receptors (ERs). Consequently, the estrogen-dependent fluid reabsorption (a significant function of the rete testis, efferent ducts, and initial segment of the epididymis) will be impeded, which in turn, leads to tubular fluid accretion and hence increases the epididymal and testicular weights (Lazari *et al.*, 2009; Hess *et al.*, 2011; Joseph *et al.*, 2011). Nevertheless, estrogens play an important role in modulating the functions of the efferent ductules. The epididymis and ERs are essential for maintaining a luminal environment that permits normal development of sperm motility and volume regulation.

## CONCLUSION

In conclusion, the present study results provided evidence that long-term (prenatal and early postnatal) exposure to synthetic progesterone has an exacerbated negative impact on sperm production and function and reproductive hormone levels, which worsens the fertility rate. Given the complexity of progesterone activity, it is important to re-evaluate the safety profile of progesterone use as a contraceptive during lactation, particularly in the case of prenatal exposure. Nevertheless, recognizing the molecular processes driving the sperm's vesicle-dependent maturation and other luminal components will increase the perception of epididymis's role in sperm maturation and discover new references to preserve male fertility.

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**ABDALLA, A. M. E.** La exposición posnatal a la progesterona exógena exagera la producción y función anormales de espermatozoides inducidas prenatalmente en ratas. *Int. J. Morphol.*, 41(4):1261-1266, 2023.

**RESUMEN:** Este estudio tuvo como objetivo aclarar el impacto de la exposición prenatal y posnatal a largo plazo a la progesterona exógena en la producción y función de los espermatozoides, el peso relativo de los órganos sexuales y los niveles de las hormonas relevantes en ratas. Sesenta ratas macho Wistar fueron incluidas y clasificadas en tres grupos (n=20 en cada uno). Un grupo de prueba I tenía ratas maduras nacidas de madres tratadas con progesterona prenatalmente. Un grupo de prueba II incluyó ratas expuestas a progesterona durante los períodos prenatal y posnatal, y un grupo de control tenía ratas tratadas con un placebo (aceite de oliva). Los grupos de prueba revelaron una reducción significativa en el recuento, la motilidad y la viabilidad de los espermatozoides con formas anormales más altas que el grupo de control (P < 0,05). De manera similar, los grupos de prueba revelaron niveles significativamente más bajos de testosterona sérica y niveles más altos de FSH y LH (P < 0.001). Curiosamente, el grupo de prueba II mostró anomalías espermáticas pronunciadas, una disminución alarmante en la viabilidad y motilidad de los espermatozoides y una acumulación significativa en el peso testicular relativo en comparación con el grupo de prueba I (p < 0.001). El tratamiento a largo plazo (prenatal y posnatal temprano) con progesterona sintética daña la cantidad y la calidad del espermatozoide, lo que afecta negativamente la futura fertilidad masculina.

**PALABRAS CLAVE:** Ratas Wistar; Progesterona; Esperma; Viabilidad; Motilidad.

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