Effects of Prenatal and/or Lactational Exposure to Synthetic Progesterone (P₄) on Pubertal Testicular Structure and Functions of Rat Offsprings

A Thesis Submitted for PhD in Human and Clinical Anatomy

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Dedication

To My Parents,

My wife and kids,

Brothers

Sisters

&
Acknowledgements

My grateful thanks and great appreciation with all respect to my spiritual and moral father and a great educator my supervisor **Professor Tahir Osman Ali**, the man who was credited us after the God for everything we have achieved. Thanks deeply for all his support and I am very proud to work with him.

My deepest appreciation with a lot of thanks and respect to my co-supervisors **Professor. Adil Salim El Sheikh El Garai** and **Dr. Gamal Ali Atia**, for their support, encouragement and motivation all through my work.

All the respect and thanks for my co-supervisor, for his helpe
I would like to thank my Najran Medical College wonderful administrators, as I found myself repeatedly helped with their ideas. Also I would like to express my deep gratitude to, Professor. Mohamed Bakheit, Dr. Abd Elhafeez Yagoub, Dr. Aymen Nasr Eldeen, Dr. Omer Eltayb, Dr. Elfatih Yagoub, Dr. Amar Babiker, Dr. Hassan Gumaa, Dr. Ahmed Elbashir Dr. Ehab Hamed for their endless help.
Truly, I was blessed to have worked with such creative, open-minded, and brilliant staff. Special thanks to **my family**, for their prayers, encouragement and support.

**Abstract**

**Introduction**
Progesterone is one of the most effective and widely used drugs in medical practice for many prenatal therapeutic purposes and during lactational as single contraceptive. Recent epidemiological studies have shown remarkable deterioration in animal reproductive health, which have been attributed to prenatal / and lactational exposure to female hormones.

**Objective**
This study was designed to evaluate the effects of gestational / and lactational exposure to synthetic progesterone (P₄) on pubertal testicular structure and functions of rat offspring.

**Literature Review**
Exposure to the natural or synthetic chemical substance (P₄) alters the hormonal and homeostatic systems of the animal and renders it incapable to respond to the surrounding environmental conditions. The United States Environmental Protection Agency (EPA) defined that exogenous agent interferes with synthesis, secretion, transport, metabolism, binding and elimination of natural blood hormones that are present in the body and are responsible for homeostasis, reproduction and developmental processes.
Study Field
The study was carried out in the laboratories of the Department of Anatomy, Faculty of Medicine, Najran University, Kingdom of Saudi Arabia between October 2012 and March 2014.

Experimental Design
Twenty seven mature male and female albino wistar rats, ten weeks old were purchased from the animal house of the Faculty of Pharmacy –Jazan University –Kingdom of Saudi Arabia. The rats were grouped into 3 groups each group consisted of seven females and two males. Then pregnant rats were grouped into 3 groups. Group 1 was kept as controls and groups 2 and 3 as experiment animals.

The experiment groups were injected with synthetic progesterone (hydroxyprogesterone; 10 mg/ kg body weight) subcutaneously on day 1, 7th and 14th of gestational period. After delivery, mothers of group 3 were further injected with the same dose of the synthetic progesterone (medroxyprogesterone; 10 mg/ kg maternal body weight) on day 1, 7th and 14th 19th of lactational period. Whereas the control group was injected with olive oil only as experiment groups received.

The dose of chemical test was adjusted daily to body weight for each dam before dosing. Twenty male puppies born to each of the experiment groups and control (n=60 puppies) were allowed to grow for 90 days where they reached maturity. Male rats born to dams treated with synthetic progesterone during pregnancy were named (experiment I) and those
who were treated during both pregnancy and lactation were named (experiment II).

Animals were anesthetized with chloroform, blood samples were collected from each animal through cardiac puncture to determine the serum levels of testosterone, luteinizing (LH) and follicle stimulating hormones (FSH).

After rat’s scarification, tissue samples were collected from the testes of all animals and processed for light microscopy, ultrastructural and morphometric studies. Semen samples were obtained from the caudal epididymis to evaluate the sperm quality and quantity.

**Results**

The testes of the control group revealed normal structure of approximately all seminiferous tubules. While the testes of the experiment groups revealed shrinkage of most seminiferous tubules which led to an increase in the interstitial spaces. Detachment of the basal lamina was noticed resulting in the formation of narrow spaces. Also some tubules showed folding of the basal lamina. Degeneration of the germ cells was observed in many tubules. Cellularity decrease was remarkable in most seminiferous tubules. The germinal epithelium showed more vacuolation and the cells became disorderly arranged. Also some vacuoles appeared in the interstitial spaces. Tubular diameter and height of the epithelium were decreased. Reduction in the concentration of luminal spermatozoa was evident in most of the seminiferous tubules.

The testes of the control rats did not reveal obvious ultra-structural change. Electron microscopic examination of the seminiferous tubules of the control animals showed that the epithelia were composed of two types of cells, spermatogenic as well as Sertoli cells. The Sertoli cells extended
radially from the basement membrane to the lumen of the seminiferous tubule. They appeared irregular, with basally located, oval, indented nuclei and prominent nucleoli. Their cell membranes showed complex infoldings. They have an abundant cytoplasm rich in smooth endoplasmic reticulum, free ribosomes, numerous mitochondria; a small Golgi complex, many lipid droplets and lysosomes. Spermatogenic cells were noticed in various stages of maturation.

Testes of the experiment groups showed vacuolation and lipid droplets in the Sertoli cells as well as in the interstitial tissue. Mitochondria appeared distended and seem to have lost their cristae. Degenerated spermatogonia with condensed chromatin were observed in most experiment groups. Some degenerated spermatogonia with pyknotic and vacuolated nuclei lost attachment to surrounding Sertoli cell and basal lamina. A significant reduction in the germ, Sertoli and Leydig cells count as also observed.

Rats exposure to synthetic progesterone during prenatal/and lactational showed a significant reduction in the sperm count, sperm motility, sperm viability, relative testicular and epididymal weights as well as a significant increment in the total sperm abnormalities which included headless, tailless and coiled tail sperm.

The serum testosterone levels were significantly decreased in the experiment groups as compared to control. Meanwhile, FSH and LH serum levels were significantly increased.

Compared to experiment group I, experiment group II showed statistically significant decrease in motility and viability; and increase in the total sperms abnormalities and epididymal weight.

**Conclusion**
Exposure to P4 during pregnancy (embryonic period) affects the testicular development precursors which results in significant testicular structural and functional disorders at puberty. While long-term exposure to the progesterone (during pregnancy and lactation) could exacerbate the problem by affecting sperm development and maturation.

ملخص الأطروحة

البروجسترون هو واحد من أكثر الأدوية فعالية ويستخدم على نطاق واسع في الممارسة الطبية لكثير من الأغراض العلاجية أثناء فترة الحمل، كما يستخدم كمإنج للحمل في فترة الرضاعة. وقد أظهرت الدراسات الوبائية الأخيرة تدهورة ملموسة في مجال الصحة التناسلية للذكور. وقد نسب هذا التدهور إلى التعرض إلى الهرمونات الأنثوية أثناء فترة الحمل و/أو أثناء الرضاعة.

صممت هذه الدراسة لقياس تأثير هرمون البروجسترون المستخدم أثناء فترة الحمل و/أو أثناء الرضاعة.

التعرض لبعض المركبات الكيميائية الطبيعية أو الاصطناعية مثل البروجستيرون يغير الأنظمة الهرمونية والتماثل الحيوي الداخلي لجسم الكائن الحي ويجعله غير قادر على الاستجابة للبيئة المحيطة به.

وقد بُنيت و كلّالة حماية البيئة الأميركية أن التعرض لمثل هذه المركبات الكيميائية يتعارض مع صناعة، و إفراز، و نقل، و استقلاب، و توازن هرمونات الدم الطبيعية الموجودة في الجسم والمسببة عمليات التوازن الداخلي للجسم، و الإنجاب، و نمو تطور أنسجة الكائن الحي.
وقد أجريت هذه الدراسة في مختبرات قسم التشريح البشري بكلية الطب، جامعة نجران، المملكة العربية السعودية بين أكتوبر 2012 ومارس 2014.

استخدم في هذه الدراسة سبعة وعشرون جزءاً من الذكور والإناث الناضجة من فصيلة ويستار البيضاء و التي تبلغ عشرة أسابيع من العمر و التي تم شراؤها من كلية الصيدلة جامعة – جازان - المملكة العربية السعودية. قسمت هذه الحيوانات إلى ثلاث مجموعات. تتألف كل مجموعة من سبع إناث واثنين من الذكور.

قسمت الجرذان الحوامل إلى 3 مجموعات منفصلة: كانت الأولى مجموعة قياسية (مجموعة تحكم)

و الثانية و الثالثة مجموعتي تجارب.

حُفِّظت مجموعتي التجارب (الثانية و الثالثة) بهورمون الهيدروكسي بروغسترون المخفف بزيت الزيتون (0.1ملغم / كغم من وزن الجسم) تحت الجلد في اليوم الأول، و السابع، و الرابع عشر من فترة الحمل. وبعد الولادة، تم حقن أمهات المجموعة الثالثة مرة أخرى بهورمون الميدروكسي بروغسترون(0.1ملغم / كغم من وزن الجسم الأم) المخفف بزيت الزيتون، في اليوم الأول، و السابع، و الرابع عشر، و التاسع عشر من فترة الرضاعة. في حين أن مجموعة التحكم تم حقنها بنفس الجرعة من زيت الزيتون فقط.

تعدل الجرعة عند الحقن على حسب زيادة أو نقصان وزن الجرذ لكل المجموعات.

ترك عشرون من الجرذان الذكور الذين ولدوا لكل مجموعة من المجموعات الثلاثة (عدد = 60 جرذاً) لنمو حتى 90 يوماً بعد الولادة لتصبح بالغة.
الجرذان الذكور المولودة لأمهات المجموعة الأولى سمت مجموعة التحكم (تعرضت لزيت الزيتون خلال فترة الحمل)، والجرذان الذكور المولودة لأمهات المجموعة الثانية (تعرضت للهرمون خلال فترة الحمل) سمت مجموعة التجربة الأولى، والجرذان الذكور المولوده لأمهات المجموعة الثالثة (تعرضت للهرمون خلال فترتي الحمل والرضاعة).

ُجَمْعَت عِيْنَات الْدِم مِن قَلب كُل حِيْوَانٍ فِي الْيَوْم الـ٩، وَبَعْدِ تَخْدِيرِ الحِيْوَانَات بِالكَلُورُفُورم لِتَحْدِيد مِستَوَيْ هُرُومُ التَّستوُسِتيُرِن، وَالْهُرُومُ المَصْفُرِ، وَالْهُرُومُ المَنَعِ نَضْوَجِ الْجَرْبِيَّة.

ُجَمْعَت عِيْنَات مِن أَنْسَجَة خَصِيَّة الْجَرْذَان مِن جِمْعِ الحِيْوَانَات وَعُوْلِجِت لِدِرَاسَةٍ تَرْكِيبَ الْخَصِيَّة النسجية عَلَى مِسْتَوَى المَجِهِر العَادِي، وَالمَجِهِر الْأَلْكْتَرَنِيَّ، وَقِيَاسُ تَرْكِيبِ الْأَنْبِيِبَات المَنْوِية وَقَد تَمّ الْحُصُول عَلَى عِيْنَات السَّائِلَ المَنْوِي مِن الْبَرْيْح الْدِّينِي لِفَحْصٍ عَدْد وَجِوْدَةِ الْحِيْوَانَات المَنْوِية.

أَظْهَرَ الفَحْصُ تَحْتِ المَجِهِر الْضَّوْنِي لَخَصِيَّةِ مِجمَعَةِ التَّرْكِيبِ النسجِيَّ الطَّبِيعِي لِمَعْظَمِ الْأَنْبِيِبَات المَنْوِية وَمُكوَّنَاتِها.

كَشْفَت خَصِيَّة مِجمَعَتِي جَرْذَانَ التَجَارِب عَن اِنْكَماشٍ مُعْظَمِ الْأَنْبِيِبَات المَنْوِية مَمَا أَدَى إِلَى زِيَادَةِ البَلْغَة الأَهْمَى فِي مَسِاحَات النسِجِ الخَلاَلِي. وَقَد لُوْحَظ انفُصَال السَّفِيحة القَادِمَة فِي العَدْد مِن الْعِيْنَات مَمَا أَدَى إِلَى تَشْكِيْلِ السَّمَائَسِ الضِيْقِة. كَمَا أَظْهَرَت بَعْضِ الْأَنْبِيِب تَعْرِجَاتِ فِي السَّفِيْحَة القَادِمَة. وَقَد لُوْحَظ تَدهْرُ الخَلاَيا الجَرْثوُمِيَّة فِي العَدْد مِن الْأَنْبِيِب. كَمَا أَنَّهُ يَوْجَد انْفَخَاضٌ فِي عَدْد الخَلاَيا فِي كَثِيرٍ مِن الْأَنْبِيِب المَنْوِية. وَأَظْهَرَت الْظِهَارَة المَنْوِيَّة كِثَّيْراً مِن الْفَجُوَاتِ وَالْخَلاَيا أَصْحَب تَرْتِيبُهَا غَيْر مُحِضَت. كَمَا ظَهَرَت بَعْضِ الْفَجُوَاتِ فِي النسِجِ الخَلاَلِي.
أيضاً ، كان هناك انخفاض في أبعاد الأنانيب المنوية، سمنة الظهرة المنوية وتركيز الحيوانات
المنوية في تجاوز معظم الأنانيب المنوية.

لم يظهر أي تغيير تركيبي عند فحص خصية الجنذان في مجموعة التحكم بواسطة المجهر
الإلكتروني النافذ. وأظهر فحص الأنانيب المنوية أنها تتألف من الخلايا المنوية وكذلك خلايا
سيرتولي. تمتد خلايا سيرتولي من الغشاء القاعدي إلى تجفيف الأنانيب المنوية. ويدعو أنها غير
منظمة، مع وجود أنوية قاعدية ببضاوية مسندة تحتوي على نوبة واضحة. يعارض غشاء الخلية
الرئيسي كل الخلايا المنوية وقد أظهر تعرجاً معتقاً من الداخل.

كان السيتوبلازم كثيفًا و غنيًا بالشبكة الاندوبلازمية المسيلة ، الريبوسومات الحرة، والعديد من
الميتوكوندريا ، و جهاز قولجي، و العديد من قطرات الدهون و الجسيمات المحتلة. وقد لوحظت
الخلايا المنوية في مراحل مختلفة من النضج.

أما عند فحص خصية الجنذان مجموعتي التجارب بالمجهر الإلكتروني فقد تبين ظهور فجوات و
قطرات الدهون في خلايا سيرتولي وكذلك في النسيج الخلائي. و ظهرت الميتوكوندريا منتفخة، و
يدعو أنها فقدت أعرافها. وقد لوحظ تناكس بعض أمهات المنى (الخلايا المنوية الأولية) بدأت كثيفة
الكروماتين. بعض أمهات المنى بأنويتها النغطية و المجوزة فقدت الاتصال بخلايا سيرتولي
المحيطة و الصفيحة القاعدية. وقد لوحظت المساحات التي خلفتها الخلايا الجرثومية المتنكسة.

مقارنة بمجموعة التحكم، أظهر تحليل السائل المنوي لمجموعتي التجارب (التي تعرضتا لهورمون
البروجسترون أثناء الحمل/ الرضاعة) انخفاضاً بليغاً في عدد، وحركة، و حيوية الحيوانات
المنوية بالإضافة إلى الإنخفاض البليغ في الوزن النسبي لخصية وبربخ مجموعتي التجربة مقارنة بالوزن النسبي لمجموعة التحكم.

أوضحت الدراسة أيضاً أن نسبة الحيوانات المنوية الغير طبيعية الشكل كانت أعلى بشكل ملحوظ في جرذان مجموعات التجارب بالمقارنة مع جرذان مجموعة التحكم وقد شملت هذه التسهؤات:

الحيوانات المنوية مقطعة الرأس، مقطعة الدليل وملفوفة الذيل.

أيضاً هنالك انخفاض ملحوظ في مستوى هرمون التستوستيرون في الدم وفي الوقت نفسه وجدت زيادة بالغة الأهمية في الهورمون المصفر والهرمون المنبه لنضوج الجرذية في مجموعتي التجارب مقارنة بمجموعة التحكم.

مقارنة بالمجموعة التجريبية الأولى ( تعرضت للهرمون أثناء فترة الحمل)، أظهرت المجموعة التجريبية الثانية ( تعرضت للهرمون خلال فترتي الحمل الرضاعة) انخفاضاً ملحوظاً في حركة وحيوية الحيوانات المنوية وزيادة بالغة الأهمية في إجمالي تشهوات الحيوانات المنوية وزن البربخ.

خلصت هذه الدراسة إلى أن التعرض لهورمون البروجسترون أثناء الحمل يؤثر سلباً على التركيب النسيجي للخصيوي و القدرة الإنجابية لدى المواليد الذكور عند وصولهم لسن البلوغ بينما التعرض الطويل الأمد لهورمون البروجسترون (خلال فترتي الحمل و الرضاعة) يمكن أن يفاقم من المشكلة و ذلك بتأثيره السلبي على تطور و نضوج الحيوانات المنوية.
### List of contents

<table>
<thead>
<tr>
<th>No</th>
<th>contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dedication</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td>Acknowledgement</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>Abstract (English)</td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td>Abstract (Arabic)</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>List of contents</td>
<td>xii</td>
</tr>
<tr>
<td></td>
<td>List of tables</td>
<td>xiv</td>
</tr>
<tr>
<td></td>
<td>List of figures</td>
<td>xv</td>
</tr>
<tr>
<td></td>
<td>Abbreviations</td>
<td>xx</td>
</tr>
<tr>
<td>1</td>
<td>Introduction and Objectives</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Rational &amp; justification</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>Objectives</td>
<td>5</td>
</tr>
<tr>
<td>1.3.1</td>
<td>General objectives</td>
<td>5</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Specific objectives</td>
<td>5</td>
</tr>
<tr>
<td>1.4</td>
<td>Hypothesis</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Literature Review</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>Testes overview</td>
<td>6</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Anatomy of the testis</td>
<td>6</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Histological background</td>
<td>7</td>
</tr>
<tr>
<td>2.1.2.1</td>
<td>Seminiferous tubules</td>
<td>7</td>
</tr>
<tr>
<td>2.1.2.1.1</td>
<td>Seminiferous epithelium</td>
<td>7</td>
</tr>
<tr>
<td>2.1.2.1.1.1</td>
<td>Spermatogonia</td>
<td>8</td>
</tr>
<tr>
<td>2.1.2.1.1.2</td>
<td>Primary and secondary spermatocytes</td>
<td>8</td>
</tr>
<tr>
<td>2.1.2.1.1.3</td>
<td>Spermatids</td>
<td>9</td>
</tr>
<tr>
<td>2.1.2.1.1.4</td>
<td>Spermatids</td>
<td>10</td>
</tr>
<tr>
<td>2.1.2.1.1.5</td>
<td>Sertoli cells</td>
<td>11</td>
</tr>
<tr>
<td>2.1.2.1.2</td>
<td>Boundary tissue</td>
<td>13</td>
</tr>
</tbody>
</table>
## 2.1.2.2 The Interstitial tissue

## 2.1.3 Development of testicular structure

## 2.1.3.1 Germ cells

## 2.1.3.2 Somatic cells

## 2.1.4 Endocrine regulation of spermatogenesis

## 2.1.5 Steroid feedback mechanism

## 2.1.6 Endocrine function of the testes

## 2.2 Previous studies

## 2.2.1 Endocrine disruptors

## 2.2.2 Effects of prenatal and / or postnatal progesterone on the testicular structure and function

### 3 Material and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Study field</td>
</tr>
<tr>
<td>3.2</td>
<td>Study design</td>
</tr>
<tr>
<td>3.3</td>
<td>Sample species</td>
</tr>
<tr>
<td>3.4</td>
<td>Materials</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Animals</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Chemicals</td>
</tr>
<tr>
<td>3.5</td>
<td>Methods</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Experimental Design</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Tissue collection and preparation</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Semen analysis</td>
</tr>
<tr>
<td>3.5.3.1</td>
<td>Sperm counts</td>
</tr>
<tr>
<td>3.5.3.2</td>
<td>Sperm motility</td>
</tr>
<tr>
<td>3.5.3.3</td>
<td>Sperm viability</td>
</tr>
<tr>
<td>3.5.3.4</td>
<td>Sperm morphology</td>
</tr>
<tr>
<td>3.5.4</td>
<td>Histometry</td>
</tr>
<tr>
<td>3.5.5</td>
<td>Cells quantitation</td>
</tr>
<tr>
<td>3.5.6</td>
<td>Ultrastructure of the testis</td>
</tr>
<tr>
<td>3.5.7</td>
<td>Serum collection &amp; hormonal Assay</td>
</tr>
<tr>
<td>3.5.8</td>
<td>Statistical analysis</td>
</tr>
</tbody>
</table>

### 4 Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Light microscopic results</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Control group</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Experiment I and II groups</td>
</tr>
<tr>
<td>4.2</td>
<td>Electron microscopic results</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Control group</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Experiment I and II groups</td>
</tr>
<tr>
<td>4.3</td>
<td>Semen analysis</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Sperm Counts</td>
</tr>
</tbody>
</table>
4.3.2 Sperm Motility
4.3.3 Sperm Viability
4.3.4 Sperm Morphology
4.4 Histometry
4.4.1 Testicular weight
4.4.2 Epididymal weight
4.4.3 Relative testicular weight
4.4.4 Relative epididymal weight
4.4.5 Testicular diameters
4.4.6 Diameter of the seminiferous tubules
4.4.7 Epithelial height
4.4.8 Thickness of the interstitial space
4.5 Stereological study
4.5.1 Quantitation of seminiferous tubule cells
4.5.2 Quantitation of seminiferous tubules and Leydig cells
4.6 Hormonal assay

5 Discussion
5.1 Histological findings
5.2 Spermatogenic parameters
5.3 Testicular and epididymal parameters
5.4 Hormonal Changes

6 Conclusion and recommendations
6.1 Conclusion
6.2 Recommendations

7 References

List of tables

<table>
<thead>
<tr>
<th>No</th>
<th>Table title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Effects of synthetic progesterone on the sperm count, motility, viability and total abnormality (%)</td>
<td>73</td>
</tr>
<tr>
<td>4.2</td>
<td>Effects of synthetic progesterone on the sperm abnormalities (%)</td>
<td>77</td>
</tr>
<tr>
<td>4.3</td>
<td>Effects of synthetic progesterone on the testicular, Epididymal and relatives weight (gm)</td>
<td>83</td>
</tr>
<tr>
<td>4.4</td>
<td>Effects of synthetic progesterone on the testicular diameters (mm)</td>
<td>85</td>
</tr>
<tr>
<td>4.5</td>
<td>Effects of synthetic progesterone on the seminiferous tubule diameter, height of germinal epithelium and thickness of interstitial space (µm)</td>
<td>87</td>
</tr>
<tr>
<td>4.6</td>
<td>Effects of synthetic progesterone on the seminiferous tubule cells count</td>
<td>88</td>
</tr>
<tr>
<td>4.7</td>
<td>Effects of synthetic progesterone on the total numbers of seminiferous</td>
<td>90</td>
</tr>
</tbody>
</table>
tubules and Leydig cells

4.8 Effects of synthetic progesterone on the serum hormones level (ng/ml) 91

List of figures

<table>
<thead>
<tr>
<th>No</th>
<th>figure title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>(A&amp;B) Light photomicrographs of control rat testis.</td>
<td>56</td>
</tr>
<tr>
<td>4.2</td>
<td>Light photomicrographs of control rat testis.</td>
<td>57</td>
</tr>
<tr>
<td>4.3</td>
<td>Light photomicrographs of control rat testis.</td>
<td>57</td>
</tr>
<tr>
<td>4.4</td>
<td>Light photomicrographs of control rat testis.</td>
<td>58</td>
</tr>
<tr>
<td>4.5</td>
<td>(A), Light photomicrographs of experiment (I) group testis.</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>(B), Light photomicrographs of experiment (I) group testis.</td>
<td>59</td>
</tr>
<tr>
<td>4.6</td>
<td>Light photomicrographs of experiment (II) group testis.</td>
<td>59</td>
</tr>
<tr>
<td>4.7</td>
<td>Light photomicrographs of (A); experiment (I) &amp; (B); experiment (II) group testes.</td>
<td>60</td>
</tr>
<tr>
<td>4.8</td>
<td>Light photomicrographs of (A); experiment (I) &amp; (B) experiment (II) groups testes.</td>
<td>61</td>
</tr>
<tr>
<td>4.9</td>
<td>Light photomicrographs of experiment (I) group testes</td>
<td>62</td>
</tr>
<tr>
<td>4.10</td>
<td>Light photomicrographs of experiment (I) group testes.</td>
<td>62</td>
</tr>
<tr>
<td>4.11</td>
<td>Light photomicrographs of experiment (II) group testes.</td>
<td>63</td>
</tr>
<tr>
<td>4.12</td>
<td>Light photomicrographs of experiment (I) group testes.</td>
<td>63</td>
</tr>
<tr>
<td>4.13</td>
<td>Light photomicrographs of experiment (II) group testes.</td>
<td>64</td>
</tr>
<tr>
<td>4.14</td>
<td>Electron photomicrograph of seminiferous tubule of control group.</td>
<td>64</td>
</tr>
<tr>
<td>4.15</td>
<td>Electron photomicrograph of seminiferous tubule of control group.</td>
<td>65</td>
</tr>
<tr>
<td>4.16</td>
<td>Electron photomicrograph of spermatid At acrosomal phase of control group.</td>
<td>65</td>
</tr>
<tr>
<td>4.17</td>
<td>Electron photomicrograph of Sertoli cell of control group.</td>
<td>66</td>
</tr>
<tr>
<td>4.18</td>
<td>Electron photomicrograph of Leydig cell (LC) of control group.</td>
<td>66</td>
</tr>
<tr>
<td>4.19</td>
<td>Electron photomicrograph of seminiferous tubule of experiment (I) group.</td>
<td>68</td>
</tr>
<tr>
<td>4.20</td>
<td>Electron photomicrograph of seminiferous tubule of experiment (II) group.</td>
<td>68</td>
</tr>
<tr>
<td>4.21</td>
<td>Electron photomicrograph of seminiferous tubule of experiment (II) group.</td>
<td>69</td>
</tr>
<tr>
<td>4.22</td>
<td>Electron photomicrograph of seminiferous tubule of experiment (I)</td>
<td>69</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>4.23</td>
<td>Electron photomicrograph of seminiferous tubule of experiment (II) group.</td>
<td></td>
</tr>
<tr>
<td>4.24</td>
<td>Electron photomicrograph of seminiferous tubule of experiment (I).</td>
<td></td>
</tr>
<tr>
<td>4.25</td>
<td>Electron photomicrograph of Sertoli cell of experiment (I).</td>
<td></td>
</tr>
<tr>
<td>4.26</td>
<td>Electron photomicrograph of Leydig cell (LC) of experiment (I) groups.</td>
<td></td>
</tr>
<tr>
<td>4.27</td>
<td>Electron photomicrograph of Leydig cell (LC) of experiment (II) groups.</td>
<td></td>
</tr>
<tr>
<td>4.28</td>
<td>Epididimal sperm count levels in the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.29</td>
<td>Epididimal sperm motility in the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.30</td>
<td>Epididimal sperm viability in the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.31</td>
<td>(A) Photomicrograph of sperm viability analysis of control group.</td>
<td></td>
</tr>
<tr>
<td>4.31</td>
<td>(B) Photomicrograph of sperm viability analysis of experiment group.</td>
<td></td>
</tr>
<tr>
<td>4.32</td>
<td>Total of epididimal sperm abnormalities in the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.33</td>
<td>Types of others epididimal sperm abnormalities in the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.34</td>
<td>The most prominent types of epididimal sperm abnormalities in the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.35</td>
<td>Photomicrographs of sperm morphology analysis obtained from the control group.</td>
<td></td>
</tr>
<tr>
<td>4.36</td>
<td>Photomicrograph (A and B) of sperm morphology analysis obtained from the experiment (I) group.</td>
<td></td>
</tr>
<tr>
<td>4.37</td>
<td>Photomicrograph (A and B) of sperm morphology analysis obtained from the experiment (II) group.</td>
<td></td>
</tr>
<tr>
<td>4.38</td>
<td>Testicular and Epididymal weight of the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.39</td>
<td>Relative Testicular and Epididymal weight of the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.40</td>
<td>Testicular diameters of the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.41</td>
<td>Seminiferous tubule diameter, height of germinal epithelium and interstitial space thickness of the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.42</td>
<td>Seminiferous tubule cells percentage of the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.43</td>
<td>Seminiferous tubule and Leydig cells percentage of the control and the experiment groups of rats.</td>
<td></td>
</tr>
<tr>
<td>4.44</td>
<td>Serum hormones level of the control and the experiment groups of rats.</td>
<td></td>
</tr>
</tbody>
</table>
Abbreviations

Ad : Spermatogonia; dark type A.
AP : Spermatogonia; pale type A.
cAMP : Cyclic adenosine monophosphate.
DES : Diethylstilbestrol
CYP11A and CYP17A : Cytochrome P450 enzymes
DMPA : Depot-medroxyprogesterone acetate.
EDs : Endocrine disruptors
EE : Ethinyl Estradiol.
EPA : Environmental protection agency (United States)
ERs : Estrogen receptors.
FSH : Follicle stimulating hormone.
GBG : Gonadal steroid-binding globulin.
GnRH : Gonadotrophin-releasing hormone.
GPR54 : G-protein-coupled receptor.
HPT : Hypothalamic pituitary- testicular.
HSD : Hydroxy-steroid dehydrogenase enzymes.
INSL3 : Insulin-like factor 3.
IVF : In-vitro fertilization.
KiSS-1 : Hypothalamic kisspeptin-1.
LH : Luteinizing hormone.
OS : Oxidative stress.
P4 : Progesterone.
CHAPTER ONE
INTRODUCTION AND OBJECTIVES

1.1. Introduction

Deterioration of male reproductive health in humans and wildlife has been reported during the last few decades. While the reasons for this problem is currently unknown, both clinical and laboratory research suggest that the adverse changes may be interrelated and may have a common origin in fetal life \[^{[1]}\].

Fertility of male and female is a major concept in reproduction, gynecology and obstetrics. Recently, fertility has become an increasingly important issue over the last years because data suggest that
there has been a progressive increase in disorders of male and female reproduction and there is a great concern as to whether man-made drugs and chemicals could have contributed to these changes [2].

Maintenance of steroid hormone homeostasis within the organism is critical to ensure normal sexual functions in males and females, including sexual differentiation. Recently, concern has been raised that humans are being exposed to higher concentrations of steroids in the form of oral contraceptives and hormone injections to protect pregnancy or for family planning [3]. These chemicals may be responsible for eliciting adverse effects on development and fertility of the next generation [3].

Testes are the primary reproductive organ in the male which have a dual function: the production of germ cells (gametogenesis) and the secretion of sex hormones. They secrete large amounts of androgens, principally testosterone, but they also secrete small amounts of estrogens [4,5].

The progesterone (pregn-4-ene -3, 20-dione; abbreviated as P₄) is an endogenous steroid hormone involved in the menstrual cycle, pregnancy and embryogenesis of humans and other species. It is the major progestogen in the body that belongs to a group of steroid hormones called the progestogens.

The progesterone is a crucial metabolic intermediate in the production of other endogenous steroids including the sex hormones and the cortico-steroids and plays an important role in brain function as a neurosteroid [7].

Endogenous progesterone plays a role in maintaining pregnancy, preparing the body for conception, the uterus for implantation, regulating the menstrual cycle and plays a role in sexual desire. The
hormone is produced in the ovaries, adrenal glands and corpus luteum and placenta (when a woman gets pregnant) \[^{8}\].

Synthetic and semisynthetic progesterone (progestin) are group of uniform progestational compounds that have been manufactured and all of them differ from the natural progesterone in one or more aspects \[^{9}\]. In general, the 21-carbon compounds that were used in the present study, hydroxyprogesterone and medroxyprogesterone are pharmacologically and chemically related to the progesterone \[^{10, 11}\]. Following their administration; via any route; these compounds are rapidly absorbed and stored in the body fat \[^{12}\].

Exogenous progesterone compounds are capable to cross the human placenta efficiently and are detectable in both maternal and fetal blood for at least 44 days after the last injection \[^{13}\]. With repeated injections; even when the doses are administered as much as week apart; their plasma concentration continues to increase. This is because these exogenous progesterones are slowly released from their oil depots and maternal fat \[^{14}\].

Synthetic progesterone is one of the most effective and widely used drugs in medical practice for many therapeutic purposes. It is traditionally used to support pregnancy during the first trimester especially in cases of early threatened abortions, abnormal uterine bleeding \[^{15}\] and for luteal support for in-vitro fertilization (IVF) pregnancies \[^{16}\].

Progesterone is also used to induce ovulation to treat the primary infertility and decreases the contractility of the smooth muscle of the uterus, for prevention of preterm delivery for almost more than 40 years, to cure breast pain in women with noncancerous breast disease, to prevent and treat abnormal thickening of the uterus endometrium (endometrial hyperplasia), heavy menstrual bleeding, endometriosis,
pelvic pain syndromes and is used topically in certain skin diseases \cite{17}. Progesterone alone is used as a contraceptive especially during lactation period \cite{18}.

Depot-Medroxyprogesterone Acetate (DMPA) is one of the most used contraceptives and it is currently used by more than 30 million women in most of the developed and developing countries for their longstanding regulation of menstrual cycle and high efficacy in inhibiting ovulation as well as sperm penetration \cite{19}. Synthetic progesterone is one of the most effective and widely used drugs in medical practice for many therapeutic purposes \cite{19, 20}.

Recent epidemiological studies have shown decreased semen concentration, sperm motility with increased percentages of abnormal sperm and increased congenital abnormalities like hypospadias and cryptorchidism in many countries \cite{20-24}. These abnormalities have been attributed to deterioration in environment quality and increased exposure to xenoestrogens and antiandrogens \cite{25}.

The use of synthetic and semi-synthetic progesterone compounds in early stages of embryogenesis and during lactation may carry hazardous effects on the testes structure of male offspring \cite{26, 27}. Prenatal and gestational exposure to hydroxyprogesterone caproate caused a significant decrease in the activity levels of steroidogenic enzymes, decreased sperm count, sperm motility and the sperm viability \cite{15}. The circulatory levels of testosterone, follicle stimulating hormone and luteinizing hormone in adult male rats exposed to hydroxyprogesterone during embryonic development was also significantly altered \cite{28}.

Histopathological studies following P$_4$ administration revealed severe testicular damage, degeneration of the seminiferous tubules
cells, widening of interstitial spaces, widening of the seminiferous tubules lumen with necrozoospermia \cite{29}.

1.2. **Rationale and Justification**

In order to establish a balance between the advantages and the disadvantages of P$_4$, the current study is designed to investigate the effects of P$_4$ administration; during gestation and lactation; on the structure and function of the testicles of pubertal off-spring reared on their treated mothers until weaning. This will be accomplished by measurement of changes in hormonal levels, testicular cells and seminiferous tubules morphometric parameters that may occur after P$_4$ administration during the critical period of embryogenesis and lactation.

Over the years, rats have been used in many experimental studies which have added to our understanding of genetics, disease, the effect of drugs and other topics in health and medicine. Rats had well characterized reproductive processes and general use in toxologic studies. Thus albino rats will serve as a good model to study the possible changes that might occur in testes of pubertal rats after pre and postnatal P$_4$ exposure.

On rats, you can accomplish different experimental tasks because of their large size, easiness to performing surgical procedures and monitoring their physiological states \cite{30}.

1.3. **Objectives**

1.3.1. **General Objectives**

To assess the effects of prenatal and / or lactational exposure to synthetic progesterone (P$_4$) on pubertal testicular structure and functions of rat offspring.

1.3.2. **Specific objectives**
The specific objectives are to:

2. Assess the prenatal exposure effect of (P₄) on the testis structure (histology, histometry and ultra-structure) and functions.

3. Clarify the effect of (P₄) as female contraceptive during lactational period on the reared rat testis structure (histology, histometry and ultra-structure) and functions.

4. Investigate the effect of prenatal and/ or lactational exposure to (P₄) on the sperm quantity and quality.

5. Evaluate the effect of prenatal and/ or lactational exposure to (P₄) on the level of Testosterone, FSH and LH hormones.

1.4. Hypothesis

Exposure to the progesterone (anti-androgen) during prenatal and / or lactation periods may impair pubertal testes structure and function.

CHAPTER TWO

LITERATURE REVIEW

2.1. Testes overview

2. 1. 1. Anatomy of the testis

The testes are the primary reproductive organs or gonads in the male. They are ovoid reproductive and endocrine organs responsible for sperm production and are suspended in the scrotum by scrotal tissues, including the non-striated dartos muscle and the spermatic cords. Average testicular dimensions in man are 4-5 cm in length, 2.5 cm in breadth and 3 cm in anteroposterior diameter; their weight varies from 10.5 -14 gm \[31\].
The surface of the testis is covered closely by the visceral tunica vaginalis, a layer of flat mesothelial cells similar to and continuous with the peritoneal lining. It is separated from the parietal tunica vaginalis, the outer layer of the double fold of peritoneum, which accompanies the descending testis by a potential space containing serous fluid, which acts as a lubricant and allows the movement of the testis within the scrotum. The testicular capsule proper, the tunica albuginea, is tough and collagenous and thickened posteriorly as the mediastinum testis \[^{31}\].

Beneath the tunica albuginea is a thin layer of connective tissue containing the superficial blood vessels. Blood vessels, lymphatics and the genital ducts enter or leave the body of the testis at the mediastinum. Septa from the mediastinum extend internally to divide the testis into about 250 lobules which differ in size, the largest and longest are in the centre \[^{31}\].

Each lobule contains one to four convoluted seminiferous tubules, which are much-coiled loops the free ends of which open into channels (the rete testis) within the mediastinum. The loose connective tissue between the seminiferous tubules contains several layers of contractile peritubular myoid cells and clusters of steroid producing interstitial (Leydig) cells \[^{31}\].

### 2.1.2. Histological Background

#### 2.1.2.1. Seminiferous tubules

There are 400-600 seminiferous tubules in each testis and the length of each is 70-80 cm. Their diameter varies from 0.12-0.3 mm. They are pale in early life, but in old age, they contain much fat and are deep
yellow. Each tubule is surrounded by a basal lamina, on which rests a complex, stratified seminiferous epithelium consisting of spermatogenic cells and supporting Sertoli cells.

When active, the spermatogenic cells include basally situated spermatogonia and their progeny in the adluminal compartment, spermatocytes (primary and secondary), spermatids and mature spermatozoa. Among the spermatids may be found residual bodies, which are spherical structures, derived from surplus spermatid cytoplasm shed during maturation and phagocytised by Sertoli cells \cite{31, 32}.

2.1.2.1.1. Seminiferous epithelium
The seminiferous tubules are lined by a complex stratified epithelium composed of two major types of cells, spermatogenic cells and Sertoli cells.

2.1.2.1.1.1 Spermatogonia
According to Witschi \cite{32}, the spermatogonia are the stem cells for all spermatozoa, there are derived from primordial germ cells, which migrate into the genital ridge of the developing testis. In the fully differentiated testis they are located along the basal laminae of the seminiferous tubules. Maturation in man covers a period of $70 \pm 4$ days. Several types of spermatogonia are recognized on the basis of cell and nuclear dimensions, distribution of nuclear chromatin (dark, condensed or pale, euchromatic) and histochemical and ultrastructural data \cite{33}. 
There are three basic groups of spermatogonia; dark type A (Ad), pole type A (AP), and type B. Ad cells divide mitotically to maintain the population of spermatogonia which, before puberty, is small but increases under androgenic stimulation. Some divisions give rise to a cell, which also divide mitotically but remain lined with clusters by fine cytoplasmic bridges. These are the precursors of type B cells, which are committed to the spermatogenic sequence. At about the time, type B cells enter a final round of DNA synthesis, without undergoing cytokinesis, they leave the basal compartment and cross the blood-testis barrier to enter meiotic prophase as primary spermatocytes. These coordinated processes are under the control of Sertoli cells\textsuperscript{31}.

2.1.2.1.1.2. Primary and secondary spermatocytes

Primary spermatocytes have a haploid chromosome number, but duplicated sister chromatids (DNA content is thus 4N, where N is the DNA content of haploid spermatozoa). They are all at some stage of a long meiotic prophase of three weeks. Primary spermatocytes are characteristically large cells with large round nuclei in which the nuclear chromatin is condensed into dark, threadlike, coiled chromatids at different stages in the process of crossing over and genetic exchange between chromatids of maternal and paternal homologues. These cells give rise to secondary spermatocytes with a haploid chromosome complement (2N DNA content), the reduction division is designed as meiosis I\textsuperscript{31-33}.

Few secondary spermatocytes are seen in tissue sections because they rapidly undergo the second meiotic (equational) division, where sister chromatids separate (DNA content is now N), to form haploid
spermatids. Theoretically, each primary spermatocyte produces four spermatids, but some degenerate during maturation so that the yield is lower \[^{31-33}\].

2.1.2.1.3. Spermatids

Spermatids do not divide again but gradually mature into spermatozoa by a series of nuclear and cytoplasmic changes known as spermiogenesis. All of these maturational changes take place while the spermatids remain closely associated with Sertoli cells and linked by cytoplasmic bridges with each other \[^{31-33}\].

The first phase of spermiogenesis is the Golgi phase, during which hydrolytic enzymes accumulate in the Golgi vesicles, which coalesce into a single large acrosomal vesicle, close to the nucleus. The pair of centrioles migrates to the opposite posterior pole. The distal centrioles begin to generate the axoneme, a circular arrangement of nine microtubules doublets surrounding a central pair. In the cap phase, which follows, the acrosomal vesicle flattens and envelops the anterior half of the nucleus to form an acrosomal cap. This comes to occupy the presumptive anterior pole of the spermatozoon, furthest from the tubule lumen \[^{31-33}\].

During the acrosome phase, nuclear chromatin condenses and nucleus elongates to a shape characteristic of species; in man for example, is almond shape. The anterior cytoplasmic volume reduces considerably, bringing the wall of the acrosomal vesicle into contact with the plasma membrane. A perinuclear sheath of microtubules develops from the posterior edge of the acrosome to the manchette, extending towards the posterior pole \[^{31-33}\].
The Axonemal complex continues to extend into the developing tail region, which protrudes into the tubule lumen. Prominent mitochondria migrate through a neck region, which forms at the posterior pole of the nucleus and contains the centrioles, and along the axoneme into the developing middle piece. Here they assemble into a helical sheath of mitochondria, which surrounds a ring of nine coarse fibres forming around the Axonemal complex along its length in the developing tail [31-33].

In the final phase of maturation, excess cytoplasm is detached as a residual body, which is phagocytised and degraded by Sertoli cells. During the formation of the residual bodies, spermatids lose their cytoplasmic bridges and separate from each other before being released into their tubules [31-33].

2.1.2.1.4. Spermatozoa

As they are released from the wall of the seminiferous tubule into the lumen, the spermatozoa are non-motile but structurally mature. The expanded head contains little cytoplasm and is connected by a short, constricted neck to the tail. The tail is a complex flagellum, which greatly exceeds the head region in volume, and is divided into middle, principal and end pieces.

In man for example, the head has a maximum length of about 4µm and a maximum diameter of 3µm and contains the elongated, flattened nucleus with condensed, deeply staining chromatin and the acrosomal cap entirely. The latter contains acid phosphatase, hyaluronidase, neuraminidase and proteases necessary for fertilization [31].

2.1.2.1.5. The Sertoli cells:
Sertoli cells are also known as supporting or sustentacular cell, provide mechanical support and protection for the developing germ cells and play an active role in the release of the mature spermatozoa \[^{34}\]. They are also responsible for degradation and disposal of residual bodies of the developing spermatozoa. In tangential sections, the supporting cells of Sertoli are polygonal in shape. In transverse sections, however, they appear irregular but basically columnar in shape. Resting upon the basal lamina and extending through the thickness of the epithelium to the lumen \[^{35, 36}\].

Sertoli cells in the normal adult testis are nondividing cells, which are relatively inconspicuous on a cross section of the seminiferous tubule and comprise about 10 -15% of the tubular cellular elements \[^{37}\]. A system of thin processes from the Sertoli cells extend between the spermatogenic cells forming pockets for the spermatogenic cells, helping in their differentiation, transport and release, some of these processes are connected to the neighborhood cells forming the blood-testis barrier.

The blood testicular barrier bound the earliest spermatogenic cells, spermatogonia (Type A and B) and the preleptotene primary spermatocytes to the basal lamina, while the more advanced developing cells are found successively in higher levels in the epithelium in the adluminal compartment of the seminiferous tubules \[^{38, 39}\].

Bloom and Fawcett \[^{35}\], described the Sertoli cell nucleus as ovoid in shape while Williams et al \[^{36}\], described it as irregular in outline and has infoldings on its surface. It is about 9-12 μm in diameter with relatively homogenous nucleoplasm, except for a large
and highly characteristic nucleolus consisting of a round or oval central body flanked by two rounded basophilic masses. The two darker masses of finely granular material appear to be nucleolus-associated chromatin.

Williams et al \cite{36}, described the cytoplasmic organelles being numerous, particularly the mitochondria, and a well developed agranular smooth endoplasmic reticulum (SER) and free ribosomes. There are also, a large Golgi complex, lysosomal system, microfilaments, microtubules; and the latter help in formation of the cytoskeleton.

The Sertoli cell cytoplasm forms a meshwork closely surrounding the developing germ cells. At puberty, a tight junction complex forms between adjacent Sertoli cells, dividing the tubule into basal and adluminal compartments. The former contains spermatogonia and pre-leptotene spermatocytes; the latter holds the remaining primary spermatocytes, secondary spermatocytes and spermatids \cite{37}.

A transient, intermediate compartment is formed by adjacent Sertoli cells as germ cells that move from the basal compartment to the adluminal compartment. Tight junction complex forms behind the germ cells to seal off the intercellular space, thereby insuring the integrity of the Sertoli cells barrier \cite{40}. This junction forms the true blood-testis barrier, preventing access of blood borne constituents to the luminal compartment except through the Sertoli cell cytoplasm. Other cell connections between adjacent Sertoli cells include gap junctions, desmosomes (rarely) and ectoplasm specialization sites. Intercellular junction like-structures have also been described
between Sertoli cells and germ cells, presumably serving as the conduit by which the Sertoli cell plays a role in the regulation of spermatogenesis \cite{41}.

In addition to their phagocytic capacity and role in modulating spermatogenesis, the Sertoli cells are active in the synthesis of a wide variety of proteins and other substances, including transferrin, growth factor ceruloplasmin testibumin and inhibin \cite{42}. The Sertoli cell contains a large amount of steroid-binding protein that serves to transport testosterone to the tubule lumen for use by the epithelial cells of the rete testis and the remaining portion of the excretory duct \cite{43}.

**2.1.2.1.2. Boundary tissue**

The components of the boundary tissue, invest the seminiferous tubules of the testis in the form of concentric layers, displaying certain differences of the species \cite{44}. Moniem et al \cite{45}, described the boundary tissue of seminiferous tubule being constituted of four basic components, a homogenous matrix, collagenous fibres, elongated contractile (Myoid) cells and fibroblasts.

According to Bloom and Fawcett \cite{35}, the seminiferous tubules are enclosed by one or more layers of adventitial cells derived from primitive tissue elements of the interstitium.

Ultrastructurally, the basement membrane is multi-layered with frequent splitting and knobby thickenings. These knobs are intimately associated with the basal portion of Sertoli cells. The lamina propria consists of an inner zone of collagen fibres and an outer zone of spindle shaped cells. These cells have the capacity to produce collagen and other extracellular fibrils and also have contractile
ability. They are referred to as fibromyocytes or myoid cells and play an important role in the movement of spermatozoa toward the rete testis \cite{46,47,48}.

2.1.2.2. The Interstitial tissue

According to Miller et al \cite{46}, the interstitium of the testis accounts for 25-30\% of the testicular mass. It can be divided loosely into intertubular and peritubular regions. Within the former are clusters of epithelioid interstitial cells, called Leydig cells, blood vessels, lymphatics, nerves, macrophages and mast cells. The macrophages are often found in close association with Leydig cells. Interstitial cells of Leydig are the endocrine components of the testis. They are mesenchymal in origin and may arise from mesonephric blastema \cite{47}. They are located in the angular interstices between the convoluted seminiferous tubules. Surrounding each seminiferous tubule in a sheath-like fashion (peritubular) are the lamina propria and an intervening homogeneous basement membrane, which together measure 0.3 to 0.4 \textmu m in width, \cite{35}. In addition to the clusters of Leydig cells \cite{36}.

Elastic fibres, as demonstrated by Verhoeff’s, iron hematoxylin stain, first appear at puberty in the outermost layer of the lamina propria \cite{48}.

Leydig cells are irregularly polyhedral in shape and closely packed but at the periphery of the clusters, or where occurring individually, they may be elongated or spindle shaped. Their large, spherical nuclei contain small amounts of peripherally disposed heterochromatin and one or two nucleoli \cite{35,47}.
The cytoplasm is acidophilic with vacuoles where lipid droplets have been extracted. Ultrastructurally, there is a large clear area occupied by the Golgi complex and the most striking feature is the extensive development of smooth endoplasmic reticulum and the abundance of mitochondria with tubular cisternae \[^{36}\].

2. 1.3. Development of testicular structure

The testes are derived from three sources, the mesothelium lining the posterior abdominal wall, underlying mesenchyme and primordial germ cells. The initial stages of gonadal development occur during the fifth week, when a thickened area of mesothelium develops on the medial side of the mesonephros. Proliferation of this epithelium and the underlying mesenchyme produces a bulge on the medial side of the mesonephros, which is called the genital or gonadal ridge \[^{49}\].

At first, the gonadal primordial is colonized by the primordial germ cells. They originate from the epiblast, migrate through the primitive streak, and, by the third week, reside among endodermal cells in the wall of the yolk sac close to the allantois. They migrate along the dorsal mesentery of the hindgut during the fourth week, arrive at the primitive gonad at the beginning of the fifth week, and invade the genital ridges in the sixth week \[^{50}\].

Fingerlike epithelial gonadal cords grow into the underlying mesenchyme. The indifferent gonad now consists of an external cortex and an internal medulla \[^{50}\]. If the germ cells fail to reach the ridges, the gonads do not develop. Hence, the primordial germ cell shaves an inductive influence on development into testes. As the embryo grows, the gonadal ridge gradually becomes pinched off from the mesonephros. However; some cells of mesonephric origin join the
gonadal ridge. Furthermore, the gonadal ridge remains connected to the remnant of mesonephros by a fold of peritoneum, the mesorchium or mesovarium\[^{49}\].

During the seventh week, sexual distinction in the gonadal ridge becomes perceptible. The differentiation of the gonadal ridge into a testis is a rapid phenomenon, in contrast to the slow and late development of the ovary\[^{49, 50}\].

Seminiferous tubules originate from the mesonephros, the medulla. Whereas ovarian tissue originates from the secondary sex cords formed from the germinal epithelium and the cortex. Inside the seminiferous tubules, the germ cells are large. They divide actively but do not enter meiosis\[^{50, 51}\].

The Sertoli cells are smaller than the germ cells, tend to surround them and prepare the future seminiferous tubules. A basal membrane is formed, isolating the tubules from the surrounding mesenchyme tissue\[^{50, 51}\].

The individualization of tubules and the synthesis of anti-Müllerian hormone precede Leydig cell differentiation. Between the eighth and ninth weeks, the Leydig cells differentiate from interstitial tissue and then spread progressively in the intertubular spaces between the 14th and 18th weeks. They secrete testosterone from the eighth week of development\[^{51}\].

2.1.3.1. The Germ cells

Primordial germ cells arise from the epiblast and migrate from the extra-embryonic mesoderm to colonize the genital ridge. The germ cells are named gonocytes once they reach the gonad, in which they
proliferate until fetal day 17.5 in the rat. At birth, the gonocytes of the rat are located centrally in the seminiferous cords and are mitotically quiescent until they resume mitosis on postnatal day 3 and begin to migrate to the basement membrane. Relocated gonocytes form the spermatogonial stem cell pool and differentiate further into type A spermatogonia. Proliferation of type A spermatogonia indicates the beginning of spermatogenesis \[^{52-54}\].

In rats, the end of the neonatal period overlaps with the beginning of puberty. In humans, there is a long delay between birth and onset of puberty. This prolonged prepubertal period not quiescent although some developmental events remain clinically unnoticed. As in rodents, human fetal gonocytes are located at birth in the central part of the cord and migrate towards the basement membrane. During childhood (0 -10 years) the number of germ cells, tubular length and testis size increase \[^{55}\]. At the beginning of puberty germ cells, now called spermatogonia, begin to proliferate at a much higher rate \[^{56}\].

The adult testis contains stem spermatogonia, differentiating spermatogonia, primary and secondary spermatocytes and spermatids. Spermatogonia are located next to the basement membrane and in progressing towards tubular lumen spermatogonia develop into spermatocytes and finally spermatids. This developmental process can be divided into three phases; spermatogonial multiplication under which spermatogonia go through a series of mitotic division, meiosis where first meiotic division forms secondary spermatocytes and the second meiotic division during which the spermatids are formed and spermiogenesis where spermatids transform into sperms \[^{57}\].
In rats, the duration of spermatogenesis takes 52 days \[^{57}\], in monkey 40 days \[^{58},^{59}\] and in human 74 days \[^{60}\]. Spermatogenic cells are arranged in distinct cellular associations that follow each other in the tubules in a wave-like fashion and cyclically in time. These are known as the stages of the seminiferous epithelial cycle. In the rat, there are 14 stages \[^{61}\], in rhesus monkey 12 \[^{58}\] and in humans 6 stages \[^{62}\].

2.1.3.2. The Somatic cells

The Sertoli cell has a supportive role in the developmental process of germ cells by providing nutrients and growth factors. Since the number of Sertoli cells is known to correlate closely with testicular size and sperm output \[^{63}\], the number of these cells is important for future fertility.

Sertoli cells are the first to differentiate. From 13.5 prenatal day in the rat, and 42–45 prenatal day in human, they surround the germ cells to form the seminiferous cords. Sertoli cells divide actively after birth, but cease dividing in the rat at postnatal days 18 \[^{64}\]. Thereafter, the number of Sertoli cells is considered to be constant. Postnatally, Sertoli cells are first distributed unevenly in the testicular cords but as development proceeds they become more organized, columnar and acquire features of the adult Sertoli cells. In rats, by postnatal day 35 Sertoli cells have gained all the characteristics required for adult Sertoli cells \[^{65}\].

In primates, infancy has been classically described as a quiescent period during which the size of the testis or the number of Sertoli cells do not increase \[^{66},^{67}\]. However, when using variables that take into account the increase in the length of the seminiferous cord and volumetric growth of the testis, the active proliferation and
differentiation of Sertoli cells during this period has been revealed by Cortes et al \cite{68} and Ray et al \cite{69}.

Higher primates have been reported to have two periods of Sertoli cell proliferation. The first begins during fetal life and continues through the prepubertal period and the second begins at puberty \cite{68,70}. It is important to notice that until puberty testicular growth results mainly from increased tubular length as Sertoli cells proliferate. During puberty, testis growth is mainly due to an increase in the number of germ cells. The diameter of seminiferous tubules increases as germ cells proliferate and the tubular lumen begins to develop \cite{68}.

Leydig cells secrete testosterone and insulin-like factor 3 (INSL3) required for development and maintenance of the male phenotype. There are two generations of Leydig cells in mammals; fetal and adult types of Leydig cells. In rats, the fetal-type Leydig cells differentiate soon after Sertoli cells on 15.5 prenatal day \cite{71,72}. The origin of these cells remains uncertain but it has been suggested that they originate from mesenchymal-like cells which have migrated into the developing testis from the mesonephros or gonadal ridge \cite{73}.

Adult-type Leydig cells begin to differentiate and replace fetal-type Leydig cells after the second postnatal week \cite{74}. It is still under debate if adult-type Leydig cells are derived from the same pool as the fetal-type Leydig cells or if they actually differentiate from fetal-type Leydig cells \cite{73,75}. The number of these adult-type Leydig cells increases until the age of 5 weeks \cite{74} and testosterone production follows a similar pattern, although in a slightly delayed manner \cite{76}.

Leydig cell development in humans has long been described to be a biphasic developmental phenomenon. However, testosterone
production in humans clearly shows a triphasic pattern. The first peak occurs during week 14-18 of fetal life, the second 2-3 months after birth and the third one lasts from puberty throughout adulthood [77]. It has been suggested that the first population of Leydig cells is referred to as fetal Leydig cells, the second, as neonatal Leydig cells and finally as adult Leydig cells [77].

As in rats, in humans there is also uncertainty whether fetal Leydig cells regress or if they serve as a precursor population for the next developing Leydig cell population. Mesenchyme originated peritubular myoid cells surround the seminiferous tubules in all mammalian species. In rats, mice and hamsters it is a uni-cellular layer, but in humans it can be formed from one up to six layers [78]. Peritubular myoid cells are contractile and their movement facilitates the transport of spermatozoa and testicular fluid in the seminiferous tubules. In rats, peritubular myoid cells begin to contract around postnatal day 15 [79]. In addition to their role in structural support and movement, myoid cells also actively participate in the regulation of the seminiferous epithelium.

Many factors secreted by peritubular myoid cells affect Sertoli cell function and it has been suggested that communication between these two cell types is required for the proper formation of basal lamina during postnatal development [80]. Proliferation of peritubular myoid cells is also known to coincide with Sertoli cell proliferation during the growth phase of postnatal testis in rats [81], which further supports the idea of their close interaction during development. In rats, plasma levels of FSH decline after birth reaching the lowest point around postnatal day 15 [82, 83].
2.1.4. Endocrine regulation of spermatogenesis

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are the key regulators of spermatogenesis. The pituitary secreting these hormones is regulated by hypothalamic gonadotrophin-releasing hormone (GnRH). FSH binds to G-protein coupled receptors located on Sertoli cells [84] and controls spermatogenesis through Sertoli cell function. FSH has an important role in the proliferation of Sertoli cells during postnatal period [70,85,86] and it also regulates the secretion of a number of Sertoli cell products such as transferrin, androgen binding protein, Müllerian inhibiting substance, stem cell factor (SCF) and inhibin B [84].

Inhibin B consists of α and β subunits [87] and immature Sertoli cells secrete both these subunits [88]. At least in humans, at puberty localization of the expression of inhibin β subunit changes; Sertoli cells cease to produce inhibin β and germ cells from pachytene stage spermatocytes to round spermatids begin to secrete inhibin β. Sertoli cells also continue to secrete inhibin α-subunit in adulthood. This maturational change makes inhibin B as a good marker for germinal epithelial function [88].

FSH production is regulated via inhibin B by negative feedback [84]. LH binds to G-protein-coupled receptors located in Leydig cells and stimulates the secretion of testosterone. Testosterone in turn has both paracrine and autocrine effects and it binds to nuclear androgen receptors, which is expressed in Leydig, Sertoli and peritubular myoid cells [84].
In rats, plasma levels of FSH decline after birth reaching the lowest point around postnatal day 5-15\textsuperscript{[82, 83]} Then FSH levels begin to rise again, and it peaks on postnatal day 45 in rats\textsuperscript{[83, 89]}. Thereafter, FSH levels fall again and reach a plateau from postnatal day 50 onwards. LH levels are regulated via testosterone by negative feedback. Changes in the plasma levels of LH are less marked during the postnatal period; LH levels are quite constant and have a tendency to increase as the rat matures\textsuperscript{[83, 89]}.

In humans, levels of FSH and LH increase directly after birth and reach a peak during the first three months\textsuperscript{[90-92]}. Levels of these hormones decline and reach the level at which they remain during childhood. Whereas, during puberty secretion of LH and FSH increases. The level of testosterone follows the pattern of LH, suggesting that the immature testis responds to LH stimulation\textsuperscript{[92-94]}. Gonadotropins are secreted in a pulsative manner both in prepubertal and adult humans\textsuperscript{[92-94]}.

### 2.1.5. Steroid feedback mechanism

Functions of the testes are regulated by steroids. Testosterone inhibits LH secretion by acting directly on the anterior pituitary and by inhibiting the secretion of GnRH from the hypothalamus. Inhibin acts directly on the anterior pituitary to inhibit FSH secretion. In response to LH, some of the testosterone secreted from the Leydig cells bathes the seminiferous epithelium and provides the high local concentration of androgen to the Sertoli cells that is necessary for normal spermatogenesis.

Systemically, administered testosterone does not raise the androgen level in the testes to a great degree, and it inhibits LH secretion. Consequently, the net effect of systemically administered
testosterone is generally a decrease in sperm count. Testosterone therapy has been suggested as a means of male contraception. However, the dose of testosterone needed to suppress spermatogenesis causes sodium and water retention. The possible use of inhibin as male contraceptives has been being explored[4,5].

2.1.6. Endocrine function of the testes

Testosterone, the principal hormone of the testes, is a C 19 steroid with a hydroxyl group in the 17 position. It is synthesized from cholesterol in the Leydig cells and is also formed from androstenedione secreted by the adrenal cortex. The biosynthetic pathways in all endocrine organs that form steroid hormones are similar, the organs differing only in the enzyme systems they contain. In the Leydig cells, the 11- and 21-hydroxylases found in the adrenal cortex are absent, but 17α-hydroxylase is present[94].

Pregnenolone is therefore hydroxylated in the 17 position and then subjected to side chain cleavage to form dehydroepiandrosterone. Androstenedione is also formed via progesterone and 17hydroxy-progesterone, but this pathway is less prominent in humans[94].

Dehydroepiandrosterone and androstenedione are then converted to testosterone. The secretion of testosterone is under the control of LH, and the mechanism by which LH stimulates Leydig cells involves increased formation of cyclic adenosine monophosphate (cAMP) via the G-protein coupled LH receptor. cAMP increases the formation of cholesterol from cholesterol esters and the conversion of cholesterol to pregnenolone via the activation of protein kinase A[94].

The testosterone secretion rate is 4–9 mg/d (13.9–31.33 μmol/d) in normal adult males. Small amounts of testosterone are also secreted
in females, with the major source being the ovary, but possibly from the adrenal as well.

Ninety-eight percent of the testosterone in plasma is bound to protein: 65% is bound to a β-globulin called gonadal steroid-binding globulin (GBG) or sex steroid-binding globulin, and 33% to albumin. GBG also binds estradiol. The plasma testosterone level (free and bound) is 300–1000 ng/dL (10.4–34.7 nmol/L) in adult men, compared with 30–70 ng/dL (1.04–2.43 nmol/L) in adult women. It declines somewhat with age in males. A small amount of circulating testosterone is converted to estradiol, but most of the testosterone is converted to 17-ketosteroids, principally androsterone and its isomer etiocholanolone, and excreted in the urine[^94].

About two thirds of the urinary 17-ketosteroids are of adrenal origin, and one third is of testicular origin. Although most of the 17-ketosteroids are weak androgens (they have 20% or less the potency of testosterone), it is worth emphasizing that not all 17-ketosteroids are androgens and not all androgens are 17-ketosteroids. Etiocholanolone, for example, has no androgenic activity, and testosterone itself is not a 17-ketosteroid. In addition to their actions during development, testosterone and other androgens exert an inhibitory feedback effect on pituitary LH secretion; develop and maintain the male secondary sex characteristics; exert an important protein-anabolic, growth-promoting effect; and, along with FSH, maintain spermatogenesis[^4,^5].

Over 80% of the estradiol and 95% of the estrone in the plasma of adult men is formed by extragonadal and extraadrenal aromatization of circulating testosterone and androstenedione. The remainder comes from the testes. Some of the estradiol in testicular
venous blood comes from the Leydig cells, but some is also produced by aromatization of androgens in Sertoli cells [95].

Estrogen's primary function in the male tract appears to be the regulation of fluid reabsorption in the efferent ductules via the estrogen receptors.

Disruption of the receptor, in the rats treated with a pure antiestrogen, results in dilution of epididymal sperm, disruption of sperm morphology, inhibition of sodium transport and subsequent water reabsorption, and eventual decreased fertility. In addition to this primary regulation of luminal fluids, estrogen is also responsible for maintaining a differentiated epithelial morphology. Thus, estrogen or its receptor is an absolute necessity for fertility in the male [96].

2.2. Previous Studies

2.2.1. Endocrine Disruptors (EDs)

Endocrine disrupting agents affecting the male reproductive system can be broadly classified into estrogenic (those acting like a natural estrogen), antiestrogenic (ability to suppress the action of natural estrogen) androgenic (those acting like a natural androgen), antiandrogenic (ability to suppress the action of a natural androgen) and steroidogenesis inhibitors (ability to suppress steroidogenesis). However, the patterns or profiles of morphological changes that occur in the various tissues of the reproductive tract in response to chemicals altering estrogen and androgen pathways do not fit so neatly into these broad categories [97].

The endocrine system regulates adjustments through slower internal processes, using hormones as messengers. The endocrine system secretes hormones in response to environmental stimuli and to orchestrate developmental and reproductive changes. The adjustments
brought on by the endocrine system are biochemical, changing the cell's internal and external chemistry to bring about a long term change in the body. These systems work together to maintain the proper functioning of the body through its entire life cycle. **Sex steroids** such as **estrogens** and **androgens**, as well as **thyroid** hormones, are subject to **feedback** regulation, which tends to limit the sensitivity of these glands. Hormones work at very small doses (part per billion ranges)\(^{[98,99]}\).

Endocrine disruption can thereby also occur from low-dose exposure to exogenous hormones or hormonally active chemicals that can interfere with receptors for other hormonally mediated processes. Furthermore, since **endogenous** hormones are already present in the body in biologically active concentrations, additional exposure to relatively small amounts of **exogenous** hormonally active substances can disrupt the proper functioning of the body's endocrine system. Thus, an endocrine disruptor can elicit adverse effects at much lower doses than a toxicity, acting through a different mechanism\(^{[98,99]}\).

The timing of exposure is also critical. Most critical stages of development occur in utero, where the fertilized egg divides, rapidly developing every structure of a fully formed baby, including much of the wiring in the brain. Interfering with the hormonal communication in utero can have profound effects both structurally and toward brain development. Depending on the stage of reproductive development, interference with hormonal signaling can result in irreversible effects not seen in adults exposed to the same dose for the same length of time. Experiments with animals have identified critical developmental time points in utero and days after birth when exposures to chemicals
that interfere with or mimic hormones have adverse effects that persist into adulthood \[^{100-101}\].

Fetuses and embryos, whose growth and development are highly controlled by the endocrine system, are more vulnerable to exposure and may suffer overt or subtle lifelong health and/or reproductive abnormalities \[^{102}\]. Prebirth exposure to endocrine disruptors, in some cases, can lead to permanent alterations and adult diseases \[^{103}\].

Mammalian spermatogenesis is a complicated cascade process that is under the tight control of the hypothalamus-pituitary gonadal (HPG) axis as well as the auto/paracrine circuit \[^{104}\]. The primary role of the hormones involved is to enable a coordinated regulation of the process that allows the development of highly differentiated spermatozoa within the seminiferous tubules. The process depends on a functional hypothalamic pituitary-testicular (HPT) axis. The hypothalamic kisspeptin-1 (KiSS-1) and its G protein-coupled receptor (GPR54) act as the gatekeeper to control the secretion of gonadotrophin releasing hormone (GnRH), which regulates the anterior pituitary hormones— luteinizing hormone (LH) and follicle stimulating hormone (FSH), and testicular hormones—testosterone, activin and inhibin B \[^{104-106}\].

Since neuroendocrine actions of endocrine disruptors have been shown, HPG circuitry signaling can be the EDs target during prenatal development. Any interruption on the hypothalamic circuitry, hormonal mediated regulation or on the constituents at the microenvironments in seminiferous tubules may result in a transient/long-term modification of the hormonal feedback circuitry, leading to the disturbance of spermatogenesis \[^{107,108}\].
Effects of EDs on animal reproductive function can be multi-faceted and pleiotropic. Exposures to EDCs can interfere with cell signaling via direct/indirect “hormonal” related pathways in HPG axis. These direct and indirect effects disrupt the homeostasis at different levels of feedback regulatory mechanisms (neuron communication, endocrine, autocrine and paracrine) for the regulation of testicular development and functions (steroidogenesis and spermatogenesis)\[109]\.

Steroidogenesis is the process for steroid hormone production. It is an enzymatic-mediated process catalyzed by several enzymes from two main categories: the cytochrome P450 enzymes (CYP11A and CYP17A), and hydroxysteroid dehydrogenase (HSD) enzymes (3β-HSD and 17β-HSD)\[110]\]. Negative influences of EDCs exposure on steroidogenesis have been reported in both in vivo and in vitro studies\[107,111]\]. Mostly down regulation of the expression levels of CYP11A and CYP17A were observed, resulting in the reduction of testosterone production. This hypothesis is rational as receptor binding affinities of most EDs are generally low as compared to the endogenous ligands\[112]\.

Although the additive/synergistic effects of mixture of EDs cannot be neglected, it seems unlikely that EDs can compete with the endogenous ligands protein for receptor binding. Retrospectively it is more likely that EDs interfere with steroidogenesis and modulate the release of endogenous steroid hormones. The altered serum levels of the steroid hormones may cause subsequent reproductive dysfunction by interfering with the feedback regulatory mechanisms of the HPG axis\[113,114]\.
2.2.2. Effects of prenatal / and lactational exposure to synthetic progesterone (P₄) on pubertal testicular structure and functions:

Exposure to the natural or synthetic chemical substance alters the hormonal and homeostatic systems of the animal and renders it incapable to respond to the surrounding environmental condition. The United States Environmental Protection Agency (EPA) defined that exogenous agent interferes with synthesis, secretion, transport, metabolism, binding and elimination of natural blood hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process[115].

Hydroxyprogesterone caproate is one of the widely prescribed drugs to prevent threatened pregnancy and abnormal uterine bleeding in women. The effect of gestational exposure to hydroxyprogesterone on the fertility of progeny has been investigated. Fertility has become an increasingly important issue over the past 50 years because data suggest that there has been a progressive increase in disorders of male and female reproduction and there is concern as to whether synthetic compounds could have contributed to these changes [2].

In Harini et al. [116] study that aimed to examine whether transplacental exposure to progesterone caused male reproductive abnormalities and whether the changes can be reversed after testosterone administration. Progesterone was injected to mice on day 1, 3, and 7 of pregnancy. The male pups (first generation) were allowed to grow for 50 days and assessed for reproductive performance. This study concluded that gestational exposure to progesterone (7 mg/kg body weight) resulted in significant body
weight gain with a decrease in weight of the reproductive organs including the testis. Total sperm count, viable sperm, and motile sperm decreased in experimental mice.

Hypo-osmotic swelling test revealed that experimental mice sperm membrane integrity was severely altered. The activity levels of testicular steroidogenic marker enzymes (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase cluster (HSD3B) and hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B)) decreased significantly in mice exposed to progesterone during embryonic development when compared with the controls. The levels of serum testosterone decreased with an increase in serum FSH and LH in mice exposed to progesterone during embryonic development [116].

Prenatal exposure to progesterone caused significant reduction in the number of spermatozoa and increase in the lumen of seminiferous tubule and the experimental mice that cohabited with normal females showed fertility reduction. A significant decrease in sperm volume and progressive forward motility and percentage of viable sperm and coiled sperm indicated that exposure to progesterone during early stages of development severely affected not only the production of sperm but also affected their quality [116].

Neonatal exposure to estrogen suppressed reproduction, as evident from the absence or reduced number of copulatory plugs with increased pre- and post-implantation losses [117]. Gestational and lactational exposure of mice to Diethylstilbestrol (DES) at 10 ml/kg body weight/day resulted in decreased fertilizing ability in mice [118].

In view of the deterioration of male reproduction in recent past, an elaborative program was initiated to evaluate the gestational exposure to synthetic chemicals on reproductive abnormalities in rats. In order to assess the reproductive potential of first generation, rats
were selected as test animal since the pubertal stage can be reached within 60 - 90 days and serves as good model for reproductive studies [28].

Hydroxyprogesterone caproate was administered to pregnant rats at a dose level of 10 and 25 mg/kg body weight on 1st, 7th and 14th gestational day and the male pups (offspring) were allowed to grow for 90 days. The effect of gestational exposure to hydroxyprogesterone caproate on fertility was assessed by breeding offspring male rats with control female rats besides analyzing sperm quality and quantity in them. The number of implantation sites and viable fetuses was significantly reduced in females mated with those males that were exposed to hydroxyl-progesterone caproate during embryonic development. The decrease in sperm function was associated with a decrease in sperm motility, sperm viability and sperm count. And clearly indicates that in utero exposure to hydroxyprogesterone caproate affects fertility in male rats and interpreted the significant reduction in epididymal index, sperm count, sperm motility, sperm viability and sperm function could be due to sperm toxic effects [2, 28].

A significant decrease in epididymal index 22.35, sperm viability, sperm count, sperm motility and sperm coiling percentage which observed in the first experimental rats when compared with the control rats, these changes were more pronounced in second experimental group of rats which received larger dose of hydroxyprogesterone caproate exposed rats. The first experimental rats generation male rats showed significant decrease in reproductive performance than the control rats. This effect is more pronounced in the second experimental group rats when compared to
control group rats. There was a significant decrease in number of implantation sites; number of normal fetuses in normal female rats mated offspring males from experimental groups. The mean weight of embryos in females mated with offspring males from experimental groups was also significantly decreased with no mortality and no behavioral abnormalities were recorded in experimental rats indicating that the hydroxyprogesterone caproate does not show acute toxicity at the dose levels of 10 and 25 mg/kg body weight. Hydroxyprogesterone caproate exposed male rats were able to impregnate female but comparatively in lower number\textsuperscript{[2, 28]}.

Hydroxyprogesterone caproate is one of the most widely used anti-abortive drugs and exposure to this drug during embryonic development significantly affects male reproduction. This significantly decreased the epididymal sperm count and sperm motility in rats. The data also revealed elevated FSH and LH levels, associated with significantly reduced circulating testosterone levels in rats exposed to hydroxyprogesterone caproate during embryonic development, indicating a probable inhibition of androgen synthesis. Thus the decreased testosterone levels might also be responsible for the decreased sperm count and sperm motility in the experimental rats. It is also suggested that the observed impairment of male reproductive performance could be mediated through the inhibition of testosterone production in hydroxyprogesterone caproate-exposed rats\textsuperscript{[28]}. It is well known that androgens are essential for normal spermatogenesis, deficiency in androgen levels of hydroxyprogesterone exposed rats led to an androgen deprivation effect on target organ\textsuperscript{[15]}.
Evidence indicates that changes in the concentrations of sex hormones (androgen and oestrogen) result in permanent changes in cell function. For example, the higher circulating levels of oestradiol (about 50 pg/ml) in male mouse fetuses result in an alteration in prostate size \cite{15}.

In the study done by (Pushpalatha et al \cite{29}, transverse section of the testis of the rat exposed to 10 mg hydroxyprogesterone/kg body weight showed symptoms of arrest of spermatogenesis. The germinal epithelium, spermatogonia, spermatocytes and spermatids were affected and the diameters of seminiferous tubules and tubular lumens were decreased in experimental rat testis. Whereas transverse section of the testis of rat exposed to 25 mg hydroxyprogesterone/kg body weight showed complete arrest of spermatogenesis. The seminiferous tubules were disorganized. The epithelium, spermatogonia, spermatocytes and spermatids were severely damaged and degenerated and the tubules showed necropsied spermatogenic cells and the lumen was empty of active sperms.

Marked degenerative changes were observed in testes of mice exposed to progesterone during embryonic development. These changes include damaged seminiferous tubules that showed decreased spermatogenic activity; the lumen was large and devoid of sperm \cite{116}.

Since the hydroxysteroid dehydrogenase enzymes (3b-HSD and 17b-HSD) are important in the synthesis of most biologically active steroids, determination of the activity levels of these enzymes can be an invaluable aid in subjectively assessing the state of the steroid biosynthesis. Decreased hydroxysteroid dehydrogenase enzymes activity levels in hydroxyprogesterone injected rats, lead to lesser quantities of steroid substrate material to be synthesized \cite{3,119}.
Experimental study done by Pushpalatha et al. 2002 \cite{15}, on the pregnant wistar strain albino rats administered hydroxyprogesterone and the steroidogenic potential of the testis was analysed in the next generation adult male rats concluded that in utero exposure to supra-normal levels (10 mg/ kg body weight or 25 mg/kg body weight) of hydroxyprogesterone suppresses testicular steroidogenesis by decreasing the activity levels of steroidogenic enzymes (3b-hydroxysteroid dehydro-genase and 17b-hydroxysteroid dehydrogenase) of adult mature rats testis which in turn may suppress the reproductive activities and can be correlated to the observed decrease in male reproductive potential in mammals exposed to female hormones during embryonic development.

The effect of maternal exposure to progesterone upon the fetal pituitary-gonadal axis and the sexual behavior of the male offspring of mice were studied by Pointis et al \cite{120}. They reported, daily injection of progesterone from days 14 to 16 of pregnancy reduced testosterone production in the fetus but caused a significant increase in circulating LH levels. Progesterone-exposed males showed no alteration in anogenital distance or in body weight at any time from birth to adulthood. At 80-90 days of age males from control and progesterone-exposed groups did not differ from each other in testis and seminal vesicle weights. However, in the latter group, there was a marked reduction in the percentage of males that displayed mount, intromission and ejaculation patterns. These findings indicate that in utero exposure to pharmacological doses of progesterone that do not cause abnormalities of male internal and external genitalia may interfere with masculine behavior in adulthood. This alteration could partially be due to diminished peripheral testosterone levels during the prenatal period.
Brady et al \cite{121} investigated the effect of progesterone on gonadotrophin secretion in normal healthy men and compared it to that produced by desogestrel, a synthetic progestagen. The results showed that treatment with progesterone for seven days caused significant decreases in mean concentrations of LH, FSH, and testosterone. The frequency and amplitude of LH secretion pulses were significantly reduced. Mean concentrations of LH and FSH during the 2-hr post-GnRH period were significantly lower after 7-day progesterone treatment than before the treatment, suggesting a significant attenuation of the response to GnRH in men treated with progesterone, and the effects of desogestrel on gonadotrophin secretion were similar to that caused by progesterone. The authors concluded that progesterone suppressed gonadotrophin secretion via a progesterone receptor-mediated mechanism and the synthetic gestogens exert their antigonadotrophic effect through their progestogenic properties.

The production of estrogen by the germ cells and secretion into the seminiferous tubule fluid may be important for the function of the efferent ductules and epididymis Hess \cite{122, 123}. Although estrogen biosynthesis by the testis is necessary for proper spermatogenesis to occur, inappropriate exposure to estrogen can be deleterious to testis development. Exposure to estrogen during the fetal and neonatal period can impact the development and organization of the hypothalamic-pituitary-testis (HPT) axis \cite{124, 125}. Exogenous estrogen exposure has been shown to inhibit proliferation of precursor Leydig cells during the neonatal period, thus altering steroidogenic capacity of adult testis \cite{124, 125}. Neonatal exposure to even 3 weakly estrogenic substances can elevate FSH
levels during puberty in rats and cause long-term changes in the testis \cite{126}. Exposure to Diethylstilbestrol (DES) during the neonatal Sertoli cell proliferative period decreases Sertoli cell number and it is thought to be through a reduction of FSH, which is a mitogen for Sertoli cell proliferation \cite{127}.

Estrogen may also inhibit Sertoli cell maturation, preventing premature Sertoli cell differentiation during the proliferative period and causes a maturational delay in Sertoli cells in rats. In the adult, maintenance of spermatogenesis is dependent on normal levels of FSH, LH and testosterone, and it is important to consider that estrogen is a component of the negative feedback action on gonadotropin secretion at the level of the hypothalamus and pituitary. Therefore high doses of estrogen administered to adult rats are expected to decrease serum FSH, LH and testosterone \cite{127}.

Epidemiological studies \cite{20-24} have shown decreased semen concentration, sperm motility with increased percentages of abnormal sperm and increased congenital abnormalities like hypospadias and cryptorchidism in many countries. These abnormalities have been attributed to deterioration in environment quality and increased exposure to xeno-estrogens and anti-androgens.

The transplacental effects of diethylstilbestrol (DES), a synthetic therapeutic agent, on the fetuses of both humans and experimental animals have been confirmed. Children exposed in utero to (DES) experience congenital abnormalities of their reproductive system and reduced fertility \cite{128}. Endogenous hormones exert effects throughout embryonic development, after birth, and into adulthood. Altered hormone levels in response to embryonic exposure to xenoestrogens have been reported in rats \cite{129}. 

55
It has been hypothesized that exposure to supra-normal levels of estrogen-like chemicals during embryonic development may interfere with mechanisms involved in the development of the male reproductive system and in determining sperm numbers \[^{130}\].

More than five million pregnant women were given diethylstilbestrol (DES), a synthetic estrogen to prevent spontaneous abortions from 1948 until 1971, when its use for this purpose was banned \[^{3}\]. Daughters whose mother took diethylstilbestrol suffer reproductive organ dysfunctions, abnormal pregnancies and a reduction in fertility \[^{131},^{132}\]. It was found in the early 1960s, that treatment of neonatal mice with estrogenic steroids or diethylstilbestrol led to pathological changes in female reproductive tract, beginning with persistent vaginal stratification and comification and developing in to a variety of dysplastic and possibly neoplastic lesions. Estrogen administered in the first few days after birth were large enough, to induce persisted changes lasted even after ovariectomy and thus, once induced, were estrogen-independent \[^{133}\]. Long-term and permanent effects were detected in the adult as a result of exposure to diethylstilbestrol during development, which can occur without apparent birth defects in the neonate \[^{134}\].

The weight of the testis is largely dependent on the mass of differentiated spermatogenic cells and it has been used as a measure of spermatogenesis in rats \[^{135}\]. A positive correlation was observed between weight of testis and number of germ cells \[^{136}\]. The reduction in the testicular weights could be due to the germinal cell loss in hydroxyprogesterone- exposed rats \[^{29}\].

Atanassova et al study \[^{124}\] aimed to identify the mechanism(s) for impairment of spermatogenesis in adulthood in rats treated neonatally with estrogens. Rats were treated (days 2-12) with 10
micrograms diethylstilbestrol (DES), 10 micrograms ethinyl estradiol (EE). They concluded that neonatal estrogen treatment results in Sertoli cell numbers and testicular weight reduction, germ cell volume alterations, efficiency of spermatogenesis, germ cell apoptosis and reduced Leydig cell volume per testis in adulthood. The relatively poor spermatogenesis in estrogen-treated animals is most likely due to altered testis fluid dynamics and/or altered Sertoli cell function. Plasma FSH level as elevated and testosterone level decreased but did not alter LH level.

Experimental study done by Kizilay and Uygu[137] on mature male wistar albino rats weighing 200g to examine the effects of ethynyl-estradiol (0.0005 mg/kg) which administered every other day for two months on the ultrastructure of testis germ cells. This study revealed that experimental group showed a decrease in the number of germ cells layers and an increase of smooth endoplasmic reticulum, hydration in mitochondria and vacuoles in cytoplasm of Sertoli cells were observed. Thickening of tubule basal lamina, a decrease in the number of dark spermatogonia, spermatids and secondary spermatocytes, on the contrary an increase in the number of light spermatogonia, and degeneration in the Leydig cells were noticed. Decrease in the diameter and irregularity in the shape of tubule, marked decrease in levels of LH, testosterone and in weights of male genital organs which were targets of androgen were observed. Too much degeneration in Sertoli cells occurred, that is why they could not support the germ cells. As a result cytoplasmic materials of Sertoli cells immature and degenerated germ cells in lumen of tubule were observed.

Sprague-Dawley (S-D) male rats with previously known DES-sensitivity were exposed in utero to diethylstilbestrol (DES,
100–300 g/kg) on 13.5, 15.5, and 17.5 prenatal days to elucidate estrogenic effects on testosterone (T) and luteinizing hormone (LH) production. A significant depression in testicular and plasma T content and male body weight gain are seen in the DES-exposed S-D rats when compared to the untreated rats. The pituitary LH levels did not alter considerably. Apparently prenatal DES-exposure impairs testicular steroidogenesis and pituitary function.

Proliferation of Sertoli cells in the rat testis occurs only during the perinatal period and is maximal during fetal life. This interval is thus of critical importance in establishing the complement of Sertoli cells that populates the adult testis\[^{[138]}\]. FSH has been implicated in this process, but direct evidence in support of its involvement is lacking. This study used in vivo and in vitro approaches to determine whether FSH produced by the fetal pituitary has a role in regulating Sertoli cell division in the fetal testis of the rat. On day 18 of gestation, just before the onset of maximal Sertoli cell proliferation, fetuses were either decapitated in utero or given antiserum to FSH. Both treatments produced dramatic and equal reductions in the percentages of Sertoli cells preparing to divide on day 19, suggesting that FSH from the fetal pituitary stimulates Sertoli cell proliferation in fetal testes. However, after exposing these testes to either FSH or dibutyryl cyclic adenosine monophosphate (Bu\(_{2}\) cAMP for 28 h, the percentage of Sertoli cells labeled was greatly enhanced. Taken together, the data obtained from these experiments identify FSH as a major factor in controlling expansion of the Sertoli cell population during fetal development of the rat and effect of FSH is mediated by cAMP. These findings confirm our earlier suggestion that production of FSH by the pituitary of the prenatal rat is important in establishing a
Sertoli cell population of adequate size and imply that this period of development is crucial in insuring fertility of the adult testis \[138\].

In vitro studies in the rat are based on organ cultures and primary cell cultures. These techniques are useful to detect short-term effects and have shown that estrogenic molecules (DES, 17\(\beta\)-estradiol, etc.) may alter cord formation early in testis development and disrupt the development of germ cells, Leydig cells, and Sertoli cells, and result in rat testicular cells that are more sensitive to estrogens during the early fetal period \[139, 140\].

Experimental study done on Sixty-day-old rats treated for 30 days with medroxyprogesterone acetate (Provera), resulted in; lowering the plasma levels of testosterone, androstenedione, and LH, and reduce the epididymal sperm counts and accessory sex organ weights. This study also showed markedly lower levels of testicular 17\(\beta\)-hydroxysteroid dehydrogenase and 3\(\beta\)-hydroxysteroid dehydrogenase activities \[141\].

A prospective case control study of Seema \[142\], aimed to evaluate impact of short term use of injectable depot medroxyprogesterone acetate (DMPA) if given in early post partum period on infant health and breast feeding performance. The study was conducted on 250 women immediately after delivery, 100 post partum women, not using hormonal contraception were taken as controls while 150 women received DMPA 150 mg intra muscularly after initiation of lactation before discharge from hospital (day 2-day 10 of their delivery). All the subjects were followed to complete a full 6 months follow up. Revealed that injectable DMPA use as a contraceptive in the immediate post-partum period was found to be a
safe and effective alternate method with no deleterious effect on mother’s milk and infant’s growth.

Dahlberg [143] reported that, in women using depotmedroxy-progesterone during breast feeding the amount of hormone found in the breast milk is very small. Many studies have shown that the hormone in breast milk appears to have no effect on the baby’s long term growth and development. The use of depot medroxyprogesterone has been approved by the American Academy of Pediatrics for use in breast feeding mothers.

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study Field

The study has been carried out in the laboratories of the department of anatomy, Faculty of Medicine, Najran University, Kingdom of Saudi Arabia between October 2012 and March 2014.

3.2. Study Design: Experimental interventional study.

3.3. Sample Species: wistar albino rats.

3.4. Materials

3.4.1 Animals

Twenty seven mature male and female albino wistar rats, eight to ten weeks old were purchased from the animal house of the faculty of pharmacy –Jazan University –Kingdom of Saudi Arabia. The rats were transferred to the animal house of the department of anatomy, faculty of medicine, Najran University. The rats were grouped into 3 groups. Each group consisted of seven females and two males. They
were kept in separate cages at 12:12 hours Light/Dark cycle, 28 ± 7 °C temperature and fed with commercial pellet and water ad libitum.

3.4.2. Chemicals

Synthetic progesterone: hydroxyprogesterone caproate (trade name: Proluton Depo ®) and medroxyprogesterone (trade name: Depo-provera ®) (250 mg in 1.0 ml) are available in an oily solution diluted by pure Spanish olive oil (1:4ml). They were obtained from Najran Maternity Hospital. Hormone Assay ELISA kit for determination of serum levels of Rat luteinizing hormone (LH) (Catalog No: E-EL-R0026, testosterone (Catalog No: E-EL-R0033) and follicles stimulation hormone (FSH) (Catalog No: E-EL-R0391).

3.5. Methods

3.5.1. Experimental design

The female rats were examined daily for the presence of cervical plugs and those proved mated and later on confirmed as pregnant were grouped into 3 groups. Group 1 was kept as control animals and groups 2 and 3 as experiment animals. The animals were kept separate away from any stress in (90×45×15 cm) sterilized polypropylene cages lined with woody husk.

The experiment groups were injected with synthetic progesterone (hydroxyprogesterone; 10 mg/ kg body weight) subcutaneously on day 1, 7th and 14th of gestational period. After delivery, mothers of group 3 were further injected with the same dose of the synthetic progesterone (medroxyprogesterone; 10 mg/ kg maternal body weight) daily for 15 postnatal days of lactational period, whereas the control group was injected with olive oil only at the same dose as experiment groups received. The dose of test chemical was adjusted daily to body weight for each dam before
dosing. Twenty male puppies born to each of the experiment groups and control (n=60 puppies) were allowed to grow for 90 days where they reached maturity. Male rats born to dams treated with synthetic progesterone during pregnancy were named (experiment I) and those who were treated during both pregnancy and lactation were named (experiment II). These periods were needed to assess the effect of the synthetic progesterone on the morphology of their testis and the spermatogenic parameters.

3.5.2. Tissue collection and preparation

Animals were anesthetized with chloroform. Blood samples were collected from each animal through cardiac puncture to determine the serum levels of testosterone, luteinizing and follicle stimulating hormones. The rats were sacrificed with cervical dislocation and the testes were then dissected. The dissected left testes were weighed and fixed immediately in aqueous Bouin’s solution for 18 hours, dehydrated into 70, 90 and 100% ascending grades of alcohol, cleared with xylene and embedded in paraffin wax. Sections were cut at 5µm thickness by using an American Optical microtome (A0-821. USA). The mounted tissue samples were deparaffinized and stained with haematoxylin and eosin (H&E) method for histological observations using light microscopy \cite{144}. The dissected right testes were cut into small pieces and fixed in 2.5% buffered gluteraldehyde with cacodylate for electron-microscopic examination.

Each right testis was separated from its adjacent epididymis and the diameter and length are measured. The right cauda epididymis of each rat was cleaned from surrounding adipose tissues, connective tissues and blood vessels and then weighed. The mid-to-
distal region of the epididymis was perforated in a petridish by 3 mL needle; the oozed semen was diluted in 1 mL physiological saline solution (0.9% NaCl). The clear sperm suspension was kept at 37°C for 5 minutes to allow for the sperm to disperse in the medium. After thorough mixing, the sperm suspension was used to assess the sperm count, motility, viability and morphology. Later, relative testis and epididymis weights were then calculated per final body weight [144].

3.5.3. Semen analysis

3.5.3.1. Sperm Counts

A drop of the diluted semen was thoroughly mixed, transferred to a Neubauer's hemocytometer using a micropipette and a cover glass was overlaid. The total numbers of the sperms were then observed and counted under a Carl Zeiss (Germany) Axio 2 Plus microscope at x400 magnification. Sperm were counted in 5 small squares of the main large central square, each square consisting of 16 smaller squares [145]. Therefore, a correction factor of 50 was applied to calculate the total number of sperm per milliliter and converted to 0.1 g / epididymis weight. Two samples from each epididymis were examined and average counts were scheduled [146, 147].

3.5.3.2. Sperm Motility

The sperm motility was assayed microscopically, within 5 minutes following their isolation from cauda epididymis at 37°C and the data were expressed as percent motility. A drop of sperm suspension was placed on a Neubauer's hemocytometer using a micropipette and then observed under a Carl Zeiss (Germany) Axio 2 Plus microscope at x400 magnification. Non-motile sperm numbers were first determined, followed by the total sperm counts (sperm with any of the different
motility type was recorded as motile sperm). Sperm motility was expressed as percentage of motile sperm of the 200 sperm counted in 10 randomly selected fields for each rat \(^{[146]}\). For each animal two separate hanging drop preparations were made, and two independent observers assessed the motility. The data from each animal were used to obtain the average.

### 3.5.3.3. Sperm Viability

This technique is used to differentiate between live and dead sperms. A drop of the diluted semen was transferred to an eppendorf tube (1mL) containing one drop of 1% Eosin stain. The contents were mixed gently, left for 5 minutes at 37°C and about 10µL of the sample were then observed under a Carl Zeiss (Germany) Axio 2 Plus microscope. The head of dead spermatozoa stained red while the live spermatozoa were unstained with Eosin. Sperm viability was expressed as percentage of live sperm of the 200 sperm counted in 10 randomly selected fields for each rat \(^{[146]}\). For each animal two separate hanging drop preparations were made, and two independent observers assessed the viability. The data from each animal were used to obtain the average.

### 3.5.3.4. Sperm Morphology

A drop of 1% Eosin stain added to a drop of the sperm suspension in an eppendorf tube (1mL), mixed gently, placed on a clean slide was gently spread to make a thin film. The film was air dried and then observed under a Carl Zeiss (Germany) Axio 2 Plus microscope using x400 and x1000 magnifications. The relative percentage of abnormal sperms was counted by observing 10 different optical fields randomly selected for each rat sample and scoring out the normal and abnormal sperms. Abnormal sperms having the following abnormalities were
counted: absence of head, absence of tail, tail bending, tail coiling, tail curving, mid-piece curving and mid-piece bending [145, 148]. For each animal two separate hanging drop preparations were made, and two independent observers assessed the abnormalities. The data from each animal were used to obtain the average.

3. 5.4. Histometry

According to Batra et al [149] and Thienpot et al [150] morphometric measurement technique was followed in the assessment of testicular parameters of the different groups. Using H&E stained, longitudinal sections, for each rat, ten tubular profiles that were round or nearly round were chosen randomly to measure the diameter of seminiferous tubules, height of germinal epithelium, thickness of interstitial spaces and numbers of germinal epithelial cells by using 100 x magnifications with the Image Pro Plus program associated to an Olympus BX-40 microscope.

Two tubular diameters for each tubule were mapped, measured and the averages were calculated. The thickness of interstitial space was measured by measuring three dimensions. Those assumed to connect the centre of each space to the basement membrane of the surrounding seminiferous tubules and the averages dimensions were calculated and multiplied 2 times. The germinal epithelium height was obtained with the same tubules used to determine tubular diameter. Germinal epithelium was assumed from the basement membrane to the latest stage of germinal cells (spermatids).

3.5.5. Cells Quantitation
The left testis from each rat was prepared for light microscopy. After animal sacrifice, a testis was fixed by perfusion with Bouin's fixative for 30 minutes. The testis was then cut into 3 vertical longitudinal slices, the middle slice including the mediastinum. After immersion fixation in Bouin's for another 1.5 hours, the slices were dehydrated in ethanol, cleared with xylene and embedded in paraffin wax. Five sections were cut at 5 µm from each slice, thus fifteen interstitial space sections from each rat were examined and the sections were mounted individually on slides.

The average of Leydig cell number of the entire fifteen interstitial space sections for each rat was counted by using 100 x magnification with the Image Pro Plus program associated to an Olympus BX-40 microscope. Consequently the mean Leydig cell value for each group was calculated. And as such, the Sertoli and germinal epithelial cells (spermatogonia type A & B and primary spermatocytes cells) were counted and mean values were calculated.

3.5.6. Ultrastructure of the testis

The exposed dispersed seminiferous tubules from the decapsulated right testes were cut into small pieces and rapidly fixed in 0.1M cacodylate buffered 3% gluteraldehyde at 4°C then washed in the 0.1M cacodylate buffer overnight before they were post-fixed in cacodylate buffered 1% osmium tetroxide for 2 hours. Dehydration was carried out in an ascending series of ethyl alcohol cleared in propylene oxide and embedded in epoxy resin in an oven at 60°C for 20h to produce affirm block.

Semithin sections were cut with glass knives in an American Optical Corporation-USA, ultra-microtome and stained with toluidine blue. Ultrathin sections were cut from the desired parts,
mounted on perforated copper grids (Plano, Wetzlar, Germany),
stained with uranyl acetate and lead citrate and examined in the
transmission electron microscope (JEOL, 1200EXII,Tokyo, Japan) at
80 KV acceleration voltages\textsuperscript{[144]}.

3.5.7. Serum Collection & Hormonal Assay

The rats were anesthetized using chloroform and afterward
sacrificed by cervical dislocation and blood sample collected
directly by cardiac puncture using 3 ml syringe and placed in 5 ml
plain blood collection tube. Serum was separated by centrifugation
at 4,000 rpm for 5 min after overnight storage at 4\textdegree C and then stored
at 20\textdegree C to assess the testosterone, FSH and LH serum level by using
specific commercially available ELISA kits prescribed above,
purchased from Elabscience Biotechnology Co., Ltd (Elabscience)
China, according to their manufacturer's method instructions.

3.5.8. Statistical analysis

Statistical analyses for all obtained parameters were performed by
using Stat Veiw statistical software package (version 5.0.1.0) and
SPSS-16.020 (Chicago, USA) statistical analysis software.
The data were expressed as (mean ±SD). Statistical evaluation of
significant difference between means was performed with one-way
analysis of variance (ANOVA) with the level of significance set at
P<0.05. A regression model was fitted considering histopathological
scores.
CHAPTER FOUR

RESULTS

4.1. Light microscopic results

Paraffin sections stained by Hematoxylin and Eosin, 5 micron thickness were examined under light microscope and revealed the following:

4.1.1. Control group

The testicular parenchyma appeared formed of seminiferous tubules and interstitial tissue. Both of these components were separated from each other by an intact basement membrane. The cut sections of the seminiferous tubules appeared rounded or oval in shape containing spermatogenic and Sertoli cells and resting on well-formed basement membrane formed of thin elongated simple epithelial cell layer and fibrous tissue (Fig.4.1). The different types of the spermatogenic cells were easily identified and so was the mitotic activity as revealed by the different stages of the spermatogenesis (Fig.4.1). The individual cells of the seminiferous tubule and interstitial tissue were seen having the following features:

Spermatogonia type (A) appeared rounded or oval shaped, pale-stained, have wide contact area with the basement membrane and their nuclei were placed toward the upper poles of the cells. Spermatogonia type (B) appeared smaller in size and have little contact with the basement membrane, darkly stained cytoplasm and centrally placed nucleus with multiple nucleoli (type B) (Fig. 4.2).
Primary spermatocytes, were large in size and slightly away from the basement membrane (constituted the inner layer after spermatogonia). They appeared rounded in shape having large spherical nuclei and acquiring various features of nuclear chromatin material according to their multiple stages of development (Fig. 4.2).

Secondary spermatocytes were hardly seen because they had passed this stage readily and converted to spermatids. They were smaller than the primary spermatocytes and have slight similarity with the rounded stage of spermatid.

Spermatid cell stage is easily identified when in their elongated stage but not in their rounded stage because of great similarity with secondary spermatocytes. They constituted more than two layers lying nearer to the lumen of the seminiferous tubules. The elongated stage is generally characterized by deeply-stained flattened oblong thin nuclei. The spermatids has different successive staged that is difficult to be distinguished from each other under light microscope (Fig.4.2).

Spermatozoa: completely well-formed sperms were well observed filling the lumens of the seminiferous tubules as filamentous structures having thick headed regions and tapering tail regions. The central lumina could easily be delineated in almost all tubules and the majority of them was occupied by spermatozoa with darkly-stained elongated heads and long tails (Fig.4.1 & 4.2).

Sertoli cells were greatly elongated extending from the basement membrane toward the lumen of the seminiferous tubule. Their nuclei lie beneath the basement membrane. Their cell membranes are difficult to distinguish from the surrounding cells
because of the numerous lateral processes that surround spermatogenic cells (Fig. 4.2).

The main structural components of the interstitial tissue were the Leydig cells, together with other connective tissue elements and blood vessels, lying between the cut sections of the seminiferous tubules. This tissue occupies irregular triangular and quadrangular shaped spaces containing an eosinophilic Leydig cells which were conspicuous and found singly or in clusters. These cells appeared having foamy washed out cytoplasm (seen as vacuoles) due to their high lipid content (cholesterol) used for testosterone synthesis. Large groups of cells gave the appearance of an epithelial complex. The cells were polygonal in shape with large and rounded nuclei (Fig. 4.3 & 4.4).

4.1.2. Experiment I and II groups

Many prominent abnormal features were observed as well as some survival normal features. Most sections taken from these experiment groups showed; slightly to moderately diminished cellular walls, the seminipherous tubules appeared to lose their usual configuration, detached and folded basal laminae (Fig. 4.5).

Many seminiferous tubules sections showed large blood vessels and wide interstitial space due to tubules shrinkage and destruction (Fig. 4.5 & 4.6).

Degenerative spermatogenic cells and apoptotic changes in the form of intercellular and intracellular cavitations indicating either cells shrinkage or destruction of formed cellular components of the seminiferous epithelium were observed (Fig.4.7).

There was obvious reduction in the diameter of the seminiferous tubule, reduction in the epithelial height and
seminiferous tubular lumens appeared wider and lighter than in control animals. These lumens revealed necrotic cellular debris and great reduction in the luminal spermatozoa as well as a reduction in the number of early and late spermatids (Fig. 4.8 & 4.9).

The surviving cellular components have the same features described in control animals but all types were apparently fewer in number. The individual cell components appeared having the following criteria:

Spermatogonia cells were fewer and appeared in most sections, disintegrated, having pyknotic faintly stained nuclei and narrow cytoplasmic shell and some cells were detached from the basement membrane (Fig. 4.10).

Primary spermatocytes: The primary spermatocytes appeared round in shape with small oval nuclei, fewer in number and smaller in size (Fig. 4.10).

Secondary spermatocytes: Although they could not be easily identified as they rapidly passed this stage and changed to spermatids, a few number of these cells were identified in their relative normal position within the seminiferous epithelium but they have smaller size, smaller nuclei with thin faintly stained chromatin relative to the control cells (Fig. 4.11).

Spermatid: Early stages of spermatids were rare, some sections showed little number of distorted elongated spermatid that are characterized by oblong deeply stained nuclei but no visible cell membranes or cytoplasmic shell around them (Fig. 4.10 & 4.11).

Spermatozoa: the experimental group’s tubular lumens showed remarkable decrease in the concentration of spermatozoa. Some
sections revealed necrotic cellular debris within their lumens (Fig.4.10 & 4.11).

Sertoli cells fewer in most sections and great vesicular cytoplasm cavities resulting in the abnormal spacing (vacuoles) between the germ cells of the seminiferous epithelium (Fig. 4.11).

Most interstitial spaces of these groups appeared wider, contained large vessels and semi-empty cells except for very little number of Leydig cells, connective tissue cells and interstitial vacuoles (Fig. 4.12 & 4.13).

4.2. Electron microscopic results

Ultrathin sections prepared for examination by transmission electron microscope revealed the following:

4.2.1. Control group

The testis of the control rats did not reveal obvious ultra-structural changes. Electron microscopic examination of the seminiferous tubules of the control animals showed that the epithelia were composed of two types of cells, spermatogenic as well as Sertoli cells. Spermatogenic cells were noticed in various stages of maturation. Spermatogonia were located in the basal compartment of the seminiferous tubules. Type-A and type-B spermatogonia were recognized. Type-A spermatogonia were characterized by large pale ovoid nuclei horizontally oriented and scanty cytoplasm while type-B spermatogonia were slightly smaller than type A and had rounded nuclei occupying the basal cytoplasm (Fig. 4.14).

The primary spermatocytes, the largest of the spermatogenic cells, were characterized by the presence of intercellular bridges, spherical nuclei with finely granular nucleoplasm and chromatin accumulation (Fig. 4.15).

Secondary spermatocytes were short-lived and become round
Spermatids. Many spermatids were observed near the lumen of the seminiferous tubules. They were in different normal maturation stages. They possessed numerous mitochondria and a well-developed Golgi complex. Spermatids have developed an acrosomic system (Fig. 4. 16).

The basal lamia surrounding the seminiferous tubule consisted of four to six thin layers of myoid cells packed together; they contain spindle-shaped nuclei with delicate cytoplasmic processes (Fig. 4. 15).

The Sertoli cells extended radially from the basement membrane to the lumen of the seminiferous tubule, and their numerous cytoplasmic processes surrounded each germ cell. They appeared irregular, with basally located, oval, indented nuclei and prominent nucleoli. Their cell membranes showed complex infoldings. They have an abundant cytoplasm with cell membrane apposed to the spermatogenic cells (Fig 4.17). The cytoplasm is rich in smooth endoplasmic reticulum, free ribosomes, numerous mitochondria, a small Golgi complex, and lysosomes (Fig. 4.17).

Leydig cell is a polyhedral epithelioid cell with a single eccentrically located ovoid nucleus surround by macrophage cells (Fig. 4.15). The nucleus contains one to three prominent nucleoli and large amounts of dark-staining peripheral heterochromatin. The acidophilic cytoplasm usually contains numerous membrane-bound lipid droplets and large amounts of smooth endoplasmic reticulum (SER). Besides the obvious abundance of SER with scattered patches of rough endoplasmic reticulum, several mitochondria are also prominent within the cytoplasm (Fig. 4. 15 & 4. 18).
Figure 4.1: (A&B) Light photomicrographs of control rat testis showing: (A) normal appearance of circumscribed seminiferous tubules (ST) and interstitial spaces (IS) (X 200). (B) Seminiferous tubules (ST) with intact basement membrane (BM), normal lumen (LU) filled with spermatozoa (SM), interstitial spaces (IS) containing Leydig cell (LC), Sertoli cell (SC) and blood vessels (BV) (H&E X 400).
Figure 4.2: Light photomicrographs of control rat testis showing: All successive stages of spermatogenesis are seen; spermatogonia type A (SPA) & type B (SPB), dividing primary spermatocyte (DPS), round spermatids (RS), elongated spermatids (ES), Sertoli cell (SC) and basement membrane (BM). The lumen (LU) is filled with spermatozoa (SM), (H&E X 1000).

Figure 4.3: Light photomicrographs of control rat testis showing: interstitial space (IS) containing numerous single Leydig cell (LC), Leydig cell clusters (LCC), vacuoles (arrowed), connective tissue (CT) with connective tissue cells (CTS) and surrounding seminiferous tubules (ST) (H&E X 1000).
Figure 4.4: Light photomicrographs of control rat testis showing: seminiferous tubule (ST), tubular lumen (LU) filled with spermatozoa (SM), interstitial space (IS) and numerous Leydig cells (LC) (H&E X 400).

Figure 4.5: (A), Light photomicrographs of experiment (I) group testis showing: wide interstitial space (WIS), large blood vessels (LBV) and folded basal laminae (arrowed) (H&E X 50).
Figure 4.5: (B), Light photomicrographs of experiment (I) group testis showing: wide interstitial space (WIS), seminiferous tubules appeared to lose their usual configuration, detached basement membrane (asterisk), detached basal laminae (arrowed), blood vessels (BV) and folded basal laminae (FBL) (H&E X 100).

Figure 4.6: Light photomicrographs of experiment (II) group testis showing: seminiferous tubules (ST) shrinkage and destruction, wide interstitial space (WIS), large blood vessels (LBV) and detached (arrowed) and folded (FBL) basal laminae (H&E X 100).
Figure 4.7: Light photomicrographs of (A); experiment (I) & (B); experiment (II) groups testes showing: disintegrated disorderly arranged spermatogenic cells with intercellular and intracellular cavitations (C), wide tubular lumen (WLU), few luminal spermatozoa (SM), wide interstitial space (IS) containing few Leydig cells (LC) and large blood vessels (BV) (H&E X 400).
Figure 4.8: Light photomicrographs of (A); experiment (I) & (B) experiment (II) groups testes showing: disintegrated disorderly arranged spermatogenic cells and Sertoli cells (SC) with intercellular intracellular cavitations (C), interstitial space (IS) containing few Leydig cells (LC), large blood vessels (BV), cellular debris in the lumen (CD), and few luminal spermatozoa (SM) (H&E X 400).
Figure 4.9: Light photomicrographs of experiment (I) group testes showing: wider tubular lumen (LU), decreased germinal epithelium height (small arrow) and tubular diameter (long arrow), few number of elongated spermatids (ES), round spermatids (RS), detached basal laminae (DPL), large blood vessels (BV) cellular debris in the lumen (CD) and intercellular cavitations (C) (H&E X 400).

Figure 4.10: Light photomicrographs of experiment (I) group testes showing: seminiferous tubules with few and small size of spermatogenic cells and Sertoli cells (SC) primary spermatocyte (PS), spermatogonia (SP) possessing pyknotic nucleus (PN), intercellular cavitations (C), myoid cells (MYC), few number of elongated spermatids (ES), tubular lumen (LU) containing few luminal spermatozoa (SM) (H&E X 1000).
Figure 4.11: Light photomicrographs of experiment (II) group testes showing: few number of Sertoli cell (SC) with vacuolated cytoplasm (C), spermatogonia (SP), primary spermatocyte (PS), small size secondary spermatocyte (SSP) with faintly stained chromatin, distorted elongated spermatids (DRS), little number of elongated spermatids (ES), rounded spermatids (RS) and tubular lumen (LU) with few luminal spermatozoa (SM) (H&E X1000).

Figure 4.12: Light photomicrographs of experiment (I) group testes showing: wider interstitial spaces (WIS) containing fewer number of Leydig cells (LC) and interstitial vacuoles (V) (H&EX400).
Figure 4.13: Light photomicrographs of experiment (II) group testes showing: wider interstitial spaces (WIS) contain fewer number of Leydig cells (LC), interstitial vacuoles (V) (H&E X 400).

Figure 4.14: Electron photomicrograph of seminiferous tubule of control group showing: normal histological structure with different normal cell stages. Spermatogonia type A (SPA) & type B (SPB), primary spermatocyte (PS), Sertoli cell (SC), round spermatids (RS), and basal lamina (BL) (X3000).
Figure 4.15: Electron photomicrograph of seminiferous tubule of control group showing: normal spermatogonia (SP), primary spermatocyte (PS), Basal lamina (BL) contains myoid cells (MYC), Sertoli cell (SC) surround by macrophages (MA), Interstitial lipid droplets (arrowed) and Leydig cells (LC) (X2000).

Figure 4.16: Electron photomicrograph of spermatid at acrosomal phase of control group showing: one-third of the nucleus was covered by the acrosomal cephalic cap (AC) and dark acrosomal granule (DAG), ovoid nucleus (N) with central rounded nucleolus (NU). The Golgi complex (GC) and mitochondria (M) occupy a peripheral site in the cytoplasm (CYT) and cell membrane (CM) (X4000).
Figure 4.17: Electron photomicrograph of Sertoli cell of control group showing normal histological structure: oval nucleus (N) with indented nucleus membrane (INM). The cytoplasm contains normal micro-organelles including mitochondria (M), folded cell membrane (CM) and well defined nucleolus (NU) (X 4000).

Figure 4.18: Electron photomicrograph of Leydig cell (LC) of control group showing: normal single eccentrically located ovoid nucleus (N), prominent nucleoli (NU) with large amounts of dark peripheral heterochromatin. The cytoplasm contains lipid droplets (L), smooth endoplasmic reticulum (short arrow), rough endoplasmic reticulum (long arrow), Lysosomes (double arrow) and mitochondria (M) (X 4000).

4.2.2. Experiment I and II groups
The experimental rats testes, (exposed to synthetic progesterone prenatal/and lactational) showed atrophied degenerated germ cells which lost their attachment to surrounding Sertoli cell and basal membrane (Fig. 4. 19). Spermatogonia have vacuolated cytoplasm and small pyknotic nucleus (Fig. 4. 20). Empty spaces left by atrophied degenerated germ cells and nuclear envelop invaginations were found occasionally in primary spermatocytes (Fig 4. 20 & 4. 21).

The Pachytene stage primary spermatocytes appeared smaller in size and the basal lamina appeared thick, folded and contained hypertrophied myoid cells (Fig. 4. 22 & 4. 23). Some section showed the germinal cells lost their normal shape and integration (Fig. 4. 22 & 4. 23) and presence of cytoplasmic cavitations in the Leydig and Sertoli cells (Fig. 4. 24).

Sertoli cell showed small invaginated nucleus with peripheral nucleolus, folded cell membrane and large lipid droplets. The cytoplasm has small cavities as a result of degenerative processes affecting its micro-organelles. Mitochondria appeared distended and seem to have lost their cristae (Fig. 4. 25).

Leydig cell appeared to have elongated nucleus, indented nuclear membrane, three prominent nucleoli and dense scattered chromatin. The cytoplasm contain: abundant lipid droplets, numerous mitochondria and cytoplasmic vacuoles (Fig. 4. 26 & 4. 27).
Figure 4.19: Electron photomicrograph of seminiferous tubule of experiment (I) group showing: atrophied spermatogonia (AT.SP), folded basal lamina (BL), degenerative cells vacuoles (V) between tubule cells, spermatogonia (SP), primary spermatocyte (PS), round spermatids (RS) and Sertoli cell (SC) (X 3000).

Figure 4.20: Electron photomicrograph of seminiferous tubule of experiment (II) group showing: cells disintegration due to presence of degenerative vacuoles (V) between the different types of cells, small size primary spermatocyte (PS), degenerated spermatogonia (DSP) with pyknotic nucleus (PN), Sertoli cell (SC) and folded basal lamina (BL) (X 3000).
Figure 4.21: Electron photomicrograph of seminiferous tubule of experiment (II) group showing: degenerated tubular cells (DGC), thick basal lamina (BL), degenerative cells vacuoles (V), between tubule cells, spermatogonia (SP) and primary spermatocyte (PS) with nuclear envelop invagination (NEI) (X 3000).

Figure 4.22: Electron photomicrograph of seminiferous tubule of experiment (I) group showing: small size Pachytene primary spermatocyte (PS) separated from adjoining cell by empty spaces (V), spermatogonia (SP), thick folded basal lamina (BL) and hypertrophied myoid cell (MYC) (X 3000).
Figure 4.23: Electron photomicrograph of seminiferous tubule of experiment (II) group showing: spermatogenic cells disintegrated disorderly arranged, spermatogonia (SP) degenerated cell cavitations (V), small size primary spermatocyte (PS) with narrow cytoplasm and thick basal lamina (BL) (X 3000).

Figure 4.24: Electron photomicrograph of seminiferous tubule of experiment (I) showed: degenerative vacuoles (V) between the different types of cells, Sertoli (SC) and Leydig (LC) cells have cytoplasmic cavitations (V), spermatogonia (SP), thick basal lamina (BL) and hypertrophied myoid cell (MYC) (X 1500).
**Figure 4.25:** Electron photomicrograph of Sertoli cell of experiment (I) group showing: small size indented (IND) and invaginated nucleus (N), ill-defined nucleolus (NU) and folded cell membrane (CM). The cytoplasm contains large and small vacuoles (V), distended mitochondria (M) and lipid droplet (LD) (X 4000).

**Figure 4.26:** Electron photomicrograph of Leydig cell (LC) of experiment (I) groups showing: elongated nucleus (N) with indented nuclear membrane (NM). The cytoplasm contains large amounts of lipid droplets (L) and numerous mitochondria (M) (X 4000).
Figure 4.27: Electron photomicrograph of Leydig cell (LC) of experiment (II) groups showing: ovoid nucleus (N) with indented nuclear membrane (NM), three prominent nucleoli (NU) with dense scattered chromatin. The cytoplasm vacuolated (V) and contains large amounts of lipid droplets (L) and numerous mitochondria (M) (X4000).

4.3. Seminal analysis

4.3.1. Sperm count

As shown in (Table 4.1&Fig.4.28), the mean epididymal sperm count of male rats born to dams treated with synthetic progesterone during pregnancy (experiment I) and during both pregnancy and lactation (experiment II) were significantly different from that of the control (p<0.001). The epididymal sperm count per 0.1 g / epididymis in the control, experiment I and experiment II were; 113.55 ± 10.46 × 10⁶, 81.72 ± 5.61× 10⁶ and 77.17 ± 5.03 × 10⁶ respectively that reflects a clear difference in spermatozoa density between the treated and the control groups.

4.3.2. Sperm motility
As shown in (Table 4.1, Fig.4.29 & Fig.4.31), a significant decrease was observed in sperm motility in both experiment groups as compared to the control (P<0.001). Whereas the sperm motility showed more decrease in the experiment group II. The mean percentage of motile sperms in the control, experiment I and experiment II were; 82.42± 6.62%, 63.65 ± 6.49% and 55.33± 6.10% respectively.

### 4.3.3. Sperm viability

The mean percentage of viable spermatozoa in the control was 83.82± 6.54%, while that of the experiment groups I and II were 64.90 ± 6.10% and 56.60 ± 6.04% respectively (Table 4.1 & Fig.4.30). Reduction of viability revealed a significant difference between the different groups (P<0.001). Also, sperm viability showed more decrease in the experiment II group.

**Table 4.1:** Effects of synthetic progesterone on the sperm count, motility, viability and total abnormality:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Synthetic Progesterone Injected(10 mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td>Sperm count(million/ml)</td>
<td>(113.55 ±10.46) &lt;sup&gt;a&lt;/sup&gt;</td>
<td>(81.72 ± 5.61) &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>(82.42± 6.62) &lt;sup&gt;a&lt;/sup&gt;</td>
<td>(63.65 ± 6.49) &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>(83.82± 6.54) &lt;sup&gt;a&lt;/sup&gt;</td>
<td>(64.90 ± 6.10) &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Treatment (I): denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while treatment (II) denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. <sup>a, b, c</sup>. Values with different superscripts in the same raw are significantly different (P<0.001). <sup>c&b</sup> Value with same superscripts in the same row is not significantly different.
Figure 4.28: Epididimal sperm count levels in the control and the experiment groups of rats. Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.05.

Figure 4.29: Epididimal sperm motility in the control and the experiment groups of rats. Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.001.
Figure 4.30: Epididimal sperm viability in the control and the experiment groups of rats. Values are expressed as Mean ± SD for 20 rats in each group. \textsuperscript{a,b,c} Values were considered significantly different at P < 0.001.

Figure 4.31: (A) photomicrographs of sperm viability analysis of control group showing; few dark stained dead sperms (arrowed) in photomicrographs (X200).
4.3.4. Sperm morphology

A significant increment in the total number of abnormal spermatozoa in both experiment groups was observed compared with control. The percentage of the total abnormal sperms was; 15.10 ± 1.42%, 41.16 ± 3.93% and 58.28 ± 5.10% for the control, experiment I and experiment II respectively. Whereas, there is a significant difference between experiment groups in the total sperm abnormalities (Table 4.2, Fig 4.32). The recorded abnormalities were headless, tailless, coiled tail, curved tail, bent tail, bent mid-piece and curved mid-piece of the sperms. The most prominent abnormalities were the headless, tailless and bent tail sperms.

Figure 4.31: (B) photomicrographs of sperm viability analysis of experiment group showing; increased number of dark stained dead sperm (arrowed) (X200).
The curved tail, bent tail, bent mid-piece and curved mid-piece sperms abnormalities showed no significant differences in all groups (Fig 4.33).

The headless and tailless abnormalities were significantly different ($P<0.005$) between experiment and control animals. The percentage of headless and tailless abnormalities in the control were; 1.42±.41% and 1.91±.48%, in experiment I were; 9.78±1.05% and 16.42±2.78% and in experiment II were; 9.88±1.56% and 16.44±2.45% respectively. Whereas coiled tail abnormality differed significantly ($P<0.001$) in the experiment groups when compared to the control. The percentage of coiled tail abnormality in the control, experiment I and experiment II were; 2.86±.52%, 16.38±3.89% and 17.26±4.52%, respectively (Table 4.2, Fig 4.34, 4.35, 4.36 & 4.37).

**Table 4.2: Effects of synthetic progesterone on the sperm abnormalities (%):**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Synthetic Progesterone Injected (10 mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td>Total sperm abnormality (%)</td>
<td>(15.10 ± 1.42)$^a$</td>
<td>(41.16 ± 3.93)$^b$</td>
</tr>
<tr>
<td>Headless</td>
<td>(1.42±.41)$^a$</td>
<td>(9.78±1.05)$^b$</td>
</tr>
<tr>
<td>Tailless</td>
<td>(1.91±.48)$^a$</td>
<td>(16.42±2.78)$^b$</td>
</tr>
<tr>
<td>Coiled Tail</td>
<td>(2.86±.52)$^a$</td>
<td>(16.38±3.89)$^b$</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. $^a$, $^b$, $^c$. Values with different superscripts in the same raw are significantly different ($P<0.005$). $^{c&b}$ Value with same superscripts in the same row is not significantly different.
Figure 4.32: Total of epididimal sperm abnormalities in the control and the experiment groups of rats. Values are expressed as Mean ± SD for 20 rats in each group. a,b,c Values were considered significantly different at P < 0.05.

Figure 4.33: Types of others epididimal sperm abnormalities in the control and the experiment groups of rats. Values are expressed as Mean ± SD for 20 rats in each group. Values were not significantly different P > 0.05.
Figure 4. 34: The most prominent types of epididimal sperm abnormalities in the control and the experiment groups of rats. \textsuperscript{a,b,c} Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.05.

Figure 4. 35: Photomicrographs of sperm morphology analysis obtained from the control group; showing normal sperm morphology with few numbers of sperms with abnormal morphologies including headless (short arrow), tailless (long arrow) and coiled tail (arrow head) (X100).
**Figure 4.36:** photomicrographs (A and B) of sperm morphology analysis obtained from the experiment (I) group showing increased number of sperm with abnormal morphologies including headless (short arrow), tailless (long arrow) and coiled tail (arrow head) (X100).
Figure 4.37: Photomicrographs (A and B) of sperm morphology analysis obtained from the experiment (II) group showing increased number of sperm with abnormal morphologies including headless (short arrow), tailless (long arrow) and coiled tail (arrow head) (X100).

4.4. Histometry

4.4.1. Testicular weight
A significant (P<0.005) reduction in the mean testicular weight of the male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) was observed. The mean testicular weights were; 2.11± 0.33gm, 1.58± 0.14gm and 1.46 ± 0.18gm for the control, experiment I, experiment II respectively (Table 4.3&Fig.4.38).

4.4.2. Epididymal weight
The mean epididymal weight was significantly differed (P<0.01) between all groups. The epididymal weight reduction more increased in the experiment I group and experiment II group show significant increase in the epididymal weight. The mean epididymal weight in the control, experiment I and experiment II were; 0.67 ±0.10gm, 0.43 ± 0.06gm and 0.49 ± 0.05gm respectively (Table 4.3&Fig.4.38).

4.4.3. Relative testicular weight
As shown in (Table 4.3&Fig.4.39), a significant difference (P<0.001) was found in the mean relative weight of the testis of the male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) and the control. The mean relative weight of the testes in the control, experiment I and experiment II were; 1.08 ± 0.14gm, 0.82 ± 0.13gm and 0.77 ± 0.12gm respectively.

4.4.4. Relative epididymal weight
A significant difference (P<0.01) was found in the mean relative epididymal weight of all groups. Whereas the relative epididymal weight reduction more increased in the experiment I and experiment II group showed significant increase in the relative epididymal
weight. The mean relative epididymal weight in the control, experiment I and experiment II were; 0.34 ± 0.04gm, 0.23 ± 0.4gm and 0.28 ± 0.4gm and, respectively (Table 4.3 & Fig.4.39).

**Table 4.3**: Effects of synthetic progesterone on the testicular, Epididymal and relatives weight (gm):

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Synthetic Progesterone Injected(10 mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experiment I</td>
</tr>
<tr>
<td>Testicular Weight</td>
<td>(2.11 ±0.33) (^a)</td>
<td>(1.60 ± 0.14) (^b)</td>
</tr>
<tr>
<td>Epididymal Weight</td>
<td>(0.67± 0.10) (^a)</td>
<td>(0.43± 0.06) (^b)</td>
</tr>
<tr>
<td>Relative Testicular Weight</td>
<td>(0.01 ± 0.001) (^a)</td>
<td>(0.007 ± 0.001) (^b)</td>
</tr>
<tr>
<td>Relative Epididymal Weight</td>
<td>(0.0036 ± 0.0004) (^a)</td>
<td>(0.002 ± 0.004) (^b)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. \(^a, b, c\). Values with different superscripts in the same row are significantly differ (P<0.001). \(^{c&b}\) Value with same superscripts in the same row is not significantly differ.
Figure 4.38: Testicular and Epididymal weight of the control and the experiment groups of rats. \textsuperscript{a,b,c} Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.01.

Figure 4.39: Relative Testicular and Epididymal weight of the control and the experiment groups of rats. \textsuperscript{a,b,c} Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.01.

4.4.5. Testicular diameters:
As given in (Table 4.4 & Fig. 4.40) the mean diameters (length and width) of the right testis in (experiment I and II) rats significantly decreased (P<0.01) when compared to the control group. While, there is no significant difference between the two experiment groups.

**Table 4.4:** Effects of synthetic progesterone on the testicular diameters (mm):

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Synthetic Progesterone Injected (10 mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Experiment I</td>
</tr>
<tr>
<td>Right testis width</td>
<td>10.87±0.041</td>
<td>8.49±0.018 b</td>
</tr>
<tr>
<td>Right testis length</td>
<td>17.85±0.036</td>
<td>15.57±0.044 b</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. a, b, c. Values with different superscripts in the same raw are significantly differ (P<0.01). c&b Value with same superscripts in the same row is not significantly differ.

**Figure 4.40:** Testicular diameters of the control and the experiment groups of rats. a,b,c

Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.05.

**4.4.6. Diameters of the seminiferous tubules**
The mean diameter of seminiferous tubule appeared significantly different (P<0.001) from male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) and the control. The mean diameter of seminiferous tubules in the control, experiment I and experiment II were; 281.45 ± 17.16µm, 255.47 ± 14µm and 253.13 ± 13.71µm respectively (Table 4.5 & Fig. 4.41).

4.4.7. Epithelial height

As shown in (Table 4.5 & Fig.4.41) a significant difference (P<0.001) was found in the mean thickness of seminiferous epithelium of male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) and the control. The mean thickness of the seminiferous epithelium in the control, experiment I and experiment II were; 93.70 ± 9.56µm, 72.58 ± 7.69µm and 71.42 ± 7.17µm respectively.

4.4.8. Thickness of the interstitial space

As shown in (Table 4.5 & Fig.4.41), a significant difference (P<0.001) was found in the mean thickness of the interstitial space of male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) and the control. The mean thickness of the interstitial space in the control, experiment I and experiment II were; 110.47 ± 11.34 µm, 132.11 ± 6.11µm and 133.10 ± 5.53µm respectively.

Table 4.5: Effects of synthetic progesterone on the seminiferous tubule diameter, height of germinal epithelium and thickness of interstitial space (µm):-
### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seminiferous tubule diameter</td>
<td>(281.45 ±17.16)</td>
<td>(255.47 ± 14.36)</td>
<td>(253.13 ± 13.71)</td>
</tr>
<tr>
<td>Height of germinal epithelium</td>
<td>(93.70 ± 9.56)</td>
<td>(72.58 ± 7.69)</td>
<td>(71.42 ± 7.17)</td>
</tr>
<tr>
<td>Thickness of interstitial space</td>
<td>(110.47 ± 11.34)</td>
<td>(132.11 ± 6.11)</td>
<td>(133.10 ± 5.53)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. a, b, c. Values with different superscripts in the same raw are significantly differ (P<0.001). c&b Value with same superscripts in the same row is not significantly differ.

![Graph showing seminiferous tubule diameter, height of germinal epithelium, and thickness of interstitial space for control and experiment groups.](image)

**Figure 4.41:** Seminiferous tubule diameter, height of germinal epithelium and interstitial space thickness of the control and the experiment groups of rats. Values are expressed as Mean ± SD for 20 rats in each group. a,b,c Values were considered significantly different at P < 0.001.

### 4.5. Stereological study

#### 4.5.1. Quantitation of seminiferous tubule cells
As shown in (Table 4.6 & Fig.4.42), a significant difference (P<0.001) was found in the mean seminiferous tubule cells count in the male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) when compared with the control rats. Spermatogonia type (A) counted in the groups were; control: 38.55±3.47, experiment I: 27.40±3.65 and experiment II: 27.00±3.73. Spermatogonia type (B) counted in the groups were: control: 36.50±3.07, experiment I: 21.80±4.53 and experiment II: 21.35±4.11.

Primary spermatocyte counted in the all groups were: control 37.50±4.02, experimental I: 26.45±4.52 and experimental II: 28.10±4.08. Whereas the sertoli cells counted in the all groups were: control: 17.65±2.32, experiment I: 9.75±2.77 and experiment II: 9.65±2.50.

Table 4.6: Effects of synthetic progesterone on the seminiferous tubule cells count:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Synthetic Progesterone Injected(10 mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td>Spermatogonia Type (A)</td>
<td>(38.55±3.47)</td>
<td>(27.40±3.65)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Spermatogonia Type (B)</td>
<td>(36.50±3.07)</td>
<td>(21.80±4.53)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Primary Spermatocyte</td>
<td>(37.50±4.02)</td>
<td>(26.45±4.52)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Sertoli</td>
<td>(17.65±2.32)</td>
<td>(9.75±2.77)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. Values with different superscripts in the same row are significantly different (P<0.001). Values with same superscripts in the same row is not significantly different.
Values were considered significantly different at P < 0.05.

4.5.2. Quantitation of seminiferous tubules and Leydig cells

A significant reduction (P<0.01) was found in the mean total number of seminiferous tubules counted in the entire histological section of the male rats born to dams treated with synthetic progesterone during pregnancy (experiment I), and during both pregnancy and lactation (experiment II) when compared with the control: control: 98.35±9.47, experiment I: 81.11±8.55 and experiment II: 79.77±9.73. However, a significant reduction (P<0.001) was observed between control and experiment groups in the mean total number of Leydig cells: control; 132.77±23.4, experiment I; 80.5±23.5 and experiment II: 78.5±27.2 (Table 4.7 & Fig.4.43).

Table 4.7: Effects of synthetic progesterone on the total numbers of seminiferous tubules and
Leydig cells:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of Seminiferous tubule</td>
<td>(98.35±9.4) ^a</td>
<td>(81.11±8.5) ^b</td>
<td>(79.77±9.7) ^c&amp;t</td>
</tr>
<tr>
<td>Total No. of Leydig cells</td>
<td>(132.77±24.4) ^a</td>
<td>(80.5±23.5) ^b</td>
<td>(78.5±27.2) ^c&amp;t</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P 4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P 4 during pregnancy and lactation period. ^a,b,c: Values with different superscripts in the same raw significantly different (P<0.001). ^c&t Value with same superscripts in the same row is not significantly different.

Figure 4. 43: Seminiferous tubule and Leydig cells percentage of the control and the experiment groups of rats. ^a,b,c: Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.05.

5.6. Hormonal Assay
The serum testosterone levels were significantly decreased (P<0.001) in male rats born to dams treated with synthetic progesterone during pregnancy (experiment I) 1.04±0.06 ng/ml, and during pregnancy and lactation (experiment II) 0.81±0.05 ng/ml when compared with the control rats 2.33±0.09 ng/ml. However, FSH serum levels (experiment I: 5.0±0.10 ng/ml, experiment II: 5.30±0.10 ng/ml, control 3.90±0.14 ng/ml) and LH (experiment I: 3.9±0.10 ng/ml, experiment II: 4.20±0.14 ng/ml, control 3.23±0.10 ng/ml) increased significantly (P<0.001) (Table 4.8 & Fig. 4.44).

**Table 4.8:** Effects of synthetic progesterone on the serum hormones level (ng/ml):

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control</th>
<th>Synthetic Progesterone Injected (10 mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(3.23±0.10) ^a</td>
<td>(3.9±0.10) ^b</td>
</tr>
<tr>
<td>LH</td>
<td></td>
<td>(5.0±0.10) ^b</td>
</tr>
<tr>
<td>FSH</td>
<td>(3.90±0.14) ^a</td>
<td>(1.04±0.06) ^b</td>
</tr>
<tr>
<td>Testosterone</td>
<td>(2.33±0.09) ^a</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Experiment I: denotes male rats reared by females dams injected with synthetic (P4) during pregnancy while Experiment II: denotes male rats reared by females injected with synthetic P4 during pregnancy and lactation period. ^a, ^b, ^c Values with different superscripts in the same raw significantly different (P<0.001).

**Figure 4.44:** Serum hormones level of the control and the experiment groups of rats. ^a,b,c Values are expressed as Mean ± SD for 20 rats in each group. Values were considered significantly different at P < 0.05

**CHAPTER FIVE**
DISCUSSION

The possible effects of prenatal and/or neonatal exposure to the female sex hormones on male reproduction have recently been studied. Several researchers observed a severe decline in human male reproductive function. Vandenbergh et al.\textsuperscript{[153]} attributed this decline to abnormal alterations in the testicular structure, male hormones level, reduction in the sperm concentrations and seminal quality.

The present study investigates the effects of progesterone administration in both prenatal and lactational periods. Clarification of the adverse effects of progesterone DMPA during lactational period is very important because it is widely used as a contraceptive method during this time.

The dose of progesterone used in this study was in the range of regular clinical use and the administrative schedule is similar to humans during pregnancy. Pregnant women were prescribed with progesterone at a dose of 5–10 mg/kg body weight per injection. Intramuscular injections were given weekly up to 12 weeks of pregnancy\textsuperscript{[116]}.

5.1. Histological findings

Results of the study provided clear evidence that prenatal exposure to progesterone resulted in statistically significant (P < 0.001) histological abnormal changes seen by both light and electron microscopy in the experiment groups compared with control. The seminiferous tubules appeared to lose their usual configuration as the basal laminae became folded, the germinal epithelium showed vacuolation which was also observed in the Sertoli cells and all the
cells were disorderly arranged. There was a significant reduction (P < 0.001) in the number and diameter of the seminiferous tubule, the thickness of the epithelial height, the seminiferous tubules cell count, the luminal spermatozoa, the leydig cell count. However, the interstitial space was significantly increased (P < 0.001).

These histological abnormalities could be due to hormones level alteration that may affect the testicular germ cells development and functions which in turn may cause germ cells death and seminiferous tubules shrinkage.

Within the testicular epithelium, two major changes were observed in the Sertoli cells and in their vicinity. One was the presence of cytoplasm vacuolization, numerous lipid droplets in Sertoli cells of experimental rats. These are in agreement with the reports showing that exogenous stimulants may cause progressive apoptosis of the Sertoli cells, which affect spermatogenesis and sperm parameters [154]. The second major change was the empty vacuolar spaces between Sertoli cells that are regarded to be the place where spermatogonia and spermatocytes should be located. In addition, the present study of some studies showed that apoptosis occurred in all germ line cells especially in spermatogonia and spermatocyte. The presence of apoptotic germ cells is supported by Blanco et al [155] finding that has described apoptosis in hamster testis following treatment with anabolic androgenic steroids.

Spermatogenesis is a complex and dynamic process that results in the continual production of spermatozoa in mammals. Sertoli cells are largely responsible for orchestrating the germ cells through sequential phases of mitosis, meiosis, and differentiation. The Sertoli cells accomplish this task by providing hormonal, nutritional, and physical support. Apoptosis of germ cells that occurs
in the testicular epithelium serves as a mechanism to reduce the germ cell population to the level that the Sertoli cells can support. Some drugs and toxic agents injure or disrupt the function of Sertoli cells and can effectively reduce their supportive role, resulting in an increase in the elimination of the germ cell numbers via apoptosis \[156\]. In present study, observation of detached germ cells and mislocation of spermatid and spermatozoa to positions that are closely related to the basement membrane may caused by a rapid disruption of Sertoli-germ cell interaction. Which intern let to the sloughing of the germ cells from the seminiferous epithelium \[156\].

On the other side, toxic agents can result in an enhanced production of reactive oxygen species (ROS) in cells/tissues and exert oxidative stress (OS), which, in turn, increases the rates of cellular damage \[157\]. Therefore, it seems logical that the physical/chemical-induced oxidative stress may affect the testicular antioxidant system and lipid peroxidation \[158\].

The present finding agreed with that of Harini et al \[116\] and Pushpalatha et al \[29\] who studied the effects of synthetic progesterone exposure during gestational period on male reproduction in mice and rat respectively. They have linked the main cause of observed testicular alteration in the form of germinal and somatic cell degeneration to the reduction of the intra-testicular testosterone level.

The present study revealed that there was a significant reduction in the Leydig and Sertoli cells, which supported by O'Donnell et al \[125\] and Abney \[159\] who found a significant reduction in both leydig and Sertoli cells (P < 0.01) in the experiment groups compared to controls after prenatal exposure to estrogenic compounds and concluded that prenatal exposure to estrogen has been shown to
inhibit proliferation of precursor Leydig cells, thus altering steroidogenic capacity of adult testis. In addition, Sharpe \textsuperscript{127} reported that exposure to diethylstilbestrol (DES) during the neonatal Sertoli cell proliferative period decreases Sertoli cell number.

The present study showed that exposure to the synthetic progesterone during specific developmental period (critical time point in utero) has adverse effects that may persist to adulthood. This fact was strongly supported by Colborn \textit{et al}\textsuperscript{102} and Rogan \textit{et al}\textsuperscript{103} who studied the effects of environmental chemicals on the sexual development, reproduction and fertility. They concluded that prebirth exposure to endocrine disruptors can lead to permanent alterations and adult diseases. Likewise, the results agreed with Allison \textit{et al}\textsuperscript{160} prospective study on children exposed in utero to 17-hydroxyprogesterone caproate compared to placebo in order to assess if there were evident adverse effects. They concluded that 17–hydroxyprogesterone caproate seemed to be safe for the fetus when administered during the second and third trimesters rather than the first trimester.

Compared to the experiment I, this study revealed similar histological changes among the experiment II group. However, the changes in experiment II were not statistically significant which suggests that maternal exposure to the synthetic progesterone (medroxyprogesterone) as a contraceptive during lactational period appear to have no significant effects on the germ cell development and morphology of male neonates. This finding was supported by Singhal \textit{et al}\textsuperscript{142} who documented the safety profile of using injectable medroxy-progesterone as a contraceptive in the immediate postpartum period. Their study concluded that the
contraceptive was a safe and effective alternate method with no deleterious effect.

Our results in line with Castro et al [99] study which was conducted to assess the effects of in utero and lactational exposure to endocrine disrupters. Fielden et al [118] and Delbe’s et al [169] studies have demonstrated a long-term decrease in the number of Sertoli cells, epididymal sperm count, and in vitro fertilizing ability in mice after gestational and lactational exposure to 10 mg/kg body weight diethylstilbestrol. On the other hand, the decrease in spermatocytes and increase in the lumen of the seminiferous tubules in the present study were consistent with the study done by Goyal et al (2003) [117].

5.2. Spermatogenic parameters

An important finding of the present study was the statistically significant reduction in the sperm count, sperm motility and sperm viability among the two experiment groups compared to the control group (P< 0.001) which could be due to sperm toxic effects. This finding is in agreement with previously recorded observations by Harini1 et al [116] and Pushpalatha et al [2] who investigated the effects of prenatal exposure to progesterone on male mice and rat reproduction respectively. In addition, Thayer et al [168] confirmed that there was a significant change in weights of the reproductive organs, quality and quantity of sperms, serum reproductive hormone concentrations and fertility at maturity following prenatal and neonatal exposure to progesterone compounds in rats.

Compared to experiment 1 group, the sperm count in experiment II group was not significantly different. This suggested that the exposure to P4 during embryonic development will affect the reproductive potential of adult male rats. However, in terms of
viability and motility, there were statistically significant differences (P< 0.001). These findings suggest that the adverse effects of P4 are more evident when it was administered in both prenatal and lactational periods. On the other hand, these findings suggested that administration of P4 during lactational period may affect sperm fertilizing ability due to direct effects on the development and maturation of the sperm, and epididymal functions. Fielden et al\textsuperscript{[1]} reported the same findings but he was testing the effects of gestational and lactational exposure of male mice to diethyl-stilbestrol. Therefore, the present study findings also may question the safety profile of P4 use as a contraceptive during lactation particularly in the presence of any testicular abnormality.

In addition, the significant increment in the percentages of the total abnormal spermatozoa in the two experiment groups compared to the control group (P < 0.005) suggested that administration of P4 during pregnancy may affect sperm development and maturation by reducing testosterone production and disrupting cell function. These findings were supported by studies done in mice in order to assess the effects of gestational and lactational exposure to progesterone Carlsen \textit{et al}\textsuperscript{[1]} and estrogen Fielden \textit{et al}\textsuperscript{[1]}.

Furthermore, there was statistically significant increase in the total percentages of abnormal spermatozoa in the experiment II group compared with experiment I group. This finding suggested that long-term exposure to P4 has more adverse effects on the sperm development and maturation. Moreover, study suggests that lactational administration of synthetic progesterone may interfere with the normal sperm production and function.

5.3. Testicular and Epididymal parameters
A significant decrease in the relative testicular and epididymal weights (P<0.01) and testicular diameters (P<0.05) in the experiment groups compared to control were documented in the present study. This result could be explained by germinal and somatic cell loss or hypotrophy; decrease in the sperm count; and seminiferous tubules shrinkage due to prenatal exposure to P4. These finding have been reported by many similar studies [28, 29, 116]. The weight of the testis is largely dependent on the mass of differentiated spermatogenic cells and it has been used as a measure of spermatogenesis in rats [134]. A positive correlation was observed between weight of testis and number of germ cells [135].

Compared to experiment I group, the experiment II group showed no statistically significant difference in the relative testicular weight and diameters in the present study. However, there was statistically significant difference in epididymal weight (p<0.05) in experiment II group compared to experiment I group despite the presence of insignificant sperm count reduction and significant increase in the total abnormal sperm. Exposure to P4 during lactational period could affect estrogen receptors (ERs) and inhibit the seminiferous tubular fluid reabsorption (major functions of the rete, efferent ducts and initial segment of the epididymis) which in turn increases the epididymal weight. The existence of ERs in the testis is the clear indication of the important physiological role that estrogens play in the histophysiology of the male genital tract and sperm production. This justification is supported by Hess et al study [170] who reported that estrogens play an important role in modulating functions of efferent ductules and the epididymis and ERs essential
for the maintenance of a luminal environment that permits normal development of sperm motility and volume regulation.

Over 98% of the seminiferous tubular fluid is reabsorbed as the sperms pass through the rete, efferent ducts and initial segment of the epididymis \([171, 172]\). The lack of ERs severely impairs epithelial development, alters epithelial morphology and inhibits fluid reabsorption \([172, 173]\). ERs are present in the undifferentiated rat testis as early as 10.5 day after conception and their distribution in germ cells, Sertoli cell, and Leydig cell has been extensively studied \([174, 175]\). ERs are localized in the fetal Leydig cells until birth in rodents \([125]\). Consequently, testicular progesterone-oestrogen imbalance and/or ERs disruption could lead to reproductive function impairment \([165 - 167]\).

5.4. Hormonal Changes
The present study also included the effect of prenatal and/or lactational progesterone treatment on the reproductive hormones namely testosterone, LH and FSH serum levels. Compared to controls, a significant reduction in the serum testosterone level (P<0.001) as well as a significant increase in the serum FSH and LH level (P<0.001) were observed in both experiment groups. These finding have been reported in rat following prenatal exposure to hydroxyprogesterone \([28, 116]\). Similar results to the present study were obtained by Atanassova et al \([124]\) who studied the effect of neonatal estrogen exposure on rat’s reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. The decrease in the serum testosterone levels has been explained by reduction in the Leydig cells count; diminished
responsiveness of Leydig cells to LH or direct inhibition of testicular steroidogenesis [28,116].

A study by Pushpalatha et al [15] reported a significant decrease in the 3b-hydroxysteroid dehydrogenase (3b-HSD) and 17b-hydroxysteroid dehydrogenase (17b-HSD) activities and steroidogenesis in the testes of mice exposed to progesterone prenatally. The increase in serum FSH levels in the present study reflected the germ cell loss in the spermatogenic compartment or damage to the Sertoli cells which is expected to decrease inhibin hormone, thereby affecting the negative feedback regulation of FSH secretion [171]. Similarly, the present study suggested that increased levels of LH together with decreased levels of serum testosterone in the experiment groups are indicative of loss of the negative feedback of testosterone on LH secretion caused by impairment of Leydig cells structure and/or function. Earlier studies also reported that neonatal exposure to P4 or DES results in suppression of androgen action in addition to abnormalities in the male reproductive tract [162-164]. Nevertheless, the increased LH serum level was found to delay the onset of mesenchymal cell differentiation into Leydig cells [164].

The present study did not find significant differences in the testosterone, LH and FSH serum level between the two experiment groups. Therefore, this unique result suggests that the embryonic exposure to P4 is the main implicating factor in the adverse effects.

CHAPTR SIX
CONCLUSIONS AND RECOMMENDATIONS
6.1. Conclusions
Exposure to P4 during pregnancy (embryonic period) affects the testicular development precursors which results in significant testicular structural and functional disorders at puberty. The disorders include form of decreased testicular and epididymal weights; decreased sperm count, viability and motility; increase in sperm abnormalities; as well as hormonal disturbances (low testosterone and high LH and FSH).

Long – term exposure to P4 (during pregnancy and lactation) exacerbates the male reproductive disorders particularly the sperm development and maturation.

Exposures to synthetic progesterone during early pregnancy can interfere with cell signaling via direct / indirect hormonal pathways in hypothalamic–pituitary–gonadal (HPG) axis. This will disrupt the homeostasis at different levels of feedback regulation of testicular development and functions. Hence, the level of FSH, LH and testosterone will fluctuate depending on the numbers and functions of Leydig and Sertoli cells.

6.2. Recommendations

Animals in this study were only observed till 90 days of age, and it is possible that other testicular disorders /recovery may become evident with ageing. Therefore, longer term studies may be necessary to identify the full effect of these endocrine disruptors.
• More experimental studies must be taken to find out the underlying mechanisms explaining the effects observed on this study as well as the recovery routes after prenatal and / or postnatal progesterone exposure.

• Prenatal effects of progesterone on the Leydig, Sertoli and germ cells differentiating factors are needed to be identified in future studies.

• More studies should also be conducted to help identify safety profile of P4 use as a contraceptive during lactation particularly in the presence of any testicular abnormality.

• The progesterone-estrogen imbalance and fertility index after long-term exposure to progesterone is a rich area to be investigated by researchers.

• An elaborative program should be initiated to evaluate the effects of gestational / and lactational exposure to man-made chemicals on human reproduction.

CHAPTER SEVEN

REFERENCES


40. Dym M. The fine structure of monkey (Macaca) Sertoli cell and its role in maintaining the blood testis barrier. The Anatomical Record. 1973; 175: 639-56


100. Recabarren SE, Rojas-García PP, Recabarren MP, Alfaro VH, Smith R, Padmanabhan V, Sir-Petermann T. Prenatal testosterone


139. Cupp A, Skinner M. Actions of the endocrine disruptor methoxychlor and its estrogenic metabolite on in vitro embryonic rat


148. Pizzi W J, Barnhart J E, Fanslow D J. Monosodium glutamate
administration to the newborn reduces reproductive ability in female and male mice. Science. 1977; 196: 452-54.


164. Siril Ariyaratne H B, Ian Mason J, Mendis-Handagama SM. Effects of Thyroid and Luteinizing Hormones on the Onset of Precursor


173. Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL. Estrogen receptor (alpha and beta) expression
