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ORIGINAL ARTICLE

Histological Sequel Following Exposure to Levonorgestrel on Wistar Rat Ovary

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KEYWORDS

Histological;
Sequelae;
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Ovary

ABSTRACT: The action of sex hormones could be altered through the introduction of exogenous synthetic substances, which have been shown by several studies to possess deleterious sequelae on the ovaries. Reports on the detailed histology following the exposure to Levonorgestrel (L) are scanty and none has espoused the use of progestin-only contraceptives (post-coital pills) on the ovary. The study, therefore, evaluated the histological sequelae following exposure to Levonorgestrel on the Wistar rat ovary. Ethical approval for the study was granted by the Research and Ethics Committee in the Faculty of Basic Medical Sciences of Delta State University, Abraka. Forty Adult female Wistar rats, grouped into A and B were investigated. A, control, consisting of 20 rats, and B, test group, 20 rats. Both groups were further divided into 10 subgroups respectively, with each consisting of 20 rats. Subgrouping was done accordingly into 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} 18^{th}, 21^{st} 24^{th} 27^{th} and 30^{th} days respectively. Group A received food and distilled water only while group B rats received 0.025mg kg^{-1} of (L), food and water. Ovary revealed untoward effects of Levonorgestrel, seen at day 6 to day 30 for routine staining pattern. Also seen is a gradatory staining intensity of brown nuclei for progesterone and estrogen receptors for immunochemical staining pattern. This treatise underscored the deleterious outcomes in female gonads exposed to Levonorgestrel and cytoarchitectural peculiarities in ovaries exposed to either combined or emergency oral contraceptives were well espoused.

INTRODUCTION

Steroids are cholesterol derivatives that can be classified into natural and synthetic forms [1]. (L), a single-use post-coital pill has served extensive patronage in recent times. A study revealed that the agent functions by interrupting follicular development and inhibition of ovulation due to the suppression of gonadotropins in the preovulatory period. The study also demonstrated that fertilization was prevented through decreased tubal motility and inhibition of implantation in the post-ovulatory period [2]. Hormonal mechanisms of the reproductive system can be altered medically through the exogenous introduction of synthetic hormones to inhibit the normal physiological process resulting in contraception. This is coordinated through the channels of the hypothalamic-pituitary axis and is regulated by a negative feedback mechanism [3, 4].

Contraceptive use is a key factor in preventing unwanted pregnancies and reducing population [5, 6]. However, there are several health challenges that can be attributed to lack of awareness and low educational level associated with contraceptive use [7, 8]. Studies have also shown that oral contraceptives have numerous health benefits, but are associated with untoward effects on users, society and well-being thereby affecting the anatomical architecture of the ovary in women [6, 9, and 10].

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Contraceptive pills have many unavoidable side effects on the body leading to several diseases and complications [6, 9, and 10]. Most common side effects that are associated with contraceptive use have a greater impact on the ovary in women with several pathologic conditions like; reduced degree of follicular activity leading to atrophic ovary, and other secondary changes seen in the liver and kidney, depending on the dose of ethinyloestradiol (EE) rather than the dose of progestin administered [11]. A study revealed that low doses of ethinyloestradiol supplements effectively inhibited ovarian activity and suppresses ovulation [12]. It has also been reported that long-term use of oral contraceptive (L) in women has led to ovarian cancer, cyst, and change in ovarian function, suppressed endometrial growth and increased viscosity of cervical mucus [13 - 17]. Furthermore, studies have also reported that sex steroid has also caused several other effects on the female reproductive system like; altering early embryo transport in fallopian tube, impairing corpus luteal formation and decreasing endometrial receptivity [18, 19]. It is also observed that oral contraceptives suppress endometrial growth and change its conformation in three phases, which may lead to infertility [20, 21]. However, in considering the rate at which women continuously ingest emergency oral contraceptives, the fear of infertility is bound to be assumed in the future. However, report on the gross, histology, estrogen receptor and progesterone receptor following the exposure to (L) are scanty and none has elucidated the single use of progestin-only contraceptive (post-coital pills) on the ovary. This study therefore espoused the histological sequelae following exposure to (L) on Wistar rat ovary.

MATERIALS AND METHODS

Experimental animals and management

Forty Adult male Wistar rats with a body weight ranging 180g -200g were purchased from animal house of Delta State University Abraka. All animals were fed with commercially formulated rat chow and water. The animals were acclimated for two weeks according to [22]. They were fed twice daily with unrestricted access to food and water under standard conditions (12 hours of light and dark cycle and temperature of about 26 - 28°C) [23]. The experiment was for 4 weeks (30 days).

Drug

(L) is an oral emergency contraceptive pill with a formulation of 1.5mg tab^{-1}. Manufacturer: Gedeon Richter Pic, Budapest Hungary

Experimental design

This study adopted an experimental and observational study design. The behavioral changes of the animals were observed before, during and after administration of the emergency contraceptive on daily basis, after which the ovary will be harvested and examined histologically.

The forty (40) female Wistar rats were divided into two main groups; group A and B. Group A serve as control consisting of 20 rats and group B will serve as treated group consisting of 20 rats. Both groups were further divided into 10 sub-groups respectively, with each sub-section having 2 rats [24]. Sub-sections were labeled accordingly into 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} 18^{th}, 21^{st} 24^{th} 27^{th} and 30^{th} days respectively for easy identification and treatment or administration.

Group A: Twenty (20) Wistar rats received food and distilled water only (Control)

Group B: Twenty (20) Wistar rats received 0.025 mg kg^{-1} of (L), food and water only (Table 1).

Table 1. Grouping of Rats.

Administration days	3^{rd} day	6^{th} day	9^{th} day	12^{th} day	15^{th} day	18^{th} day	21^{st} day	24^{th} day	27^{th} day	30^{th} day
Group A (Control)	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats
Group B	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats	2 rats

Determination of administered concentration of the drug

The Drug will be dissolved in distilled water and administered orally to the animals [25 - 28]. The dose of (L) that was used for this study is 0.025 mg kg^{-1}; this was selected based on the normal prescription of the drug [29].

Animal euthanasia

Experimental rats were euthanized at the end of each experimental day which was stipulated for 3 days intervals of 30 days, by cervical dislocation [30] to prevent possible effect of anesthetic agent on samples to be collected. The ovaries were dissected, weighed and fixed immediately in 10% formal-saline.

Histological procedures

At the end of each experimental day, the animals were weighed and euthanized by cervical dislocation. Ovary were grossed, placed in tissue cassette and processed manually under standard histological procedures which entail several stages, from fixation using 10% formal saline, tissue processing comprises four sections: dehydration (series changes of the tissue in alcohol with time dependency), clearing (series changes of the tissue in xylene also with time-dependent), infiltration (series changes of the tissue in paraffin wax also with time dependency) and embedding (preparation of tissue block, using a mold), sectioning (using a microtome at 5 to 7um), staining (using H and E and special stains), mounting (using DPX) and photomicrography [31, 32].

Procedure to determine estrogen and progesterone receptor

Immunohistochemistry kits were purchased from a chemical shop. Routine paraffin-embedded ovary tissues were sectioned at a thickness of 4µm. After deparaffinization and incubation with 3% H_2O_2 for 15 min, routine antigen retrieval was carried out by microwaving the sections in citrate buffer. Sections were incubated with primary ER or PR antibody (1:50 dilution) overnight at 4°C. After rinsing with 1 × phosphate-buffered saline (PBS), and then incubated with horseradish peroxidase-conjugated anti-rat secondary antibody for 30 mins at room temperature. After treatment with 3, 3'-Diaminobenzidine (DAB) substrate, sections were counter-stained with haematoxylin, dehydrated, and soaked in xylene. The negative control was made by replacing the primary with 1 × PBS antibody. Immunohistochemistry was further evaluated via a semi-quantitative approach involving H-score. H-score is based on a predominant staining intensity in a fixed field [27, 33].

Photomicrography

Prepared Slides were viewed and tissue images were captured using digital microscope "CARL ZEISS (Primo Star)" of about 8.3 mega-pixel camera, connected to computer. Obtained micrographs were interpreted to know the histological and cytological effects of (L) on the ovaries.

RESULTS

Effect of (L) on the Histoarchitecture on the Ovary of Adult Wistar Rats Assessed Using H and E and Special Staining Patterns

Figure A1: Sections of the ovary show a cortex with several corporal luteal (5) with a graffian follicle showing smaller primordial follicle and secondary follicle also in the cortical region, blood vessels are also present in the medullary region of the ovary. The corporal luteal are composed of luteinized granulosa cell, which possesses abundant deeply eosinophilic cytoplasm and round to oval vesicular nuclei posited centrally. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward and separates corporal in a lobular fashion. In a pole of these sections is the fimbriated end of the fallopian tube. These features are those of normal ovarian tissue

Figure B1: In this section of the ovary, there was a lining germinal epithelium of cuboidal epithelial cells in its

outermost part. The stroma located immediately beneath this epithelium contains several sections including vascular corporal luteal and follicles at different stages of maturation, which are contained in the cortex of the gland. The corpus (yellow body) contains luteinized granulosa cells that display amphophilic cytoplasm round to oval vesicular nuclei, prominent nucleoli and condensed chromatin. The cell membrane is poorly defined with each cell separated from its neighbor by capillaries disposed in thin connective tissue stroma (fibro-vascular stroma).

The primordial follicles are lined by a single layer of flattened epithelium unlike the stratification present in the growing follicle. Both however possess an oocyte with polar body present in the periphery of the nucleus in both maturity and tertiary follicles, antral spaces are seen. The presence of an oocyte surrounded by corona radiata with cumulus oophorus forming the floor and a large antrum containing fluid enclosed by strata of granulosa cells is indicative of a Graafian follicle. Also present in the ovary are several blood vessels, small arteries and veins distributed toward the medulla of the gland, it's obvious that the macrophages are clustered within corporal albuginea. These features therefore depict normal ovarian tissue.

Figure C1: Sections show ovarian tissue composed of a cortex in which are several variably sized corporal luteal and some atrophied follicles. The corpora are composed of luteinized granulosa cell, disposed in both clusters and trabeculae with loose fibro-vascular stroma separating the cell group of individual luteinized cell possessing single nuclei which is vesicular, round to oval with a prominent nucleolus. The pale to mildly eosinophilic cytoplasm is bounded by a plasma membrane with ill-defined borders. The atrophied follicle containing granulosa cells lacks antra and is enclosed by fibrous bands.

Blood vessels specifically veins, are present in the medulla separated by a loose connective tissue stroma. The outermost part of the ovarian section is composed of one or more layers of germinal epithelial cells (cuboidal cells), which are composed of adipose tissue constituting a false capsule of the ovary. These features are in keeping with a mildly atrophied ovarian tissue

Figure D1: Sections of the ovary show a cortex, lined by one or more layers of cuboidal epithelial cells and medullar obliterated by a single and large Graafian follicle. These follicles possess a cumulus oophorus that is proliferating as is in the corona radiate. There is suppression of primary and primordial follicles, disposed between corpora amnesia with these cells which are displayed in trabeculae and fibro-vascular channels, displaying some atretic properties. Present also in one of the corporal luteal were hemosiderin-laden macrophages with blood vessels. These features are those of moderate atrophic changes in the ovary.

Figure E1: A Section of the ovary shows several variably sized luteinized corporal disposed to its cortical aspect. A few atrophic follicles are present in the section especially in the cortical aspect. Blood vessels (veins) were also seen in the tissue CT of the medulla ovarian biopsy. Present in one of the corpora luteal are hemosiderin laden macrophages. These features are those of ovarian atrophy.

Figure F1: Section of the ovary show several variably sized luteinized corporal disposed to its cortical aspect. A few atrophic follicles are present in the section, especially in the cortical aspect. Blood vessels (veins) were also seen in the connective tissue of the medulla ovarian biopsy. These features are those of ovarian atrophy.

Figure G1: Section of the ovary show ovarian follicles with a prominent antrum containing granulosa cells floating in the antral cavity. The follicle is enclosed by a bound of fibrous tissue containing hemosiderin-laden macrophages. There is compression of the granulosa cell separating the antrum from the fibrous band patch of germinal epithelium present in the outermost aspect of the ovary. Corpus luteum containing venules and blood vessels are also seen in the fibrotic stroma. These features are those of moderate ovarian atrophy

Figure H1: Ovarian section reveals a capsule of cuboidal epithelial cells, underneath which are proliferating fibroblasts extending to the medulla through the cortex. The spindled cells possess dense but band nuclei and they separated the follicles and corpora lobular fashion extensively distorting the normal architecture

Some of the follicles, especially the growing follicle are surrounded by these spindled cells completely enclosing

and separating them from the adjacent structures. Even so, the follicles seen appear to be arrested in early prophase with intact nuclei or nucleoli found in some with no sign of further progress into the metaphase of mitosis.

Hemosiderin-laden macrophages are prominent in some albican and atretic corpora. These cells reveal pale-blared single nuclei with abundant vesicular cytoplasm containing degenerating hemoglobin pigment. These features are those of moderate fibrosis, follicular arrest and follicular cyst at early prophase of first meiotic division

Figure I1: Sections of the ovary show a cortex and a medulla, and within the medulla are some blood vessels enmeshed in loose connective section stroma. Growing follicles containing oocytes arrested in the first meiotic division, with reduced atrophic sizes are seen approaching the medulla and occasionally in the cortical aspect of the glands one of these in the cortical aspects is cystically dilated and lined by a stratified layer of granulosa cells. Thick bands of fibro tissue are apparent around these follicles, which are extending into the ovarian stroma and around the variable size large corporal luteal.

Small-sized arteries, clustered of hemosiderin laden macrophage and the fimbriated end of the fallopian tube (adjacent to the hilum) is also seen, High magnification within the corporal reveal luteinized granulosa cells, with a round to oval pale vesicular nuclei disposed of with an abundant pale cytoplasm these cells were distributed in single and separated by non-luteinized cells within the corporal luteal. These features depict an ovary with cyst follicle.

Figure J1: Ovarian section reveals a capsule of cuboidal epithelial cells, underneath which are proliferating fibroblasts extending to the medulla through the cortex. The spindled cells possess dense but band nuclei and they separated the follicles and corpora lobular fashion extensively distorting the normal architecture

Some of the follicles, especially the growing follicle are surrounded by these spindled cells completely enclosing and separating them from the adjacent structures. Even so, the follicles appear to be arrested in early prophase with intact nuclei or nucleoli found in some with no sign of further progress into the metaphase of mitosis.

Hemosiderin-laden macrophages are prominent in some albican and atretic corpora. These cells reveal pale-blared single nuclei with abundant vesicular cytoplasm containing degenerating hemoglobin pigment. These features are those of moderate fibrosis, follicular arrest and follicular cyst at early prophase of first meiotic division.

Figure K1: Sections of the ovary showed a cortex and a medulla, and within the medulla are some blood vessels enmeshed in loose connective section stroma. Growing follicles containing oocytes arrested in the first meiotic division, generally with reduced atrophic sizes are seen approaching the medulla and occasionally in the cortical aspect of the glands, one of these cortical aspects is cystically dilated and lined by a stratified layer of granulosa cells. Thick bands of fibro tissue are apparent around these follicles, which are extending into the ovarian stroma and around the variable size large corporal luteal.

Small-sized arteries, clustered of hemosiderin-laden macrophage and the fimbriated end of the fallopian tube (adjacent to the hilum) is also seen, High magnification within the corporal reveal luteinized granulosa cells, with round to oval pale vesicular nuclei disposed with an abundant pale cytoplasm these cells are distributed in single and separated by non-luteinized cells within the corporal luteal. These features are those of moderate fibrosis, follicular arrest and corpus luteum cyst at early prophase of first meiotic division.

Figure A2 and A3: Sections of the ovary show a cortex with several corporal luteal (5) with a Graafian follicle showing smaller primordial follicle and secondary follicle also in the cortical region, blood vessels were also present in the medullary region of the ovary. The corporal luteal is composed of luteinized granulosa cell, which possesses positive brown-colored PR immuno-stained nuclei, which is round to oval vesicular nuclei posited centrally. These cells were separated by a thin fibro-vascular stroma.

Theca cells are also present around the corpora luteal, possessing positive brown-coloured ER immuno-stained nuclei, which are flattened in structure, with oval vesicular nuclei. The external part of the ovary is lined by an irregular dense connective tissue that extends inward and

separates corporal in a lobular fashion. These are features of normal ovarian tissue.

Figure B2 and B3: Sections of the ovary showed a cortex with several corpora luteal (5) with a growing follicle showing smaller primordial follicle and secondary follicle also in the cortical region, blood vessel were also present in the medullary region of the ovary. The corpora luteal are composed of luteinized granulosa cells, which possess slightly reduced positive brown colored PR immuno-stained nuclei, which is round to oval vesicular nuclei posited centrally. These cells were separated by a thin fibro-vascular stroma.

Luteinized theca cells present around the corpora luteal, possess unremarkable positive brown colored ER immuno-stained nuclei showing no sign of reduced cells with flattened nuclei. The external part of the ovary is lined by an irregular dense connective tissue that extends inward and separates corporal in a lobular fashion. These are features of a slightly decrease level of progesterone antibody receptor, with a stable estrogen antibody receptor level

Figure C2 and C3: Sections of the ovary show a cortex with several corporal luteal lined by germinal epithelium, with a Graafian follicle and a growing follicle seen in the cortical region The corpora luteal are composed of luteinized granulosa cell, which possesses slightly reduced positive brown colored PR immune-stained nuclei, which is round to oval vesicular nuclei posited centrally. These cells are separated by a thin fibro-vascular stroma.

Luteinized theca cells present around the corpora luteal, possessing positive brown colored ER immuno-stained nuclei but showed no sign of reduction in number and are flattened in structure. The external part of the ovary is lined by an irregular dense connective tissue that extends inward and separates corporal in lobular fashion. Blood vessels were also present in the medullary region of the ovary. These are features of a slightly decrease level of progesterone antibody receptor, with a stable estrogen antibody receptor level

Figure D2 and D3: Sections of the ovary showed a cortex possessing cuboidal epithelial lining with several corpora luteal with a Graafian follicle and a growing follicle seen in the cortical segment. The corpora luteal are composed of luteinized granulosa cells, which possess gradual reduction in the staining intensity (positive brown) of PR immuno-stained nuclei. These nuclei are round to oval vesicular nuclei posited centrally. These cells are separated by a thin fibro-vascular stroma.

Theca lutein cells are present around the corpora luteal, possessing positive brown-colored ER immuno-stained nuclei, which were unremarkable in numbers with flattened nuclei. These cells were separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward and separates corporal in a lobular fashion. Blood vessels were seen in the medullary region of the ovary. These are features of a moderately atrophied follicle; slightly decrease level of progesterone antibody receptor, with a stable estrogen antibody receptor level

Figure E2 and E3: Section of the ovary shows germinal epithelium which bounds the cortex with several corpora luteal. The cortical part is composed of several numbers of secondary and growing follicles. Composed in the corpora luteal luteinized granulosa cell, with an obvious reduction in number and staining intensity (positive brown) of PR immuno-stained nuclei, which are round to oval vesicular nuclei posited centrally. These cells are aggregated by a thin fibro-vascular stroma.

Theca lutein cells present surrounding the corpora luteal, possess reduced positive brown-colored ER immuno-stained nuclei, flattened in orientation. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in lobular fashion, with few atrophic and arrested follicles. The medullary region is also shown composed of vascular spaces and blood vessels. These are features of atrophied follicles; with a gradual decrease in the level of progesterone and estrogen antibody receptors.

Figure F2 and F3: Sections of the ovary show a cortex, bounded by cuboidal epithelial lining, composed of several corpora luteal with a Graafian, growing, secondary and primordial follicle seen in the cortical region. The corporal luteal is composed of luteinized granulosa cell, which

possesses decreased numbers of nuclei and reduced staining intensity of positive brown-colored PR. These cells are round to oval vesicular nuclei posited centrally. These cells are separated by a thin fibro-vascular stroma.

Theca lutein cells are present around the corpora luteal, possessing positive brown-colored ER immuno-stained nuclei, which are unremarkable with flattened nuclei. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in lobular fashion, with few atrophic and arrested follicles. The medullary region is also shown composed of vascular spaces and blood vessels. These are features of atrophied follicles; with steadily decreasing levels of progesterone and estrogen antibody receptors.

Figure G2 and G3: In the sections of the ovary which is lined by germinal epithelium show cortex with several corpora luteal composed of an obvious Graafian follicle and several growing follicles seen in the cortical region. The corpora luteal are composed of luteinized granulosa cells, which possess decreased numbers of nuclei and reduced staining intensity of positive brown-colored PR immuno-stained nuclei. These nuclei are round to oval posited centrally. These cells are separated by a thin fibro-vascular stroma.

Theca lutein cells present around the corpora luteal reveal reduced staining intensity (positive brown) colored ER immuno-stained nuclei, which are remarkable with flattened nuclei. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in lobular fashion, with few atrophic and arrested follicles. The medullary region is also shown composed of vascular spaces and blood vessels. These are features of atrophied follicle; gradatory decreased level of progesterone and estrogen antibody receptor.

Figure H2 and H3: In the section of the ovary showed a cortex with several corpora luteal with a Graafian follicle and a growing follicle seen in the cortical region. The corpora luteal is composed of luteinized granulosa cells, which possesses decreased numbers of nuclei and reduced staining intensity of positive brown-colored PR. These nuclei are round to oval vesicular nuclei posited centrally. These cells are separated by a thin fibro-vascular stroma.

Theca lutein cells present around the corpora luteal, possess disappearing positive brown-colored ER immuno-stained nuclei, which are remarkable with flattened nuclei. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in a lobular fashion, with few atrophic and arrested follicles. The medullary region is also composed of vascular spaces and blood vessels. These are features of an atrophied follicle; slightly decrease levels of progesterone and estrogen antibody receptors.

Figure I2 and I3: Sections of the ovary show a cortex over-lined by cuboidal epithelial cells possessing several corporal luteal with a several growing follicles seen in the cortical region. The corpora luteal are composed of luteinized granulosa cells, which possesses decreased numbers of nuclei and reduced staining intensity of positive brown-colored of progesterone receptor. These nuclei are round to oval posited centrally. These cells are separated by a thin fibro-vascular stroma.

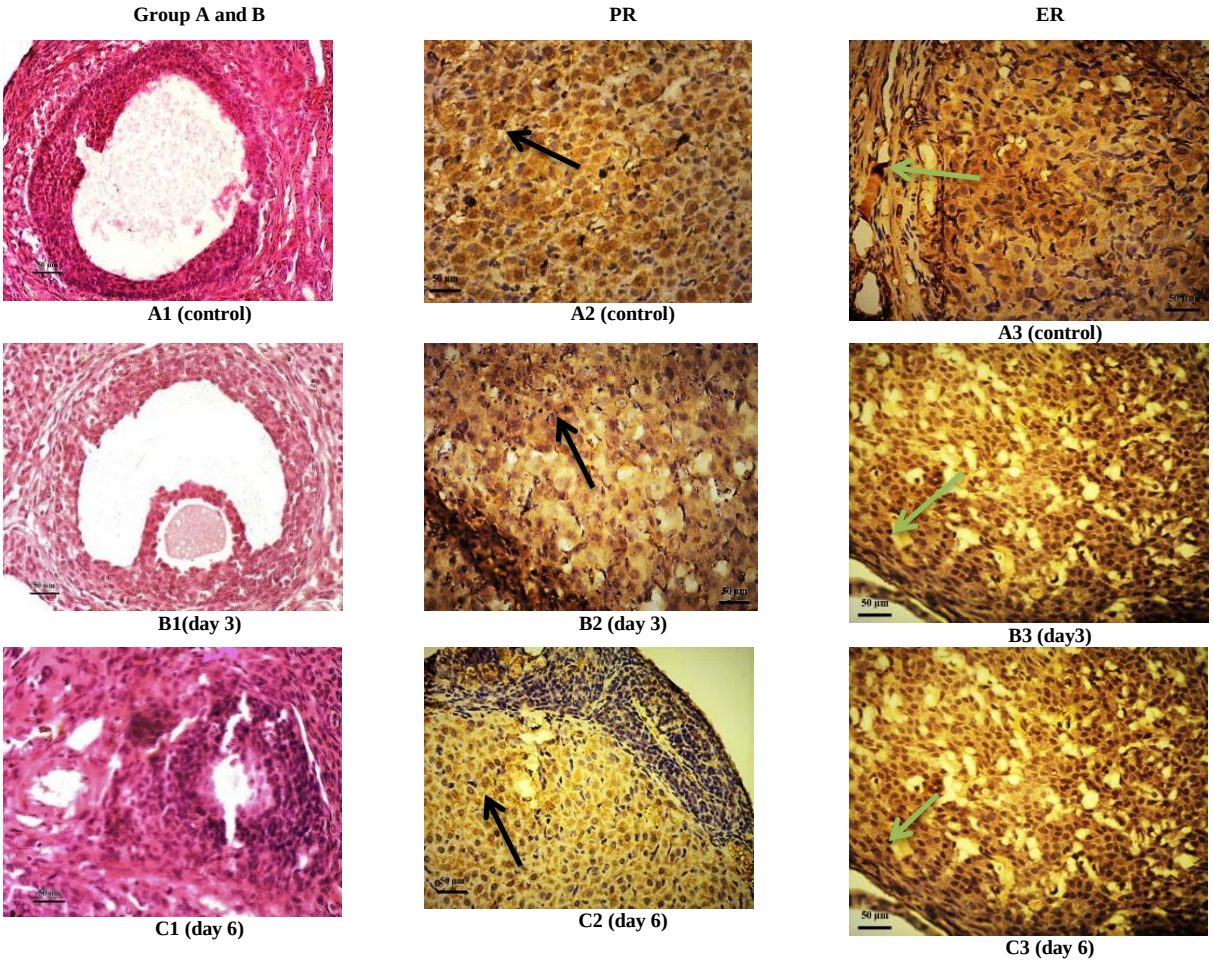
Luteinized theca cells present around the corpora luteal, possesses disappearing positive brown colored ER immuno-stained nuclei. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in lobular fashion, with arrested follicles. The medullary region is also composed of vascular spaces and blood vessels. These are features of atrophied follicle; slightly decrease level of progesterone and estrogen antibody receptor.

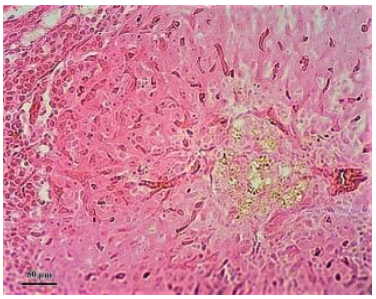
Figure J2 and J3: Sections of the ovary show a cortex with several corpora luteal lined externally with germinal epithelium with a several growing follicle seen in the cortical region The corporal luteal composed of luteinized granulosa cell, which possess decreased numbers of nuclei and reduced staining intensity of positive brown colored of PR. These nuclei which appear round to oval vesicular nuclei posited centrally. These cells were separated by a thin fibro-vascular stroma.

Theca lutein cells are present around the corpora luteal, possessing disappearing positive brown-colored ER immune-stained nuclei, which are remarkable with flattened nuclei. These cells are separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in a lobular fashion, with aggregate of granulosa cells. The medullary region is also shown composed of vascular spaces and blood vessels. These are features of corpus luteum cyst with reduced levels of progesterone and estrogen antibody receptors which appeared diminished.

Figure K2 and K3: Sections of the ovary show a cortex with several corpora luteal with several growing follicles seen in the cortical region. The corpora luteal are composed of luteinized granulosa cells, which possess decreased numbers of nuclei and degraded staining intensity of positive brown-colored progesterone receptor immune-stained nuclei. These cells appear round to oval vesicular nuclei posited centrally. These cells were separated by a thin fibro-vascular stroma.

Theca lutein cells were present around the corpora luteal, possessing disappearing positive brown-colored estrogen receptor immuno-stained nuclei, which are remarkable with flattened nuclei. These cells were separated by a thin fibro-vascular stroma. The external part of the ovary is lined by an irregular dense connective tissue that extends inward separating the corporal in lobular fashion, with arrested follicles. The medullary region is also shown composed of vascular spaces and blood vessels. These are features of follicular arrest; diminished levels of progesterone and estrogen antibody receptor





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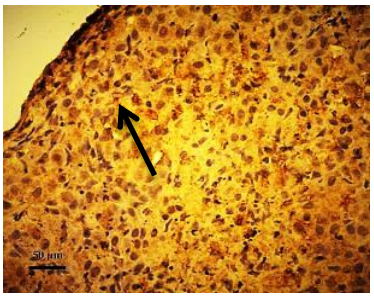

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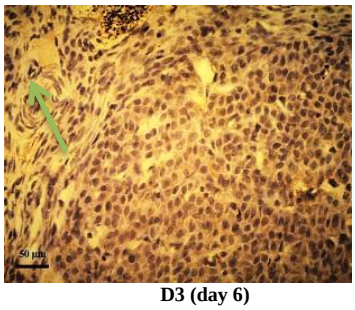

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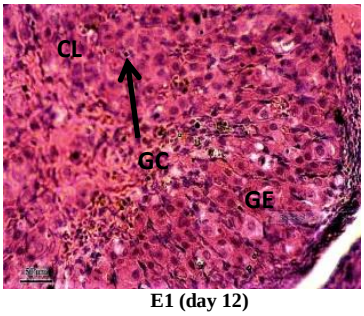

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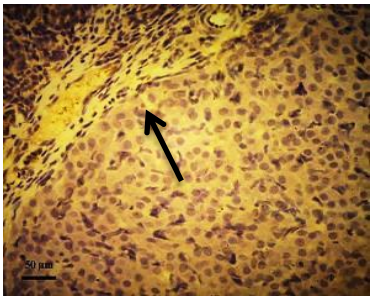

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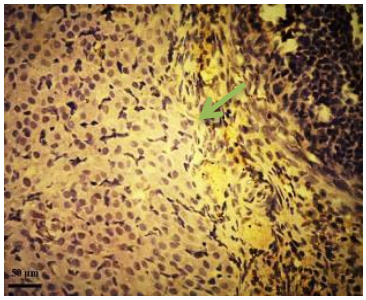

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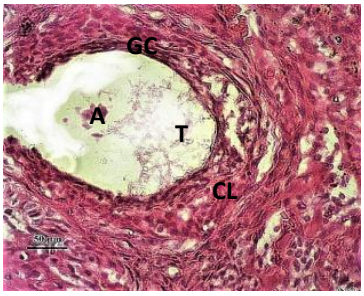

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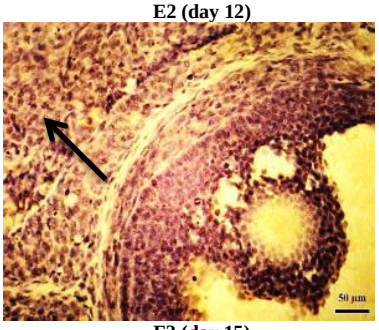

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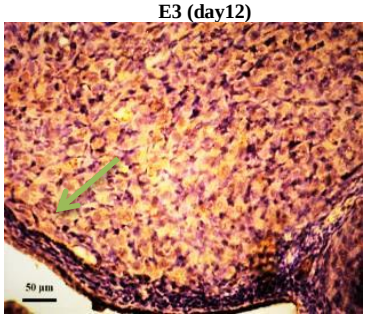

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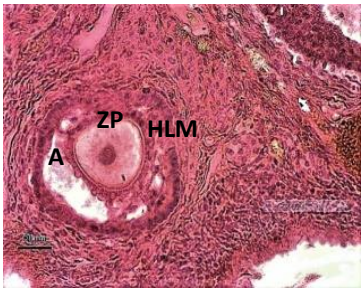
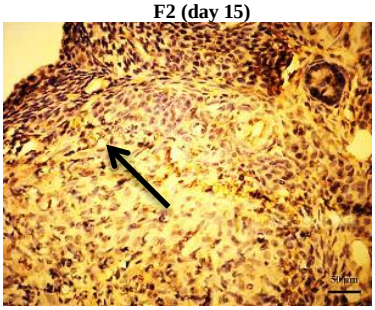
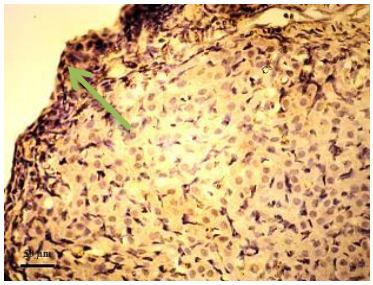


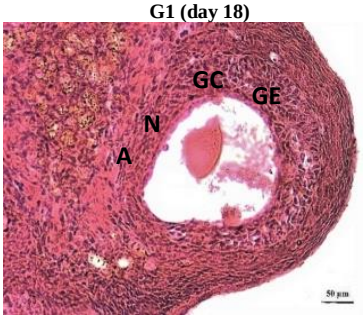
G1 (day 18)



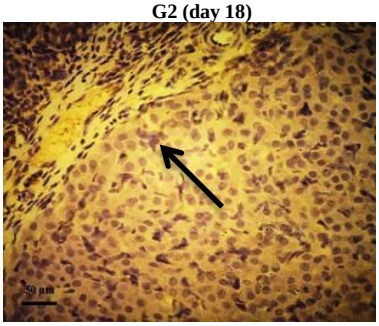
G2 (day 18)



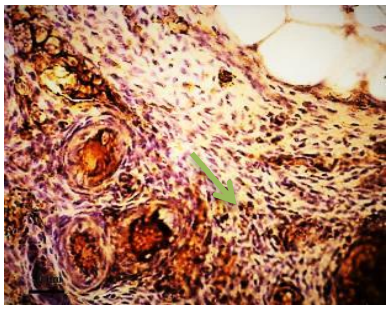
G3 (day 18)



H1(day 21)



H2 (day21)



H3 (day 21)

Figure A1 - D1. Sections of the ovary for H and E (x400), A2 - AD2: Sections of the ovary for immunochemistry staining pattern for PR (x400) and A3 - D3: Sections of the ovary for immunochemistry staining pattern for ER (x400)**Figure E1 - H1.** Sections of the ovary for H and E (x400), E2 - H2: Sections of the ovary for immunochemistry staining pattern for PR at (x400) and E3 - H3: Sections of the ovary for immunochemistry staining pattern for ER at (x400)

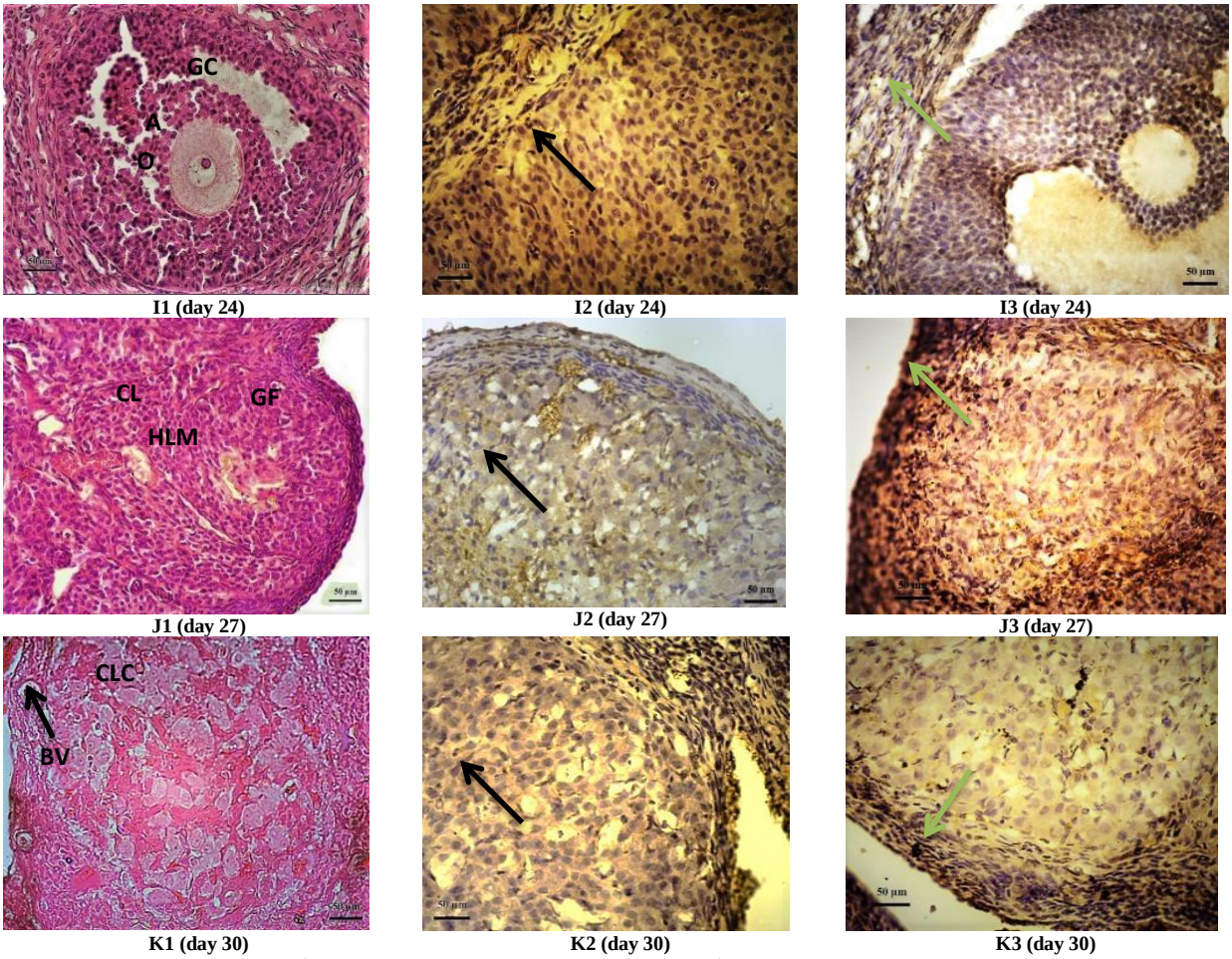


Figure 2. K1: Sections of the ovary for H and E (x400), A2 – K2: Sections of the ovary for immunochemistry staining pattern for PR (x400) and A3 – K3 show sections of the ovary for immunochemistry staining pattern for ER (x400).

Keys: CL- corpus luteum, GC – granulosa cell, M – medulla, GE – germinal epithelium, SF, secondary follicle, GF- growing follicle, T – theca cell, CR – corona radiate, CO – cumulus *oophorus*, A – antrum, BV – blood vessel, CLC – corpus luteum cyst, HLM – hemosiderin-laden macrophages, O – oocyte

Positive brown-coloured PR immuno-stained nuclei

Positive brown-coloured ER immuno-stained nuclei

DISCUSSION

Oral contraceptive pills exist in various forms, including emergency and combined, which are used extensively for the prevention of unwanted pregnancies [5, 6]. These pills, which have patronage globally, have been demonstrated to be associated with several side effects [34]. Despite these observations which have been extensively elaborated, reports on the outcomes following repeated exposure to (L) have been scarcely documented [6, 9, and 10]. The thrust of this treatise therefore was to determine the possible histological sequelae following exposure to (L) on Wistar rat ovary.

Exposure to (L) at day 3 displayed unremarkable ovarian changes in the index study. These changes might be attributed to the minimal dose of the test agent (L) administered, which is considered as second-generational progestin noted by [35]. Also, Regidor opined in his study that progestin could be classed into first, second, third and fourth generations, stating that (L), as a second-generational progestin, is known for its minimal side effects as compared with other generational progestin [2]. More so on this day, assessment of (L) on the ovary, stained with immuno-histochemical stains revealed slightly decreased staining intensity of PR immuno-stained nuclei while ER staining intensity remained unremarkable. Meng

in his study obtained a similar report where the effect of an oral contraceptive pill was assessed on the endometrium with closely aggregated cells and reduced staining intensity of positive PR immuno-stained nuclei however, the ER show no significant changes with regards to its reactivity [36]. This can also be explained by the mechanism of the drug's action on tissue. The biological response to the drug diminishes (tolerance) following exposure to a drug. This phenomenon then causes a reduction in the number of hormonal receptors produced by the ovary [37]. Therefore (L) enhances contraception by inhibiting hypothalamic-pituitary gonadal axis, activating a negative feedback mechanism as explained earlier [6].

The ovarian response to (L) on the 6th day exhibited atrophic ovarian changes, which had been earlier reported by D'Arpe who demonstrated the occurrence of suppressed ovarian functions, which further suggests inhibition of luteinizing hormone surge [3]. This was explicated in Massawe's report in 2018 which highlighted that both injectable and oral contraceptives produced a negative feedback response on the hypothalamus. The resulting response thereby decreases the pulse frequency of gonadotropin-releasing hormone. Ultimately decreased GNRH distorts the ovarian histoarchitecture by asphyxiating follicular development, through decreased estradiol levels [38]. A similar report by Regidor demonstrated that high concentration of (L) suppresses ovulation and is characterized by poor follicular growth. Furthermore, the determination of (L) on the ovary, using immuno-histochemical stains revealed slightly decreased staining intensity of PR and unremarkable staining intensity for ER. This is in keeping with a study conducted by Christie *et al.*, 2008 where he observed the staining intensity of PR and ER and both mean hormonal receptor immuno-reactivity in epithelial cells were significantly higher in normal ovaries than in benign, borderline and malignant ovarian tumors. This can be ascribed to the molecular mechanism of drug action on biological tissue, that continuous and repeated administration of a drug to the ovary, diminishes ovarian response to the drug effect (desensitization or down-regulation). This phenomenon then causes a reduction in the target receptor numbers, which are produced by the ovary as was described previously [39].

Repeated exposure of the ovary to (L) on the ninth day of exposure, was observed to initiate ovarian histoarchitectural changes, with distinct hemosiderin-laden macrophages. Urzua et al., made similar observations with distorted ovarian tissue exposed to oral contraceptives and age-related factors. They documented evidence of differential accumulation of hemosiderin, formed as a consequence of micro-hemorrhages (red blood cell phagocytosis) in corpus luteum formation known as corpus hemorrhagicum [40]. Furthermore, exposure of levonorgestrel on the ovary was also assessed using immuno-histochemical staining pattern, with findings which were synonym to results obtained on day 3 and 6, showing no significant effect of levonorgestrel on the nuclei of the granulosa and theca cell located in the corpus luteum and around the follicles respectively, except for a slight decrease in the staining intensity for PR. These findings concur with the investigation conducted by Orbo, where the exposure to (L) in women with endometrial hyperplasia resulted in decreased immuno-histochemical detection of metallothionein protein [41]. This was also clearly explained in his study that continuous exposure to levonorgestrel in the ovary can also cause tachyphylaxis or tolerance, leading to receptor down-regulation, as seen in the result obtained earlier [42].

Evidence from this study clearly revealed the continuous exposure of (L) to the ovary on the 12th day, which depicts resultant effect of ovarian histomorphology traced with atrophic changes. A similar finding was also observed in a study conducted by de Medeiros where progestin administration affected, a luteinizing hormone pulsatile pattern before the distortion of a luteinizing hormone surge which is a result of the negative feedback mechanism of the exogenous test agent on the hypothalamo-pituitary system, thereby eliminating ovulation [43]. In situ to the assessment using the immuno-histochemical staining technique, the result revealed steadily decreasing granulosa and theca cell staining intensity of PR and ER immuno-stained nuclei respectively. These findings are synonymous with the upshot cited in Lindgren's study conducted in 2004, where

there was a significant reduction of PR and ER immuno-reactivity found in epithelial cells of ovarian malignant tumors than in normal ovaries [44]. This was further proven by the desensitization phenomenon which is clearly expressed towards the reduction of hormonal receptors noted in a treatise conducted by [45].

Continuous exposure to (L) at day 15 displayed unremarkable changes marked with obvious atrophic changes. This finding concurred with the study where the daily oral administration of progestin-only pill display endometrial atrophy using the ovarian architecture as a tool marker to confirm. This was established from the ovary by reduced estradiol secretion from developing follicles and reduction in the progesterone from the corpus luteum [46]. Furthermore detection of progesterone and estrogen receptors, following the continuous exposure of (L) on the ovary was also assessed. Reduced staining intensity of both PR and ER immuno-stained nuclei at different sites of the ovary where progesterone and estrogens are produced, was assessed. Upshot from in vivo study were similitude to a treatise where there was a significant decrease of PR immuno-reactivity, following treatment with repeated progestin oral pills however, ER immuno-reactivity was not assessed [20]. He further states that the delayed recovery of some histological and immuno-histochemical changes observed in this study, could be explained by the cumulative effect of the drug. This cumulative effect is seen to cause a down regulatory effect of the drug on the uterus.

Infertility and ovarian torsion activity of (L) were recorded in this treatise, following continuous administration of (L) on the 18^{th} day. These ovarian distortions seen on this day can be attributed to the accumulative effect of repeated exposure to (L). Similar observations were also highlighted in a study, where the ovary of quinestrol gerbils inhibit the maturation and ovulation of follicles in the ovary, ultimately leading to reduced fertility [47]. In addition, assessment with a special staining technique display a gradatory decrease in the staining intensity of PR and ER immuno-stained nuclei, which appears somehow rapid. The outcome obtained were synonyms to findings in a study conducted in 2017, which displays a cut-off point of 1% positive cells for selecting tumorigenic cells, which is considered for PR or ER immuno-reactivity [48]. These findings however, is cognitive to a study that reflects that continuous administration of levonorgestrel inhibits the pre-ovulatory luteinizing hormone surge, thereby leading to a negative feedback response resulting in the reduced effect of a hypothalamic-pituitary gonadal axis, with an obvious reduction in progesterone and estrogen [3, 17]. This mechanism was further explained by an author that prolonged and repeated drug stimulation on the receptors, wanes the receptors [49].

Subsequent exposure at day 21 resulted in a more severe change in ovarian histomorphology which coincided with a report conducted by D'Arpe *et al* where he demonstrated a decrease in the numbers of follicles and cystic change following the administration of progestin-only pill [3]. Cystic change may be ascribed to the accumulation of fluid in the bilayer of target cell plasma membranes, potentially altering the fluidity and function of the membrane by hormonal steroids, which are lipophilic. Also, lipid fluidity influences protein activity and mobility within the bilayer which is a primary determinant of enzyme activity in the membrane for most proteins [50]. However, administration (continuous) of (L) on the ovary using an immuno-histochemical technique was also demonstrated (PR and ER antibody markers). The outcome revealed a gradatory decrease of PR and ER immuno-stained nuclei in the site of progesterone and estrogen location in the ovary. Naik's study in 2015 critically explains the decreased positivity of immuno-stained PR or ER in ovarian cancer (benign tumor), with no significant correlation between expression of ER, PR, and severity of malignant tumor [51]. This was also clearly justified in Goodman and Gilman's pharmacological basis of therapeutics that continuous stimulation of receptors by agonists generally results in a state of desensitization, a phenomenon which is referred to as "Tachyphylaxis" [52]

Exposure to (L) at day 24 displayed remarkable changes with cysts displayed in the follicle. The result obtained was similitude to the investigation, conducted by Jeong in 2000 where he use ultrasonography assessment to visualize the presence of cyst in the follicle showing a thin wall, vascular

nature and not resorbed with proliferating sizes [53]. The mechanism for the cystic change was made possible by steroid hormones intercalating into the membrane bilayer of the cell, with their planar ring system in the hydrophobic core of the bilayer, lying parallel with the phospholipid molecules noted in a study conducted by Kuhn-Velten. Further assessment was also conducted using immunochemical staining pattern, with an upshot revealing both PR and ER with a gradatory decrease to immuno-stained nuclei. The obtained results can be ascribed to the continuous stimulation of receptors by the drug. Thence it is essential to note that continuous exposure to levonorgestrel decreases the immuno-reactivity of progesterone and estrogen receptors present in the ovary following a phenomenon known as down-regulation [37].

Ovarian histoarchitecture evaluated following the continuous administration of (L) on the twenty-seventh day, exhibited several ovarian torsions. A similar report was elucidated by Maqueo's study where he assessed several histologic changes in the ovaries following (L) administration (cortical fibrosis, follicle degeneration, decreased luteinization, cyst formation and increased vascularity). He also observed that changes are reversible with restoration of follicle growth and maturation occurring after cessation of (L) administration [54]. Ohvo-Rekila *et al.,* noted in their study the mechanism by which cell membrane fluidity and permeability are affected by sterols [55]. Further investigation also displays the down regulatory effect of the receptors by the continuous administration of the drug [56, 57]. This was also clearly explained by Grundy that the immunoreactivity of these receptor experience hyposensitivity due to continuous stimulation of these receptors [58].

The regimen continuous administration of (L) to the ovary of adult female Wistar rats was assessed at the last exposure. Findings of ovarian tissue reveal features of moderate fibrosis, follicular arrest and corpus luteum cyst at early prophase of first meiotic division. This finding were in keeping with a study conducted by von Hertzen where he randomly assessed 4000 healthy women of reproductive age with emergency contraceptives, with upshot displaying ruptured corpus luteum cyst, cortical fibrosis and other ovarian tissue distortion [59]. Similarly, Dupuis and Kim assess the ovarian tissue using ultrasonography which was followed with repeated use of (L), outcome revealed cyst in both the follicle and corpus luteum [60]. Cyst formation can be attributed to the increase in cell membrane fluidity and permeability as a result of repeated use of hormonal steroids [61].

CONCLUSIONS

The observed histological distortions of the gonads have most likely arisen from repeated exposure to exogenous progestin. Previous reports have established that extensive and repeated doses of (L) result in the suppression of trophic and releasing hormones from the pituitary and hypothalamic glands respectively. These effects can explain or account for the observed ovarian changes recorded in the index study.

Recommendations

Finding from the study will help to be a educate women of reproductive age on the accurate, safe and effective use of emergency oral contraceptive. Also the result obtained will enlighten the public on contraception, which should start from antenatal and gynecological clinics. This will aid to reduce the misconception or misinformation on the use of emergency oral contraceptive, to minimal level.

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ETHICAL CONSIDERATION

Ethical clearance was sought from the Research and Ethics Committee in the Faculty of Basic Medical Sciences of the Delta State University prior to the commencement of this research. Ethical reference number: REC/FBMS/DELSU/21/102

Conflict of interests

The authors declare no conflict of interest

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