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Effects of Crude Aqueous Extract of *Origanum vulgaris* in Developing Ovary of Rabbits Following *in Utero*, Adolescent and Postpubertal Exposure

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ABSTRACT

We evaluated the impact on rabbits after having been treated with *Origanum vulgaris* (Lamiaceae) (OV) at a dose level known to adversely affect ovarian functions in rodents without causing systemic toxicity. The choice of rabbits has been guided by the fact that rabbits have a relatively long phase of reproductive development and hence simulation of reproductive development is better as opposed to dealing with rodents. The use of rabbits facilitates multiple evaluations of mating ability. An attempt has also been made at determining whether OV affected ovarian development and hence the use of animal model. Rabbits were exposed to 80 mg OV/kg/day *in utero* (gestation days [GD] 0 to23) or during adolescence (postnatal weeks [PNW] 4 by breast feeding and orally from 4w to 12 w), and the offspring were examined at the end of the 12 W period. Another group was treated after puberty (for 12 weeks) till age of 24 [PNW] of age and examined at the conclusion of exposure and follicles were categorized as primordial, primary, small preantral, large preantral or small antral follicles. The most pronounced reproductive effects were in female rabbits group which had been exposed from *in utero* till post-puberty period, in weights of ovaries (at 12 and 24 weeks, down 23%; $p < 0.05$). Serum Gonadotropin levels were down (at 24 weeks, 32%; $p < 0.05$); a slight increase in histological alterations of the ovaries ($p < 0.05$) at 24 weeks, of abnormal follicles.

Keywords: *Origanum vulgaris (OV), ovarian functions.*

Introduction

Pregnancy and lactation diet have been shown to have a significant effect on offspring as adults. Exposure to toxicants during the critical periods of oogenesis and follicular formation could have profound effects on ovarian function and maturity in the adulthood. Maternal nutrition during pregnancy and lactation has a significant impact on maternal care during lactation. Maternal and postweaning nutritional signals, independent of maternal care, alter offspring pre-puberty and female reproductive function in adulthood, which may be associated with advanced ovarian ageing and altered fertility [1].

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Oregano (*Orinagum vulgaris L., Lamiaceae*) (OV) is a commonly used herb, light green in colour and with a strong aromatic odour but with a pleasantly bitter taste. It blends well with tomato sauces, pizza and vegetables [2].

Some researchers have confirmed that oregano improves growth retarded animals and has nonspecific immunostimulatory effects on porcine immune cells [3].

Origanum. vulgare is widely distributed throughout Asia, Europe and North America [4] and is commonly known as Wild Marjoram and Winter Sweet [5, 6].

It has a wide traditional therapeutic usage to treat kidney stones, as diuretics, antispasmodics, antibacterial, anticancer, anti-inflammatory, antioxidant and laxative [7-9].

However, the long term effect of OV consumption on female fertility and ovarian function has not been fully established. We hypothesized that chronic exposure of rabbits to oregano would alter gametogenesis and cause ovary toxicity.

In all female mammals, the period of germ cell proliferation and follicular formation is strictly limited to fetal or neonatal life [10].

The pool of primordial follicles that is formed during this period comprises the sole source of female gametes and when depleted, reproductive senescence occurs. In smaller mammals (mouse, rat, rabbit), follicular formation does not begin until the perinatal period and is completed within one to two weeks of birth) [11].

Therefore, the objective of the present study was to examine the effects of chronic, relatively low dose exposures to OV on folliculogenesis in prepubertal and adult female rabbits.

Materials and Methods

Rabbit husbandry

Three months old, specific-pathogen-free, white New Zealand (NZW) rabbits were obtained from animal house of faculty of medicine Zagazig University. Rabbits were individually housed in stainless steel cages with wire-mesh flooring. The rooms were maintained at a 12 h light-dark cycle at approximately 20–22°C and ~40% humidity. Rabbits were fed certified rabbit ration (#5322 Purina Certified Rabbit Ration). After acclimatization for 3–4 weeks, female rabbits were intramuscular injected with 10 µg of GnRH 2 days prior to mating to induce ovulation. Each female was then mated with different male rabbits to ensure successful pregnancy. The day on which the copulation plug was found was designated as gestational day (gd0). On the following morning and each morning thereafter, the females were examined for the presence of vaginal copulation plug. All animals were checked daily for clinical signs, mortality and evidence of abortion. Rabbits were palpated for pregnancy 14 days after mating. Body weights and food consumption were measured at predetermined intervals throughout the course of the studies. Rabbits were randomly assigned to treatment groups for appropriate exposures, they were individually housed. Experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [12].

O.V. Extraction and preparation

O.V. was bought from Seekam Company and identified by taxonomist at the (which institution). The aerial part of the plant material was cleaned of adulterants and soaked for three days in the crude aqueous (30:70) with occasional shaking, at room temperature. The filtration was carried out using a muslin cloth and then through Whatman qualitative Grade 1 filters paper. This procedure was repeated twice and then all the filtrates obtained were combined and concentrated on a rotary evaporator (RE-111, Buchi, Flawil, Switzerland) accompanied with B-700 recirculation chiller and a water bath model 461 at 40 °C to a thick pasty mass as crude extract [13, 14]. The active ethanolic extract of *OV.* was classified for different phytochemical groups such as alkaloids, saponins, coumarins, sterols, terpenes, tannins and flavonoids according to methods described by Matsuura [10] and Ocana-Fuentes [15].

Treatments:

The animals were randomly separated into three different treatment groups: first group (A) were exposed to O Vulgare *in utero*, starting one day after mating until gestational day (GD) 23 to ensure that major organogenesis was completed within the start of the treatment and that the window of exposure corresponded with the onset of gonadal differentiation [16] and development of female reproductive tract. The second group (B), adolescence were treated from day 28 gd and continued until 12 weeks postnatal (PNW 12), which encompassed the infantile period of ovarian differentiation—differentiation of germ cells in female, onset of oogenesis, and initiation of puberty [17, 18]. The third treatment group (C), post-puberty received O Vulgare from PNW 12 for another 12 weeks (24 PNW).

A- Early embryonic development study in rabbits

For *in utero* exposure, rabbit does were dosed with rabbit ration. Control group animals in this study, received a volume of 2 ml deionized water [*n* =20 (10 /sex)] and treatment group received a daily oral dose of 80 mg OV/kg/day(*n*=20, 10 animals/sex/dosage group). Animals were examined daily and clinical signs were recorded. Body weight and food consumption data were collected throughout the study for both control and treated females. Treatment for rabbits continued throughout mating until the F0 females reached gestation day (gd23). A total of 10 F0 females from 2 groups were sacrificed within 24 h after the last dose via CO_2 asphyxiation, and necropsied with evaluation of uterine contents. The ovarian corpora lutea were counted and the uterine contents recorded. The histopathological assessment was performed on the reproductive organs of either sex (left genital ridge). The specimens were divided into two halves, one was fixed in 10% neutral buffered formalin, processed, embedded in paraffin wax, sectioned at 5 µm and stained with

Haematoxylin and Eosin (H&E) for light microscopic examination and the other half in gluteraldehyde for electron microscope.

B- Postnatal study in rabbits

Upon birth, a total of 30 F1 pups from each group were weighed, and examined externally. Pups were observed daily and body weight was collected thorough out the duration of the study. During the pre-weaning period, up to postnatal day 3 (pnd 23), pups were observed for developmental landmarks: age at acquisition of pinna detachment (pnd 1–4), incisor eruption (pnd 8–13), and eye opening (pnd 11–16). Observation continued daily and the number of pups achieving each landmark was recorded until all pups of the same sex in a litter responded. A group of 10 pups from F1 females were sacrificed on pnd 23, and histopathological assessment was performed on the reproductive organs of all neonates. The specimens were divided into two half, one was fixed in 10% neutral buffered formalin, processed, embedded in paraffin wax, sectioned at 5 µm and stained with Haematoxylin and Eosin (H & E) for light microscopic examination and the other half in gluteraldehyde for electron microscope. While, at PNW 3, a total of 20 pups from F1 were randomly selected to become parents of the next generation (F2), female pups were culled, and at PNW 4 male pups were weaned and individually housed. Fourteen F1 offspring were not selected, grossly examined for external abnormalities, and individually administered 80 mg OV/kg/day from PNW 4 until PNW 24 and were then euthanized without mating and examined as adult non pregnant. The randomly selected 14/ sex/group F1 pups were held on the study, without dosing, for a minimum of 28 days. During this weaning period only under breast-feeding of 80 mg OV mothers, F1 pups were weighed weekly. The following assessments were also performed: auditory function (startle reflex and habituation) in pups 23–28 days old, acquisition of vaginal patency beginning at 22 days of age, all F1 females were individually evaluated daily for estrous cycles and were administered 80 mg OV/kg/day from PNW 4 to 12, and 6 pups from F1 were necropsied (3 males to 3 females) and prepared for histopathological assessment.

C- Post puberty study

In adolescent exposure, 8 F1 animals received 80 mg OV/kg/ day and were mated (4 male to 4 female, with brother-sister mating avoided) within groups for 14 days. F1 dams were weighed during pregnancy and allowed to deliver their F2 litters. All F2 rabbits were counted, weighed and examined grossly as soon as possible on the day of birth, and again at study termination on and 4 , the same for control group. Due to the Animal Ethics condition, as to minimize the number of animals, control rabbits from *in utero* exposure (4 each for 12- and 24-week age groups) were utilized as controls for the adolescent exposure experiment. Given the observations seen herein, follow-up studies are being designed to replicate and expand understanding of the adverse effects of OV on the male reproductive system.

Histopathology of follicles

Rabbits were euthanized by CO_2 asphyxiation at 12 or 24 weeks of age for *in utero* and adolescent exposures and at the end of a 12-week postpubertal exposure (8 months of age). Follicles were categorized as primordial (single layer of squamous granulose cells with<10 cells surrounding the oocyte), primary (less than 2 complete layers of cuboidal granulose cells),small preantral (from 2 to < 4complete layers of cuboidal granulose cells), large preantral (from 4 to 5 complete layers of cuboidal granulose cells) or small antral follicles (< 5complete layers of granulose cells and an antrum). Ovarian corpora lutea were counted and the status of uterine implantation sites was recorded. Fetuses were dissected from the uterus, counted, weighed and examined for external abnormalities. Approximately one-half of the live fetuses in each litter were examined for visceral malformations and variations. All healthy follicles present in sections were categorized into one of five specific developmental stages. A morphological classification scheme developed and used in our laboratory for morphometry of bovine ovaries was used. Criteria used to classify rabbit ovarian follicles included the shape (squamous vs. cuboidal) and number of layers of granulosa cells. Primordial follicles were characterized by a single layer of squamous granulosa cells with < 10 cells surrounding the oocyte; primary follicles had 1 but less than 2 complete layers of cuboidal granulosa cells; small preantral follicles had 2 to < 4 layers of cuboidal granulosa cells; large preantral follicles had 4 to 5 layers of cuboidal granulosa cells; and small antral follicles with >5 layers of granulosa cells and an antrum [19].

Statistical analyses

Data were analyzed using Statview (version 5.0, SAS Institute, Inc., Cary, NC). Treatment was fixed for each experiment and all parameters were random. Difference between means was determined by t-test. The level of significance was set at $p < 0.05$. This model was used to evaluate body weights, hormone concentrations, and necropsy data. In post pubertal exposure, data for repeated measures on the same animal (sexual capacity) were analyzed by comparing before- and after-treatment values. Percentage values were transformed using arcsine of the square root of the percentage/100 to account for any inequalities in variance.

Results and Discussion

23 days intrauterine sex cannot be identified only genital ridge showed by electron microscope fig1-4 and ovaries in 23 neonatal day identified by electron microscope fig 5-8 without any difference between control and treated. The primordial clusters and the primordial follicles were the only follicles observed at birth for both groups. However, all stages of the development of the ovarian follicles had been observed at infantile age 12 PNW (pre pubertal) like normal and the maturation of germ cells, in rabbits, occurred after birth where most of these cells at the infantile age (12 PNW) like normal and the yolk droplets were demonstrated at infantile age as two types within the cytoplasm of the oocyte, which observed by electron

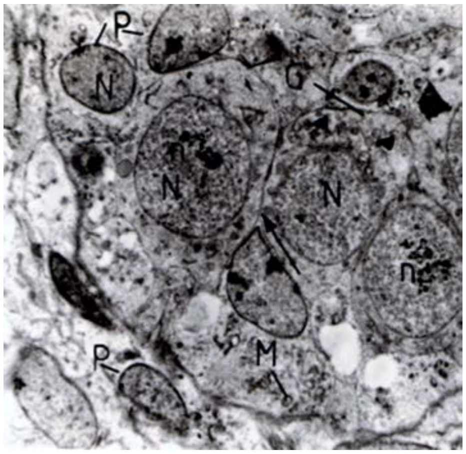

Figure 1: Electron micrograph of control rabbit ovary, prenatal day-23, showing a group of germ cell (G) surrounded with partially small sized and spindle-shaped pregranulosa cells (P).The nucleus [N) of pregranulosa cell is oval in shape and have marginally condensed chromatin. The extremities of some (P) extend around the germ cells (arrows).Notice mitochondria (M) and the discrete accumulation of small dense granules (arrowheads) (X 8000).

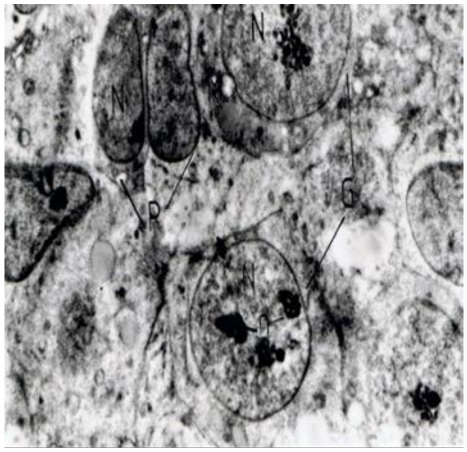

Figure 2: Electron micrograph of control rabbit ovary, prenatal day-23 showing the germ cells (G) with large round nuclei (N), having prominent nucleoli (n). One germ cell nucleus shows three nucleoli. The pregranulosa cells (P) have nearly oval nuclei (N) with marginally condensed chromatin. Notice the mitochondria (M). (X 10.800).

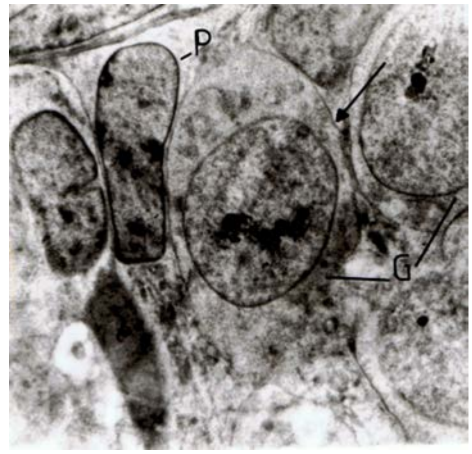

Figure 3: Electron micrograph of treated rabbit ovary, prenatal day-23, showing the germ cell (G) and the surrounding pregranulosa cells (P). The limited intercellular space between the germ cells contains pregranulosa cell process (arrows). (X 10.800).

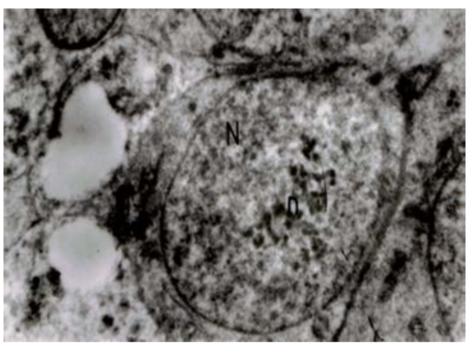

Figure 4: Electron micrograph of treated rabbit overy, prenatal day-23, shoing a germ cell with large, rounded nucleus (N). The Nucleus has prominent nucleoli (n) and focally condensed chromatin. Notice the mitochondria (M). (X 20.000).

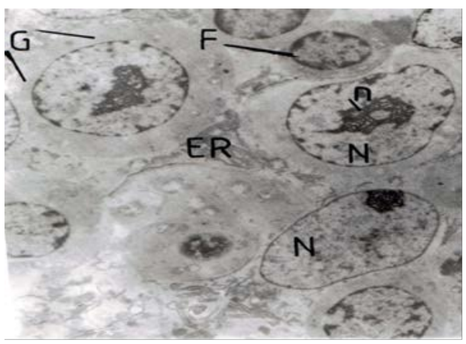

Figure 5: Electron micrograph of control rabbit overy, neontal day-23, showing clusters of germ cells (G) internixed with smaller follicular cells (F). The cells are closely opposed without any membrane specialisetion. Notice the nucleus (N), the nucleous (n), the mitochondria (M) and endoplasmic reticulum (ER). (X 8000).

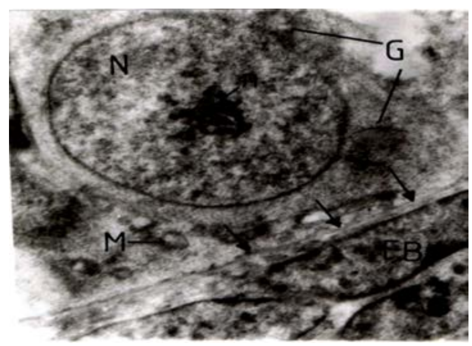

Figure 6: Electron micrograph of rabbit overy, neontal day-23, showing part of the plasma membrane of germ cells (G) resting on a basal lamina (arrows), which separtes the germ cell from the stromal fibroblast (FB). Notice the nucleus (N), the nucleous (n) and the mitochondria (M). (X 20.000).

Table 1: Number of total implant in uterus, relative weight of genital ridge in 23 days prenatal, number of follicles in non pregnant and number of corpus luteum in pregnant in both ovaries.

Sample Mean	Number of implant				Right Ovary			Left Ovary		
	None live& & mal		Live		Organ Wt	No .f	No c	Organ Wt	No. F	No. C
	Male	Female	Male	Female						
Control	1.13 ± 0.02	1.7 ± 0.21	3.2 ± 0.26	3.19 ± 0.15	0.09 ± 0.02	26.5 ± 5.4	10.1 ± 2.53	0.07 ± 0.0	27..2 ± 4.1	9.1 ± 2.3
Treated 80 mg OV	1.22 ± 0.14	1.38 ± 0.10	3.36 ± 0. 3	3.23 ± 0.3	0.08 ± 0.02	24.9 ± 2.4	9.2 ± 3.3	0.08 ± 0.01	28.5 ± 5.1	8.5 ± 2.7

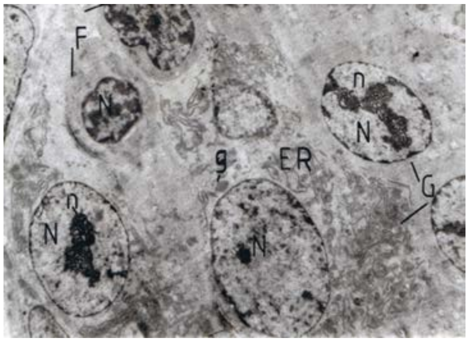

Figure 7: Eletronic micrograph of treated rabbit overy neonatal day-23, showing pale-stained germ cells (G). Nuclei (N) of germ cells are large, nearly rounded and contained prominent nucleoli (n). follicular cells (F) are smallar and more electron-dense than germ cells. they have small round nuclei (N) with randomly scattered chromatin mitochondria (M), endoplasmic reticulum (ER) and Golgi complex (g). (X 8.000).

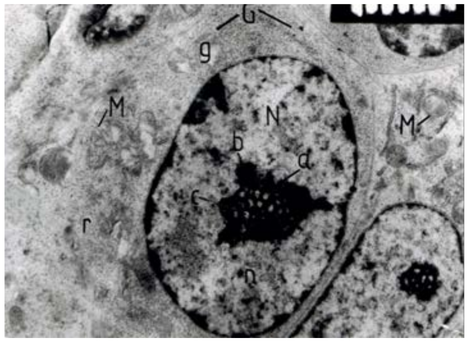

Figure 8: Eletronic micrograph of treated rabbit overy neonatal day-23, showing erm cells (G) with large rounded nuclei (N). The prominent nucleolus (n) has an electron-dense reticular network (a), a large mass of electron-sense granular material (b) and a smaller round less electron-dense ball of fine granular material (c). Mitochondria (M), ribosomes (r) and Golgi complex (g). (X 20.000).

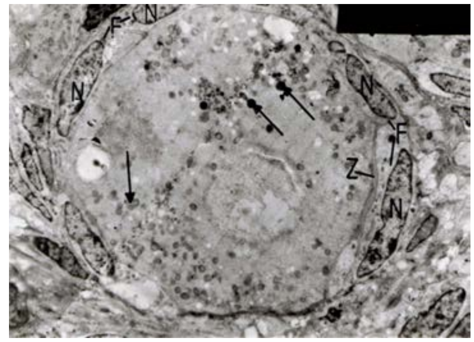

Figure 9: Eletronic micrograph of control rabbit overy, infantile pnw12, showing oocyte sourrounded by flattened follicular cells (F). Cytoplasm contains small round granules of variable densities (arrows). Zona pellucid (Z) and Follicular cell nucleus (N). (X 8.000).

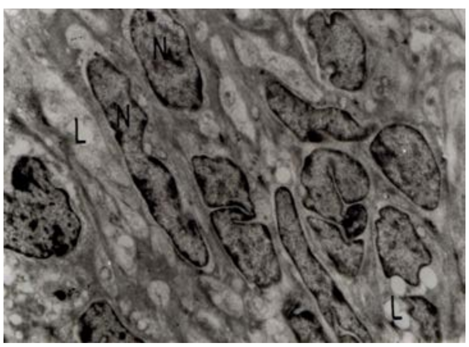

Figure 10: Eletronic micrograph of control rabbit overy, infantile post natal week 12, showing the cells of medullary cords. They have spindle shaped nuclei (N). Their cytoplasm contains lipid droplets (L). (X 10.800).

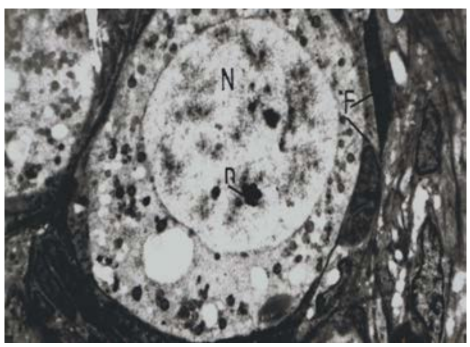

Figure 11: Eletronic micrograph of treated rabbit overy, infantile post natal week 12, showing a primordial follicle formed of an oocyte surrounded by flattened follicular cells (F). The oocyte has a large sherical shaped and eccentrically located nucleus (N) with two prominent nucleoli (n) and dispersed nuclear chromatin. (X 8.000).

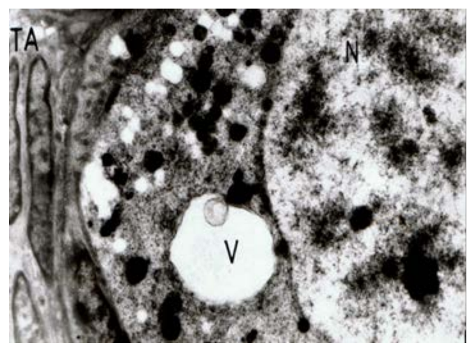

Figure 12: Eletronic micrograph of treated rabbit overy, infantile post natal week 12, showing part of the primordial follicle, very close to the tunica albuginea cells (TA). The oocyte nucleus (N) shows dispersed chromatin. Notice the presence of large cytoplasmic vacuole (v). (X 16.000).

microscope fig 9-14.However, these droplets became coalesced at adult ages to form one type only. But in OV treated group, these droplets were seen traversing the intercellular spaces between the oocytes and the surrounding follicular cells. So they appeared within the cytoplasm of the follicular cells, but became more electron-dense which observed by electron microscope fig 15-16 and light microscope fig 17-20.

There was no significant effects from the treatment on the number of, ovarian corpora lutea also the total follicle numbers (graffian, antral , uni and multilaminar) were not significant differ among the experimental groups. Also the number of implant fetuses did not significantly change between all groups. Sex ratio per litter did not exhibit a dose-related significant upward trend across groups with no significant comparisons to the concurrent control group mean. However, there was no increase in non-live implants or preferential loss of fetuses of a particular sex. Visceral malformations included bilateral hydronephrosis, reported in 1 fetus, however, no significant treatment-related changes in the incidence of pooled external, visceral, skeletal, total fetal malformations or variations, as showed in (Table 1). OV cause no developmental and/or reproductive toxicity in female Dutch-Belted rabbits exposed from in utero, during adolescence, till after puberty. The most pronounced effects were observed in rabbits exposed from in utero till post-pubertal. By using the rabbit as an animal model. We observed atypical germ cells in one rabbit exposed to OV after puberty. It is assumed that these atypical cells were derived from a more differentiated phenotype than a gonocyte since exposure began well beyond infantile and pubertal

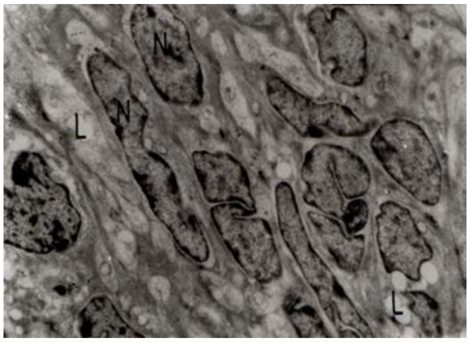

Figure 13: Eletronic micrograph of control rabbit overy, infantile post natal week 12, showing the columnar shaped granulosa cells (G) with large oval nuclei (N). These granulosa cells rest on a basal lamina (BL) separating them from the theca internaL (TI). The latter have spindle shaped nuclei and few lipid droplets (L) in their cytoplasm. Notice the presence of narrow intercellular space (arrows) between the granulosa cells (X 10.800).

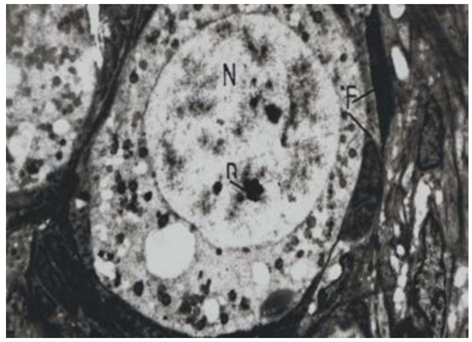

Figure 14: Eletronic micrograph of treated rabbit overy, infantile post natal week 12, showing portion of the oocyte. The nucleus (N) has a prominent nucleolus (n), leptotene chromosome core (long arrow) and nuclear pores (short arrows) are seen. Notice the prsence of numerous cytoplasmic granules (G). Insert shows leptotene chromosome core, at higher magnification, lies within an electroni dense chromosome material. (X 16.000).

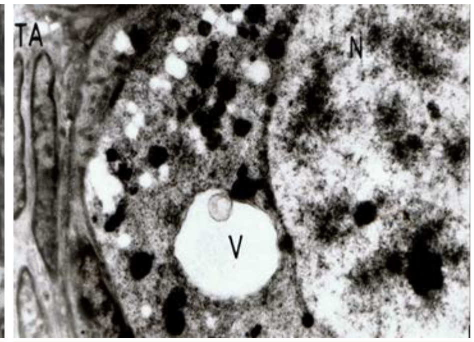

Figure 15: Eletronic micrograph of adult non-pregnant rabbit ovary showing the oocyte (O) wth large nucleus (N) with a thin rim of heterochromatin and a prominent nucleolus (n). The cytoplasm of the oocyte contains membrane bound granules (G). Notice the nuclear pores (Short arrows). (X 20.000).

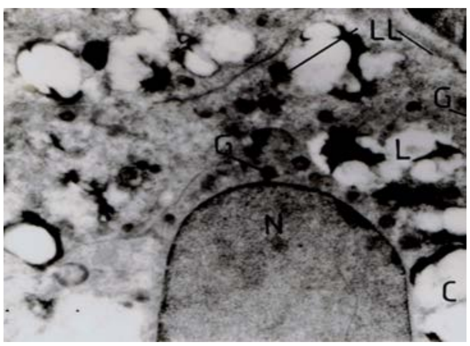

Figure 16: Eletronic micrograph of adult pregnant rabbit ovary showing the large luteal cell (LL). Its cytoplasm contains lipid droplets (L) as well as many small, round, electron-dense granules (G). Notice the presence of the capillaries (C) in the intercellular space. (X 26.800).

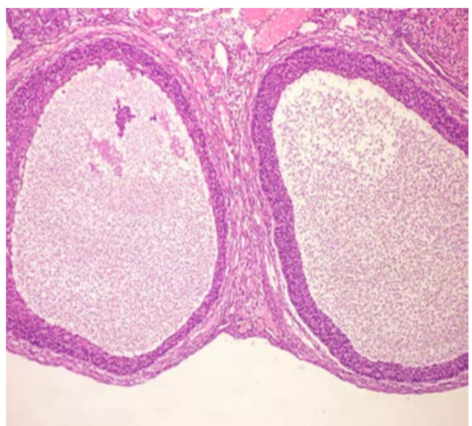

Figure 17: Ovary of rabbit from control group showing normal histopathological appearance of mature follicles (H&E X 150).

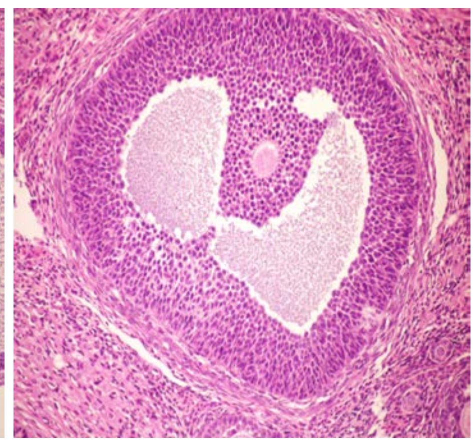

Figure 18: Ovary of rabbit from treated group showing normal histopathological appearance of graffine follicles (H&E X 150).

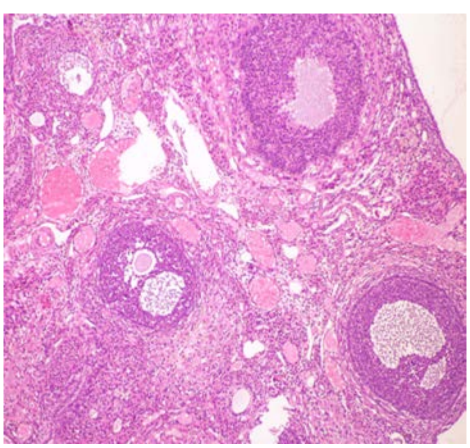

Figure 19: Ovary of rabbit from treated group showing mature follicles (H&E X 100).

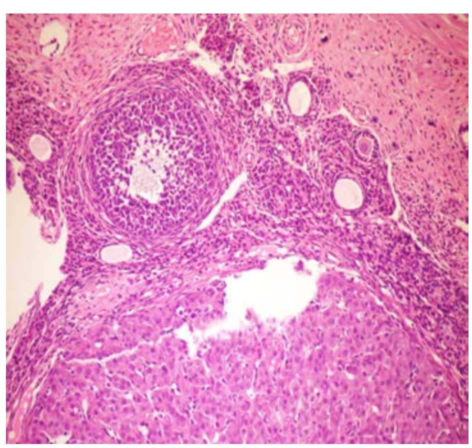

Figure 20: Ovary of rabbit from treated group showing mature follicles (H&E X 200).

development when gonocytes are not expected to be present. As with the CIS-like cells observed in in utero and adolescent exposures, the fate of atypical germ cells observed in postpubertal exposure is not known. These observations are consistent with the reports of increased germ cell loss in rodents without recovery as adults [20].

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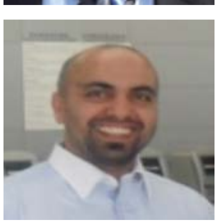

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