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Ovarian Modifications in Mice Exposed to Whole-Body Irradiation.

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TABLE OF CONTENTS:

ABSTRACT

This experiment was designed to determine the involvement of varying levels of whole-body irradiation on ovarian follicular and corpora luteal development in mice. Previous research has indicated reduced counts of ovarian follicles and corpora lutea in mice flown in space. These differences may be the result of microgravity, increased exposure to radiation, or some combination of both. Fifty-six mice were divided into three groups (apocynin-treated, nox2 knockout, and wild-type control) before exposure to 0 Gy, 0.5 Gy, or 2.0 Gy radiation. The tissues were harvested, preserved, run through the appropriate paraffin embedding procedures, serially sectioned, mounted on microscope slides, and stained using a standard H&E staining technique. Total and mean follicular and corpora luteal counts were accessed and compared across treatment groups. Mean ovarian weight, mean total reproductive weight, mean ovarian weight percentage of total body weight, mean total reproductive weight percentage of total body weight, and the apparent estrous phase of the animals were also compared. Radiation from 0.5-2.0 Gy had no significant effect on mean ovarian weight, mean total reproductive weight, mean ovarian weight percentage of total body weight, or mean total reproductive weight percentage of total body weight. Radiation from 0.5-2.0 Gy significantly increased mean early-stage follicular count in the wildtype group only. Radiation of 2.0 Gy increased late-stage follicular count across all groups after accounting for mean ovarian percent of total body weight. Radiation of 2.0 Gy significantly increased mean corpora lutea count in the wildtype group only. This result not only suggests that low-dose radiation accelerates oocyte development in the murine ovary, but also that the inaction of NADPH-oxidase (via apocynin inhibition or genetic knockout) may ameliorate some of these effects.

INTRODUCTION

Throughout its history on planet Earth, mankind has expanded its habitat to include the most extreme of environs, facing dramatic changes in temperature, molecular oxygen level, and illumination. Now man looks to conquer a new habitat, Mars and the space between, and the journey threatens to expose astronauts to the full spectrum of gravitational force. The challenges on Mars itself will resemble those most unforgiving habitats of Earth. Its surface is frozen, hypoxic, and unprotected from solar radiation. Investigation into such extreme conditions, and their combined effect upon human physiology, must be understood if spaceflight is to be a viable option for prolonging and protecting man's future.

The Space Studies Board and National Research Council convened in 1991 to address research goals for the new millennium, highlighting a need for spaceflight experimentation in the area of human viability in space. Possible reproductive pathologies associated with orbit were a primary concern. And although the recommendation by the council that spaceflight test animals remain in orbit for 2 life cycles has been largely abandoned in favor of cheaper, shorter experiments, the ongoing mission of the National Aeronautics and Space Agency (NASA) to increase spaceflight funding has been a successful one. Such progress is illustrated by the wealth of experiments that have been and are being done upon the recently constructed International Space Station (ISS). ISS is outfitted specifically for developmental biological studies. Organized by Professor Sally A. Moody of George Washington University and Dr. Catherine Golden of NASA, the International Space Life Sciences Working Group Developmental Biology Workshop aimed to further spaceflight research, specifically in reference to the processes of gametogenesis,

fertilization, organogenesis, and vestibular system development (Moody and Golden, 2000).

These findings were revisited inside the 2011 Decadal Survey on Biological and Physical Sciences in Space, which proposed a research effort to examine the efficacy of spaceflight-induced bone loss countermeasures, such as exercise and anti-osteoporosis drugs. The survey emphasizes current questions concerning the health of female astronauts, particularly the physiological impact of interrupting the menstrual cycle during spaceflight, a common practice for NASA employees (National Research Council Decadal Survey, 2011).

As Droppert (1990) suggests, the first country to resolve the deleterious effects of spaceflight will be well on its way to colonizing Mars. Chapes et al. (1993) illustrated a pronounced need for more experimentation in the area of orbit's effect on immune tissue, prompting work on the action of inflammatory neutrophils, activated macrophages, and cytokines in space. In 1992, NASA itself expressed a need for an increased biological research endeavor in space (Motabagani, 1992).

Effect of Spaceflight on Various Organ Systems

Space-related research includes data on the musculoskeletal system (Dropper, 1990; Ferguson et al., 2002; Meigal, 2012; Milstead et al., 2004; Sandona et al., 2012; Stabley, 2012), the cardiovascular system (Agarwal, 2010; Dabertrand et al., 2012; Zuj et al., 2012), the lymphatic system (Armstrong et al., 1993; Chapes et al., 1993; Chapes et al., 1999), the urinary system (Kaplanskii et al., 2008), the nervous system (Frigeri et al., 2008; Santucci et al., 2012), and the digestive system (Atlashkin et al., 2012).

Droppert (1990) reported a decreased calcium concentration in the weight-bearing bones of astronauts in orbit. Increased exercise, drugs involved in calcium storage and reuptake, high-calcium diets, and artificial gravity technologies have been used to ameliorate calcium decrease with some success. These findings are important because such calcium loss could increase the possibility of bone fractures or premature osteoporosis in astronauts returning to Earth.

Sadona et al. (2012) investigated the effects of long-term low-gravity exposure on skeletal muscle in mice. In their experiments, mice were subjected to 91 days of orbit on the ISS. The study revealed greater slow-twitch atrophy and alteration of gene expression in both the soleus and extensor digitorum longus muscles, though the effect was enhanced in the soleus. This data suggests that the extensor digitorum longus muscle may have compensatory mechanisms that allow some level of resistance to microgravity (Sadona et al., 2012).

Stabley et al. (2012) concluded that although arterial vessel wall thickness and diameter remained unchanged, orbit reduced voltage-gated calcium and norepinephrine vasocontrictor responses, linked to low ryanodine receptor subtype-2 and subtype-3 expression in spaceflight subjects. These receptors facilitate calcium release deep within smooth muscular tissue. This calcium binds to filamentous troponin, shifting the tropopin-tropomyosin complex and revealing binding sites on actin proteins to the globular heads of myosin, resulting in muscular contraction (Widmaier et al., 2011). As a result, the researchers found that smooth muscle contractility was inhibited in mice exposed to spaceflight (Stabley et al., 2012).

Natural systolic blood pressure variation, maintained by circadian rhythms over a 24-hour period, was shown to be disrupted by spaceflight (Agarwal, 2000). Blood pressure is regulated by the combined action of the suprachiasmic nucleus of the hypothalamus and peripheral vascular and nephritic organs. Spaceflights of varying lengths were shown to inhibit blood pressure regulation in both humans and mice . Whether this effect was the result of physical inactivity, whole-body fluid shifts, vasoconstriction, or a stress response while in space is unknown. Such "out-of-phase" and "phase-less" circadian activity may result in overall organ damage, cardiovascular trauma, chronic kidney disease, sleep apnea, or metabolic stress (Agarwal, 2000).

Santucci et al. (2012) noted 28 up-regulated proteins important to mitochondrial metabolism, ATP synthesis and hydrolysis, calcium and calmodin metabolism, the nervous system, and amino acid transport following three months of spaceflight. Down-regulation of some proteins involved in mitochondrial metabolism were also observed, along with a decrease in nerve growth factor in the hippocampal, cortical, and adrenal regions of the brain.

While studying the effects of spaceflight on the digestive system, Atiashkin et al. (2012) compared the jejunum wall intersticium of three groups of Mongolian gerbils: one group orbited for 12 days upon spacecraft Foton-M3, another exposed to 12 days of simulated orbital factors (excluding microgravity and cosmic radiation), and a ground control. Damage to the internal elastic membrane of submucosal vessels was observed in both the flight and simulated flight groups compared to the control. Such damage was diluted in the simulated flight group. Kaplanskii et al. (2008) also comments on the unique susceptibility of the Mongolian

gerbil to the stressful conditions of spaceflight. The animal is characterized by its large adrenal glands, an important site of stress-associated hormone secretion, including the release of epinephrine, norepinephrine, and cortisol. Mongolian gerbils also have a highly sensitive thymus and spleen, organs strongly influenced by corticosteroids (Kaplanskii et al., 2008).

Effects of Spaceflight on Non-Mammalian Reproductive Tissues

Many articles present reproductive data from non-mammalian species. Ijiri (1998) exposed 4 *Oryzias latipes* fish to 15 days of spaceflight on International Microgravity Laboratory 2. These small fish were the first vertebrates to successfully conceive in orbit, producing 8 "space-born" fry. All parameters examined in this study (gonadal germ cell concentration, genital ridge migration, and fertility) were found to be within normal limits, and insignificantly affected by spaceflight in comparison with ground controls.

During an observation of the effects of simulated microgravity on ovarian development, Skrobanek et al. (2008) described low ovarian weight, shorter oviducts, delayed egg-laying, and decreased plasma progesterone in space-flown Japanese quails, though this did not significantly affect normal development. Souza et al. (1995) showed that although *Xenopus laevis* amphibian embryos (fertilized *in vitro*) failed to undergo rotation to align the animal-vegetal pole with Earth's gravitational field, normal development was unimpeded by spaceflight. Wong and Desantis (1997) observed eye opening and walking in rat pups gestated aboard STS-66, Atlantis. These events occurred within a natural time frame, although perinatal morbidity was higher for spaceflight subjects.

Effects of Spaceflight on the Reproductive Tissues of Pregnant Mammals

Spaceflight studies focusing on mammalian reproduction have largely been limited to pregnant specimens. Burden et al. (1998) observed no significant difference between ground control mice and mice in orbit from days 11-20 of gestation in terms of fetal mass at day 20 of gestation, as well as myometrium smooth muscle volume at day 20 of gestation and postpartum. However, pup mass at birth (day 22-23) was significantly decreased in spaceflight mice, and uterine smooth muscle volume between day 20 of gestation and postpartum was decreased by 37% in spaceflight specimens.

Burden et al. (1999) showed a marked decrease in the expression of connexin 43, an integral protein building block in myometrial gap junctions, in mice exposed to spaceflight between days 11-20 of gestation. Gap junctions are important to the formation of a functional syncytium and electrical coupling between smooth muscle cells during uterine contraction. Due to this deficiency, the flight animals showed significantly more, though less efficacious, labor contractions at term (day 22-23) than controls.

The only study to examine pregnant mammals in microgravity prior to Ronca and Alberts (2000) did not observe parturition, a pattern of physiological behaviors that include labor, delivery, maternal care, placentophagia, and nursing. Parturition begins with weak, nonregular uterine contractions a few hours before birth. It has been noted that whole-body fluid shifts, calcium loss, and muscle atrophy of the transverse abdominis pose a particular risk to labor. Abdominal muscles play an important supplementary role in expulsion of the fetus. Ronca and Alberts (2000)

exposed female Norway rats to either 9 or 11 days of spaceflight beginning at either day 11 or 9 or gestation, respectively. The rats were allowed to come to term 2-3 days after return to a 1 G environment and monitored with time-lapse videography. Doubled amounts of termed "lordosis" contractions were observed in flight animals compared to ground controls. Lordosis contractions characterize the earliest contraction period during labor. The animal lies prone, arching her back and lifting her hindlimbs. These contractile episodes are spaced with longer rest intervals than vertical contractions (which occur more frequently and characterize the latter portion of labor) and are believed to facilitate movement of the fetus to the lower birth canal (Ronca and Alberts, 2000).

Effects of Spaceflight on The Reproductive System of Non-Pregnant Mammals

Only a small number of recent studies have examined non-pregnant mammalian reproductive tissues in a spaceflight environment, and some of these findings have been contradictory. Evaluation of ovaries from mice flown on shuttle mission STS-118 in 2007 indicated no gross morphological changes with regard to follicle counts and numbers of corpora lutea between spaceflight and control animals (Smith and Forsman, 2012). However, other reproductive tissues from the same animals did exhibit changes. The spaceflight environment was shown to cause a significant thickening of the apical mucin layer of the uterus (Nier and Forsman, 2011, 2013). Evaluation of the apical mucin layer of the three regions of the uterine tube (Svalina and Forsman, 2013) indicated a trend toward a thinning of the mucin layer in all three regions. Measurements of the thickness of the apical mucin layer of the vagina indicated a significant thickening of the vaginal mucin layer in the mice

subjected to spaceflight (Romer, et al, 2013). These results from the last two studies were very interesting because in both studies there was a change, although not statistically significant, in the mucin layer between animals kept in normal cages under normal laboratory animal conditions and animals kept in the same type of cages used for spaceflight but kept in normal gravity conditions. This implies that the cage environment has an effect on the animals and that some aspect of spaceflight enhances this effect.

In studies of the ovarian tissue from mice flown on shuttle mission STS-131, Gupta et al. (2010) and Tash et al. (2011) reported significant suppression of ovarian, luteal, and uteral development relative to baseline and ground control animals. In contrast to the findings of Smith and Forsman (2012), Tash and Gupta reported that examination of spaceflight ovaries indicated fewer corpora lutea and a large number of atretic follicles compared to control animals. They also reported a complete absence of corpora lutea in the spaceflight animals. They also reported that uterine horns harvested from spaceflight animals were also generally smaller than concomitant controls, although uterine gland counts were not significantly affected. Estrogen receptor, lactoferrin mRNA, and progesterone receptor expression were also found to be blunted in uteri and ovaries from spaceflight animals. The researchers concluded that spaceflight significantly disrupts the mammalian estrous cycle.

Although changes have been reported in various locations and in various forms within the female reproductive system from mice subjected to spaceflight, the causative factor is unknown. There are two main areas that distinguish the spaceflight environment from the environment on Earth. The first is the

microgravity experienced during spaceflight and the second is the high exposure to radiation associated with spaceflight.

Effects of Radiation on The Reproductive System of Mammals

While many studies have shown that spaceflight has effects on various systems of the body, it is not known if such effects are due to the exposure of microgravity. Several scientists believe that cosmic radiation may be responsible for the various documented effects. This is because astronauts are exposed to much more radiation in space than on Earth (Horneck et al., 2010). In addition, cosmic radiation in space is characterized by the increased presence of damaging high-energy heavy ions. The difference comes down to Earth's atmosphere, which contains ultraviolet (UV) radiation absorbing ozone gas, which protects Earth's inhabitants from much of the Sun's harmful UV radiation. The same protection is not afforded to astronauts and test subjects on shuttle or ISS deployments, which explains the increase in radiation exposure. Such findings have led many researchers to investigate the role of radiation in human and mammalian reproduction, comparing these effects to those observed in space (Horneck et al., 2010).

The mammalian ovary is a most fascinating organ, and has been a focal point of both whole-body and targeted radiotherapy for over a century. The structure and function of the ovary are innately linked. As follicular count decreases, the organ becomes less likely to ovulate viable eggs. Like humans, mice cannot produce new oocytes after birth, and quickly lose the majority of them in early life, and the remaining are lost steadily throughout their life cycle. As Lindop (1969) claims, only

a small amount of oocytes will ovulate during the reproductive lifespan, about 100 of 10,000 in mice, and 400 of 2 million in humans.

The majority of radiation research on mammals has followed either whole-body or targeted 250 kV X-rays and neutron exposure. The effect of irradiation on the ovary is well-documented, causing increased oocyte depletion rates, acceleration of oocyte maturity, reduced litter size, ovarian tumors, and various genetic damage. The timing of exposure, in terms of the age of the animal, is the most important factor in accessing the damage. An acute peak of sensitivity to 20 or 25 rads has been reported in mice at two and a half weeks old (Peters, 1961; Oakberg, 1962). One rad, the unit of ionic radiation absorbance, is equivalent to 0.01 Gy, or 0.01 joules of absorbed radiation for every kilogram of biomaterial (International Bureau of Weight and Measures, 2008). Mice with nitrogen-induced hypoxia show a unique resistance to low-dose radiation, possibly related to a decrease in cellular respiratory function seen in as little as 2 minutes after exposure to 200 rads (Peters, 1961; Oakberg, 1962).

However, the evidenced cell depletion may not tell the entire story of the ovary, and morphology may not be as closely linked to function as once thought. If radiative treatment is applied in early life, where the cell depletion rate is already highest, induced cell killing may be ameliorated by a decrease in natural cell depletion. Indeed, irradiated mice are more efficient at making use of the remaining oocyte population than control animals. Irradiated animals lost reproductive capability at 3-4% remaining oocyte population, while control animals lost virility at 37% oocyte population (Lindop, 1969).

Lindop (1969) also comments that interspecies extrapolation between mice and human females is justified, though damage may present in various forms. This hypothesis was reiterated in the NASA's aforementioned 2011 Decadal Survey on Biological and Physical Sciences.

"Given the typical lifespan of humans, 180 days in space may seem trivial. However, in the case of rodents, the animal model most scientists have used to study fundamental biological processes in space, such a time frame represents approximately one-fourth to one-third of the species' adult life. Thus, studies on these rodents in space have the potential to extrapolate important implications for humans living in space well beyond 6 months" (National Research Council Decadal Survey, 2011).

Human ovaries are also about six times more resistant to radiation than mice, possibly due to relative oocyte population or cell-specific sensitivity, requiring a dose of 600 rads delivered over a few hours to induce permanent sterility (Lindop, 1969).

The ultimate goal of this study was to determine the involvement of varying levels of whole-body irradiation on ovarian luteal and follicular development in mice. It hopes to provide sufficient data for an anatomical comparison with mice of actual spaceflight. It is hypothesized that the increased exposure to cosmic radiation observed in spaceflight, or some combination of both microgravity and radiation, is to blame for structural and functional differences between spaceflight and ground control tissues. This opposes the idea that microgravity, or another outside factor (such as closed life support systems as Santy et al. 1990 suggests) is solely responsible for the observed results.

Some of the mice in this experiment have been treated with apocynin, a drug that inhibits nicotinamide adenine dinucleotide phosphate-oxidase, often abbreviated "NADPH-oxidase" or simply "nox". NADPH-oxidase plays a role in radiation-induced oxidative stress by converting molecular oxygen (O_2) to

superoxide anions (O_{2^-}). Specifically, NADPH-oxidase has been shown to cause radiation-mediated upregulation of intracellular reactive oxygen species, or ROS. Therefore, the inhibition of NADPH-oxidase by apocynin should reduce radiation induced oxidative stress in tissues (Shatwell et al., 1996).

Genetic knockouts were also used in this experiment, and are invaluable to *in vivo* case studies. Cells of NADPH-oxidase knockouts are unable to manufacture the enzyme, producing similar effects as treatment with apocynin, theoretically. The two most popular methods of generating knockouts are 1, germline knockout, which is full gene inactivation of the gene in all cells at all stages of development and 2, conditional mutagenesis, which enables cell-specific gene inactivation. (Friedel et al., 2011).

The Murine Estrous Cycle

The murine estrous cycle is a cycle of hormonally-driven morphological changes in oocyte or follicular development and maturity within the ovary. Each of four phases (proestrous, estrous, metestrous, and diestrous) corresponds to a characteristic portion of the ovulatory timeline. During the proestrous phase of the estrous cycle, the growth of early-stage follicles, also known as preovulatory or Graafian follicles, begins. Deteriorating postovulatory cells, or corpora lutea, can also be seen. These atrophying lutea are marked by vacuolation within the cell (Westwood, 2008). Ovulation occurs during the estrous phase, which is marked by a higher concentration of late-stage follicles. These fluid-filled follicles are much larger, and rupture during ovulation, forming immature postovulatory or corpora lutea cells. During this roughly 24-hour period, fertilization may occur (Rugh and

Clugston, 1955). During the metestrous phase, recently-formed corpora lutea grow to their maximum size, vacuolizing during diestrous (Westwood, 2008). Parkes (1928) found the mean length of 1000 estrous cycles to be 6.213 days. Proestrous and estrous accounted for a mean of 2.494 days. Metestrous and diestrous accounted for a mean of 3.719 days.

MATERIALS AND METHODS

The tissues used in this study were provided by the laboratory of Dr. Michael Pecaut of Loma-Linda University in Loma-Linda, California. Thirty-six C57BL/6 mice were randomly split into two uniform groups of eighteen. One of these groups of eighteen was treated with apocynin, the other represented the wildtype control. A final group of eighteen B6.129S6-Cybbtm1Din/J or "nox2 knockout" mice formed the third test group for a total of 54 mice.

Within the knockout animals, the specific section of DNA responsible for coding for NADPH-oxidase2 has been inactivated or "knocked out". A noncoding replacement section causes the resultant inhibited phenotype (Friedel et al., 2011).

Each of the three groups of eighteen were further divided into three subgroups of six, and given a label A-G. Three subgroups of six, one from each of the original groups of eighteen, were exposed to no whole-body radiation other than that which is incurred normally on Earth. A second set of three subgroups were exposed to 0.5 Gy whole-body radiation for an average of 0.94 minutes, and a third set were exposed to 2.0 Gy whole-body radiation for an average of 3.37 minutes. The resultant subgroups A-G appear in the chart below.

SUBGROUP	TREATMENT
A	WT Control
B	0.5 Gy
C	2 Gy
D	Apocynin (Apo)
E	Apo + 0.5 Gy
F	Apo + 2.0 Gy
G	NOX2 Knockout (KO)
H	KO + 0.5 Gy
I	KO + 2.0 Gy

The mice in the experiment were irradiated via whole-body (excluding the head) proton radiation at Loma Linda University Medical Center Proton Treatment Facility. The animals did not receive anesthesia and were placed into identical rectangular enclosures and covered with a 400mm by 400mm polystyrene phantom prior to proton exposure. A Markers parallel plate ionization chamber from the National Institute of Standards and Technology was used to calibrate the proton dose. Water equivalent depth, or WED, is a measure of radiological thickness to which proton radiation techniques must be appropriately calibrated. A polystyrene phantom is a container used to calibrate WED.

After irradiation, the animals were euthanized at 8-9 weeks of age with one hundred percent carbon dioxide. Ovarian tissues were harvested by a lab technician within 30-60 minutes of sacrifice. After euthanasia, the specimen were fixed in four percent paraformaldehyde, and sent to the laboratory of Dr. Allan Forsman at East Tennessee State University, where the tissues were trimmed of fat and debris and stored in seventy percent ethanol solution. The ovaries, uterine horns, and uterine tubes were weighed independently before being dehydrated with increasing dilutions of ethanol, hemo-de, and paraplast in preparation for paraffin embedding.

Upon completion of the embedding process, one ovary from each animal was serially sectioned into 4 micrometer sections using a Micron HM35 microtome and mounted on glass microscope slides. These slides were stained according to standard H&E staining protocol.

After staining, the tissues were examined and photographed using a Zeiss Axioskop 4C compound microscope equipped with a Canon Powershot A640 digital camera. These photographs were used to formulate a comprehensive corpora luteal

and follicular count for each ovary. Follicles were determined to be of either late or early-stage development via the presence of a fluid-filled antrum. These counts were compared to total ovarian mass, total reproductive mass, and total body mass. Resultant ratios were compared with the apparent estrous phase.

The phase of the estrous cycle for each animal was determined by an assessment of predominant cell type within corresponding vaginal smears. These smears were performed in the laboratory of Dr. Pecaut at the time of euthanasia. Proestrous was identified by the presence of nucleated epithelial cells. Estrous was identified by the predominance of cornified squamous epithelial cells. Metestrous was characterized by a mixture of leukocytes, nucleated epithelial, and cornified squamous epithelial cells. Diestrous contains almost solely leukocytes.

RESULTS

The mean ovarian and total reproductive weight of each treatment group can be seen in Table 1. These values are representative of recorded ovarian and total reproductive weights for control tissues, 0.5 Gy irradiated tissues, and 2.0 Gy irradiated tissues shown in full in Tables 7, 8, and 9. Measurements of total reproductive weight included ovarian, uterine, and vaginal tissues (Figure 1). Measurements of ovarian weight were inclusive of both ovaries when possible. An ovary was found to be absent from the F2 animal, reflected in both the individual and mean ovarian and total reproductive weights. Table 1 also features the z-score of the ovarian and total reproductive weights for all treatment groups. The between-group standard deviation of ovarian weight for all treatment groups was 0.437 mg. The between-group standard deviation of total reproductive weight for all treatment groups is 26.81 mg. This value, and the z-score are the result of a standard T-test, and are to be used in a discussion of significance within the data set.

Mean Ovarian and Total Reproductive Weight, All Treatment Groups				
Treatment Group	**Ovarian Wt. (mg)**	**Ovarian Wt. Z-Score**	**Total Reproductive Wt. (mg)**	**Total Reproductive Wt. Z-Score**
WT Control (A)	2.8	0.917	60.9	0.552
0.5 Gy (B)	2.5	1.601	64.2	0.429
2.0 Gy (C)	3.4	0.457	61.3	0.537
Apocynin (D)	3.2	0.000	96.4	0.772
Apo + 0.5 Gy (E)	3.5	0.686	72.8	0.108
Apo + 2.0 Gy (F)	2.8	0.917	61.1	0.545
NOX2 Knockout (G)	3.5	0.686	112.2	1.361
KO + 0.5 Gy (H)	3.9	1.601	116.4	1.518
KO + 2.0 Gy (I)	3.1	0.229	36.3	1.470

Table 1: Mean Ovarian and Total Reproductive Weights for All Treatment Groups

Table 2 expresses the mean ovarian and total reproductive weights for each treatment group as percentages of total body weight. These values were obtained via

comparison of recorded ovarian and total reproductive weights (Tables 7, 8, and 9) with recorded total body weights (Table 10, 11, 12). The ovarian and total reproductive percent of total body weight for all animals can be found in Tables 13, 14, and 15. Table 2 also lists the respective z-scores of ovarian and total reproductive percentages. Between-group standard deviation for ovarian percentage of total body weight 2.36×10^{-5}% for all treatment groups. Between-group standard deviation for total reproductive percentage of total body weight was 1.4×10^{-3} % for all treatment groups.

Mean Ovarian Percent and Total Reproductive Percent of Total Body Weight, All Treatment Groups				
Treatment Group	**Ovarian Wt. (%)**	**Ovarian Wt. % Z-Score**	**Total Reproductive Wt. (%)**	**Total Reproductive Wt. % Z-Score**
WT Control (A)	1.70×10^{-4}	0.424	3.6×10^{-3}	0.429
0.5 Gy (B)	1.44×10^{-4}	1.525	3.7×10^{-3}	0.357
2.0 Gy (C)	2.08×10^{-4}	1.186	3.7×10^{-3}	0.357
Apocynin (D)	1.84×10^{-4}	0.170	5.6×10^{-3}	1.000
Apo + 0.5 Gy (E)	1.96×10^{-4}	0.678	4.0×10^{-3}	0.143
Apo + 2.0 Gy (F)	1.53×10^{-4}	1.144	3.3×10^{-3}	0.643
NOX2 Knockout (G)	2.08×10^{-4}	1.186	5.8×10^{-3}	1.143
KO + 0.5 Gy (H)	2.15×10^{-4}	1.483	6.3×10^{-3}	1.500
KO + 2.0 Gy (I)	1.74×10^{-4}	0.254	1.9×10^{-3}	1.643

Table 2: Mean Ovarian and Total Reproductive Percent of Total Body Weight for All Treatment Groups.

Early-stage ovarian follicular count means for each treatment group can be found in Table 3, along with z-scores. The between-group standard deviation of early-stage follicles for all treatment groups was 19.94. Individual recordings for control, 0.5 Gy irradiated, and 2.0 Gy irradiated animals are shown in Tables 16, 17, and 18. A representation of an early-stage follicle is pictured in Figure 2.

Mean Early-Stage Follicle Count, All Treatment Groups		
Treatment Group	**Early-Stage Follicles**	**Early-Stage Follicles Z-Score**
WT Control (A)	197	2.021
0.5 Gy (B)	140	0.838
2.0 Gy (C)	162.7	0.301
Apocynin (D)	167	0.517
Apo + 0.5 Gy (E)	146.7	0.502
Apo + 2.0 Gy (F)	159.3	0.130
NOX2 Knockout (G)	163	0.316
KO + 0.5 Gy (H)	126.3	1.525
KO + 2.0 Gy (I)	148.7	0.401

Table 3: Mean Early-Stage Follicle Counts for All Treatment Groups

Late-stage ovarian follicular count means for each treatment group can be found in Table 4 below, along with z-scores. The between-group standard deviation of late-stage follicles for all treatment groups was 10.42. Individual recordings for control, 0.5 Gy irradiated, and 2.0 Gy irradiated animals are shown in Tables 16, 17, and 18. A representation of a late-stage follicle is pictured in Figure 3.

Mean Late-Stage Follicle Count, All Treatment Groups		
Treatment Group	**Late-Stage Follicles**	**Late-Stage Follicles Z-Score**
WT Control (A)	18.7	1.056
0.5 Gy (B)	17.3	1.190
2.0 Gy (C)	30.0	0.029
Apocynin (D)	35.3	0.537
Apo + 0.5 Gy (E)	43.3	1.305
Apo + 2.0 Gy (F)	47.0	1.660
NOX2 Knockout (G)	26.7	0.288
KO + 0.5 Gy (H)	27.3	0.230
KO + 2.0 Gy (I)	21.7	0.786

Table 4: Mean Late-Stage Follicle Counts for All Treatment Groups

Mean corpus luteum counts for each treatment group were recorded in Table 4 below, along with z-scores. Individual recordings for control, 0.5 Gy, and 2.0 Gy irradiated animals are shown in Tables 19, 20, and 21. A representation of a corpus luteum is pictured in Figure 4.

Mean Corpus Luteum Counts, All Treatment Groups		
Treatment Group	**Corpus Luteum**	**Corpus Luteum Z-Score**
WT Control (A)	3.7	0.111
0.5 Gy (B)	5	0.611
2.0 Gy (C)	7	1.722
Apocynin (D)	4.3	0.222
Apo + 0.5 Gy (E)	2.7	0.667
Apo + 2.0 Gy (F)	1.3	1.444
NOX2 Knockout (G)	4	0.056
KO + 0.5 Gy (H)	5.3	0.778
KO + 2.0 Gy (I)	1.7	1.222

Table 5: Mean Corpus Luteum Counts for All Treatment Groups

Estrous phase determinations made from vaginal smears of each animal are listed in Tables 21, 22, and 23. These determinations were used to structure Table 6, which depicts the number of ovaries within each treatment group that presented in each of the estrous phases. Representations of proestrous, estrous, metestrous, and diestrous smears may be found in Figures 5, 6, 7, and 8.

Within-Group Estrous Phase Determination Totals, All Treatment Groups				
Treatment Group	**Proestrous**	**Estrous**	**Metestrous**	**Diestrous**
WT Control (A)	0	0	1	2
0.5 Gy (B)	0	1	0	2
2.0 Gy (C)	1	1	0	1
Apocynin (D)	0	2	1	0
Apo + 0.5 Gy (E)	0	2	0	1
Apo + 2.0 Gy (F)	0	1	1	1
NOX2 Knockout (G)	0	3	0	0
KO + 0.5 Gy (H)	0	2	0	1
KO + 2.0 Gy (I)	0	1	0	2
TOTAL	**1**	**13**	**3**	**10**

Table 6: Within-Group Estrous Phase Determination Totals for All Treatment Groups

Statistical significance was observed for z-scores greater than 1.645, corresponding to p-values of 0.05, or 95% confidence that the null hypothesis is false. In other words, the hypothesis that radiation has no significant effect on ovarian morphology is false. Representative ovarian sections from Treatment Group's A-I are pictured in Figures 9-17.

DISCUSSION

In regard to ovarian weight, the results of Table 1 (Mean Ovarian and Total Reproductive Weights for All Treatment Groups) reveal two points of interest: a minima in Treatment Group B (WT + 0.5 Gy) of 2.5 mg, and a maxima in Treatment Group H (KO + 0.5 Gy) of 3.9 mg. Compared to a between-group standard deviation of 0.437, both groups are well beyond one standard deviation and worth mention. However, with z-scores of only 1.601, these values are not statistically significant and may be explained by natural variation. It may be concluded from analyzing the result of the T-test that there is no significant correlation between ovarian weight and irradiation up to 2.0 Gy.

Table 1 also highlights Treatment Groups G (KO + 0.0 Gy) and H (KO + 0.5 Gy) for their high total reproductive weight. Treatment Group G has a mean total reproductive weight of 112.2 mg, much higher than one standard deviation (26.81) above the grand mean of 75.7 mg. Treatment Group H has a mean total reproductive weight of 116.4 mg, nearly two standard deviations above the grand mean. However, with z-scores of 1.361 and 1.518, respectively, neither result is statistically significant and may be explained by natural variation. From the analyzing result of the T-test, it may be concluded that total reproductive weight is not significantly affected by irradiation up to 2.0 Gy.

The results in Table 2 depict a minimum mean ovarian weight percentage in Treatment Group B (WT + 0.5 Gy) of 1.44×10^{-4}%, much lower than one standard deviation (2.36×10^{-5}). Also shown is a maximum mean ovarian weight percentage in Treatment Group H (KO + 0.5 Gy) of 2.15×10^{-4}%, much higher than one standard deviation of 2.36×10^{-5} from the grand mean of 1.80×10^{-4}%. However, with z-scores of 1.5254 and 1.4831, respectively, these endpoints of the range escape statistical

significance. From the result of the T-test, it may be concluded that radiation has no significant influence on the ovarian weight to total body weight ratio.

Table 2 also highlights a maxima in Treatment Group I (KO + 2.0 Gy), where the mean total reproductive weight percentage of total body weight was 1.9×10^{-3}%. This value is nearly two standard deviations (1.4×10^{-3}) lower than the grand mean of 4.2×10^{-3}%. However, with a z-score of 1.6429, it is not statistically significant. This suggests there is no correlation between radiation and the total reproductive weight to total body weight ratio.

Statistical analysis of the results in Table 3 (Mean Early-Stage Follicle Counts for All Treatment Groups) show that with an average early-stage follicle count of 197, and a z-score of 2.021, Treatment Group A (WT + 0.0 Gy) has a significantly high count. This result suggests that amongst the wildtype treatment group, normal or Earth-provided radiation does not inhibit early-stage follicular development. Treatment Group H (KO + 0.5 Gy) provides the minima for this data set at a mean of 126.3 early-stage follicles, more than one standard deviation (19.94) below the grand mean of 156.7 early-stage follicles. This result is surprising, given that Treatment Group H has the highest ovarian and total reproductive weights of the data set. However, with a z-score of 1.525, Treatment Group H is not statistically significant and may be explained by normal variation.

Table 4 (Mean Late-Stage Follicle Counts for All Treatment Groups) illustrates an interesting trend within the apocynin-treated group. Within this group, late-stage follicular count increased from a mean of 35.5 to 47.0 as radiation increased from 0.0 to 2.0 Gy. This result culminates with Treatment Group F (Apo + 2.0 Gy), with a significantly high late-stage follicular count mean of 47.0 follicles, nearly two standard

deviations (10.42) above the grand mean of 29.7 follicles. As shown, the z-score for Treatment Group F was 1.660. Strangely, the same trend also exists within the wildtype and knockout groups if one accounts for how the lower than average ovarian weights of Treatment Groups B (WT + 0.5 Gy) and I (KO + 2.0 Gy) may have limited follicular count. But, however interesting, no wildtype or knockout treatment group is host to significantly high or low late-stage follicular counts. This result suggests that radiation level may have a slight excitatory effect on late-stage follicular development, or an inhibitory effect on ovulation, leading to a greater late-stage follicular density.

The results in Table 5 (Mean Corpus Luteum Counts for All Treatment Groups) are also interesting. Within the wildtype treatment group, the appearance of corpora lutea increases from means of 3.7-7.0 as radiation increases from 0.0 to 2.0 Gy, culminating in significantly high corpora lutea in Treatment Group C (WT + 2.0 Gy). This trend is not seen in the knockout treatment group, and a slight opposite trend is seen in the apocynin-treated group, both of which are NADPH-oxidase inhibited. This result suggests that radiation up to 2.0 Gy significantly increases corpora luteal concentration within the ovary. As pointed out earlier, this effect could be explained by an acceleration of oocyte development, as suggested by Peters (1961) and Oakberg (1962). An increase in corpora luteal count after radiation also illustrates that radiation of 2.0 Gy or higher does not inhibit ovulation. This is because the corpus luteum itself forms as a result of ovulation. The trend is not present in the NADPH-oxidase inhibited groups, suggesting that the ability to ameliorate the effects of radiation is preventing this process in all groups other than the untreated wildtype.

Table 6 displays estrous phase characterization for each treatment group. To understand the effect of estrous phase on an interpretation of the results, the properties of

each phase must be reexamined. For example, ovary C1 from Table 24 is in the proestrous phase. Theoretically, within the same treatment group, most proestrous ovaries will have lower numbers of late-stage follicles than their estrous and diestrous counterparts. The absence of these large, late-stage follicles and mature corpora lutea also contributes to lower luteal counts and smaller organ size. Indeed, C1 has a late-stage follicular count of 23 and a corpora luteal count of 6, the lowest of any ovary in its treatment group. However, at an ovarian weight of 3.6 mg and ovarian percentage of total body mass of 2.27×10^{-4}%, C1 is the largest ovary in its group. Although this last result seems contrary to the properties of the proestrous phase, it must be considered that the C1 animal also had the largest total reproductive weight at 88.9, almost twice that of C2 and C3. This easily explains the discrepancy and is a good example of natural variation.

Likewise, mice from Treatment Groups D, E, G, and H that have a high-estrous characterization (2 or more ovaries within the group are of the estrous phase) would be expected to have larger ovaries and higher late-stage follicular counts than Treatment Groups A, B, and I (their high-diestrous-characterized counterparts) Indeed, this is the case across the board for both late-stage follicular count and ovarian weight to total body weight ratio. Groups D, E, G, and H have mean late-stage counts of 35.3, 43.3, 26.7, and 27.3, respectively. These results are much higher than the late-stage means from groups A (18.7), B (17.3), and I (21.7). The same trend is seen in ovarian size. The ovaries from groups D, E, G, and H were much larger at 1.84×10^{-4}%, 1.96×10^{-4}%, 1.79×10^{-4}%, and 2.15×10^{-4}% of total body mass, respectively.

The results of this study are somewhat corroborated by findings in previous studies on the effect of radiation on follicular development. Radiation studies by Peters (1961) and Oakberg (1962) noted an acceleration of oocyte maturation after exposure to

low-dose irradiation. This finding would explain trends toward increasing late-stage follicular count and corpora lutea count in wildtype mice, but fails to explain why this effect was not ameliorated in the late-stage follicles of apocynin-treated mice. Peters and Oakberg also noted radiation-induced cell depletion in mice 2 ½ weeks old and younger was ameliorated by the adaptive mechanism of decreased natural oocyte depletion. Because the mice of this study were 8 to 9 weeks of age at euthanasia, this effect would not be seen in any of the treatment groups. The hypothesis that increased exposure to radiation, also seen in spaceflight, may have a significant structural effect upon ovarian follicular development is supported by the data of this study.

CONCLUSIONS

Without accounting for morphological differences resulting from estrous phase variation within each group, radiation from 0.5 to 2.0 Gy had no significant effect on mean ovarian weight, mean total reproductive weight, mean ovarian percent of total body weight, and mean total reproductive percent of total body weight in any treatment group. However, radiation of 0.5 Gy or higher did significantly decrease mean early-stage follicular count in the wildtype mice, an effect that did not present in the apocynin-treated or knockout groups. Radiation of 2.0 Gy or higher also increased mean late-stage follicular count across all treatments, significantly increasing the late-stage count in apocynin-treated mice. Although, as discussed, a significant increase due to 2.0 Gy radiation is found in all treatments if ovarian percent of total body weight is taken into account. Finally, radiation of 2.0 Gy or higher significantly increased mean corpora lutea count in wildtype mice, but not in apocyin-treated or knockout mice. This suggests that treatment with apocynin or genetic knockout of the NADPH-oxidase coding region may ameliorate early-stage decrease and luteal count increase in mice exposed to radiation.

Because the phase of the estrous cycle was not the same for all animals, and varied both within and between each treatment group, any determination of trend or significance between data sets may only be viewed out of the context of estrous phase. This places a severe limitation on the data, and contributes to a decrease in the n value for the data set. Essentially, treatment groups like F, which contain one estrous, one metestrous, and one diestrous ovary see a decrease from n=3 to n=1 for the group. This n value is much too low for counts to be of statistical value. In-depth studies on the effect of radiation on ovarian morphology are required. These should examine either ovaries of a single phase only or conduct an experiment of such magnitude (high n value) that phase

is irrelevant.

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FIGURES AND TABLES

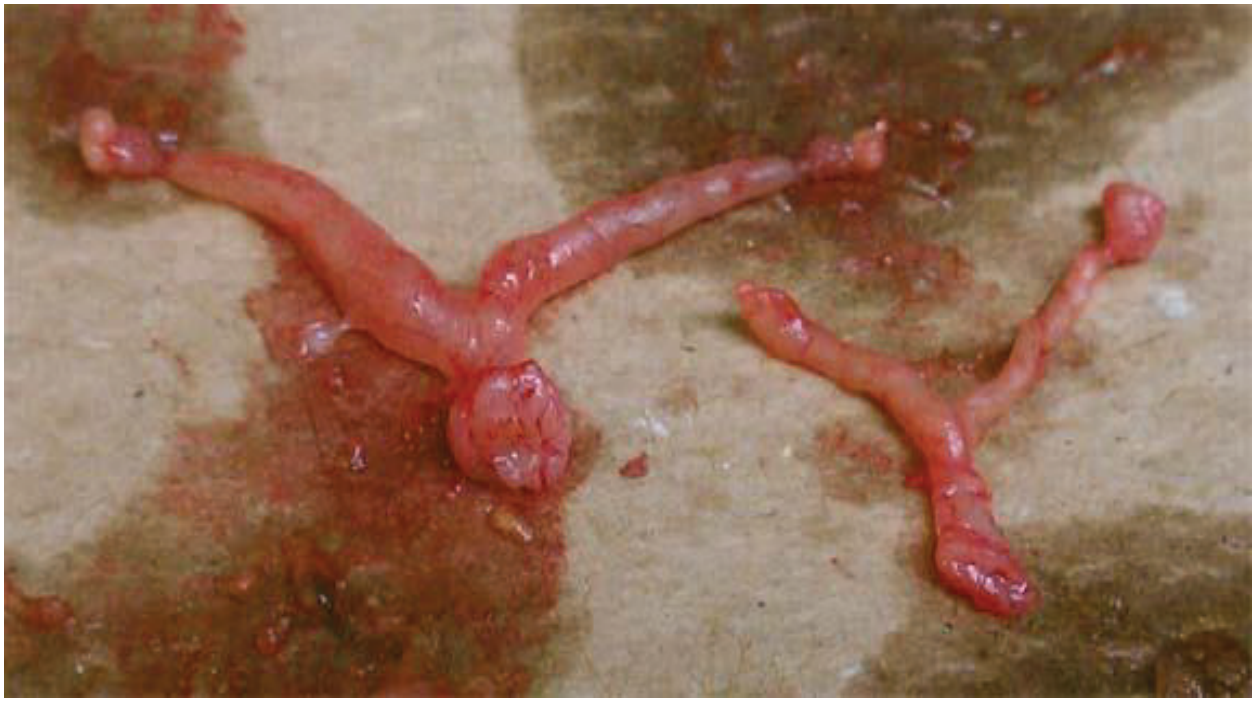

Figure 1: Two total reproductive tracts, including ovarian, uterine, and vaginal tissues. These animals also were part of the same treatment group, illustrating a remarkable range of natural variation.

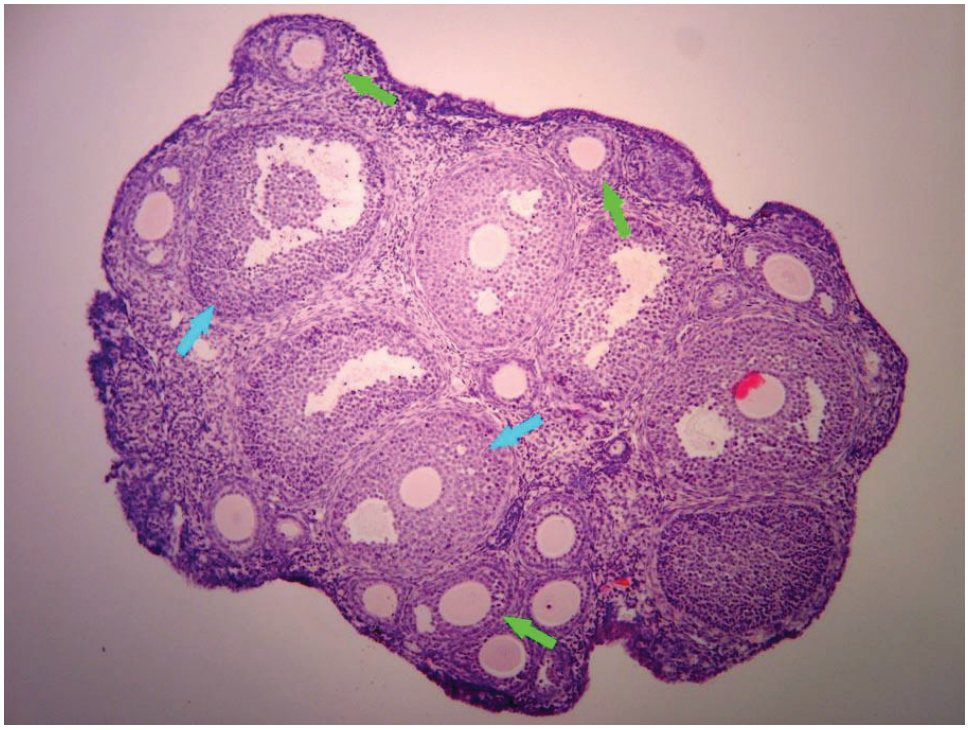

Figure 2: Early-stage follicles within an ovarian section (100X). Arrows indicate representative structures. Green Arrows = Early-Stage Follicles, Blue Arrows = Late-Stage Follicles (antrum present)

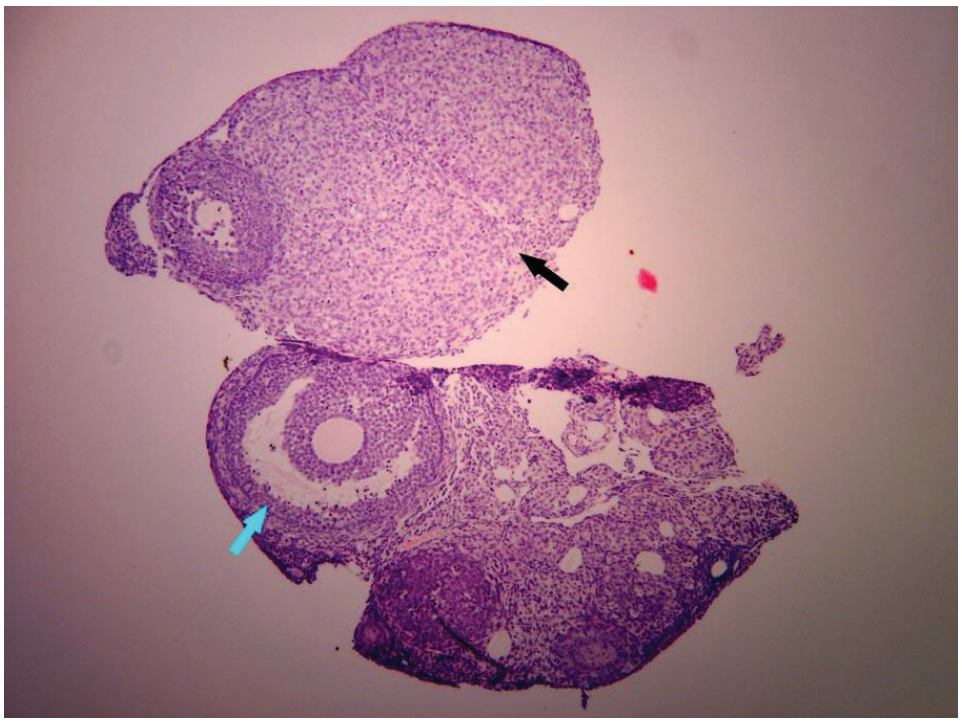

Figure 3: Late-stage follicles within an ovarian section. Arrows indicate representative structures (200X). Blue Arrows = Late-Stage Follicles (antrum present), Black Arrows = Corpora Lutea

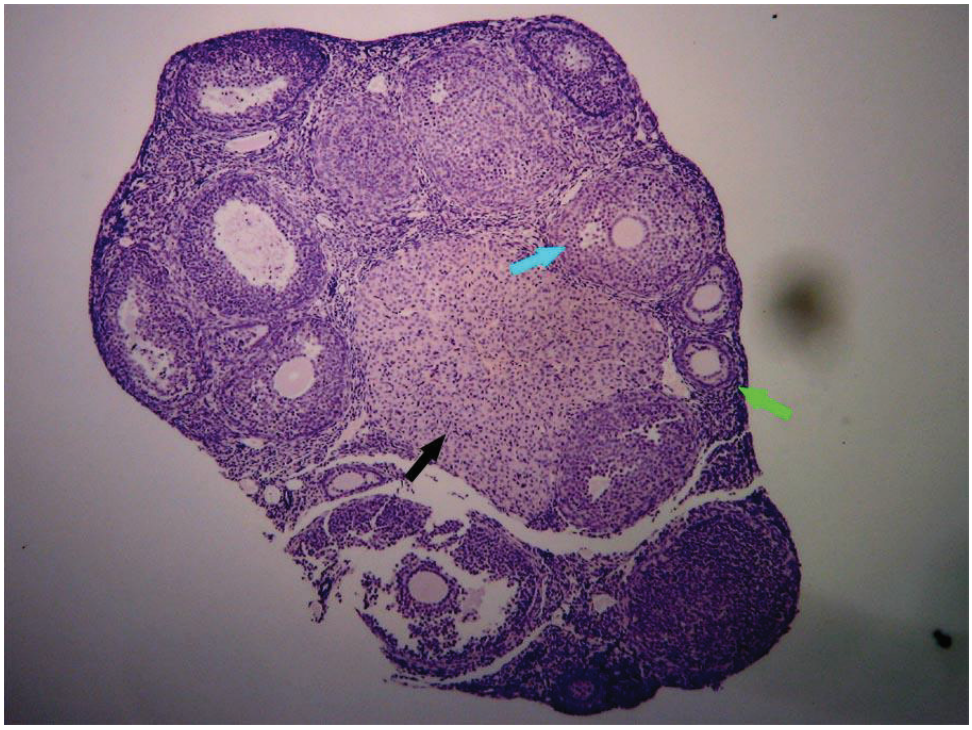

Figure 4: Corpora luteum within an ovarian section (100X). Arrows indicate representative structures. Green Arrows = Early-Stage Follicles, Blue Arrows = Late-Stage Follicles (antrum present), Black Arrows = Corpora Lutea

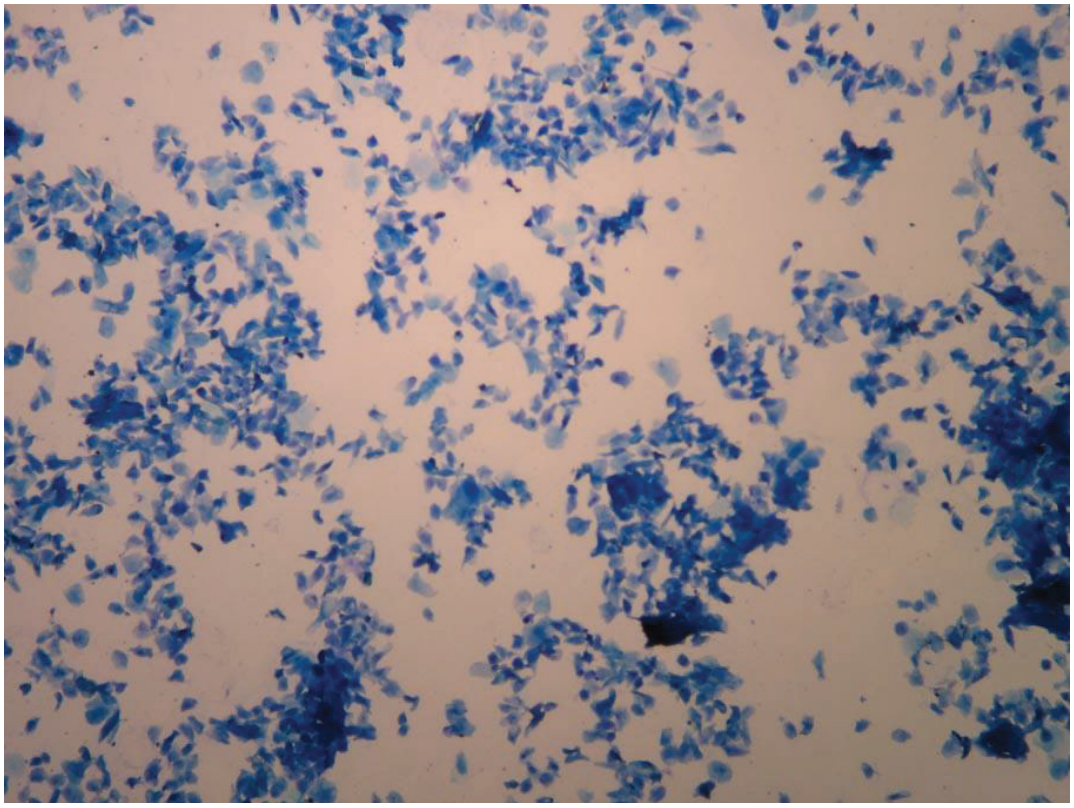

Figure 5: Proestrous phase vaginal smear from C1 animal (200X).

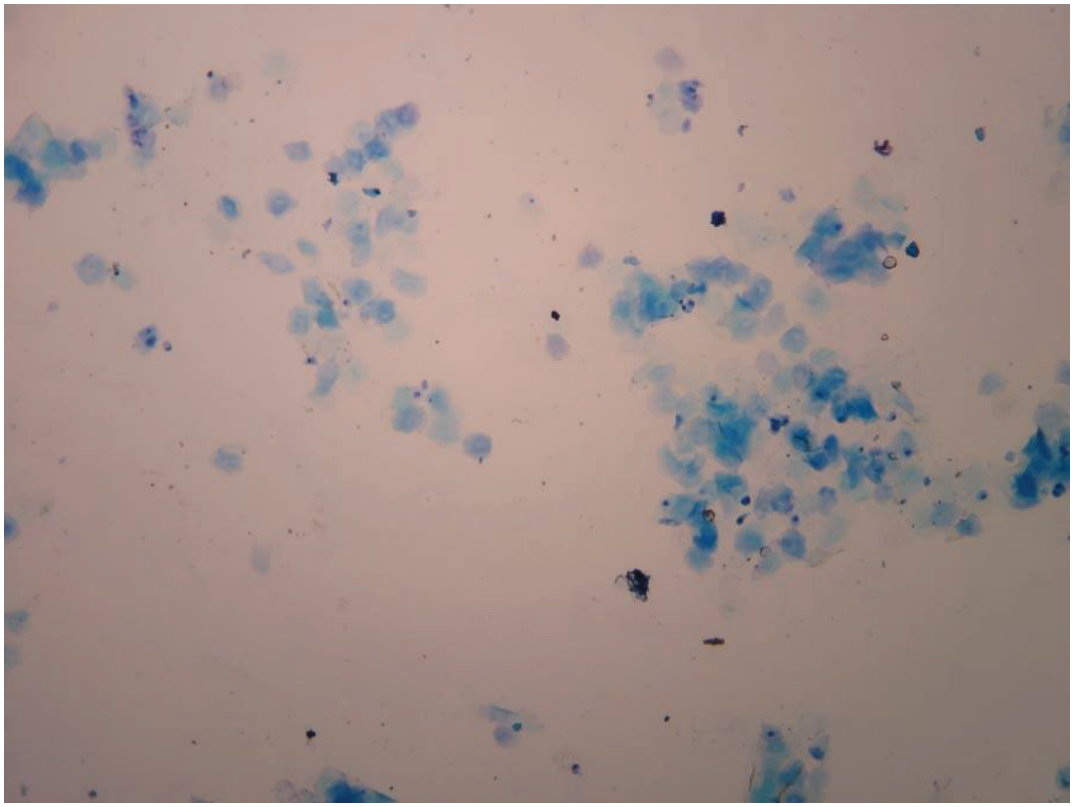

Figure 6: Estrous phase vaginal smear from E3 animal (200X).

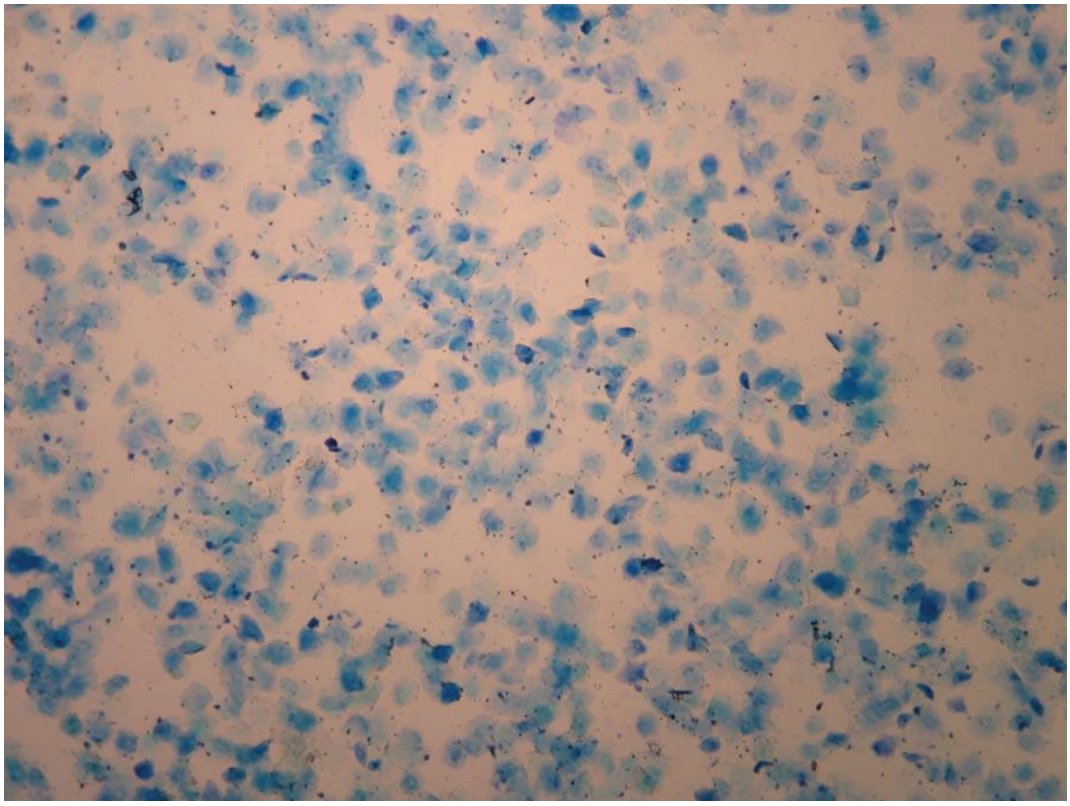

Figure 7: Metestrous phase vaginal smear from D3 animal (200X).

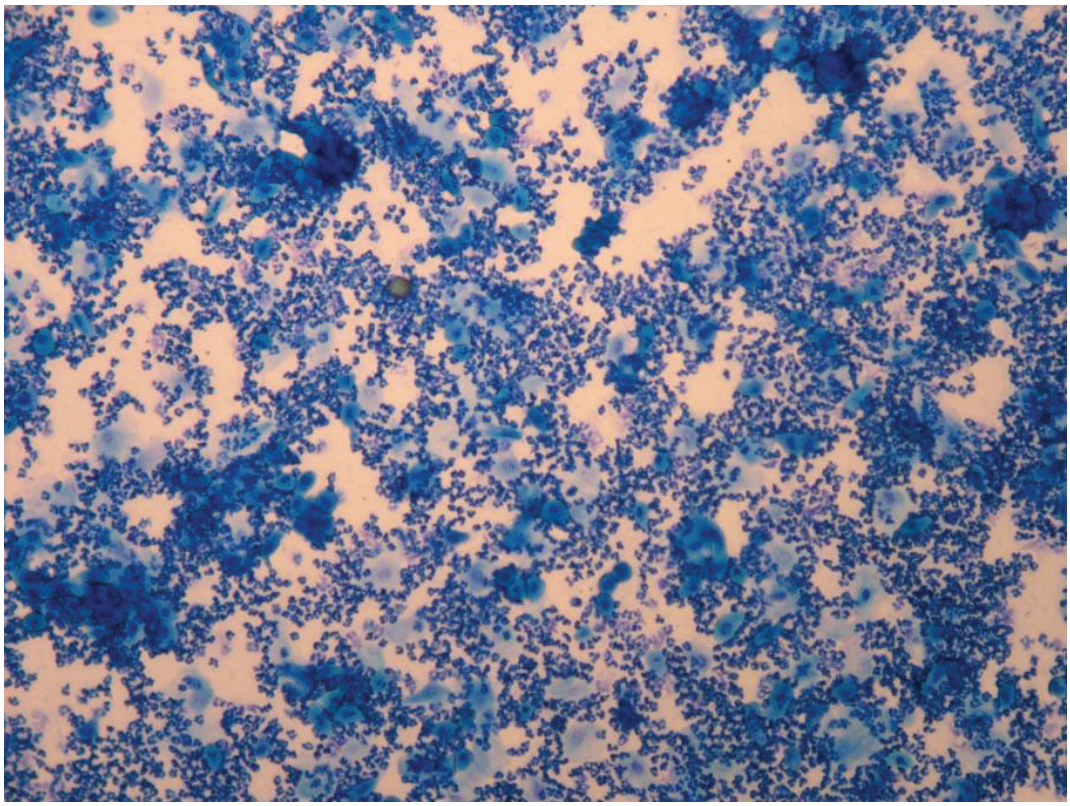

Figure 8: Diestrous phase vaginal smear from D5 animal (400X).

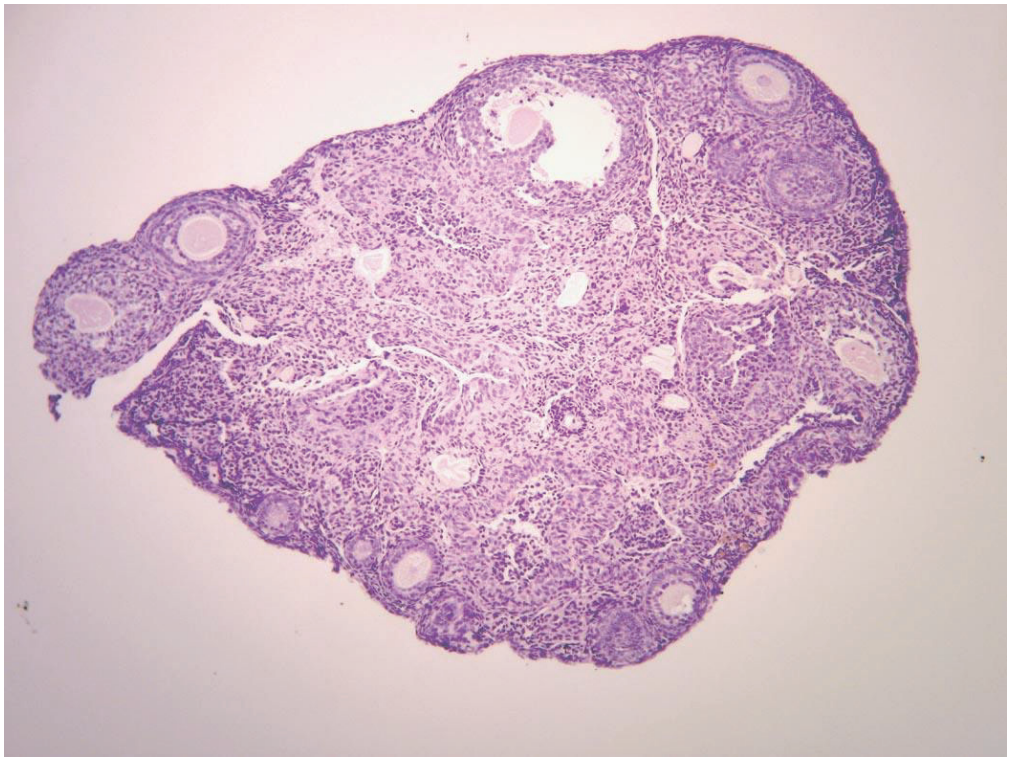

Figure 9: Ovarian section from Treatment Group A (WT + 0.0 Gy), showing early-stage and late-stage follicles (100X).

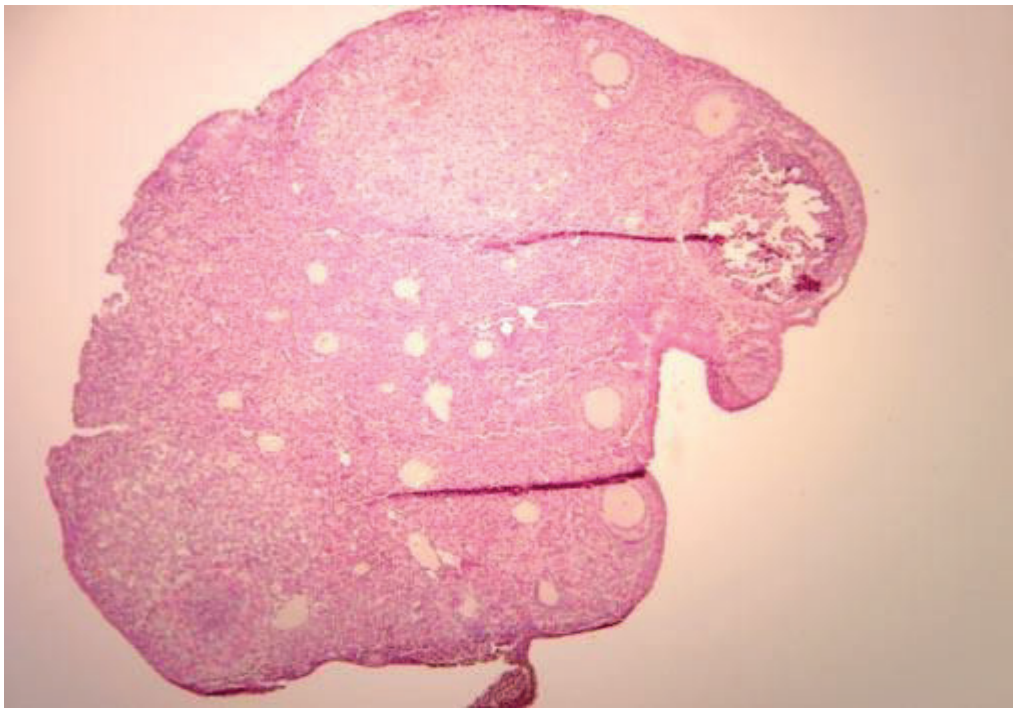

Figure 10: Ovarian section from Treatment Group B (WT + 0.5 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (100X).

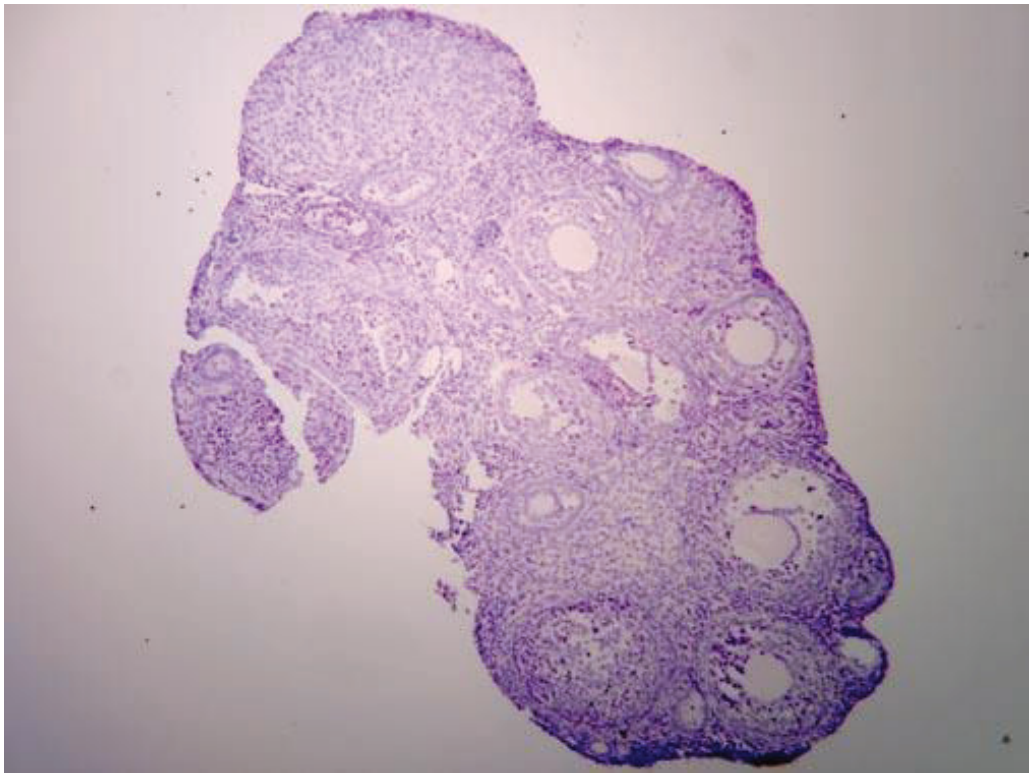

Figure 11: Ovarian section from Treatment Group C (WT + 2.0 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (100X).

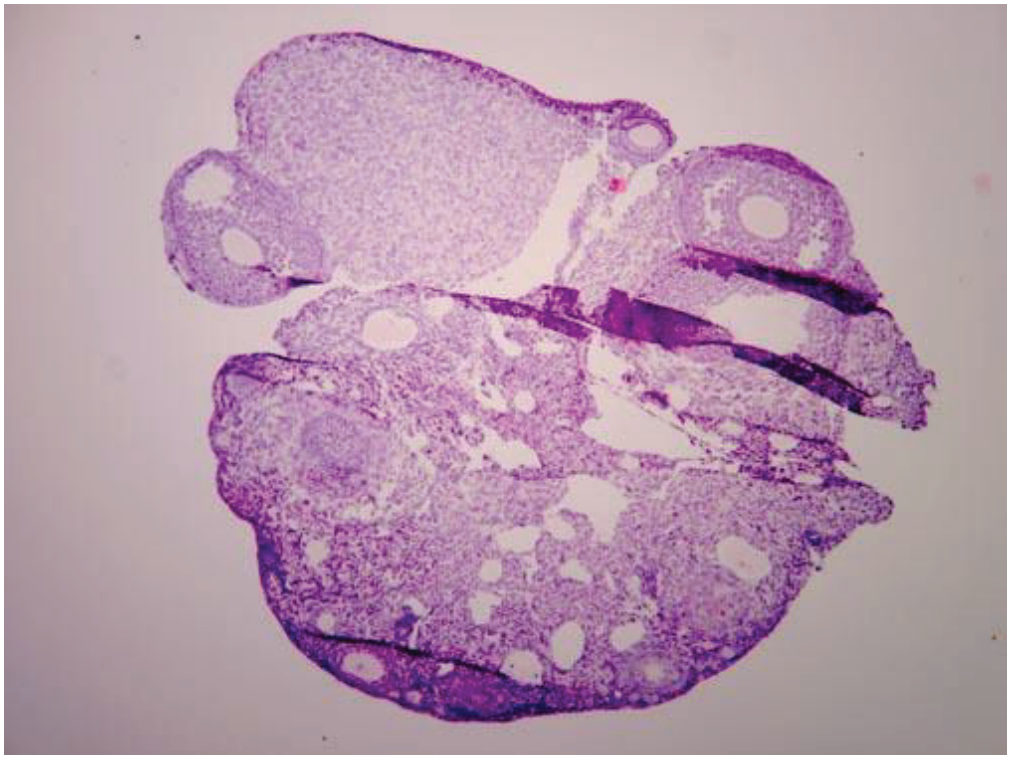

Figure 12: Ovarian section from Treatment Group D (Apo + 0.0 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (100X).

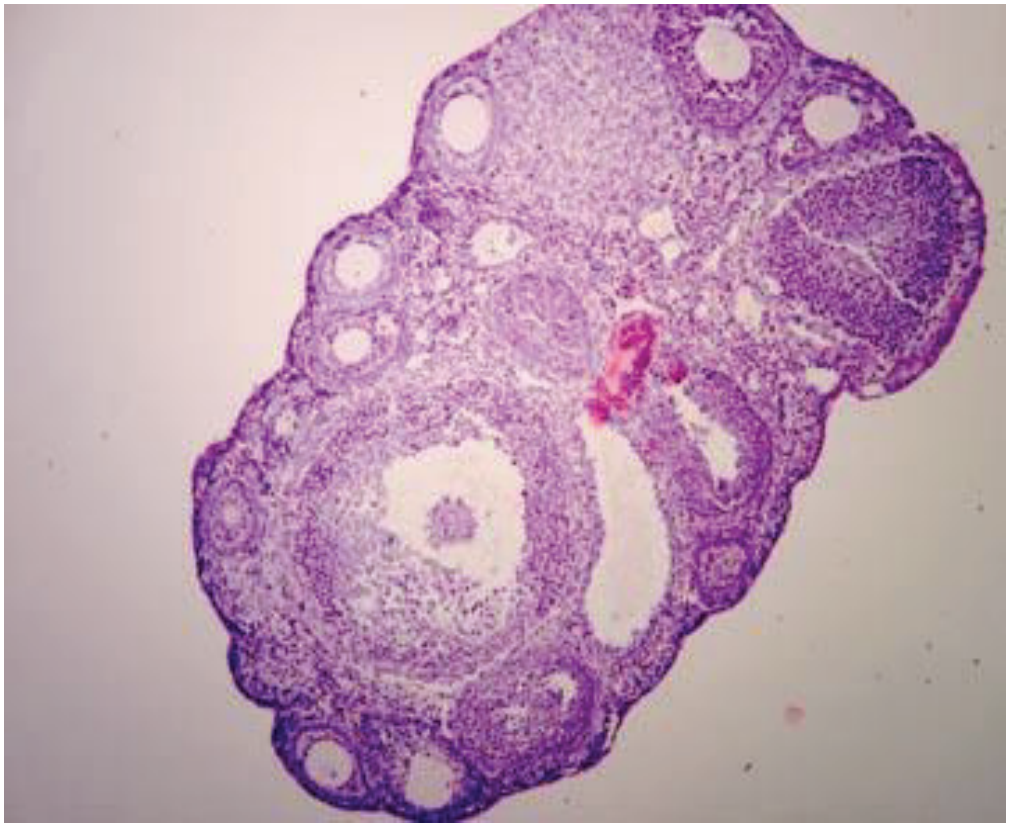

Figure 13: Ovarian section from Treatment Group E (Apo + 0.5 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (100X).

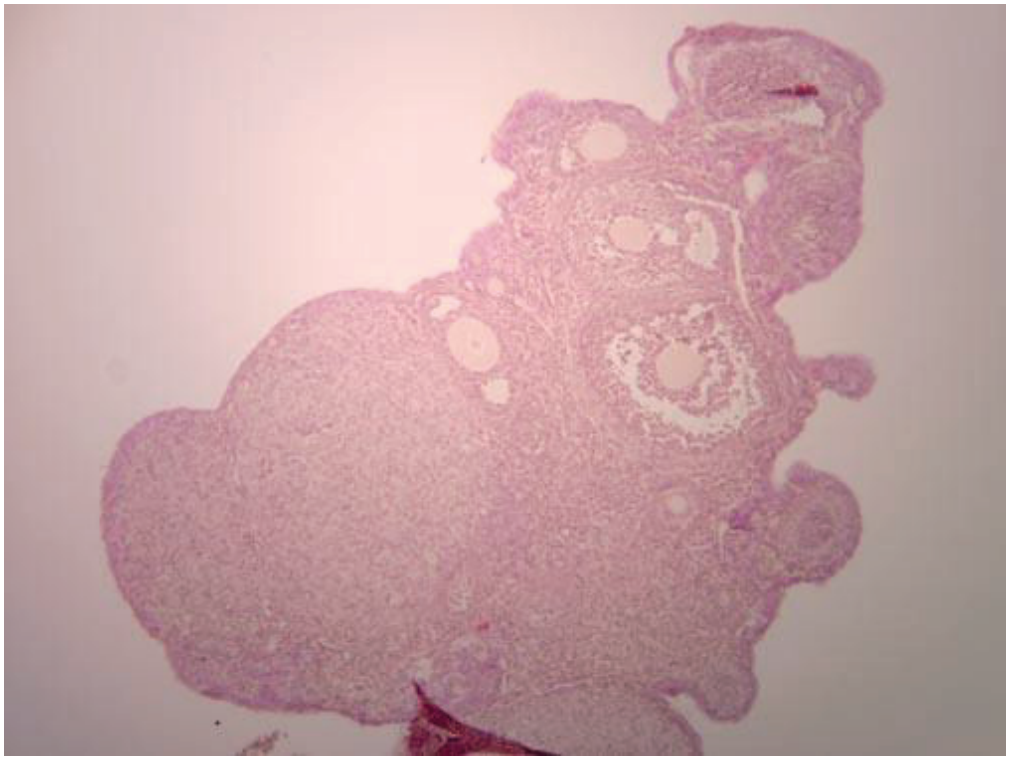

Figure 14: Ovarian section from Treatment Group F (Apo + 2.0 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (100X).

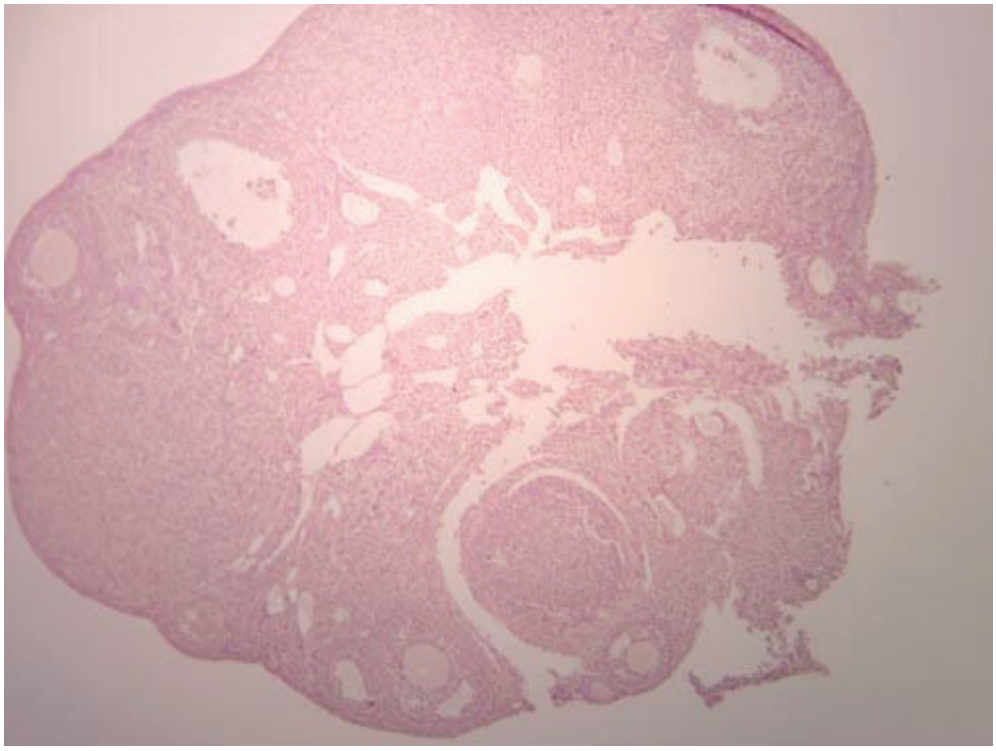

Figure 15: Ovarian section from Treatment Group G (KO + 0.0 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (100X).

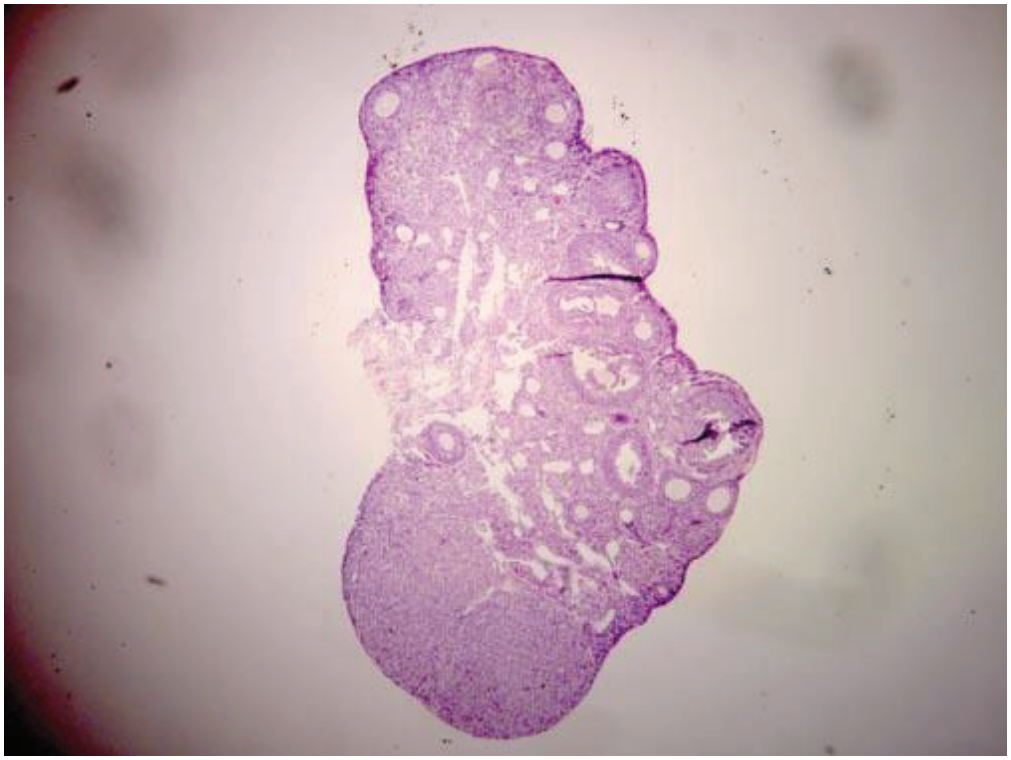

Figure 16: Ovarian section from Treatment Group H (KO + 0.5 Gy), showing early-stage follicles, late-stage follicles, and corpora lutea (50X).

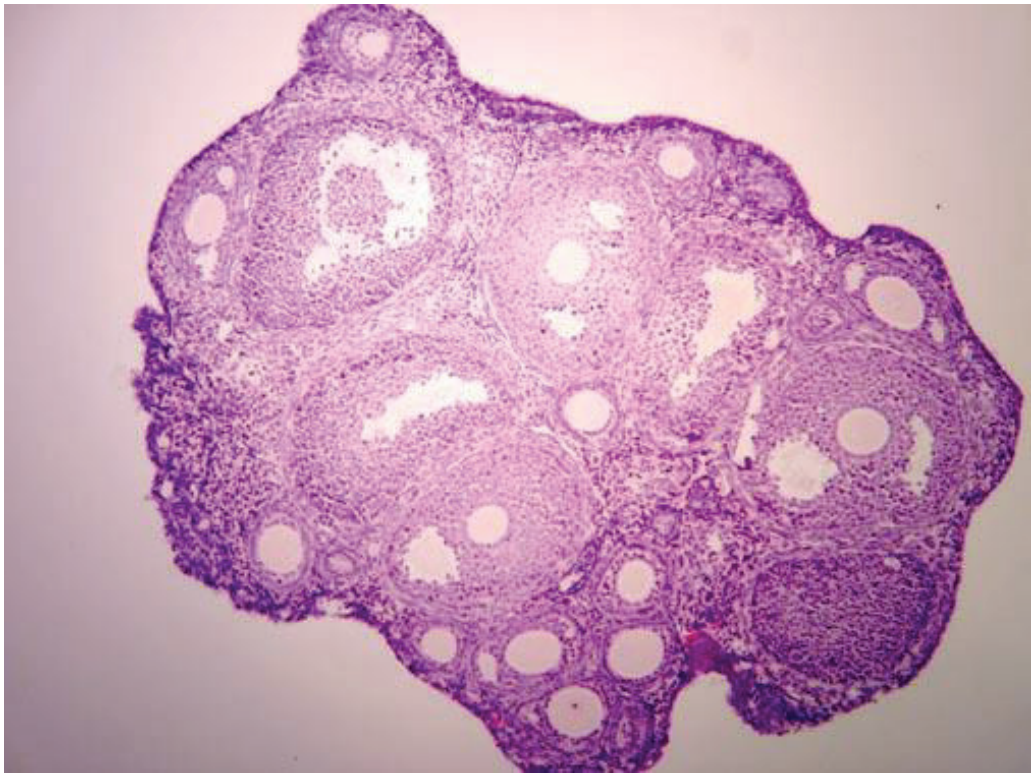

Figure 17: Ovarian section from Treatment Group I (KO + 2.0 Gy), showing early-stage follicles and late-stage follicles (100X).

Wildtype Tissues (uncolored)
Apocynin-Treated Tissues
Knockout Tissues

Ovarian, Uterine Tube, and Composite Reproductive Weight, Control Tissues			
Specimen	**Ovarian Wt. (mg)**	**Uterine Tube Wt. (mg)**	**Total Reproductive Wt. (mg)**
A2	3.1	3.6	105.9
A3	2.0	2.2	26.1
A4	3.3	2.5	50.6
D1	2.7	2.8	98.1
D2	3.2	2.0	75.6
D4	3.6	3.5	115.4
G1	3.5	1.8	68.1
G2	3.8	3.5	159.9
G3	3.1	2.6	108.7

Table 7: Ovarian, Uterine Tube, and Composite Reproductive Weight for Control Tissues (0 Gy)

Ovarian, Uterine Tube, and Composite Reproductive Weight, 0.5 Gy Tissues			
Specimen	**Ovarian Wt. (mg)**	**Uterine Tube Wt. (mg)**	**Total Reproductive Wt. (mg)**
B1	2.6	1.7	84.1
B2	2.9	2.8	50.1
B3	2.1	2.9	58.4
E1	3.3	2.1	57.6
E2	3.3	3.2	102.2
E3	3.9	2.3	58.7
H1	3.0	2.3	138.5
H2	4.3	2.8	132.9
H3	4.5	2.7	77.9

Table 8: Ovarian, Uterine Tube, and Composite Reproductive Weight for 0.5 Gy Irradiated Tissues

Ovarian, Uterine Tube, and Composite Reproductive Weight, 2.0 Gy Tissues			
Specimen	**Ovarian Wt. (mg)**	**Uterine Tube Wt. (mg)**	**Total Reproductive Wt. (mg)**
C1	3.6	2.1	88.9
C2	3.2	1.7	52.6
C3	3.5	1.6	42.5
F1	3.1	2.6	97.0
F2	1.5	2.1	52.0
F3	3.8	1.8	34.3
I1	1.5	2.0	40.9
I2	4.1	2.6	42.6
I4	3.8	0.7	25.5

Table 9: Ovarian, Uterine Tube, and Composite Reproductive Weight for 2.0 Gy Irradiated Tissues

Total Body Weight, Control Tissues	
Specimen	**Total Body Weight (mg)**
A2	17,490
A3	14,460
A4	16,920
D1	16,940
D2	17,250
D4	17,340
G1	18,990
G2	20,500
G3	18,430

Table 10: Total Body Weight for Control Tissues

Total Body Weight, Control Tissues	
Specimen	**Total Body Weight (mg)**
B1	16,970
B2	19,010
B3	16,640
E1	18,900
E2	18,430
E3	16,650
H1	18,820
H2	18,690
H3	17,600

Table 11: Total Body Weight for 0.5 Gy Irradiated Tissues

Total Body Weight, Control Tissues	
Specimen	**Total Body Weight (mg)**
C1	15,850
C2	17,390
C3	16,540
F1	18,810
F2	17,750
F3	18,090
I1	21,090
I2	17,900
I4	17,180

Table 12: Total Body Weight for 2.0 Gy Irradiated Tissues

Ovarian Percent and Total Reproductive Percent of Total Body Weight, Control Tissues		
Specimen	Ovarian Wt. (%)	Total Reproductive Wt. (%)
A2	1.77×10^{-4}	6.1×10^{-3}
A3	1.38×10^{-4}	1.8×10^{-3}
A4	1.95×10^{-4}	3.0×10^{-3}
D1	1.59×10^{-4}	5.8×10^{-3}
D2	1.86×10^{-4}	4.4×10^{-3}
D4	2.08×10^{-4}	6.7×10^{-3}
G1	1.84×10^{-4}	3.6×10^{-3}
G2	1.85×10^{-4}	7.8×10^{-3}
G3	1.68×10^{-4}	5.9×10^{-3}

Table 13: Ovarian Percent and Total Reproductive Percent of Total Body Weight for Control Tissues

Ovarian Percent and Total Reproductive Percent of Total Body Weight, 0.5 Gy Tissues		
Specimen	**Ovarian Wt. (%)**	**Total Reproductive Wt. (%)**
B1	1.53×10^{-4}	5.0×10^{-3}
B2	1.53×10^{-4}	2.6×10^{-3}
B3	1.26×10^{-4}	3.5×10^{-3}
E1	1.75×10^{-4}	3.0×10^{-3}
E2	1.79×10^{-4}	5.5×10^{-3}
E3	2.34×10^{-4}	3.5×10^{-3}
H1	1.59×10^{-4}	7.3×10^{-3}
H2	2.30×10^{-4}	7.1×10^{-3}
H3	2.56×10^{-4}	4.4×10^{-3}

Table 14: Ovarian Percent and Total Reproductive Percent of Total Body Weight for 0.5 Gy Irradiated Tissues

Ovarian Percent and Total Reproductive Percent of Total Body Weight, 2.0 Gy Tissues		
Specimen	**Ovarian Wt. (%)**	**Total Reproductive Wt. (%)**
C1	2.27×10^{-4}	5.6×10^{-3}
C2	1.84×10^{-4}	3.0×10^{-3}
C3	2.12×10^{-4}	2.6×10^{-3}
F1	1.65×10^{-4}	5.2×10^{-3}
F2	8.45×10^{-5}	2.9×10^{-3}
F3	2.10×10^{-4}	1.9×10^{-3}
I1	7.11×10^{-5}	1.9×10^{-3}
I2	2.29×10^{-4}	2.4×10^{-3}
I4	2.21×10^{-4}	1.5×10^{-3}

Table 15: Ovarian Percent and Total Reproductive Percent of Total Body Weight for 2.0 Gy Irradiated Tissues

Early and Late-Stage Follicular Counts, Control Tissues		
Specimen	**Early-Stage Follicles**	**Late-Stage Follicles**
A2	181	33
A3	234	9
A4	174	14
D1	157	35
D2	153	34
D4	191	37
G1	144	19
G2	178	31
G3	167	30

Table 16: Early and Late-Stage Follicular Counts for Control Tissues (0 Gy)

Early and Late-Stage Follicular Counts, 0.5 Gy Tissues		
Specimen	**Early-Stage Follicles**	**Late-Stage Follicles**
B1	112	29
B2	191	15
B3	117	8
E1	71	58
E2	238	46
E3	131	26
H1	150	18
H2	154	20
H3	75	44

Table 17: Early and Late-Stage Follicular Counts for 0.5 Gy Irradiated Tissues

Early and Late-Stage Follicular Counts, 2.0 Gy Tissues		
Specimen	**Early-Stage Follicles**	**Late-Stage Follicles**
C1	103	23
C2	201	37
C3	184	30
F1	197	74
F2	152	39
F3	129	28
I1	167	18
I2	175	15
I4	104	32

Table 18: Early and Late-Stage Follicular Counts for 2.0 Gy Irradiated Tissues

Luteal Counts, Control Tissues	
Specimen	**Corpora Lutea**
A2	6
A3	1
A4	4
D1	5
D2	3
D4	5
G1	4
G2	5
G3	3

Table 19: Luteal Counts for Control Tissues (0 Gy)

Luteal Counts, 0.5 Gy Tissues	
Specimen	**Corpora Lutea**
B1	6
B2	4
B3	5
E1	1
E2	3
E3	4
H1	8
H2	3
H3	5

Table 20: Luteal Counts for 0.5 Gy Irradiated Tissues

Luteal Counts, 2.0 Gy Tissues	
Specimen	**Corpora Lutea**
C1	6
C2	7
C3	8
F1	2
F2	0
F3	2
I1	3
I2	2
I4	0

Table 21: Luteal Counts for 2.0 Gy Irradiated Tissues

Estrous Phase Determination, Control Tissues	
Specimen	**Phase Determination**
A2	Diestrous
A3	Metestrous
A4	Diestrous
D1	Estrous
D2	Metestrous
D4	Estrous
G1	Estrous
G2	Estrous
G3	Estrous

Table 22: Estrous Phase Determinations for Control Tissues (0 Gy)

Estrous Phase Determination, 0.5 Gy Tissues	
Specimen	**Phase Determination**
B1	Diestrous
B2	Diestrous
B3	Estrous
E1	Diestrous
E2	Estrous
E3	Estrous
H1	Estrous
H2	Estrous
H3	Diestrous

Table 23: Estrous Phase Determinations for 0.5 Gy Irradiated Tissues

Estrous Phase Determination, 2.0 Gy Tissues	
Specimen	**Phase Determination**
C1	Proestrous
C2	Diestrous
C3	Estrous
F1	Metestrous
F2	Estrous
F3	Diestrous
I1	Diestrous
I2	Estrous
I4	Diestrous

Table 24: Estrous Phase Determinations for 2.0 Gy Irradiated Tissues