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Medical Ozone Effect on Ovary Damage in Alloxan-Induced
Diabetic Rats

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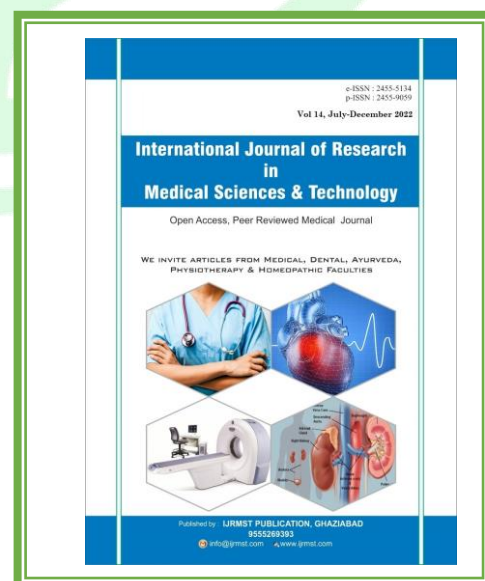
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ABSTRACT

This study aimed to investigate the therapeutic effect of Medical Ozone(MO) in combination with insulin on reproductive hormones and histopathological changes in ovaries of rats with diabetes mellitus (DM). forty eight adult female rats were randomly divided into the following six groups (n = 8): control (A), MO (B), diabetic (C), MO-treated diabetic (E), Insulin-treated diabetic (E), and MO, Insulin-treated diabetic (F). DM was induced by a single intraperitoneal (ip) Alloxan injection (150 mg/kg) .after six weeks of treatment Blood sample were collected to estimate biochemical parameters glucose, serum hormones. Also, ovary samples were obtained for histological examination in diabetic untreated rats the results showed hyperglycaemia, and significant decrease in hormones profiles. While ,MO and Insulin treated rats reported an amelioration of the most toxic effect of alloxan and returned most of these parameters nearly normal. Microscopically ovaries showed definite, degeneration in the diabetic group while when use of MO and Insulin treatment in this study showed significant improves of such histological changes when compared to diabetic untreated rats. Conclusion: it was recommended that the use of the Medical Ozone with Insulin as a supplementary agent to reduce oxidative stress damage of hyperglycaemia and recommended to use variable doses and different periods of treatment to evaluate the best dose and period.

Keywords: *Medical Ozone; Insulin; Alloxan; Diabetic; reproductive hormone*

INTRODUCTION

Ozone (O₃) gas was discovered in the 1840s, and soon after that, the scientific community began to expand past the notion that it was just another gas of the Earth's atmosphere (Elvis and Ekta, 2011). Ozone is a triatomic molecule which is blue in colour and has a characteristic pungent

smell (Santra, 2012). Ozone has been used as a therapeutic agent for treating different diseases (Inal *et al.* , 2011). Ozone has a strong oxidizing power and good antiseptic, disinfectant, and antiviral properties (Eliakim *et al.*, 2001). Furthermore, Ozone has been proposed as an immunomodulator and activator of cellular metabolism, Ozone therapy stimulates the endogenous antioxidant system activities in endotoxic and septic

shock models (Zamora *et al.*, 2005). Medical Ozone (MO) can be precisely produced with a medical generator and may be used proficiently as a real drug (Bocci, 2006a). O₃ has also proven itself beneficial as a disinfectant for drinking water and sterilization of medical instruments (Elvis and Ekta, 2011). The function of O₃ shares similarities to that of a prodrug, as it is modified upon reacting with molecules to create more active substrates, thus stimulating an endogenous cascade of responses. On the other hand, it is hard to classify O₃ as simply a prodrug, due to its capability to directly interact with phospholipids, lipoproteins, cell envelopes of bacteria, and viral capsids. (Zanardi *et al.*, 2016). O₃ therapy administration is variable based on treatment goals and location of therapy (Bocci, 2006a).

Diabetes mellitus is one of the most common endocrine disorders that affect the body's ability to make or use insulin (Mukhtar *et al.*, 2020). DM is one of the common and prominent metabolic diseases that have been diagnosed in canine and feline family after human beings. The clinical features described and investigated are rarely observed in other domestic large animals such as horse, cattle, buffalo, swine, and other small ruminants

(Robinson *et al.*, 2016). Actually in diabetes, the oxidative stress is increased because of the deficiency in the antioxidant defense, so the intake of antioxidant (powerful natural antioxidant) may reduce the oxidative stress associated with diabetes and hence help to restore the antioxidant defense system by reducing free radical (Jawad *et al.*, 2011). Alloxan is considered to be a good preliminary screening model to induce hyperglycaemia in rats. Alloxan is well known for its selective pancreatic islet cell toxicity and has been extensively used to induce diabetes mellitus in animals (Etuk, 2010), (Al-sultan, 2015). Management of diabetes without any side effect is still a challenge to the medical system; this has led to an increasing demand for natural products with antidiabetic activity and fewer side effects (Abed and Azeez, 2018)

MATERIALS AND METHODS

Animal Housing and Animal Grouping

For this experimental study, forty eight adult female rats (180±20g) from animal house of biology department, college science, Thi-Qar University and used them in a completely randomized, all the rats were kept under controlled light and dark condition randomly divided the animals into six equal groups n=8/group:

Group A Animals will daily administer intraperitoneal with citrate buffer (0.1 M, pH 5).

Group B Animals will daily administer 1.1 mg/kg of medical Ozone (MO) intraperitoneal

Group C Animals Untreated diabetic group will induce with single intraperitoneal injections of alloxan (150 mg / kg) in citrate buffer (pH 5) (Mounce & AL-Saeed, 2017)

Group D Diabetic rats will receive 1.1 mg/kg of (MO) intraperitoneal

Group E Diabetic rats will receive the dose of Mixtard insulin will be injected subcutaneously at a dose of 0.75 IU/100 g body weight (Sohair *et al.* , 2014).

Group F Diabetic rats will receive the dose of (MO) 1.1 mg/kg intraperitoneal and Mixtard insulin subcutaneously at a dose of 0.75 IU/100 g body weight

INDUCTION OF DIABETES

The baseline glucose levels were determined 12 hours before the induction of diabetes. Induction of diabetes was done by intraperitoneal injection of alloxan at a

dose of 150mg/kg body weight. The animals were fasted for twelve hours before alloxan was injected. They were left for 72 hours for diabetes to develop, after which blood glucose level were again determined to ascertain that the rats were diabetic.

After 6 week of treatment blood samples were collected from the tail vein used clean centrifuge tubes to separate the serum by centrifugation for 10 min. for 5000 rpm, and the supernatant serum was immediately separated for biochemical analysis. Samples from the ovaries were also taken, stained with Heamatoxylin and Eosin (Drury and Willington,1980). for histological study.

STATISTICAL ANALYSIS

All data of the present study were statistically analyzed by using Microsoft windows EXCEL (version2019) and SPSS version 26, based in using Paired sample t test, One Way ANOVA, Least Significant Difference at p. value < 0.001.

RESULTS AND DISCUSSION

Blood glucose levels

Table (1)shows the serum levels of FSG (mg/dl in all the study groups. As the figure shows, the mean value of serum

FSG the MO-treated rats(Group B) were not significantly different ($P < 0.001$) than the corresponding values in control rats. The table also shows that the mean value of FSG of the diabetic MO treated, Insulin treated and MO and Insulin treated groups were significantly lower ($P < 0.001$) than the corresponding values in diabetic non treated group at same time group F (diabetic treated with MO and Insulin) showed significant decrease when compared to other diabetic group. Alloxan induces diabetes through ROS that leads to a rapid destruction of pancreatic beta cells causing hyperglycemia (Stanely *et al.*, 2000). Hyperglycemia in turn increases the

generation of free radicals by glucose auto-oxidation (Bajaj and Khan, 2012). Administration of ozone (1.1 mg/kg body weight) for 60 days led to a significant decrease in FSG this effect produced by Ozone treatment seems to be associated with the antioxidant properties of Ozone. This is in agreement with Al-Dalain *et al* (2001). found a direct link between the presence of oxidative stress and impaired glucose uptake. In adipocytes, glucose uptake is rapidly decreased in the presence of hydrogen peroxide (H_2O_2), an effect that was reversed by Ozone treatment in preclinical studies

Table (1) Showed blood glucose levels associated with DM in normal, diabetic, MO and Insulin treated Female rats

Groups No. 8	Mean \pm SD
	Glucose mg/dl
Group A	84.8 \pm 3.51 ^e
Group B	84.8 \pm 3.56 ^e
Group C	488.9 \pm 18.5 ^a
Group D	318.0 \pm 35.9 ^b
Group E	203.7 \pm 18.4 ^c
Group F	185.7 \pm 14.5 ^d
p. value of ANOVA	< 0.001*
LSD	18.6

- *indicate the statistic significant at p. value less than 0.01

- The similar small litter indicate a non-significant between two compared means, while the different small letter indicates a significant difference between two compared means within same column

it is of critical importance to maintain the antioxidant potential of the pancreatic cell in order to ensure both its survival and insulin secretory capacity during times of increased oxidative stress. On the other hand, the pancreas is the main target of alloxan. The antioxidant–prooxidant balance, associated with the control of oxidative stress was favored by Ozone treatment, Ozone reduced hyperglycemia and it increased the antioxidant defenses of the pancreas. There is evidence that

hyperglycemia can lower both the activity of a number of antioxidant enzymes presumably by glycation (Yoshida *et al* .,1995). West, (2000) observed that diabetic patients have lowered antioxidant defenses, both enzymatic and non-enzymatic so that increased oxidative damage. Therefore, these results suggest that MO protective effects on antioxidant endogenous defenses improve glucose metabolism

Table (2) Showed some hormones associated with DM in normal, diabetic, MO and Insulin treated Female rats.

Groups No. 8	Mean ± SD			
	FSH	LH	Estrogen	Progesterone
Group A	1.98 ± 0.23 ^{ab}	14.6 ± 1.04 ^b	65.8 ± 3.27 ^b	21.7 ± 0.99 ^b
Group B	2.04 ± 0.50 ^a	16.2 ± 0.34 ^a	80.6 ± 2.75 ^a	25.6 ± 3.00 ^a
Group C	1.39 ± 0.48 ^c	11.1 ± 0.71 ^e	46.3 ± 3.10 ^d	18.2 ± 1.58 ^c
Group D	1.72 ± 0.45 ^b	12.5 ± 0.40 ^c	50.2 ± 2.40 ^c	20.6 ± 1.27 ^b
Group E	1.57 ± 0.23 ^c	11.2 ± 0.50 ^e	48.9 ± 3.44 ^d	20.5 ± 2.00 ^b
Group F	1.98 ± 0.40 ^{ab}	11.8 ± 0.53 ^d	52.3 ± 5.62 ^c	21.2 ± 1.02 ^b
P-value	< 0.001*	< 0.001*	< 0.001*	< 0.001*
LSD	0.32	0.51	2.9	1.46

- *indicate the statistic significant at p. value less than 0.01

- The similar small letter indicate a non-significant between two compared means, while the different small letter indicates a significant difference between two compared means within same column

Based on the results obtained in this study, the serum level of FSH, LH, estrogen and progesterone hormones significantly decreased in the diabetic group (C) in comparison with the control group ($P < 0.001$). At the same time, current study shows a significant increase in the serum level of the above mentioned hormones in the (B group) rats treated with 1.1 mg/kg dose of MO in comparison with the control group ($P < 0.001$).

While with regard to the results of diabetic groups treated with MO (D group) there was a significant increase found in the serum level of FSH, LH, estrogen and progesterone hormones. Groups (E) recorded a non-significant change in FSH, LH and estrogen serum level when compared to group (C) at the same time progesterone recorded significant increase in group (E) compared to group (C). When conducting a statistical comparison between the diabetic groups treated with MO and Insulin and diabetic group the serum levels of all hormones in this study recorded significant increase. As for the results FSH and Progesterone levels of group (F) their reading reached the

normal level of the control group. Khaneshi *et al.*, (2013) found in experimental work on rat that the hyperglycemia resulted in significant reduction in LH and FSH levels, in support for the findings of the present study. Pournaghi, *et al* (2012) concluded that there is a direct relationship

Diabetes mellitus has been shown to suppress reproductive functions in humans and animals (between experimental diabetes with blood glucose level, body weight and pituitary-gonadal axis hormones. In turn, diabetes-related alteration of female reproductive function is caused by the lack of FSH- and LH-signaling (Ballester *et al.*, 2007). Clinical and experimental studies have demonstrated that diabetes causes a defect in the activity of hormonal pituitary-testis axis, causing a reduction in the secretion of gonadotropins (Rato L.). Also, some laboratory studies showed that the lack of insulin among diabetic rats results in a reduced secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Chandrashekar 1991)

Generally, the present study indicates that alloxan-induced diabetes can decrease

serum levels of progesterone it is in accordance with the previous study that induced diabetes could reduce the serum level progesterone in female rats (Ballester *et al.*, 2007). Impaired action of LH on the gonadal organ is a suggested mechanism for decreasing the reproductive hormone levels mainly progesterone from luteal cells (Kiasalari *et al.*, 2009)

In this investigation, the serum Estradiol levels were significantly decreased in untreated diabetic group in comparison with control group. According to a report, estradiol is associated with diabetes mellitus and altered glucose tolerance. Ballester *et al.*, 2007) It has been reported that women with type I diabetes showed reduction (Salonia *et al.*, 2006) in circulating estradiol levels compared to non-diabetic women. Experimental streptozotocin-induced diabetic rodents, have shown a more consistent hormonal profile characterized by reduced estradiol levels in females. (Kim *et al.*, 2006). Oxidative stress is increased in the diabetic condition due to overproduction of reactive oxygen species and decreased efficiency of antioxidant defenses (Martin-Gallan *et al.*, 2003). Moreover, oxidative stress can generate by hyperglycemia (Ceriello and Motz, 2004). The increased hydroperoxide level in diabetic animals

can be attributed to hyperglycemia. The increase of lipid peroxide level in diabetic rats suggests that increased generation of free radicals by hyperglycemia related glucose auto-oxidation (Alhazza 2007). Free radicals have a direct toxic effect on the tissues (Sener *et al.*, 2005). The alloxan administration produced marked oxidative stress as evidenced by a significant increase lipid peroxidation as well as a significant decrease antioxidants (El-Missiry, 1999)

Ozone is formed by the combination of three oxygen atoms, and when used in appropriate concentrations, causes an enhancement in antioxidant enzymes, and increases tissue oxygenation and neoangiogenesis (Bocci, 2006 b). Based on this knowledge, Al-Dalain *et al.*, (2001) postulated that controlled Ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, thereby inhibiting the damage induced by reactive oxygen species. Moderate oxidative stress activates nuclear factor. (Sagai & Bocci, 2011)

The efficacy of MO therapy in diabetic mellitus has been attributed to its hypoglycemic effect, induction of antioxidant enzyme activities and control of their expression (Morsy *et al.*, 2010).

Rodriguez *et al.* (2009) found that controlling hyperglycemia in diabetic patients with Insulin or other hypoglycemic agent reduces oxidative stress-induced DN complication in diabetes patients. This explains the results of the current study, where the results of the diabetic group treated with MO showed a significant improvement in hormone levels. Both MO and Insulin may act synergistically to reduce ROS production while stimulating antioxidant enzymatic activities to the control levels. Both of them independently exert hypoglycemic effects (Al-Dalain *et al.*, 2001). An antioxidant-based therapy combined with glucose control will give patients more of advantage and lessen the chance of complications with diabetes (Rubin and Mordecai, 2001), this was observed through the current study through group (F) especially the results of the FSH and Progesterone hormones, whose levels were close to the normal levels of the control group.

The histopathology of rat ovary was shown in (Fig.1-6) Microscopic investigation of ovary sections of control rats showed the normal appearance of the ovary indicating normal structures, where several primary, secondary, and graafian follicles, as well as normal corpus luteum

(Fig. 1). At same time The results of group (B) showed normal structure of ovary, where many developing follicles, and corpus luteum were evident. (Fig. 2). However, The (C) group Section of ovary showed pathological changes characterized by the presence of a sparse number of follicles at the early stages of proliferation, and a lack of corpora luteum, and many congested blood vessels (Fig. 3)

On the other hand, The result of group (D) the ovarian section showed remarkable amelioration ovary showed graafian follicle, developing follicles and atretic follicles also presence of corpus luteum (Fig.4 . while, group (E) Section of ovary showed degenerated follicles and congested blood vessels (Fig.5). The group (F) showed several primary, secondary, and graafian follicles, as well as normal corpus luteum .(Fig.6)

Previous studies show that diabetes has negative effects on the growth of the ovarian follicles and decreases the rate of fertility, but the exact mechanisms are unclear (Chang *et al.* ,2005, Wu *et al.*, 2015). Alterations of glucose concentration can greatly affect some functions of reproductive system (colton *et al.*, 2002). For example, in streptozotocin (STZ)-induced diabetic rats,

high level of glucose reduces the production of progesterone and estradiol in granulosa cells and causes reproductive disorders due to changes in folliculogenesis and steroidogenesis (Chabrolle *et al.*, 2008) This explains the histopathological changes of the ovaries and the accompanying decrease in the serum levels of the progesterone and estrogen hormones of diabetic groups

Alloxan induces free radicals which play a relevant role in the etiology and pathogenesis of both experimental and human diabetes mellitus (soto *et al.*, 2004). Medical Ozone is a very reactive gas and is well known as a potent oxidant (Bocci *et al.*, 2001) However, recent studies have clearly shown that a small and precisely (MO) dose can induce up regulation of antioxidant enzymes (Bocci *et al.*, 2009). Further, other results have confirmed that neither structural nor enzymatic cell damage occurs if an appropriate Ozone therapeutic window is used (Travagli *et*

al., 2007). These findings support our hypothesis that MO has ovary protective role against oxidative damage in diabetes mellitus at same time current study agreement with a study conducted by Güçlü *et al* (2016), it was concluded ozone therapy significantly improves the histopathological changes induced by high glucose level in diabetic nephropath

CONCLUSION

This study explored the possible mechanisms by which Medical Ozone may improve oxidative stress levels and ovary antioxidant system in experimental diabetic rats. So Medical Ozone therapy may be considered as an adjuvant to insulin in the treatment of diabetes to prevent or alleviate diabetes induced pathological changes in ovaries. This opens the way for long-term studies to confirm the beneficial effects of ozone administration in diabetic animal models

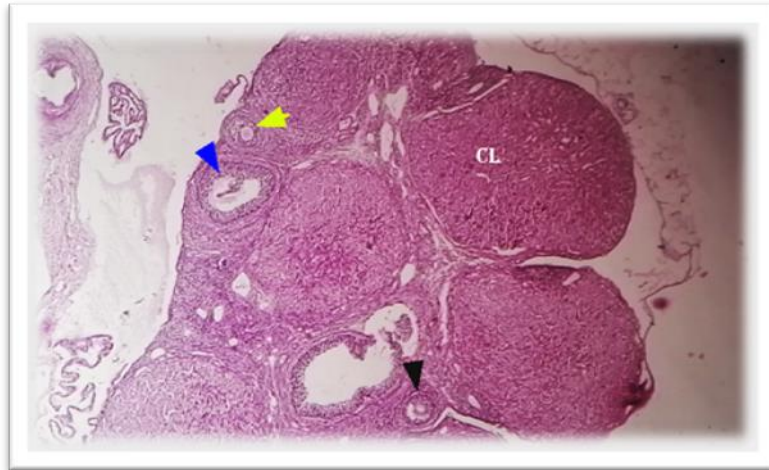


Fig. 1 (group A). Section of ovary showing several primary (yellow arrow), secondary (blue arrow), and graafian follicles (black arrow), as well as normal corpus luteum (CL). H&E, 40X

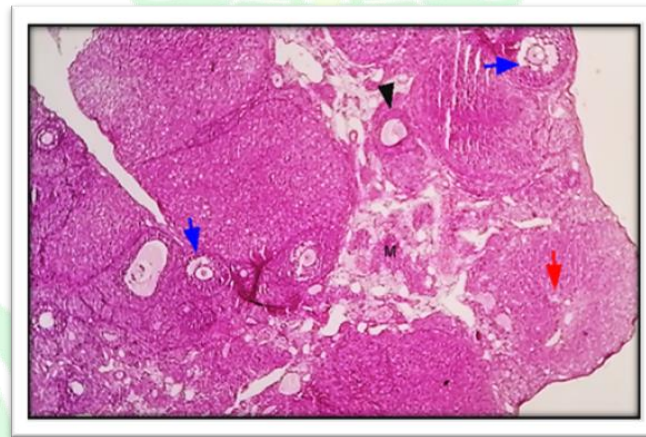


Fig.2 (group B) Section of ovary shows graafian follicles (blue arrow), corpus luteum (red arrow), and medulla (M). H&E, 100x

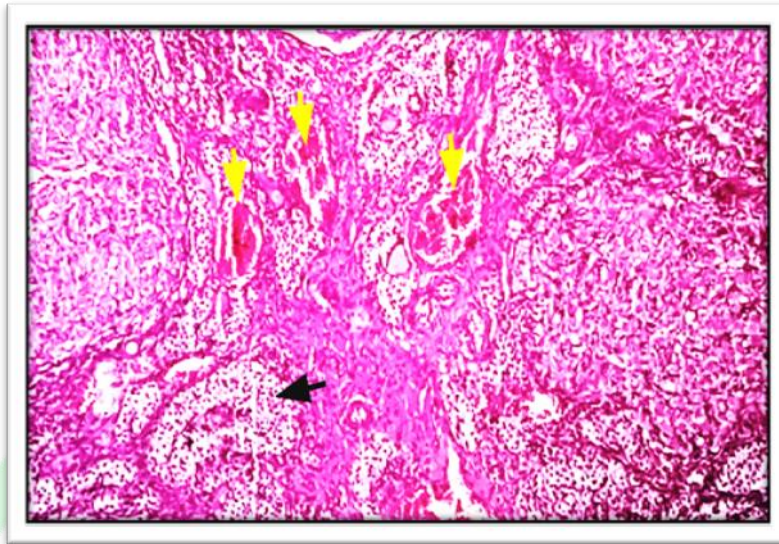


Fig.3 (group C) Section of ovary is characterized by the presence of a sparse number of follicles (black arrow) at the early stages of proliferation, and a lack of corpora luteum, and many congested blood vessels (yellow arrows). H&E, 100X.

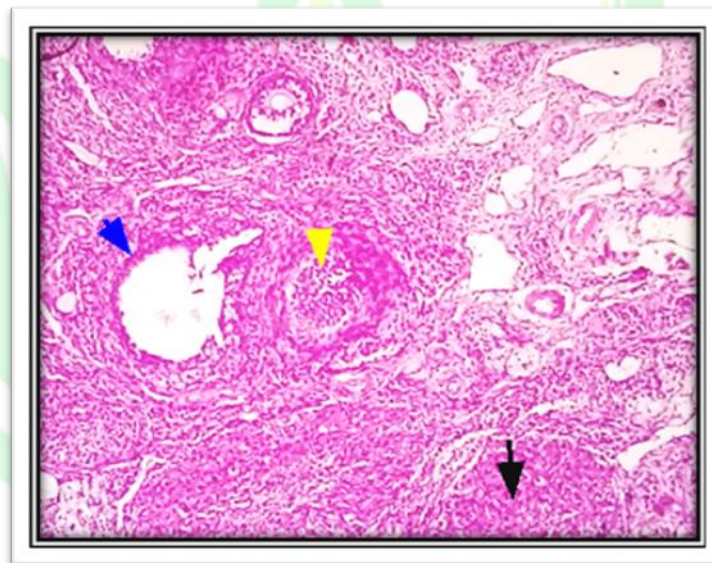


Fig. 4 (group D). Section of ovary shows developing follicles (yellow arrow) and atretic follicles (blue arrow), and corpus luteum (black arrow). H&E, 100X.

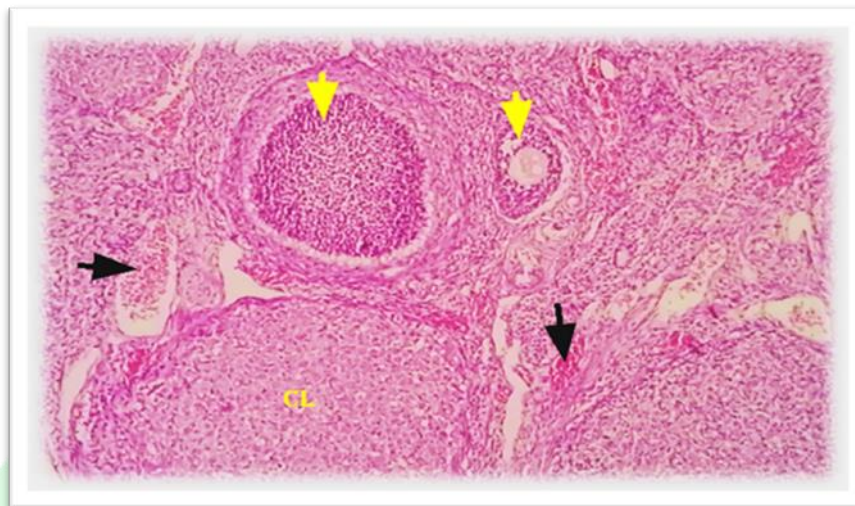


Fig.5 (group E). Section of ovary showing degenerated follicles (DF) and congested blood vessels (black arrow). H&E, 40X.

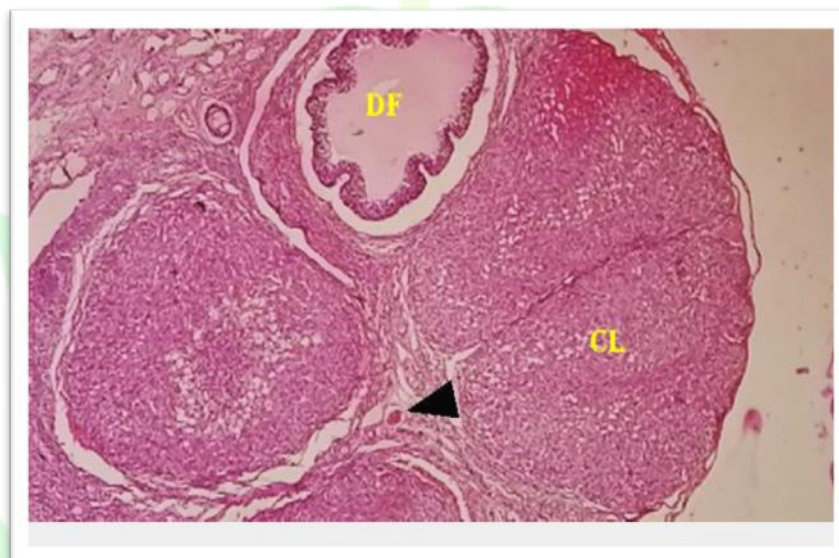


Fig.6 (group F) . Section of ovary showing several primary (yellow arrow), secondary (blue arrow), and graafian follicles (black arrow), as well as normal corpus luteum (CL). H&E, 40X.

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Conflict of Interest: None

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