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Research Article

Histological and ultrastructural study of the effect of Isoxicam on Ovary and possible protective role of Melatonin in albino rat

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Abstract

Background: Isoxicam is a member of nonsteroidal anti-inflammatory drugs (NSAIDs) that reduces pain, decreases fever, prevents blood clots, and in higher doses decreases inflammation. **Aim of the work:** The aim of this work is to investigate the harmful effect of Isoxicam on the ovary and the possible antagonistic effect of Melatonin. **Material and methods:** Isoxicam were obtained from Beecham pharmaceutical, Cairo, Egypt. Fifty-four albino rats were used throughout the study. The rats were assigned into three groups eighteen female albino rats in each group. **Group I:** Received saline intraperitoneally. **Group II:** The rats were injected with 0.7gm/kg isoxicam intraperitoneally for 20 days. **Group III:** The rats were injected with 0.7mg/kg isoxicam + 10mg/kg melatonin intraperitoneally for 20 days. Ovaries were removed and prepared for histological and ultrastructural analyses. **Results**: Isoxicam induced marked histopathological ovarian changes that were remarkably ameliorated by the prophylactic use of Melatonin. **Conclusion:** This work revealed a prophylactic role of melatonin in isoxicam induced ovarian damage. Thus, melatonin administration is recommended for female patients when isoxicam is used for long time to avoid ovarian damage.

Keywords: Isoxicam, Melatonin, Ovary

Introduction

Isoxicam is a member of the oxicam family, and its pharmacological action is related to the inhibition of the cyclo-oxygenase enzyme that is involved in the prosta-glandin biosynthesis process. This enzyme has been implicated as a regulator of several physiological processes in the human body, including inflammatory processes that are involved in the immune response, pain perception, and fever [1].

Melatonin has the capacity to inhibit the generation of reactive species and may serve as a free radical scavenger. Melatonin also has anti-inflammatory properties. Melatonin is effective in reducing the negative effects of oxidative stress because it increases gene expression in other antioxidant enzymes [2].

In this research, we want to evaluate the impact that administration of isoxicam has on the ovary as well as the potential protective effects that administration of melatonin has.

Material and Method

Isoxicam as vials were obtained from Beecham pharmaceutical, Cairo, Egypt. The drug was given intraperitoneally as 0.7gm/kg for 20 days[3] to the isoxicam group**.** Melatonin (Sigma-Aldrich, Cairo, Egypt) was co-administered with isoxicam intraperitoneally at a dose of 10mg/kg for 20 days [4].

Animals and treatments:

The Ethical Committee of Minia University **(Approval no. 693:12/2020)** gave its consent to the experiment. Fifty-four adult

female albino rats were brought from the Minia University, Laboratory Animals Unit, and were maintained in appropriate environment. Their weights were between 200-250g.

Group I (control): received normal saline intraperitoneally (0.9mg/L).
Group II (Isoxicam): were injected with 0.7gm/kg[3] intraperitoneally for 20 days.
Group III (Isoxicam-Melatonin): were injected with 0.7mg/kg Isoxicam + 10mg/kg melatonin [4] intraperitoneally for 20 days**.**

Histological Study: At the conclusion of the twentieth day, the animals were given chloroform anesthesia, the peritoneal cavity was opened, and both of each animal's ovaries were taken for histological evaluation. This process took place at the end of the study. after the tissue had been fixed in formalin at a concentration of 10 percent for 14 to 16 hours. After being dehydrated in an increasing concentration of alcohol (from 20 to 100 percent) for 45 minutes in each, the sample was after wards clarified in xylene alcohol (50:50), exelon (three times), and embedded in paraffin. After being sectioned with a microtome to a thickness of 5 micrometers and placing the sections on slides, the samples were sliced. After deparaffinization of these parts and yielding with an increasing grade of alcohol, the results were as follows: (100-20 percent). Some of the slices were clustered together in preparation for staining with hematoxylin and eosin, Van Gieson stain, and periodic acid Schiff stain.

Transmission electron microscopy (Ultrastructure) study: ovaries were cut into 1mm² pieces and fixed for 4h at 4c° in glutaraldehyde, fully washed in PBS, post fixed in 2% osmium tetroxide for 2h in 4c°, and finally dehydrated in ascending grade of alcohol, embedded in Epon 812, cut ultrathin sections (75nm) and stained with uranyl acetate and lead citrate. Sections were examined with electron microscope.
Morphometric analysis: The photos of slides enter into image j program to estimate the discolored area and its density [5].

Results

By Hematoxylin and Eosin (H&E) stain:
Group I (Control group) Sections showed an intact ovarian surface (germinal layer), tunica albuginea contains graffian follicles that surrounded by fibro vascular layer (theca folliculi), lined by outer cell layer (granulosa cells), follicular cavity and a group of cells connect the granulosa cells with the oocyte (cumulus oophorous). The oocyte appeared to be surrounded by homogenous membrane (zona pellucida) and columnar cells (corona radiata) **(Fig. 1-A).**

Group II (Isoxicam group) Sections show degenerated germinal cells on the surface the ovary, tunica albuginea contains unhealthy graffian follicles that detached from surrounded stroma with deposition of collagen fiber around it. Granulosa cells and oocyte were destructed **(Fig. 1-B).**

Group III (Isoxicam and melatonin group) show restoration of some normal architecture of ovary was observed. Intact ovarian surface (germinal epithelium layer) some healthy graffian follicle showed apparently healthy architecture with secondary oocyte that surrounded by zona pellucida, corona radiata, antrum and theca cells (stromal cells) outside the granulosa cells that formed of theca externa and theca interna **(Fig. 1-C).**

By Periodic Acid Schiff (PAS) stain:
1- Group I (Control group): A PAS-stained segment of the control rat's ovary showed a significant positive PAS response in the zona pellucida of the ovarian follicles and a modest level of reactivity in the healthy ova **(Fig. 2-A)**.
2- Group II (isoxicam group): A cystic follicle with deteriorated zona pellucida and one oocyte was seen in the isoxicam group when the slice was stained with PAS **(Fig. 2-B).**
3- Group III (isoxicam – melatonin groups the PAS-stained slice for the isoxicam and melatonin group showed a moderately positive PAS response in the zona pellucid of the ovarian follicle, with just a week's worth of ovarian responsiveness **(Fig. 2-C).**

By Van Gieson stain:

1- Group I (Control group): Presence of few collagen fibers mainly around the graffian follicle **(Fig. 3-A)**.

2- Group II (isoxicam group): An extensive deposition of collagen fibers around the graffian follicle **(Fig. 3-B)**.

3- Group III (isoxicam – melatonin group): Less deposition of collagen fibers around the graffian follicle **(Fig. 3-C)**.

B) Ultrastructural study

1- Group I (Control group): The control group's rat ovaries had normal granulosa cells and normal nuclear morphology. There was no extension into the perinuclear area, and the structure of the double nuclear membranes was continuous. Chromatin was equally distributed throughout the nuclei. In addition to this, the mitochondrial structure was normal inside the cell's cytoplasm, **(Fig. 4-A)**.

2- Group II (isoxicam group): The distorted granulose cells, decreased number of organelles with vacuolated cytoplasm, condensation and margination of the nuclear chromatin, mitochondrial enlargement, and degraded mitochondrial cristae were seen in the rat ovaries from the isoxicam group, **(Fig. 4-B)**.

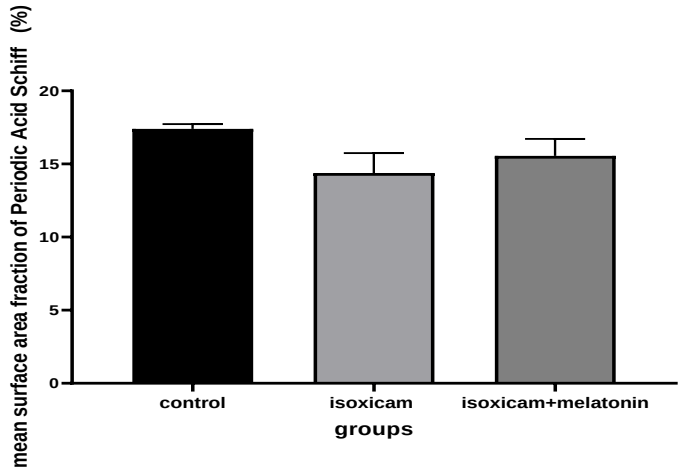
3- Group III (isoxicam and melatonin group): The rat ovaries that were given isoxicam and melatonin showed increased mitochondria in what appeared to be normal granulosa cells, as well as many cristae (with some destroyed) in part of the mitochondria. Additionally, there was less condensation and margination of nuclear chromatin, as well as less vacuolated cytoplasm in this group, **(Fig. 4-C)**.

Statistical analysis:

The mean surface fraction area ± standard deviation (SD) was used to compare the Periodic Acid Schiff, Van Gieson positive stained ovarian tissues in the three groups by One Way ANOVA test.

Table 1: displaying the mean surface area fraction amounts of PAS-positive.

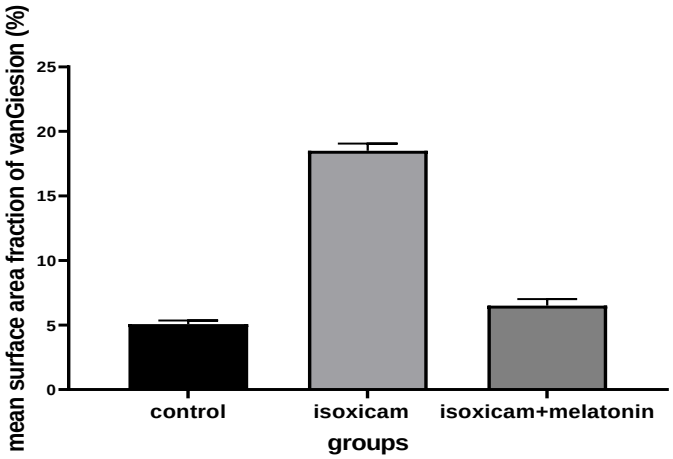
Group / parameter	Group 1 (Control)	Group 2 (isoxicam)	Group 3 (isoxicam+melatonin)
mean ± SD	17.40±0.3225	14.38± 1.361	15.55± 1.164
P- value		*0.0004	*0.0038 **0.0411



- The results are significant at *P ≤ 0.05, * comparison with control, ** comparison with isoxicam group
- Bar chart shows the percentage of glycoproteins between different groups. There was decrease in the level of glycoproteins materials present in the isoxicam group.

Table 2: displaying the mean surface area fraction amounts of Van Gieson's stain.

Group / parameter	Group 1 (control)	Group 2 (isoxicam)	Group 3 isoxicam+melatonin
mean ± SD	6.100 ±0.6603	14.60 ± 0.5477	7.500 ± 0.2898
P-value		*0.0001	*0.0008 **0.0022



- The results are significant at *P ≤ 0.05, * comparison with control, **comparison with isoxicam group.
- Bar chart shows the percentage of collagen fibers (fibrosis) between different groups. A high level of fibrosis presents in the isoxicam group**.**

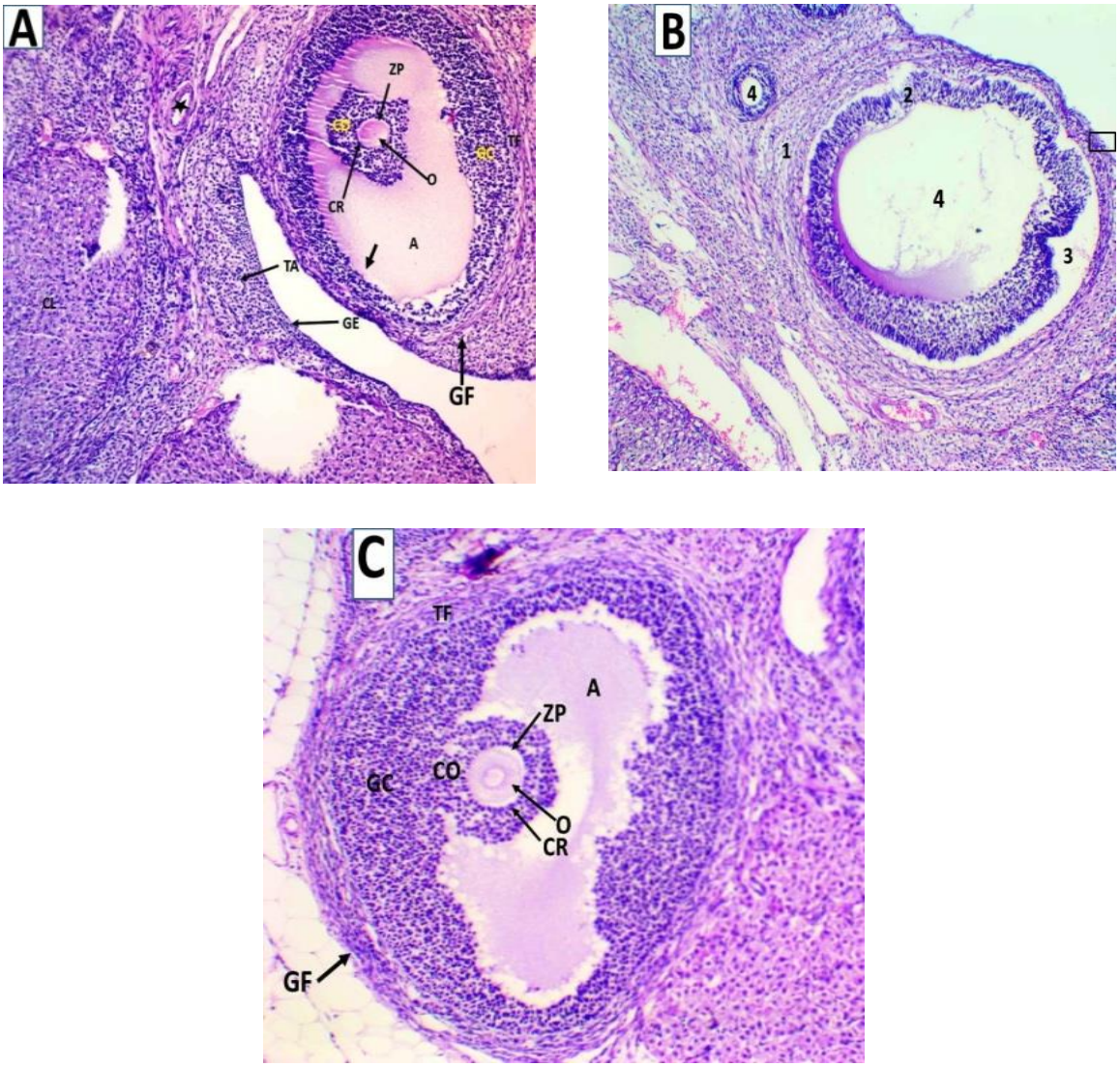


Fig. 1 (A, B, C): photomicrographs of ovarian tissue stained with Hematoxylin and Eosin of control group (A), isoxicam group (B) and Isoxicam and melatonin (C). Showing ordinary normal structure of ovarian tissue germinal epithelium (GE), tunica albuginea (TA), corpus luteum (CL), mature graafian follicle (GF) formed of granulosa cell (GC), theca folliculi (TF) and oocyte (O) surrounded by zonapellucida (ZP), corona radiata (CR), cumulus oophorous (CO) and follicular antrum (A) and blood vessels (star) in figure A and C. In the figure B, there are degenerated graffian follicle showing degeneration of germinal epithelium (square) with thickening of stromal collagen fibers (1) around ovarian follicle, degeneration of granulosa cells (2) and detachment of them from surrounded stroma (3) and absence of oocyte (4) (H &E X 100).

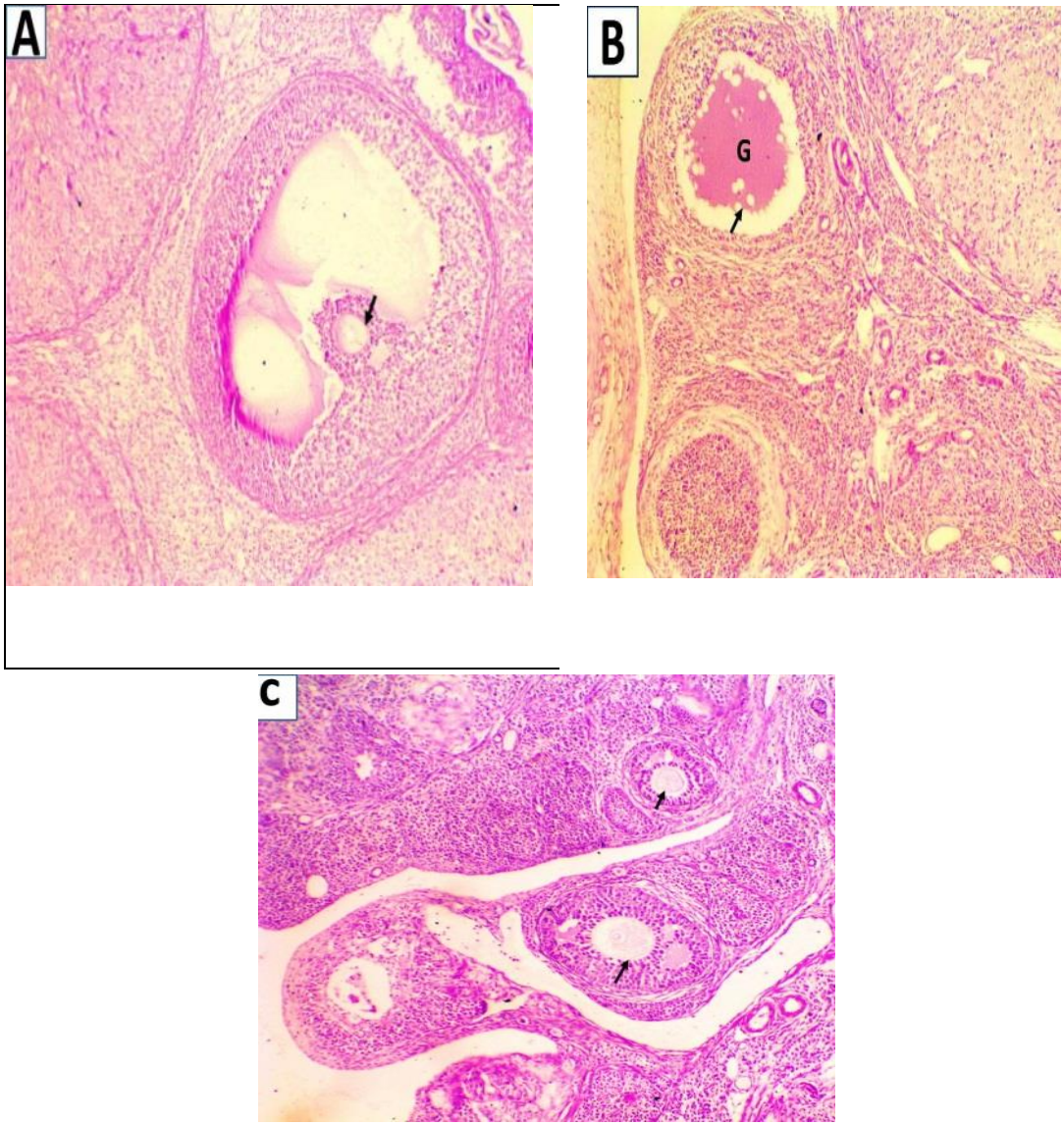


Fig. 2 (A, B, C): photomicrographs of ovarian sections that have been stained with PAS. A PAS-stained segment of the ovary from a control rat shows a significant positive PAS response in the zona pellucida (arrow) of the ovarian follicle, whereas the healthy ova show only mild reactivity (A). A cystic follicle with damaged zona pellucida and degraded oocytes can be seen in this PAS-stained slice from the isoxicam group (B). PAS-stained slice for the isoxicam and melatonin group demonstrating a moderately positive PAS response in the zona pellucida (arrow) of the ovarian follicles and a mild reactivity of the ovary (C) PAS X 100**.**

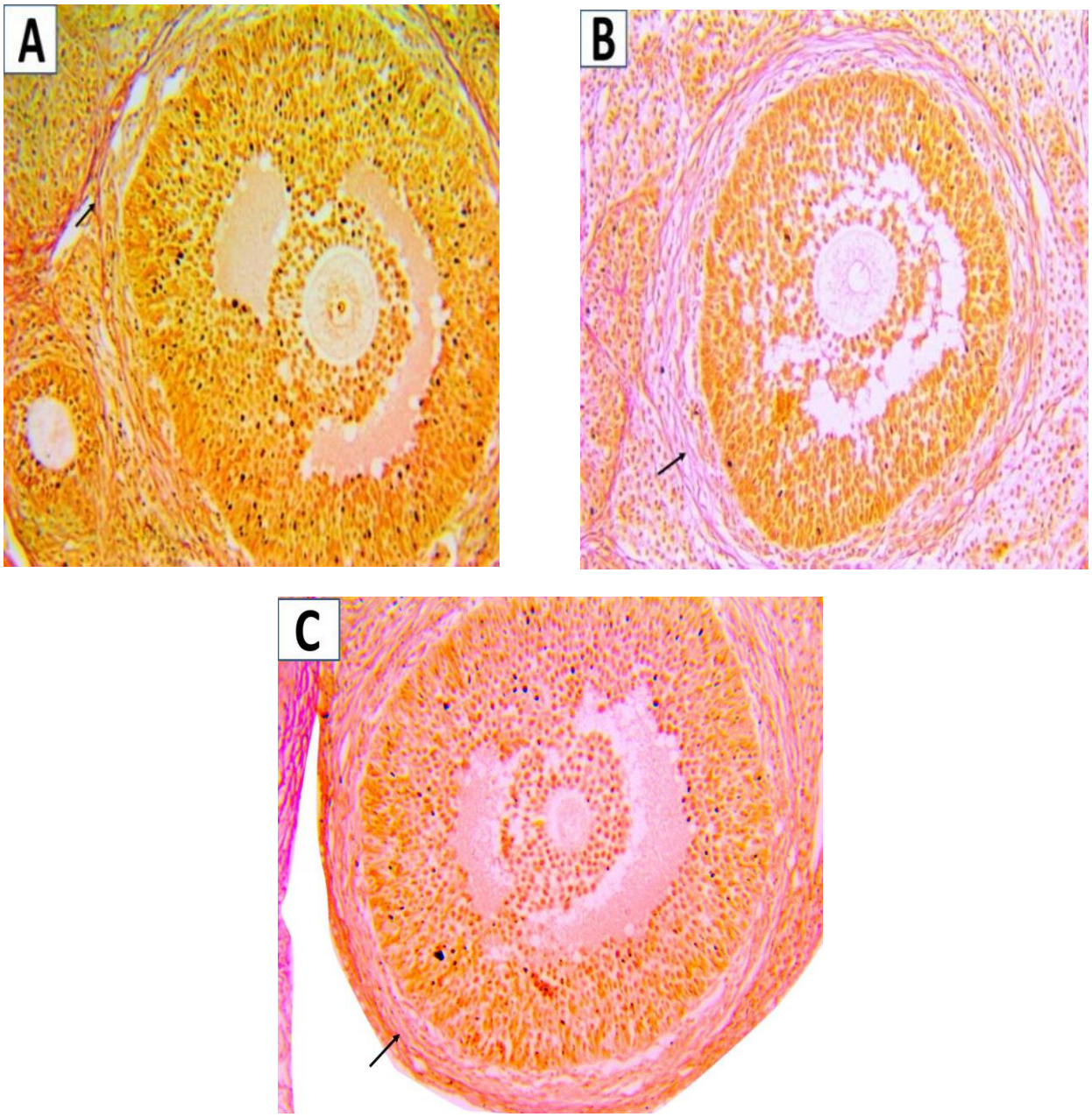


Fig. 3 (A, B, C): photomicrographs of van Gieson-stained section for control rats revealing presence of few collagen fibers mainly around the graafian follicle(arrow) (A). Isoxicam group showing an extensive deposition of collagen fibers mainly around the graafian follicle (arrow) (B). Isoxicam and melatonin group revealing slightly increase deposition of collagen fibers than control group around the graffian follicle (arrow) (C), **X200**.

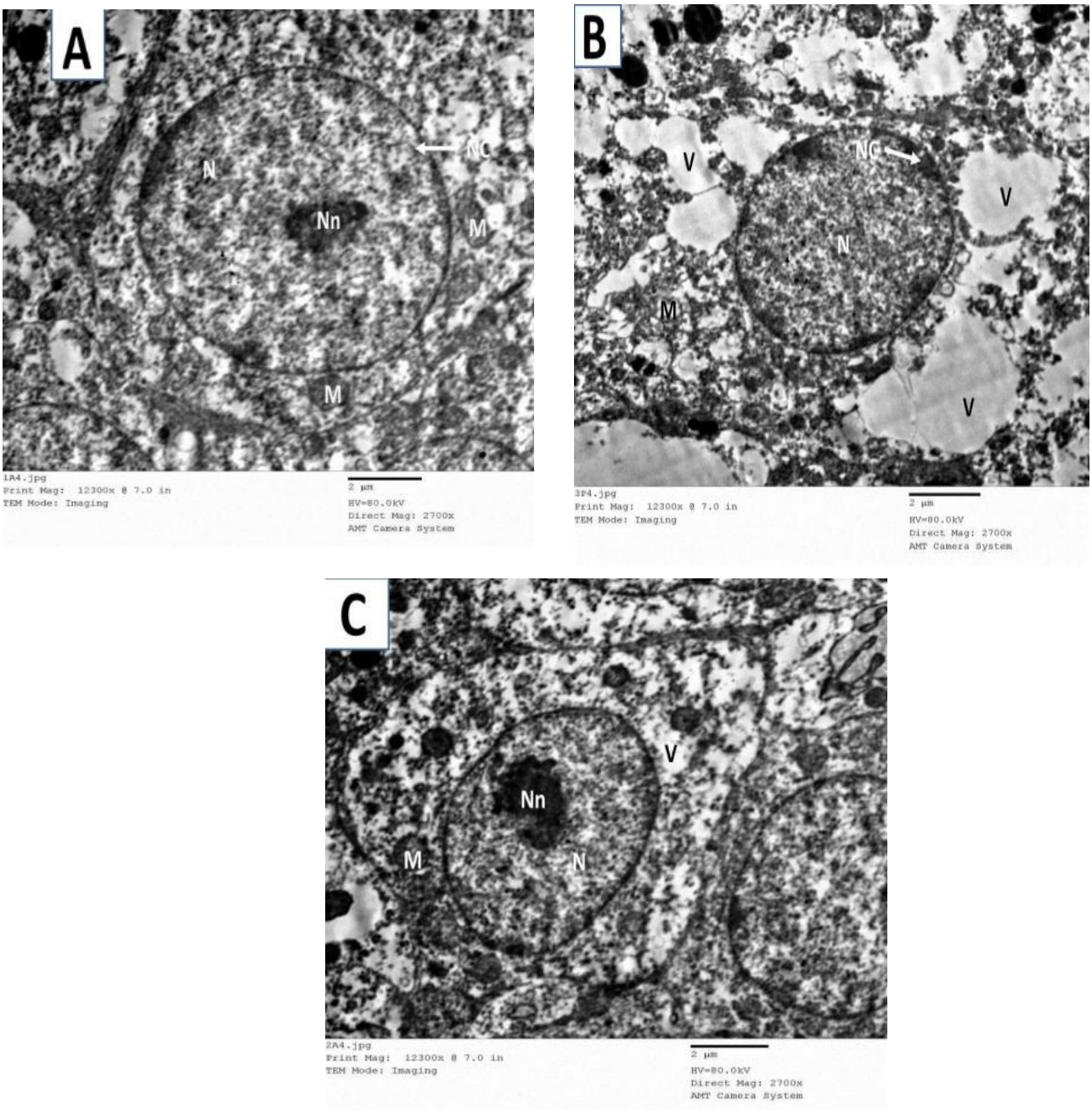


Fig. 4 (A, B, C): An electron micrographs of the follicular cells of control group (A)Note the presence of nucleus (N), nucleolus (Nn) with euchromatin distribution (NC) and mitochondria (M), treated with isoxicam group (B) nucleus (N)with heterochromatin (NC) clumps near the nuclear membrane, swollen mitochondria (M) and surrounded by vacuolated areas (V) in between and within the cells and treated with isoxicam-melatonin group (C) apparently normal follicular cells with nucleus (N), nucleolus (Nn) and less condensated nuclear chromatin. There are many mitochondria (M) that appears with many cristae and less ballooned in shape. Also, there is less vacuolated (V) cytoplasm, X2700.

Discussion

The purpose of the current investigation was to illustrate the effects of the nonsteroidal anti-inflammatory drug (NSAID) isoxicam after it had been administered intra-peritoneally to mice for a period of 20 days. Isoxicam is a member of the oxicam family of drugs, and its pharmacological effects are associated with the suppression of the cyclo-oxygenase enzyme that is responsible

for the manufacture of prostaglandins at the site of inflammation. As a result of the role that prostaglandins play as regulators of a variety of physiological processes in the human body, including inflammatory processes, the immunological response, vasodilation, vasoconstriction, pain perception, and fever, it is possible that prostaglandins have an impact on fertility [1].

Melatonin plays a significant part in the control of a wide variety of processes, including sexual maturation, reproduction, temperature regulation, circadian rhythms, and immune function. Melatonin may inhibit the production of reactive species, making it a potential free radical scavenger, and it also has the capacity to limit the generation of free radicals [2].

In the control group, ovarian tissues displayed an intact ovarian surface (germinal layer), and immediately underneath it was tunica albuginea, which included ovarian follicles at different stages of development. These phases included primary follicles, secondary follicles, developing follicles, and graafian follicles. The ovarian medulla comprised nerves, blood arteries, lymphatics, and connective tissue that were loosely organized [6].

PAS stain of control group revealed that the outer cell layer of the ovary (germinal epithelium) and stromal cells showed strong PAS positive reaction. The oocyte of the primary, secondary and graffian follicles appeared to have moderate PAS positive reaction, while the cytoplasm of these granulosa cells was slightly positive stained with PAS. The zona pellucida encircling the oocyte in the different types of ovarian follicles had marked positive PAS reaction [7].

Ultrastructurally, the oocyte cell was surrounded by zona pellucida and granulosa cells, and its nucleus had a morphology that was fundamentally normal. There was also no extension into the perinuclear region, and the structure of the double nuclear membranes was continuous [8].

In the isoxicam group, isoxicam had adverse effects on ovarian tissue and this confirmed histologically by the ovarian sections that revealed degenerative changes in the form of degeneration of germinal epithelium and ovarian follicles within the cortex with pyknotic nuclei and vacuolated cytoplasm which had foamy appearance**,** also there was hyper cellularity in follicular cells of oocyte that has been degenerated with detachment of them from surrounding stroma with many vacuoles. Some of the ovarian follicles had a protrusion that resembled a tongue and went all the way into the medulla [9].

Ultrastructural examination of ovarian sections had confirmed the destructive changes demonstrated by L.M. as degeneration of the germinal cell layer of the ovary, the oocyte was completely absent and the zona pellucida was degenerated and contained cellular debris and apoptotic cell bodies that lie within the zona pellucida, the corona radiata cells around the degenerated zona pellucida exhibited some fragmented nuclei and their cytoplasm showed many vacuolated areas in addition to many vacuoles in between the corona radiata cells and the ooplasm appeared to contain many swollen mitochondria, apoptotic bodies and dark condensed nucleus [8].

In isoxicam and melatonin group, there was restoration of some normal architecture of the ovary tissues. There was intact ovarian surface (germinal epithelium layer), some healthy graffian follicles showed apparently healthy architecture. The secondary oocyte was surrounded by zona pellucida, corona radiata, antrum and theca cells (stromal cells). Outside the granulosa cells there was theca externa and theca interna.

Melatonin is a major hormone secreted by the pineal gland, which is involved in mammalian biological clocks and reproductive cycles. It plays a crucial part as an antioxidant, an inflammatory, and an antiapoptotic. These results may be important in preventing oxidative stress-related harm to the oocyte and neighboring cells. Also, melatonin reduces damage from oxidative stress because it increases the production of antioxidant enzymes via its

effect on gene expression. Melatonin is crucial for oocyte maturation, embryonic development, and luteinizing. Increased melatonin levels inside the ovaries of infertile women were associated with less oxidative damage within the ovaries, improved fertilization rates, and a higher birth rate[10]. Melatonin ensures prostaglandin E2 synthesis and induces actin cytoskeleton rearrangement in the follicular cells at ovulation [11].

Melatonin was administered with isoxicam to reduce the damage seen at both the light and electron microscopes. It appears to establish the normal process of granulosa cells proliferation in a rat model of polycystic ovarian cyst and exerts positive effects on the protection and treatment of reproductive dysfunction [12].

Melatonin to nicotine-treated mice would preserve the ovary and lessen the severity of any harm already done. Thus, it seems that melatonin reduces the rat ovary's structural side effects from isoxicam. An increased number of mitochondria, including numerous cristae (with some damaged) in portion of the mitochondria, reduced condensation and margination of nuclear chromatin, and a less vacuolated corona radiata were noted [13].

Many studies have been published on the protective benefits of melatonin against oxidative stress induced by many commonly used medicines and on various bodily organs outside of the ovary. Exogenously administered melatonin has been shown to have large quantities in the brain, which supports the hypothesis that it acts as a free radical scavenger, has antioxidant capabilities, and readily crosses the blood-brain barrier. Thus, melatonin counteracts the neurotoxicity of diclofenac sodium [14].

Kidneys treated with diclofenac sodium and later given exogenous melatonin exhibited signs of vascular congestion, nephron degeneration, and tubule abnormalities when examined under a microscope. Despite diclofenac sodium's deleterious effects on the kidneys of rats, melatonin administration restored kidney function to normal [15].

Conclusion & Recommendations:

Isoxicam was shown to induce a substantial histological change in ovarian tissue in the current investigation. Melatonin's ability to mitigate these alterations suggests it might be employed in the future as an adjunctive treatment to isoxicam.

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