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Ameliorating potentials of N-acetyl-l-cysteine against methoxychlor instigated modulation in structural characteristics of granulosa cells of caprine antral follicles

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Follicular granulosa cells (GCs) form an important association with follicle's survival and health that determines ovulation. Apoptosis induction in GCs leads to follicular atresia and infertility problems. The present study aims at assessing the ultrastructural toxicity of methoxychlor (MXC), an organochlorine insecticide and reproductive toxin, on structural aspects of GCs owing to its potential for inducing apoptosis and the ameliorating potential of N-acetyl-L-cysteine (NAC) in this toxicity. The ultrastructural morphology revealed MXC induced degenerative changes in GCs like loss of cellular junction complexes and membrane integrity; nuclear abnormalities like occurrence of condensed and marginated chromatin, crescent shaped or blebbed nucleus; presence of disrupted mitochondria with disrupted cristae, vacuolization, empty spaces, large number of homogenous lipid droplets and extensive network of rough endoplasmic reticulum and several cytoplasmic processes at various doses of MXC with maximum degeneration observed at 100 µg/ml. NAC supplementation reduced the observed apoptotic characteristics of GCs with most significant changes at 5- and 10 mM concentration.

Thus, it is evident that MXC acts as an apoptotic inducer in GCs that influences the quality of antral follicles in mammals; however, NAC, with its anti-oxidative and anti-inflammatory properties, turns out to be a potential therapeutic and anti-apoptotic agent against MXC toxicity.

Keywords: Folliculogenesis, Granulosa Cells, Methoxychlor, N-acetyl-l-cysteine, Steroidogenesis

The indispensable role of granulosa cells (GCs) in folliculogenesis and oocyte development suggests their significant involvement in female fertility[1-3]. Though apoptosis has been found to be a contributing factor in follicular atresia[1,2]; its direct correlation with GCs apoptosis has least been focused upon till now. The degree of GCs apoptosis determines the follicular fate; as per literature cites 11–30% apoptosis in granulosa cells signifies a follicle in mid-atretic stage while apoptosis rate of more than 30% marks late atretic stages of follicular atresia[3].

Among the various causes, pesticides-induced reproductive obstructions are central to understand the follicular atresia. Although not extensively studied, organochloride pesticides are known to affect reproduction, fertility potential, pregnancy, gestation and birth of new born. Among all the pesticides used, organochlorine pesticides (OCPs) accounts to almost 40%, among which Lindane, chlordane and Methoxychlor (MXC) have been found to be associated with increased follicular atresia affecting fertility in mice and rats[4-7].

MXC acts against wide variety of insects, potentially causing irregular estrous cycle, implantation defects, occurrence of ovarian cysts and infertility[8]. In few cases, it has been found to affect pregnancy[9], decrease ovulation[10] and cause other reproductive alterations[11]. In addition, Methoxychlor is also a potent inducer of apoptosis that draws the attention towards its role in inducing follicular atresia. Although literature is scanty, few reports suggest its involvement in inducing atresia *via* apoptosis in domestic animals, baboons and mice[12-14]. However, there are no reports on MXC induced histo-morphological alterations at cellular and sub cellular level in GCs during apoptosis. Since, most of these pesticide toxicity cases increase the oxidative stress level, antioxidant N-acetyl-L-cysteine (NAC), excellent source of sulfhydral group, has been found to counteract oxidative stress mediated pesticidal toxicity, leading to decreased apoptosis rate[5-7,12,14]. *Capra hircus* forms a suitable model organism due to its socio-economically important livestock position in developing countries like India. They are seasonally polyestrous and widely affected due to pesticide poisoning as they directly feed in pesticide exposed agriculture lands.

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Thus, the present study examined various ultrastructural changes within caprine GCs due to methoxychlor toxicity and correlating them with apoptotic attributes, substantially causing follicular atresia. Besides this, the study will also assess the efficacies of NAC, a powerful antioxidant and precursor of glutathione, as a therapeutic agent against MXC toxicity in GCs.

Materials and Methods

Collection and classification of antral follicles

The ovaries from Jammanapari breed of goat (*Capra hircus*) were procured from the government and non-government slaughter houses of Chandigarh (30°43´N, 76°12´E) and around Kurukshetra (29°6´N, 76°50´E) respectively, in 0.9% ice-cold normal saline and brought to lab at 4°C. All the ovaries were cleared of extra tissue using pointed scissors and then follicles were manually separated using fine forceps and blades in PBS (pH-7.2). Follicles of diameter 3-8 mM were selected as antral follicles and were then classified on morphometric basis namely colour, turbidity of follicular fluid and vascularity as healthy and atretic[15,16].

Experimental design

Methoxychlor (MXC), an organochloride insecticide, was purchased from Sigma Chemical Co., St. Louis, MO, USA with percentage purity of 99.9%. Three experimental doses of 1 µg/ml, 10 µg/ml and 100 µg/ml were selected for treatment, in accordance with the previous studies[6]. NAC, a thiolic antioxidant was brought from HiMedia Co., India. The final concentrations of 1 mM, 5 mM and 10 mM was chosen, based on the literature, for NAC supplementation along with the test pesticide[5]. Stock solution of 0.1 M NAC was prepared by dissolving NAC in 100 µl DMSO and then the volume was made upto 1 ml with double distilled water. Final concentration of 1−, 5− and 10 mM NAC was reached by diluting stock solution directly in the media[6]. The follicles were treated with MXC and with/without NAC for 24, 48 and 72 h exposure duration.

In vitro culture of follicles

The antral follicles (n=10 per treatment group) were cultured in Dulbecco's modified eagle medium supplemented with 10% FBS, antibiotics (200-unit having concentration of penicillin 100 IU/ml and streptomycin 100 IU/ml) in a CO_2 incubator (5% CO_2, 95% humidity, 38°C) in different treatment groups of MXC at doses 1−, 10− and 100 µg/ml supplemented with NAC at 1−, 5− and 10 mM in dose and time dependent manner in comparison with no treatment controls (only culture media) for 24, 48, and 72 h. Post incubation treated antral follicles were used for electron microscopy.

Transmission electron microscopy (TEM)

TEM was performed as described by Zamboni[17]. Post treatment, antral follicles were fixed in modified Karnovsky's fixative (5% glutaraldehyde, 4% formaldehyde in 0.08 M sodium phosphate buffer, pH-7.2) for 72 h at 4°C. After fixation, follicles were trimmed into appropriate size and then transferred to Phosphate Buffered Saline pH-7.2. The follicles brought to Electron Microscopy Department were then post-fixed in 1.3% osmium tetroxide for 2 h at 4°C and then dehydrated in ascending grades of acetone, cleared with toluene and embedded in epoxy-resin. Preselected blocks were sectioned serially at 1 µM thickness and examined under light microscope for selection of the required portion. The blocks were trimmed and sections were cut at 60-90 nm thickness using glass knives and mounted on meshed copper grids. After staining with uranyl acetate and lead citrate, sections were examined using Morgagni 268 D TEM (FEI, The Netherlands) at AIIMS, New Delhi.

Results

TEM results revealed that MXC induced several cyto-pathological alterations in granulosa cells owing to apoptosis, both at cellular and sub cellular level in comparison with control group that received no treatment (Figs. 1-3) while NAC had the mitigating role (Figs. 4-6).

Observations in control groups

GCs exhibited healthy cellular morphology with intact structural integrity of cellular membranes (Fig. 1). The cells were found to display proper cellular junctions as revealed through the presence of various cytoplasmic protrusions from Granulosa cell surface (Fig. 1A). Close association between the granulosa cell membranes was observed with nucleus containing homogeneously dispersed chromatin surrounded by smooth double layered nuclear membrane interspersed by nuclear pores (Fig. 1B). Numerous round mitochondria with well-defined cristae were observed in the perinuclear region (Fig. 1B inset).

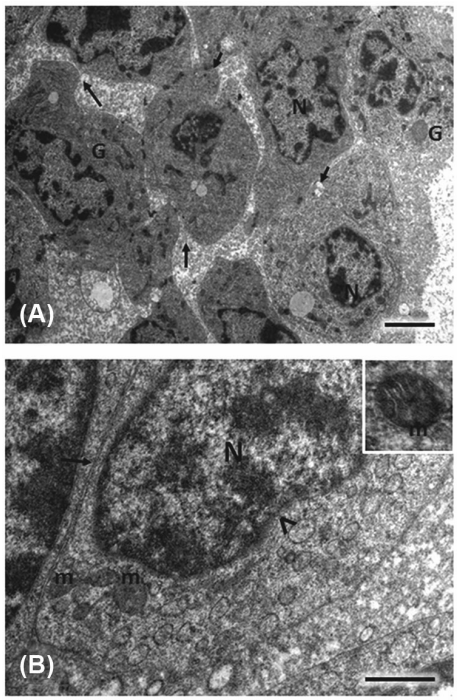

Fig. 1 — Electron micrograph showing normal granulosa cells with intact cell membrane and cytosolic structure and mitochondria (inset–b) at (A) 2500X; and (B) 3200X. Arrow-intercellular associations; arrowhead- nuclear envelop; m- mitochondria; G granulosa cell; N –nucleus

Observations in MXC treated groups

With increasing concentration and exposure duration, toxicity of MXC was found to be enhanced. After 24 h exposure duration, at lowest dose of 1 µg/ml MXC for 24 h exposure duration, treated GCs demonstrated normal membranous integrity even though, numerous features like condensed and marginated chromatin, high degree of vacuolization and presence of elongated rod like mitochondria were observed (Fig. 2A & B). With increasing dose of MXC exposure to 10 µg/ml, most prominent change observed was nuclear blebs as nucleus lost its membrane integrity, increase in extent of chromatin condensation and margination, disruption of cellular mitochondria with degenerated cristae along with presence of large number of homogenous lipid droplets and extensive network of rough endoplasmic reticulum (RER) in perinuclear region of the GCs (Fig. 2C & D). The most distinctive features observed at the highest dose of 100 µg/ml MXC were the presence of numerous cytoplasmic projections from the surface of GCs along with cellular degeneration and disruption of cell membrane in few of the treated cells (Fig. 2E & F).

The intensity of the cytotoxicity severely enhanced with increasing the exposure duration to 48 and 72 h (Fig. 3). Characteristics like crescent shaped or blebbed

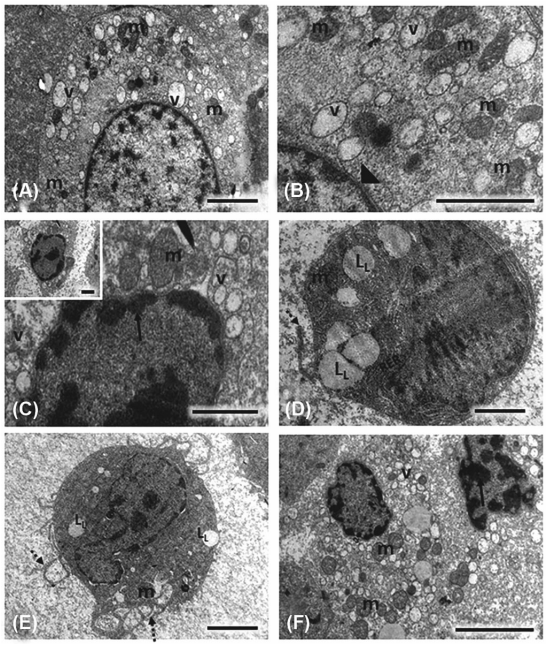

Fig. 2 — Electron micrograph showing apoptotic granulosa cells at (A, B) 1 µg/ml MXC, (C, D) 10 µg/ml MXC and (E, F) 100 µg/ml MXC treatment for 24 h exposure duration at (A, D, E) 3200X, (C, F) 4200X; and (B) 6500X. Arrow- condensed chromatin, arrowhead- nuclear envelop; dotted arrow- cytoplasmic evaginations; m- mitochondria; v- vacuoles; L_L- low density lipid droplets

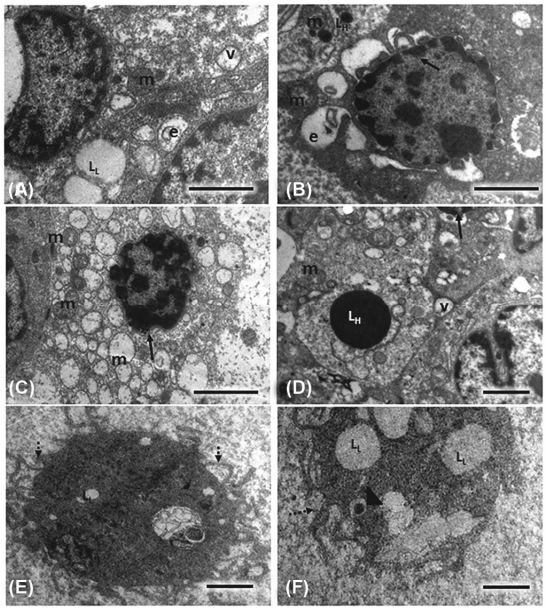

Fig. 3 — Electron micrograph showing apoptotic granulosa cells at (A, D) 1 µg/ml MXC, (B, E) 10 µg/ml MXC and (C, F) 100 µg/ml MXC treatment for (A-C) 48 h; and (D-F) 72 h exposure duration at 3200X (D-F, inset–c) and 4200X (A-C). Arrow- condensed chromatin, arrowhead- endo or exocytosis; dotted arrow- cytoplasmic evaginations; m- mitochondria; e- endosomes; v- vacuoles; L_L- low density lipid droplets; L_H- high density lipid droplets; RER- rough endoplasmic reticulum

nucleus with high rate of chromatin condensation, presence of empty spaces and electron dense homogenous lipid droplets, numerous autophagic vacuoles were noted after 48 h exposure duration at different MXC concentrations (Fig. 3A-C). However, 72 h duration treatment exhibited large lipid droplets and cytoplasmic processes besides other features indicating apoptosis at different MXC doses (Fig. 3D-F).

Observations in NAC supplemented groups

N-Acetyl-L-Cysteine was found to lower the MXC induced cytotoxicity due to initiation of apoptosis in GCs with its increasing concentration (Figs. 4-6). At 1 µg/ml MXC treatment, supplementation of NAC did not show significant changes at 1 mM and 5 mM concentration; however, at the highest dose of 10 mM, significant reduction in vacuolization has been observed with restored cell to cell association, signifying a decreased level of cellular damages (Fig. 4). NAC in 10 µg/ml MXC treated groups maintained the structural integrity of cell and nucleus and no chromatin condensation was observed. Most of the mitochondria were round and RER was extensive surrounding the nucleus, and there was scanty occurrence of lipid droplets (Fig. 5). At this concentration of MXC, 1 mM NAC supplementation showed minimum amelioration (Fig. 5A-D). Most prominent ameliorative effect of NAC

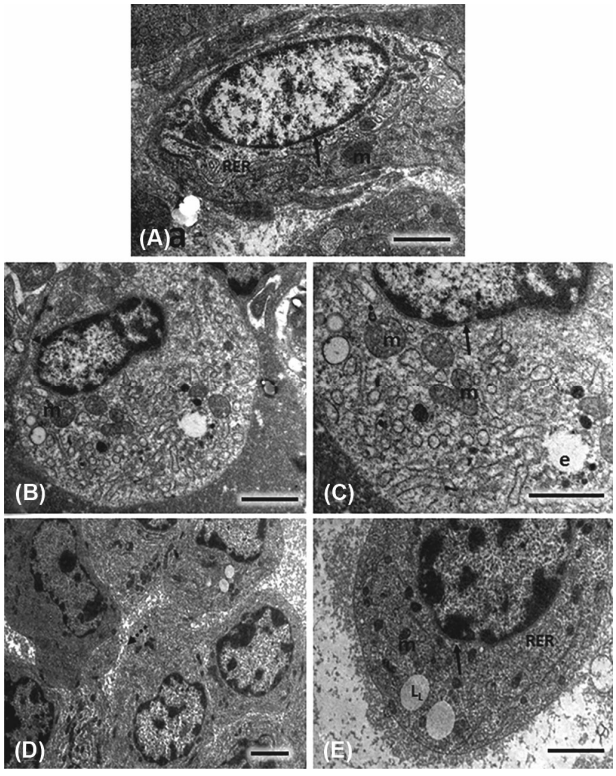

Fig. 4 — Electron micrograph showing treated granulosa cells at 1 µg/ml MXC supplemented with ameliorant NAC at (A) 1 mM, (B, C) 5 mM; and (D, E) 10 mM concentration after 24 h exposure duration at (D) 2500X, (A, B, E) 3200X; and (C) 4200X. Arrow- nuclear envelop, dotted arrow- inter cellular association; m- mitochondria; e- empty spaces; L_L- low density lipid droplets; RER- rough endoplasmic reticulum

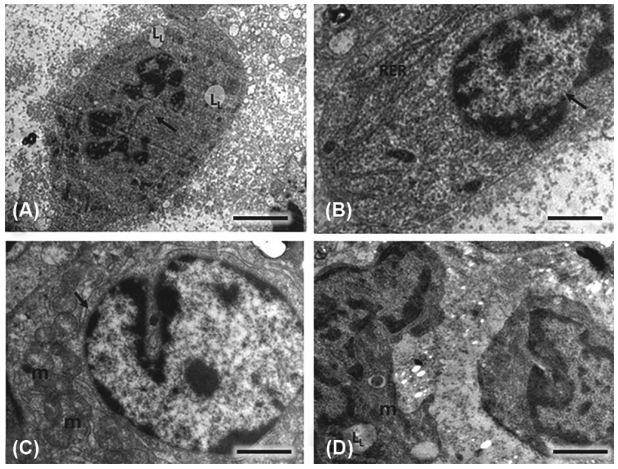

Fig. 5 — Electron micrograph showing treated granulosa cells at 10 µg/ml MXC supplemented with ameliorant NAC at (A) 1 mM, (B) 5 mM; and (C, D) 10 mM concentration after 24 h exposure duration at (A) 3200X; and (B-D) 4200X. Arrow- nuclear degeneration, m- mitochondria; L_L- low density lipid droplets; RER- rough endoplasmic reticulum

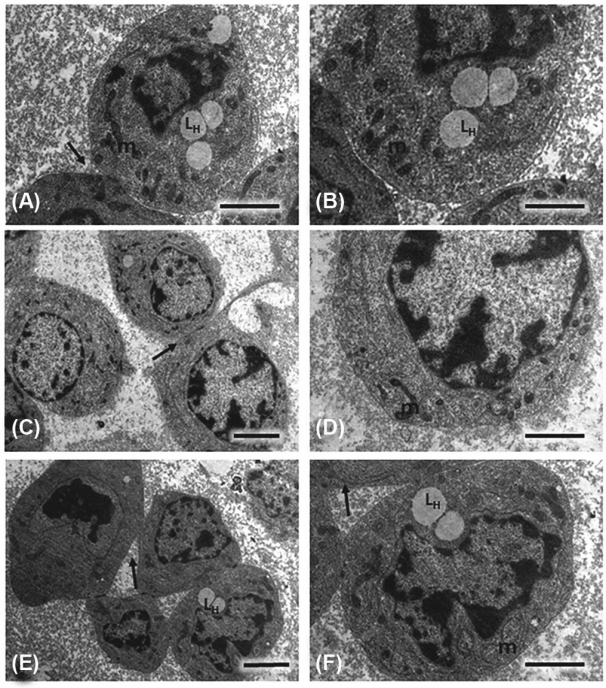

Fig. 6 — Electron micrograph showing treated granulosa cells at 100 µg/ml MXC supplemented with ameliorant NAC at (A, B) 1 mM, (C, D) 5 mM; and (E, F) 10 mM concentration after 24 h exposure duration at 2500X (C, E), (A, B. F) 3200X; and (D) 4200X. Arrow- intercellular association, m- mitochondria; L_L- low density lipid droplets

was found at highest concentration of MXC, where the cells were found to possess proper cell integrity with smooth cellular membranes that was lost in only 100 µg/ml MXC treated groups. NAC at all doses decreased the incidences of apoptotic features in comparison with only 100 µg/ml MXC treated groups. At lowest NAC dose (1 mM), presence of lipid droplets and chromatin condensation was still observed that decreased with increasing doses concentration of ameliorant (Fig. 6).

Discussion

The present study revealed that MXC induced several characteristic changes within GCs were associated with the attributes of apoptosis. The present study suggests loss of intercellular associations was the prominent and frequently noticed feature that signifies late apoptotic stage in the treated GCs, resulting in occurrence of wide intercellular spaces marking lack of intercellular communication within GC layer[18,19]. Another distinctive attribute exhibited during the study of MXC induced GCs apoptosis was the histopathological effects on nucleus form, structure and rigidity with disruption of double membrane nuclear lamina that signifies a state of genome instability that indirectly affects the metabolically active state[20,21]. These anomalies may be the resultant of instability of lamins, the intermediate filament protein present in the nuclear lamina[22]. Type A (Lamins A/ C) and Type B (lamins B1/B2) lamins is incorporated into the mesh of nuclear lamina that provides structural stability to the nuclear membrane; under pathological conditions disruption of these lamin proteins, especially lamins A/C, causes them to segregate and form nuclear blebs[22,23]. Studies have also reported that nuclear lamins interacts with chromatin and controls various aspects of nuclear function like chromatin organisation, DNA replication, transcription, and DNA repair[24].

At highest dose concentration of MXC, some GCs displayed several cytoplasmic protrusions from its surface. This was a rare phenomenon but in some studies, it was suggested that these protrusions facilitated easier binding and uptake of a apoptotic cell by phagocytes[25]; thus exhibiting correlation between microtubule-dependent apoptotic spike formation and cell fragmentation[25]. It has also been noted that such evaginations increase the surface area of the cell making it more responsive towards the gonadotropin as revealed by the increase in LH receptors corresponding to presence of these protrusions[15,16,26]. Several other studies hypothesized the reason and mechanisms involved with this peculiar observation and it has been proposed that during apoptosis the cells undergo characteristic modulations in cytoskeleton, *i.e*., in microtubule and intermediate filaments and during the execution phase of apoptosis forms apoptotic microtubule network (AMN) by reorganisation, resulting in apoptotic membrane protrusion and microtubule spikes formation giving apoptotic cell irregular morphology[27,28].

Mitochondria, being a site of oxygen radical production, are considered as the first organelle that shows degenerative effects[29]. The disrupted mitochondria with degenerated mitochondrial cristae direct towards the impaired metabolic activity of the GCs perpetuating to MXC poisoning[14]. This disruption of mitochondrial trans-membrane potential across its bio membrane hindered the electron transport chain and ATP generation creating a state of oxidative stress owing to the observed loss of structural integrity of mitochondrial membrane[30]. Moreover, it has been reviewed that mitochondrial lipid play essential role in apoptosis, alterations in cardiolipin synthesis regulate mitochondrial remodelling during apoptosis that causes release of various anti-apoptotic and pro-apoptotic molecules from the mitochondria[31]. According to Fair and co-workers, elongated mitochondria represented as mature that arise from the immature round type[32]. It has been documented that under stress condition, like starvation, cell increases cAMP that in turn phosphorylates Dynamin Related Protein, a pro fission protein, inhibiting its translocation into mitochondria, causing its elongation due to fusion; this protects the organelle from degradation and maintains ATP level[33].

Since granulosa cells play a vital role of regulating steroidogenesis, accumulation of lipid droplets in the peri-nuclear region marks the role of altered steroidogenesis in them[34,35] during MXC exposure. Increased accumulation of lipid droplets and decrease in its lysis signifies a distinctive pathological defect in response to MXC exposure[36]. It has already been documented that hypercytolipidemic metabolic state of a cell induces lipid mediated structural and chemical disruption of intrinsic nuclear DNA that promotes apoptosis initiation[37]. The extensive network of endoplasmic reticulum and mitochondria in the peripheral region of nucleus plays a vital role in lipid metabolism and ER-mitochondria calcium signalling[38]. In support of this, Paulini and co-workers[38] also suggested that the enzymatic action of mitochondria and endoplasmic reticulum mediates lipid synthesis where lipids get transported between these two organelles[39].

NAC, thiol containing compound and main precursor of glutathione[40], has reduced the cytotoxicity

and incidences of apoptotic characteristics observed in MXC treated groups in comparison with no ameliorant supplemented groups. Various pesticidal toxicity cases have used NAC to evaluate its antioxidative potency[7,14,41-44]. The antioxidant response of NAC has been found to regulate apoptosis as revealed in COV434 human granulosa tumor cells, where NAC was found to regulate GCs death *via* ROS-JNK-p53 pathway in response to hydrogen peroxide toxicity suggesting link between NAC mediated prevention in GCs apoptosis[45]. In Chinese hamster ovary cells, NAC inhibited apoptosis restoring ovarian function by preventing the failure of defence system[46]. It is also found that the NAC treatment both in *in vitro* conditions on rat antral follicles and *in vivo* conditions in mice inhibited follicular cell apoptosis[5].

Studies indicate that NAC notably prevented follicular atresia in antral follicles by decreasing lipid peroxidation and increasing the activity of various antioxidant enzymes[5,47]. Molavi and coworkers showed that antioxidants decline oxidative stress mediated caspase- 3 activation, protecting the cellular DNA and RNA content[48]; that probably explains the NAC mediated restoration of nuclear degeneration. NAC supplemented groups was found to restore the metabolic activity and mitochondrial damage signifying reduction in ROS generation and membrane permeability pores reduction due to its scavenging action as observed in Chinese Hamster ovary cells[46]. Few reports have suggested the involvement of NAC in inhibiting the disruption of steroidogenesis in arsenic-treated female rats by elevating the expression of antioxidant enzymes[49] relating with the reduction in lipid droplets accumulation in NAC treated groups.

Conclusion

The afore-mentioned characteristic alterations in ultrastructure of GCs after MXC treatment suggests MXC induced apoptosis in GCs that alters various cellular aspects; affecting follicular growth and promoting atresia, ultimately affecting the fertility of an individual. Furthermore, NAC proves to be an effective ameliorant and therapeutic agent against toxic effects of MXC in granulosa cells, combating cytotoxicity and associated infertility issues.

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Conflict of interest

All authors declare no conflict of interest.

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