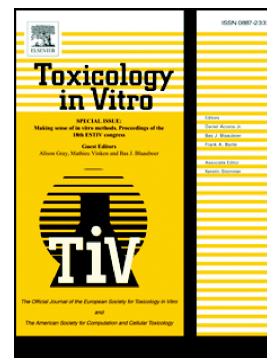


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**The role of oxidative stress in antipsychotics induced ovarian toxicity****Ekramy Elmorsy**

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**Abstract**

This study tested the hypothesis that oxidative stress could be an underlying mechanism for APs-induced ovarian cytotoxicity and reproductive dysfunction. Rat ovarian theca interstitial cells (TICs) were isolated and treated with four APs [chlorpromazine (CPZ), haloperidol (HAL), risperidone (RIS) and clozapine (CLZ)]. MTT assay was used to test the effects of these antipsychotics on TICs viability and to estimate their 50% inhibitory concentrations ( $IC_{50}$ s). The effects of APs ( $IC_{50}$ s and  $1\mu M$  concentrations) on the activities of caspases-3, -8 and -9, reactive oxygen species (ROS) production, total intracellular glutathione and lipid peroxidation (LPO) in TICs were assessed. The effect of antioxidants (reduced glutathione (GSH) and quercetin) on the APs-induced cytotoxicity on TICs was investigated. MTT assay showed all APs to reduce TICs viability. CPZ, HAL and CLZ significantly increased the activity of caspases-3, -8 and -9 ( $P < 0.0001$ ,  $< 0.0001$  and  $< 0.01$ , respectively). All APs at  $IC_{50}$ s significantly ( $P < 0.0001$ ) increased ROS production, decreased total intracellular glutathione and increased LPO. MTT assay in the presence of antioxidants (reduced GSH (5mM) or quercetin (50mM)) showed each antioxidant to significantly inhibit the effects of APs at their  $IC_{50}$ s on TICs viability. In conclusion, oxidative stress seems to be a possible mechanism for APs-induced ovarian and reproductive toxicity.

**Keywords:** Antipsychotics, Oxidative stress, reproductive toxicity, antioxidants

Abbreviations: APs, Antipsychotics; CPZ, Chlorpromazine; CLZ, Clozapine; HAL, Haloperidol; LPO, lipid peroxidation; ROS, reactive oxygen species; RIS, Risperidone; TICs, Theca interstitial cells; TBA, Thiobarbituric acid.

## 1. Introduction

Most psychotic patients require life-long treatment with antipsychotics (APs) (Peuskens et al., 1998; Dickson and Glazer, 1999). Unfortunately, this treatment has been associated with a wide range of side effects such as abnormal movements, weight gain, diabetes and reproductive disorders. Between 15% and 97% of women, receiving APs have been reported to develop menstrual irregularities or amenorrhea (Santoni and Saubadu, 1995; Crismon and Dorson, 1997). Although typical APs are generally believed to be more toxic than the atypical (second-generation) APs (Üçok and Gaebel, 2008), reproductive toxicity seems to be similar in the two groups of APs (Murke et al., 2011).

It has been suggested that APs-induced hyperprolactinemia, via inhibition of dopamine action at D2 receptors in the tuberoinfundibular system, is the main cause for the associated reproductive disorders. Wong and Seeman (2007) reported hyperprolactinaemia in 57% of patients receiving typical APs. However, atypical APs were found to have no effect on serum prolactin levels with the exception of risperidone, which has been reported to increase circulating prolactin, similar to the typical APs (Halbreich et al., 2003; Aboraya et al., 2004). On the other hand, Lee and Kim (2006) found no correlation between plasma prolactin levels and menstrual irregularities. Furthermore, Canuso et al. (2002) found similar rates of reproductive dysfunction and ovarian hormone values in women with and without APs-induced hyperprolactinemia. Moreover, irrespective of the AP type or the prolactin status, APs were associated with reduced peak peri-ovulatory estradiol levels. Therefore, APs-induced hyperprolactinemia cannot fully explain reproductive toxicity of APs.

We have recently shown APs to induce cytotoxic effects in rat's ovarian theca interstitial cells as evidenced by inhibition of mitochondrial bioenergetics (Elmorsy et al., 2017).

Furthermore, several in-vitro (Contreras-Shannon et al., 2013; Antherieu et al., 2013; Elmorsy et al., 2014) and in-vivo (Shivakumar and Ravindranath, 1992; Parikh et al., 2003; Martins et al., 2008) studies have shown APs to induce oxidative stress in non-reproductive cells. Moreover, our group has provided evidence that oxidative stress plays a role in APs-induced toxicity in human brain microvascular endothelial cells (Elmorsy et al., 2014).

Based on the above, we hypothesized oxidative stress as a possible underlying mechanism of APs-induced ovarian theca cell toxicity with subsequent reproductive dysfunction. In order to test this hypothesis, rat's ovarian theca interstitial cells (TICs) were isolated and used as a model to investigate the effects of APs. Four APs were chosen for the present study [Two typical; chlorpromazine (CPZ) and haloperidol (HAL) and two atypical; risperidone (RIS) and clozapine (CLZ)]. These APs, which are commonly used in clinical practice, are well known for their reproductive adverse effects (Spitzer et al., 1998; Feldman and Goldberg, 2002; Knegtering et al., 2003). The *main aim* of this study was to investigate the cytotoxic effects of APs on rat's TICs and to determine the possible role of oxidative stress in this toxicity.

## 2. Materials and methods

### 2.1. Chemicals and media

Chemicals, reagents and drugs, used in this study, were purchased from Sigma-Aldrich (Poole, UK) unless another source is specified. All stock solutions of drugs were made in DMSO (vehicle). Media and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY). Caspase activities were assessed by BD ApoAlert caspase fluorescent assay kits (Clontech Laboratories, Palo Alto, CA).

## 2.2. Animals

Female Sprague-Dawley rats were housed in air-conditioned rooms, which were illuminated 14 h/day. Experiments were conducted in accordance with the guidelines for United Kingdom laboratory animals' use. The protocol has been approved by the Institutional Research Animal Committee, Mansoura University, Egypt.

## 2.3. Theca interstitial cell isolation and culture:

In order to obtain rat pre-ovulatory follicles, immature 23- to 24-day-old rats were injected with equine chorionic gonadotropin (10 IU) between 09:00 and 09:30 h to enhance multiple follicular development. Rats were then anesthetized and their ovaries removed. Theca interstitial cells (TICs) were isolated from the ovaries following the procedures described by Hoang et al (2013). Briefly, ovarian follicles were punctured with needles to release granulosa cells (GCs) and oocytes. The remaining ovarian tissue was minced with a scalpel and digested in 100  $\mu$ L/ovary of M199 medium with 0.35 mg/mL collagenase type IA, 10  $\mu$ g/mL DNase, and 10 mg/mL BSA at 37°C for 30 minutes. After digestion, the tissue was centrifuged at 1,000 rpm for 4 minutes then the medium was aspirated, and cells were resuspended in 5 mL fresh M199. Debris and oocytes were subsequently removed using 100- and 40-mm cell strainers. Discontinuous Percoll gradients were used to purify TICs following Magoffin and Erickson (1988). Primary TICs were cultured in culture plates (Falcon, Meylan Cedex, France) in HEPES buffered M199 with 5% FCS for 24 h, followed by 24–48 h in serum-free medium containing 0.1% bovine serum albumin (BSA). Cultures were maintained in a humidified air/CO<sub>2</sub> atmosphere at 37°C.

#### 2.4. Antipsychotics cytotoxicity by MTT

Following the manufacturer's protocol, TICs were seeded ( $1 \times 10^4$  cells per well) in 96-well plastic plates. After reaching confluence, cells were incubated for 4, 24 and 48 hrs in the presence of the test APs at concentrations of 0.1, 1, 10 and 100  $\mu\text{M}$  or DMSO alone as a vehicle control. Each experiment was performed in triplicate with at least 3 wells of each drug concentration in each experiment. The MTT absorbance values were expressed as a percent of the vehicle control (defined as 100%) and the 50% inhibitory concentration ( $\text{IC}_{50}$ ) for each AP was determined.

#### 2.5. Caspase assay

For the apoptotic caspases, -3, -8 and -9, activity assays were carried out. Cells were grown in six-well plates ( $10^6$  cells per well) and treated with  $\text{IC}_{50}$  of each AP (CPZ,  $12 \mu\text{M}$ ; HAL,  $58 \mu\text{M}$ ; RIS,  $160 \mu\text{M}$ ; CLZ,  $44 \mu\text{M}$ ) for 2 hrs. Following the manufacturer's protocol, cells were harvested and centrifuged at 1000 rpm for 5 min and lysed in 50  $\mu\text{l}$  of chilled lysis buffer on ice for 10 mins. Cells were then centrifuged for 10 mins at  $4^\circ\text{C}$ . The supernatant (50  $\mu\text{l}$ ) was added to an equal volume of 2 $\times$  reaction/dithiothreitol (DTT) buffer supplemented with the supplied caspase-3, -8 or -9 substrates and incubated at  $37^\circ\text{C}$  for 2 hrs. Samples were transferred to a 96-well plate to measure the fluorescence intensities by a Synergy HT Fluoremeter (Bio-tek Instruments, Inc., Winooski, VT) at the appropriate excitation and emission wavelengths.

#### 2.6. Reactive oxygen species (ROS) detection

3,7-dichlorodihydrofluorescein diacetate (DCFDA) assay was used to detect the Changes in pattern of ROSs production in TICs under the effect of APs. TICs were treated with APs in both  $\text{IC}_{50}$ s and 1  $\mu\text{M}$  concentrations for 24-hrs then the assay was performed as described by our

group (Elmorsy et al., 2014). Experiments were performed in triplicate, with at least 3 wells for each treatment in each experiment.

### *2.7. Measurement of total glutathione*

Glutathione levels were assessed in the isolated TICs according to methods described by Senft et al. (2000). Briefly, TICs were exposed to APs at their estimated  $IC_{50}$ s and  $1\mu M$  concentration. After 24 hrs, TICs were scraped in ice cold phosphate buffered saline (PBS) followed by centrifugation. The pellets obtained were re-suspended in ice-cold lysis buffer and centrifuged at 15000 g for 5 mins to generate lysates and protein pellets. Lysate content of glutathione was quantified with an excitation/emission wavelength of 350/420 nm using the fluorescent substrate o-phthalaldehyde (OPT).

### *2.8. Lipid peroxidation assay*

Thiobarbituric acid (TBA)-reactive products were assayed as a markers of Lipid peroxidation (Armostrong and Brown, 1994). Briefly, TICs were treated with APs at their  $IC_{50}$ s and  $1\mu M$  concentrations for 24-hrs. Then media were removed and cells were harvested, counted and resuspended in phosphate buffer saline (PBS) at a density of  $10^6$  cells/ml. Half a ml of PBS was added to 0.5 ml of 30% (w/v) trichloroacetic acid containing 1 mM butylated hydroxytoluene. The samples were kept on ice for 30 mins and centrifuged at 1000g for 10 min. Then 700  $\mu l$  of the supernatant was added to an equal volume of TBA (182 mg/25 ml) and heated to  $100^\circ C$  for 15 min. The samples were then cooled and absorbance read at 535nm (Ottolenghi, 1959).



### 2.9. Effect of antioxidants on APs-induced cytotoxicity

MTT assay was repeated in APs-treated TICs after adding 5mM reduced glutathione (GSH) and quercetin (50mM).

### 2.10. Statistical analysis

For  $IC_{50}$  estimation and non-linear curve fitting log (inhibitors)- variable slope equation was used. For comparisons, one way ANOVA test was used with Dunnett's multiple comparisons post-test. Statistical analysis was conducted using PRISM 5 (GraphPad Software Inc., San Diego, CA) with statistical significance defined as  $P < 0.05$ .

## 3. Results

### 3.1. Antipsychotics and TICs viability

The MTT assay showed all APs to impair viability of TICs in a concentration and duration dependent manner (Fig 1). According to the estimated  $IC_{50}$  values, CPZ was the most potent, while RIS was the least toxic with the highest  $IC_{50}$  (Table 1). TICs viability was significantly decreased after four hours post-exposure to a concentration of 1  $\mu$ M of CPZ ( $p=0.008$ ) and CLZ ( $p=0.02$ ).

### 3.2. Antipsychotics and caspases in HMVECS

As shown in Figure 2, Fluorometric assay revealed a significant increase in caspases 3, 8 and 9 in TICs treated with  $IC_{50}$ s of three APs including CPZ ( $p < 0.0001$ ), HAL ( $P < 0.0001$ ) and CLZ ( $P < 0.01$ ). There was no change in caspases activities in TICs treated with RIS (Fig 3).

### 3.3. Antipsychotics and ROS production

DCFDA assay showed significantly increased release of ROS in TICs treated with all APs at their  $IC_{50}$ s ( $P < 0.0001$ ). Only typical preparations CPZ and HAL in  $1\mu M$  concentrations resulted in significant increase in ROS after 24hrs post-treatment (Dunnett's multiple comparison post test) (Fig 3A).

### 3.4. APs and TICs intracellular total glutathione

As shown in Figure 3A, intracellular total glutathione significantly ( $P < 0.001$ ) decreased in TICs treated with all APs in their  $IC_{50}$ s compared to TICs treated with vehicle control. In  $1\mu M$  concentrations, only typical preparations (CPZ and HAL), significantly decreased glutathione after 24-hrs exposure (Fig 3B).

### 3.5. Antipsychotics and lipid peroxidation in TICs

The lipid peroxidation assay showed TBA-reactive products to significantly ( $P < 0.0001$ ) increase in TICs treated with all tested APs (Fig 3C). Dunnet's posttest showed that all APs significantly increased LPO ( $P < 0.001$ ) in their  $IC_{50}$ s, while only the typical preparations in  $1\mu M$  concentration significantly (CPZ,  $p < 0.05$ ; HAL,  $P < 0.01$ ) increased LPO in TICs after 24-hrs (Fig 2C).

### 3.6. Antioxidants and APs-induced cytotoxicity

MTT assay showed anti-oxidants (reduced GSH (5mM) or quercetin (50 mM)) to significantly decrease the cytotoxic effects of all tested APs in their  $IC_{50}$ s (CPZ,  $P < 0.0001$ ; HAL,  $P < 0.01$ ; RIS,  $P < 0.01$ ; and CLZ,  $P < 0.0001$ ) (Figure 4). Reduced GSH (5mM) at a concentration of  $1\mu M$  significantly decreased the cytotoxic effect of CPZ ( $P = 0.0175$ ) with no effect on

cytotoxicity of other APs. Quercetin at a 1 $\mu$ M concentration showed no effect on the cytotoxicity of any AP (Fig 4).

#### 4. Discussion

To the best of our knowledge, this is the first study to investigate the role of oxidative stress in APs-induced reproductive toxicity. We found APs to induce cytotoxic effects on rat's TICs as shown by the impairment of TICs viability and increased caspases activities. We have also shown APs to induce oxidative stress as evidenced by increased ROSs production, decreased intracellular total glutathione and increased lipid peroxidation. Finally, we have shown for the first time that antioxidants can inhibit the cytotoxic effects of the APs on rat's TICs.

We have chosen rat's TICs to test our hypothesis as they have been shown to be a valuable in-vitro model for evaluating the safety of chemicals (Roberts, 2001) and are known to play an important role in ovarian steroidogenesis (Young and McNeilly, 2010).

For the MTT assay, we used a wide range of APs concentrations including 0.1, 1, 10 and 100 $\mu$ M. These concentrations were chosen as they correspond to therapeutic, hyper-therapeutic and toxic serum levels of APs in human (Winek et al., 2001; Van Putten et al., 1991; Chang et al., 1997; Chang et al., 1994). Therapeutic serum levels were previously reported as 0.01-0.5 mg/l (0.03-1.5  $\mu$ M) for CPZ, 0.1-0.7 mg/l (0.3-2 $\mu$ M) for CLZ, 0.006-0.24 mg/l (0.016-0.64  $\mu$ M) for HAL and 0.003-0.12 mg/l (0.007-0.28  $\mu$ M) for RIS (Winek et al., 2001). Overdose serum concentrations were 3mM for CPZ (Van Putten et al., 1991), 5mM for CLZ (Chang et al., 1997), 0.7mM for HAL (Chang et al., 1994) and for RIS 0.5-0.8mM (220 to 320 ng/mL ng/ml; Titier et al., 2003; Nishikage et al., 2002). We have used higher concentrations in order to accelerate the effects and to define the IC<sub>50</sub> values. We examined APs-induced cytotoxicity

48hrs after exposure as it was previously reported that APs take at least 48hrs to achieve maximum effect on cell viability (Erik et al., 2010).

#### *4.1. Antipsychotics cytotoxicity to rat's TICs*

Our MTT assay results are in agreement with several previous studies investigating APs effects on non-reproductive cells (Dwyer et al., 2003, Dwyer et al., 2005; Ardizzone et al., 2001; Elmorsy et al., 2014; Hampson, 2000; Bhatt and Saleem, 2004; Ahn et al., 2004; Marlowe, 2000). Regarding our IC<sub>50</sub>s data, CPZ was the most potent followed by CLZ. These findings are in agreement with previous studies investigating the effects of these APs on pC12 cell line (Dwyer et al., 2003) and on normal human fibroblast (Jones-Brando et al., 2003).

#### *4.2 Antipsychotics and Caspases*

In this study, APs-induced cytotoxic effect on TICs was associated with increased apoptotic caspases activity. It is well known from recent studies that oxidative stress plays a pivotal role in induction of apoptosis. Antioxidants such as the thiol reducing agent N-acetylcysteine have been found to block or delay apoptosis (Buttke and Sandstrom, 1994; Kannan and Jain 2000). We have investigated caspases activity in this study as a marker of cytotoxicity as well as oxidative stress. Our findings, therefore, provide further evidence to support our hypothesis that APs-induced oxidative stress may play a role in ovarian cytotoxicity by inducing apoptosis.

Our data on the effects of APs on caspases are consistent with previous studies investigating the effects of APs on non-reproductive cells. HAL was reported to increase caspase-3 activity in human neuroblastoma SK-N-SH cells (Gassó et al., 2012) and CPZ increased apoptosis in lymphoblasts by (Hieronymus et al., 2003). In addition. Elmorsy et al.

(2014) reported increased caspases -3, -8 and -9 activities in human microvascular endothelial cells of blood brain barrier in response to a 24-hours exposure to CPZ, HAL and CLZ.

#### *4.3. Antipsychotics and Oxidative stress*

Our data on the effect of APs on ROSs production are in agreement with previous in-vitro studies investigating APs effects on non-reproductive cells. We have previously reported increased ROSs production in human microvascular endothelial cells of blood brain barrier 24-hrs after exposure to CPZ, HAL, RIS and CLZ. (Elmorsy et al., 2014). Antherieu et al (2013) showed CPZ to induce oxidative stress in human hepatoma HepaRG cells. Similarly, Contreras-Shannon et al (2013) found CLZ to induce oxidative stress in several non-reproductive mouse cell lines; myoblasts (C2C12), adipocytes (3T3-L1), hepatocytes (FL-83B), and monocytes (RAW 264.7).

Our findings are also consistent with several earlier in-vivo studies investigating APs-induced oxidative stress in human (Shivakumar and Ravindranath, 1992) as well as in animals (Parikh et al., 2003 and Raudenska et al., 2013). Shivakumar and Ravindranath (1992) reported decreased human serum antioxidant enzyme activity after chronic treatment with HAL. Parikh et al (2003) and Martins et al (2008) provided evidence of oxidative stress in Wistar rat brain after chronic treatment with HAL.

#### *4.4 Implications and clinical relevance*

The protective effect of both antioxidants on the APs-induced cytotoxicity supports our hypothesis that oxidative stress plays a role in reproductive toxicity. Oxidative stress may therefore be a good therapeutic target for the management of the troublesome reproductive adverse reproductive effects of APs. In addition, antioxidant therapy may be beneficial in

treating APs overdose and toxicity. Further clinical research is required to investigate the clinical usefulness of antioxidants in patients receiving antipsychotic therapy.

### **Conclusion**

Oxidative stress seems to be a possible mechanism for APs-induced ovarian cytotoxicity with subsequent reproductive dysfunction. The protective effects of antioxidants on this ovarian cytotoxicity may pave the way for the development of an effective therapy to counteract the reproductive as well as other adverse effects of antipsychotics.

### **Conflict of interest:**

No conflict of interest.

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**Figure legends**

**Figure (1):** MTT study of the effect of APs on viability of rats isolated ovarian TICs. Viability was expressed as a percentage of the vehicle control. Data are shown as mean  $\pm$  S.E.M (n=x-y). Solid lines are fitted to a four parameter logistic equation given in the materials and methods section.

**Figure (2):** Fluorometric assay of caspases -3, -8 and -9 activities in rats isolated ovarian TICs treated with APs CPZ, HAL, RIS and CLZ. Significance is relative to vehicle. Values are represented as means ( $\pm$  S.E.M; n=9). P-values were estimated by one way ANOVA test with Dunnett's multiple comparison post-test to test the significance of difference from the control. (\* indicate p-value  $<0.05$ , \*\* indicate p-value  $<0.01$ , and \*\*\* indicate p  $< 0.001$  when compared with vehicle control treated group).

**Figure (3):** The effect of antipsychotics on isolated rats TICs ROSs production [A]. intracellular total glutathione intracellular total glutathione [b] and LPO products [C]. Samples were compared statistically with the vehicle samples using one way ANOVA with Dunnett's post-test. Data are represented as percentages of the vehicle control treated samples assuming that the vehicle control readings are equal to 100%. (\* indicates p-value  $<0.05$ , \*\* indicate p-value  $<0.01$ , and \*\*\* indicate p  $< 0.001$  when compared with vehicle control treated group).

**Figure (4):** Effect of reduced glutathione (5mM) and quercetin (50mM) on APs-induced cytotoxicity in rats isolated ovarian TICs. Readings for MTT absorbance of the treated samples (in absence or presence of reduced GSH or quercetin) were compared statistically with the vehicle samples using one way ANOVA with Dunnett's post-test. (\* indicates p-value  $<0.05$ , \*\* indicate p-value  $<0.01$ , and \*\*\*\* indicate p  $< 0.0001$  when compared with vehicle control treated group).

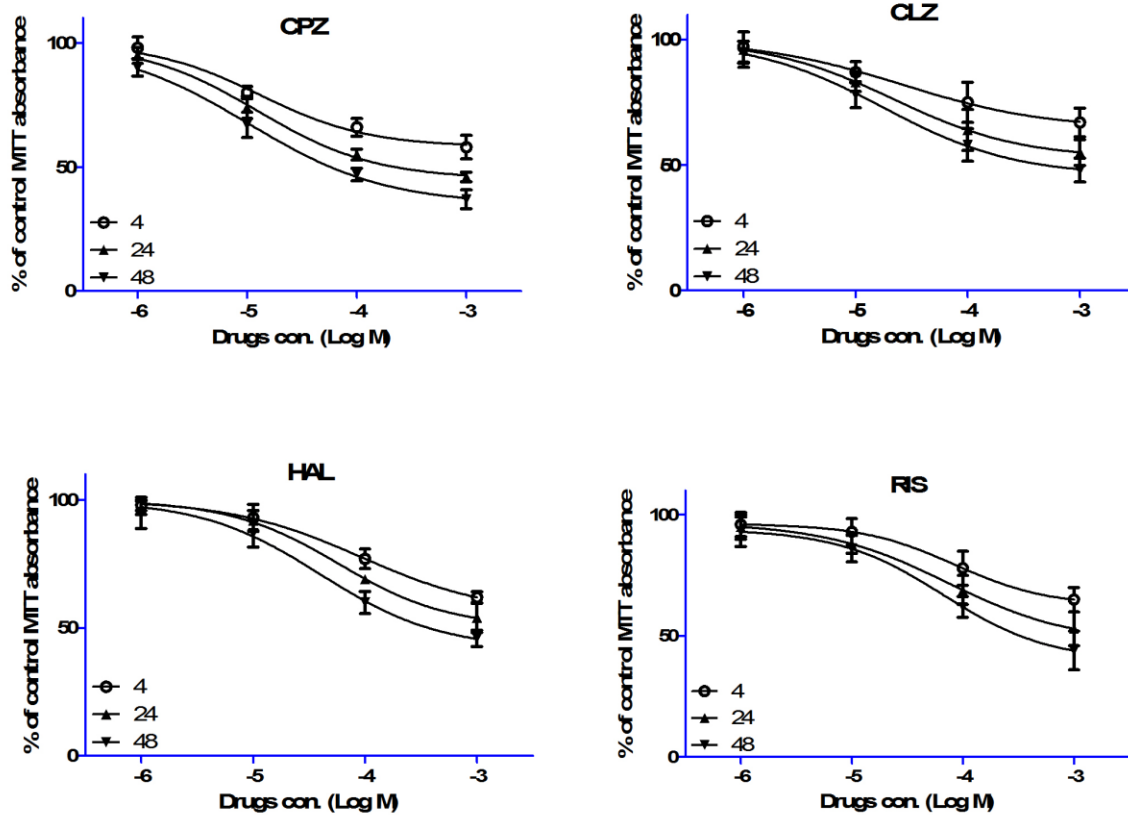


Fig. 1

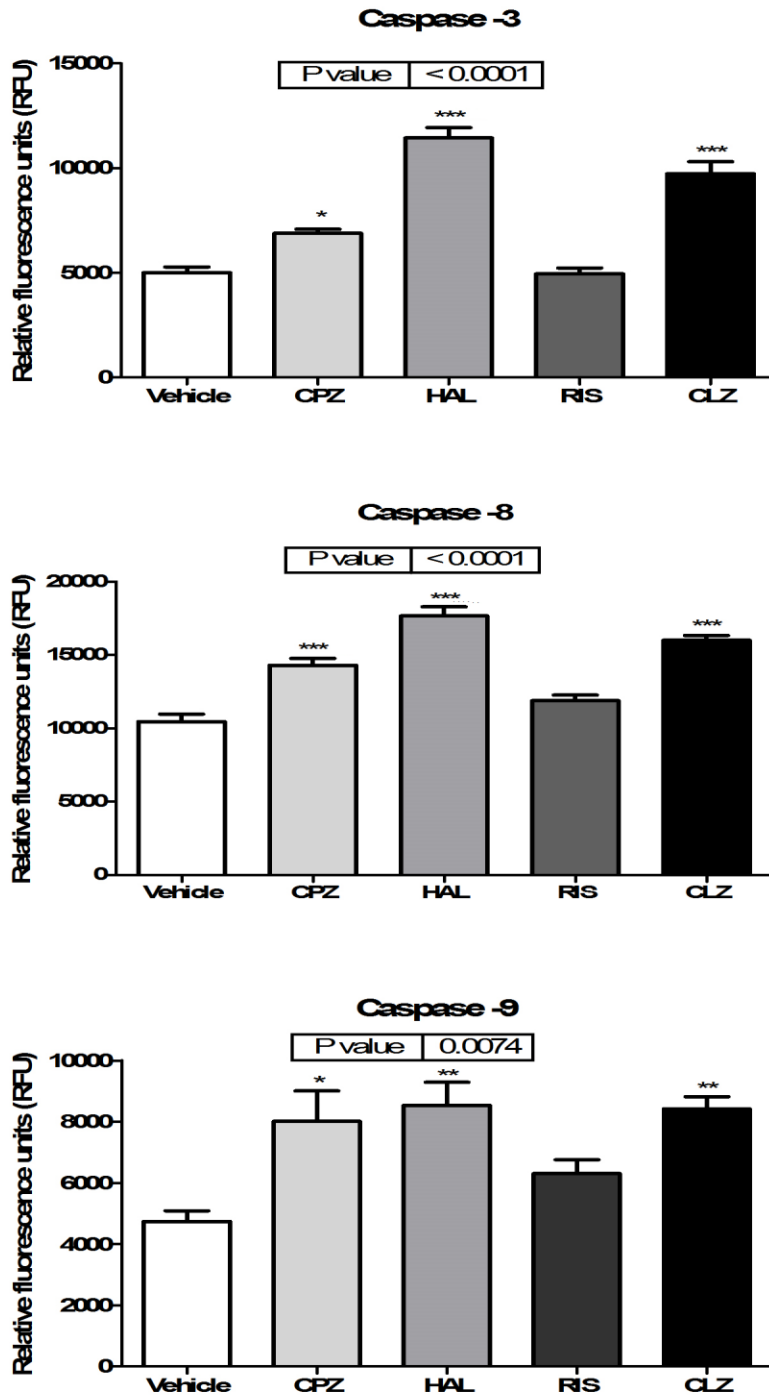


Fig. 2



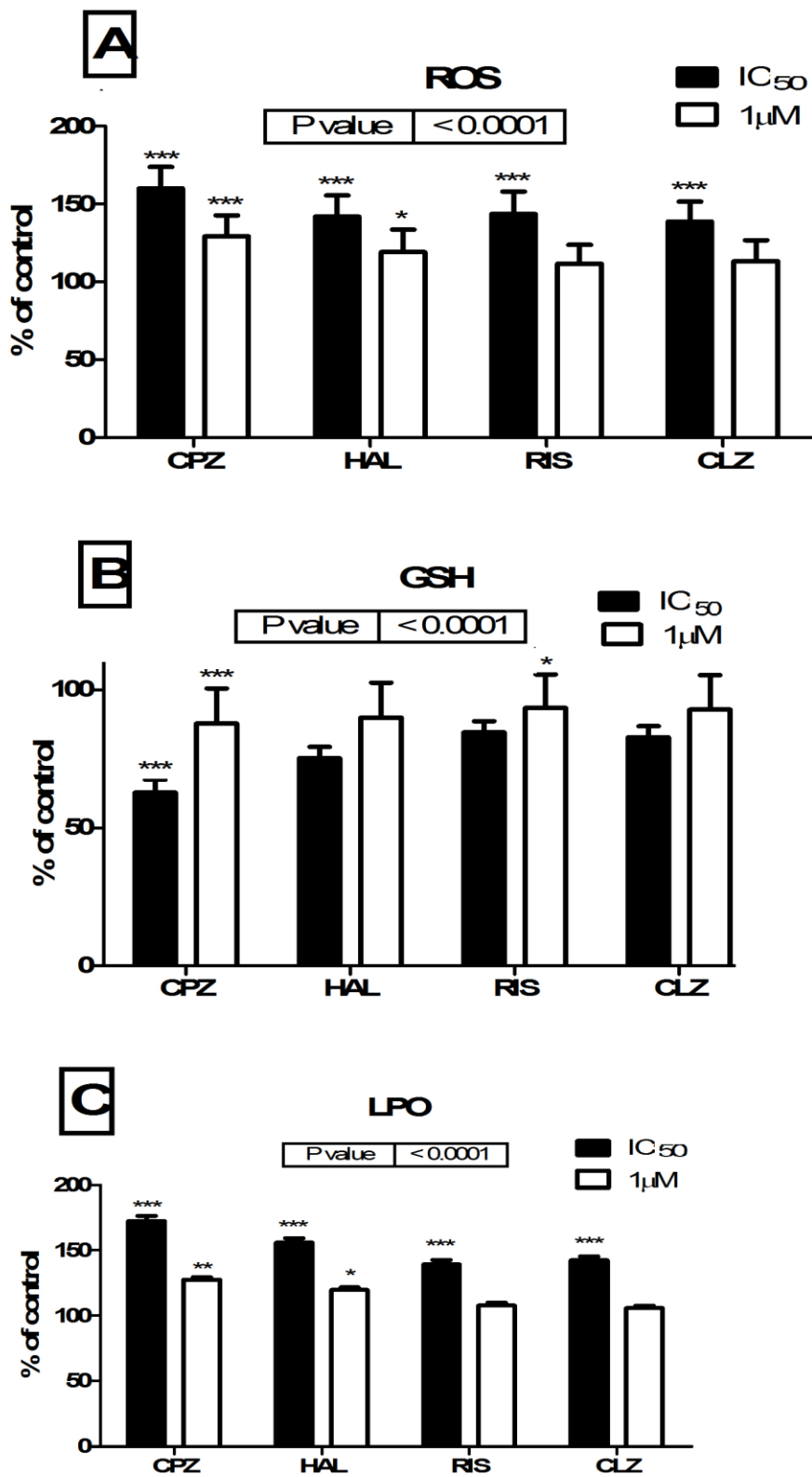


Fig. 3

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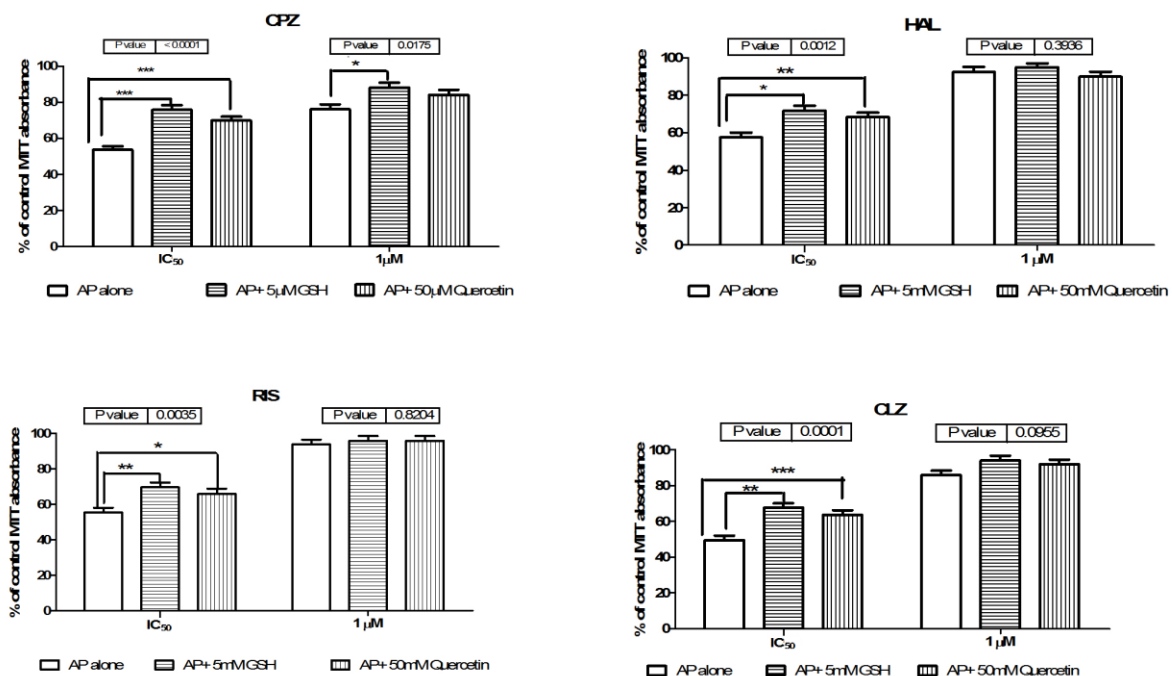


Fig. 4

Table (1) MTT assay estimated  $IC_{50}$  of antipsychotic-induced cytotoxic effect on rat isolated ovarian TICs.  $IC_{50}$ s were estimated by log inhibitors versus response (variable slope) equation.  $IC_{50}$ s are represented as means (M), upper limit (UL) and lower limit (LL) of 95% confidence interval.

Exposure	CPZ			HAL			RIS			CLZ		
	M	UL	LL	M	UL	LL	M	UL	LL	M	UL	LL
4hrs	13	19	9.5	93	152	76	84	168	45	26	78	7.6
24hrs	12	19	9.5	58	76	38	73	160	30	22	44	11
48hrs	1	13	7.7	40	76	38	67	114	38	18	29	11

## The role of oxidative stress in antipsychotics induced ovarian toxicity

### Highlights:

- Antipsychotics are cytotoxic to ovarian theca cells in a concentration dependent manner.
- Typical antipsychotics are more cytotoxic than atypical antipsychotics to the ovarian theca cells.
- Oxidative stress plays an important role in antipsychotics-induced ovarian cytotoxicity
- Antioxidants are proposed to alleviate antipsychotics-induced reproductive dysfunction.

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