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Effect of antipsychotics on mitochondrial bioenergetics of rat ovarian theca cells

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Highlights:

- This study investigated the effects of four antipsychotics (APs) on mitochondrial bioenergetics and steroidogenesis of rats isolated ovarian theca interstitial cells (TICs) as a possible mechanism of reproductive toxicity.

- The APs used in this experiment include chlorpromazine (CPZ) haloperidol (HAL), risperidone (RIS) clozapine (CLZ).
- All four APs seem to inhibit mitochondrial bioenergetics and steroidogenesis in rat's TICs.
- These findings support the hypothesis that APs-induced reproductive toxicity may be through mechanisms involving mitochondrial insult.

Abstract:

Background: Antipsychotics (APs) are widely prescribed drugs, which are well known to cause reproductive adverse effects through mechanisms yet to be determined. The purpose of this study was to investigate the effect of antipsychotics on mitochondrial bioenergetics of rat ovarian theca cells as a possible mechanism of reproductive toxicity. **Methods:** Isolated rat's theca interstitial cells (TICs) were treated with two typical (chlorpromazine [CPZ] and haloperidol [HAL]) and two atypical APs (risperidone [RIS] and clozapine [CLZ]). The effects of these APs on TICs bioenergetics (ATP content, mitochondrial complexes I and III activities, oxygen consumption rates (OCRs), mitochondrial membrane potential (MPP) and lactate production) and on steroidogenesis (androstenedione and progesterone synthesis) were investigated. **Results:** All APs resulted in a concentration-dependent decrease in the ATP content of TICs. All APs in their estimated IC_{50}s (6 µM, 21 µM, 35 µM and 37µM for CPZ, HAL, CLZ and RIS respectively) significantly decreased TICs OCRs ($p<0.0001$), MPP ($p<0.0001$) and significantly ($p =0.0003$) inhibited mitochondrial complex I activity. Only typical APs inhibited complex III ($p=0.005$). Also, APs in IC_{50}s increased TICs lactate production to varying degrees. All APs used at their IC_{50}s significantly inhibited progesterone ($p=0.0022$) and androstenedione ($p=0.0027$) production. Only CPZ was found to inhibit these hormones in the low concentration (1µM). **Conclusion:** All four antipsychotics seem to inhibit mitochondrial bioenergetics and steroidogenesis in rat's ovarian theca cells. These findings support the hypothesis that APs-induced reproductive toxicity may be through mechanisms involving mitochondrial insult. Further research is required to establish the link between APs-induced mitochondrial dysfunction and disordered steroidogenesis.

Keywords: Antipsychotics; ; ; , Ovarian cytotoxicity, Mitochondrial bioenergetics, Reproductive toxicity

INTRODUCTION

Antipsychotics (APs) are well known for their toxicity, which affects almost all systems of the body resulting in a wide range of manifestations such as extra-pyramidal symptoms, weight gain and reproductive dysfunction. Chlorpromazine (CPZ) has been shown to inhibit ovulation in rats via suppression of the LH surge (Everett and Taya, 1982). Haloperidol (HAL) has been shown to lower ovarian and uterine weights and to inhibit ovulation in rats (Boris et al., 1997). Antipsychotic-induced hyperprolactinaemia has been strongly suggested as a mechanism for these reproductive dysfunctions. Several studies have reported an association between hyperprolactinaemia and reproductive dysfunction in women receiving APs (Ghadirian et al., 1982; Meltzer, 1985; Bargiota et al., 2013). However, other studies reported no association between hyperprolactinaemia and reproductive dysfunction during AP therapy (Canuso et al, 2002). Therefore, the mechanisms explaining the reproductive adverse effects of APs remain largely uncertain.

Several APs, such as haloperidol (HAL), chlorpromazine (CPZ), thiothixine and clozapine (CLZ), have been found to inhibit mitochondrial respiratory enzyme complexes, which have a crucial role in Oxidative phosphorylation cycle for the synthesis of adenosine triphosphates (ATP) (Burkhardt et al, 1993). Inhibited respiratory enzyme complexes have been found to induce damage to the mitochondria via the release of reactive oxygen species (ROS) (Wei et al., 1998 and Harper et al., 2004, Guo et al., 2013). This mitochondrial damage has been suggested as a possible mechanism of AP-induced extrapyramidal side effects such as tardive dyskinesia (Goff et al., 1995 and Casademont et al., 2007). This hypothesis, which is widely accepted, has been supported by several studies reporting a correlation between the tardive dyskinesia and mitochondrial inhibition during APs treatment and production of ROS (Grenell et al, 1955; Guth et al, 1964; Gallagher et al, 1965, Elkashef and Wyatt, 1999).

In this study, we hypothesized that mitochondrial dysfunction is the underlying mechanism of AP-induced reproductive adverse effects. Ovarian theca interstitial cells (TICs) were isolated from rats and used as a cell model to test the effects of APs. The primary aim of this study was to investigate the cellular and functional effects of two typical APs (haloperidol [HAL], chlorpromazine [CPZ)]) and two atypical APs (risperidone [RIS] and clozapine [CLZ]) on mitochondrial bioenergetics and on steroidogenesis of rat's TICs.

MATERIALS

Chemicals and media

All chemicals and reagents including the APs were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise. Two typical (CPZ and HAL) and two atypical (RIS and CLZ) APs were obtained for this study. All stock solutions of drugs were made in DMSO (vehicle) then diluted further in media as required. Media and their additives were purchased from Gibco (Grand Island, NY). The adenosine triphosphate (ATP) assay and lactate colorimetric assay kits were purchased from Abcam (Cambridge, MA, USA).

Animals

Female Sprague-Dawley rats were used. They were provided with water and rat chow ad libitum and housed in air-conditioned rooms that were illuminated 14 h/day. The experiments were carried out in accordance with the principles and guidelines for the use of laboratory animals and approved by the institutional research animal committee, Mansoura University, Egypt.

Theca interstitial cells isolation and culture

In order to obtain rat pre-ovulatory follicles, immature 23- to 24-day-old female rats were injected with equine chorionic gonadotropin (eCG) (10 IU) between 09:00 and 09:30 h to enhance multiple follicular development. The rats were then anaesthetized and TICs were isolated according the method described by Hoang et al (2013). Briefly, ovaries were removed and follicles were punctured with needles to release granulosa cells (GCs) and oocysts. The remaining ovarian tissue was minced with a scalpel and digested in 100 μL/ovary of M199 medium with 0.35 mg/mL collagenase type IA, 10 ug/mL DNase, and 10 mg/mL BSA at 37ºC for 30 minutes. After digestion, the tissue was centrifuged at 1,000 rpm for 4 minutes then the media was aspirated, and cells were resuspended in 5 mL fresh M199. Debris and oocytes were subsequently removed using 100- and 40-mm cell strainers.

Theca cell purification was performed according to the methods described by Magoffin and Erickson (1988). Discontinuous Percoll gradients were formed using both 36% and 50% percoll solutions. Theca cells were separated in the interface between the two Percoll layers and aspirated using a 20-gauge needle and syringe. The isolated cells were then washed twice in M199 and resuspended in Hepes-buffered medium 199 with 5% FCS for 24 h, followed by 24–48 h in serum-free media containing 0.1% BSA. The primary TICs were cultured in individual wells of 24-well culture plates (4x105/ well) (Falcon, Meylan Cedex, France), in 5% CO2/air atmosphere and maintained at 37˚C.

METHODS

Intracellular adenosine triphosphate (ATP) content

Intracellular ATP was measured using Abcam assay kit according to the manufacturer's protocol. Briefly, cells ($36x10^3$) were seeded in each individual well of a 96-well plate. After overnight incubation, cells were treated with the test APs in concentrations 0.1, 1, 10 and 100µM for 24 hrs. Afterward, 50 µl of cell lysis buffer containing an inhibitor of ATPase was added to each well, and the plate was shaken gently for 5 minutes. Then the 50 µl reconstituted substrate was added to each well and the plate shaken again for another 5 minutes and kept in dark for 10 minutes before measurements. The amounts of ATP were measured with a microplate scintillation counter 'TopCount' (Perkin Elmer, Ueberlingen, Germany) enabling quantitative measurements via luminescence detected by single photon counting. Basal values in the medium were subtracted from each reading obtained in the presence of test drug then viability was expressed as a percentage from the same concentration of the vehicle control readings, presuming that the vehicle control viability is 100%.

Effect of APs on TICs oxygen consumption rates (OCRs)

TICs were incubated with the vehicle and with inhibitory concentrations 50 (IC_{50}s, defined as the concentration that inhibits up to 50% of the viability of control cells) and 1µM of the APs for 24 hrs. Then cells were harvested by trypsinization, centrifuged and re-suspended in Hank's solution [that contained (in mM): 5.6 KCl, 138 NaCl, 4.2 NaHCO3, 1.2 NaH2PO4, 2.6 CaCl2, 1.2 MgCl2, 10 HEPES (pH 7.4 with NaOH), and 0.1% (wt/vol) BSA] and counted by haemocytometer. Then oxygen consumption rats (OCR) were assessed polarographically using Clark oxygen electrodes (Rank Brothers). Firstly, OCRs were studied in the basal condition for 10 minutes then 2µL of 6mM azide solutions were added to each chamber. OCR was measured as the change in oxygen tension level over a 300-sec period. In all OCR experiments; subsequent application of 6 mm azide, a blocker of cytochrome oxidase, partly blocked cells O^2 consumption. This confirms that the majority of O^2 consumption measured reflects mitochondrial respiration. Also the TICs' response to azide confirms absence of cellular damage due to stirring. For glucose, the slope was measured 120 seconds after reagent addition, whereas for azide, the slope was measured 60 seconds after its addition.

Effect of APs on TICs Mitochondrial membrane potential (MPP)

To monitor the changes in TICs MMP we used rhodamine-123 (Rh-123) which is a well-established cationic lipophilic dye with high affinity to be localized to mitochondria. Depolarization of mitochondrial membranes, caused by uncoupling or blocking of mitochondrial respiration, results in redistribution of the dye with less amount retained inside the cells and increased measured fluorescence outside the cells and vice versa with increased hyperpolarization of the mitochondrial membranes (Duchen et al., 1993; Baracca et al., 2003). Briefly, TICs were treated with APs in both 1µM and IC_{50}s. After 24 hrs cells were harvested and counted. Then cells were loaded with 50µg/ml Rh-123 in Hank's solution (same constitutes as Hank's solution used for OCR) at 37^{o}C in a quartz cuvette in a spectrophotometer (Hitachi) for 3 minutes. After 3 minutes Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1µM) was added for each cuvette to confirm the functional integrity of the cells. The dye was excited at 480 nm and the emitted fluorescence monitored at 530 nm. Fluorescence was normalized to the number of cells.

Effect of APs on mitochondrial complexes

TICs were seeded in T75 flasks till confluence, then treated with the test APs, used at the IC_{50} concentration for 24 hrs. Complexes were then assayed using TIC mitochondrial enriched fraction for complex I and TIC lysate for complex III. Both mitochondrial enriched fraction and cell lysates were prepared following methods described by Spinazzi et al. (2012). For complex I assay, Dichlorophenolindophenol (DCIP) was used as a terminal electron acceptor (Janssen et al., 2007). Complex I oxidizes NADH, and the electrons produced reduce the artificial substrate decylubiquinone that subsequently delivers the electrons to DCIP. Complex III activities were studied as previously described by Spinazzi et al. (2012).

Lactate production assay

TICs were seeded in 24-well plates and treated with IC_{50} of the test APs. The supernatant media were collected and assayed immediately for lactate levels using glucose assay kit and lactate assay kit according to the manufacturer's instruction. Lactate production was normalized to cell number and expressed as a percentage of control lactate production. All experiments were performed in triplicate (i.e. three wells of each concentration in each experiment) and repeated three times.

Effect of APs on progesterone and androstenedione secretion

TICs were incubated with APs at both estimated IC_{50}s and 1µM for 24hrs. Enzyme linked immunosorbent assay (ELISA) was used to measure progesterone and androstenedione in the

media obtained from the cultured TICs before and after treatment with the APs. For progesterone assay, progesterone ELISA kit was purchased from (Enzo Life Sciences) with sensitivity down to 8.57 pg/ml. The results were read at Absorbance of 405 nm. While for androstenedione assay, the kit was obtained from Aviva Systems Biology with detection range of 0.312 - 20 ng/mL and 405 nm was used as the accurate absorbance to read the results.

Statistical analysis

Statistical analysis was performed using Prism V6 6 (GraphPad Software Inc., San Diego, CA). Data did not show normal distribution by as determined by D'Agostino–Pearson omnibus normality test. We therefore used Kruskall Wallis test with Dunn's multiple comparisons post-test to compare multiple treatments. The (IC_{50}) for each AP was quantified by fitting the data with the log(inhibitor) versus response -variable slope equation is Prism V6.

RESULTS

Antipsychotics and TIC viability

Intracellular ATP assay showed concentration-dependent decrease in TICs intracellular ATP concentration after 24-hr treatment with the four tested APs (figure 1A). The estimated IC_{50}s for the studied APs were 6, 21, 35 and 37µM for CPZ, HAL, CLZ and RIS respectively (24 hours post-treatment). According to these values, CPZ was the most potent, while RIS was the least toxic with the highest IC_{50} (figure 1B).

Antipsychotics decreased TICs oxygen consumption rates (OCRs)

All APs significantly ($p<0.0001$) decreased TICs' basal OCRs, as shown in figure (2A), by 56.3±14.4% ($p<0.001$), 34.5±12.5% ($p<0.01$), 36±13.5 ($p<0.01$) and 26.2±17.6% ($p<0.05$) for CPZ, HAL, CLZ and RIS respectively in their IC_{50}s. None of the APs in a concentration of 1µM had any statistically significant effect on TICs' OCRs (Figure 2B).

Antipsychotics and MMP

Figure 2C shows significant ($p<0.0001$) increase in Rhodamine-123 (Rh-123) in TICs treated with all APs (used at their IC_{50}s). This signifies a decrease in TICs' mitochondrial membrane potential. However, all APs had no significant effect on MMP of TICs in 1µM concentration (figure 2D).

Antipsychotics and mitochondrial complexes I and III functions

As shown in figure 3A, all tested APs used at their IC_{50}s significantly ($p<0.0001$) inhibited mitochondrial complex I activities. CPZ was the most potent, while CLZ was the least potent. With regards to mitochondrial complex III, only the typical preparations (CPZ and HAL) showed significant inhibitory effects (Fig 3B). On the other hand, only CPZ in 1µM concentration significantly ($p=0.032$) inhibited complex I.

APs and TICs lactate production

As shown in figure 3C, all APs at IC_{50}s significantly ($p< 0.001$) increased TIC lactate production to different extents, with typical APs having more potent effects. On the other hand APs used at 10µM concentration had no statistically significant ($p=0.67$) effect on cellular lactate production.

Antipsychotics and TIC production of progesterone and androstenedione

As illustrated in figure 4, ELISA showed that all the tested APs at the IC_{50} concentration significantly lowered TICs' secretion of both progesterone and *Androstenedione* to different extents. CPZ showed the most potent inhibitory effect on progesterone secretion. In addition, typical APs (CPZ and HAL) showed a greater inhibitory effect on androgen productions compared to the newer atypical APs (RIS and CLZ). Only CPZ in a concentration of 1µM showed significant inhibitory effect of TIC secretory functions.

DISCUSSION

To the best of our knowledge, this is the first study to investigate the effects of antipsychotics (APs) on ovarian theca mitochondrial bioenergetics and steroidogenesis as possible mechanisms of reproductive toxicity. We found that APs impaired viability of rat's TICs in a concentration dependent manner, decreased TIC OCR, decreased mitochondrial membrane potential, inhibited mitochondrial complexes I and III activities, increased TICs lactate production and lowered TICs secretion of both progesterone and androstenedione.

The rat was chosen as a model for this study as it is relatively easy to isolate and culture theca cells from its ovaries. In addition, it has been shown to be a valuable *in-vitro* model for evaluating the safety of chemicals (Roberts, 2001). Theca interstitial cells (TICs) were used as they have an important role in ovarian steroidogenesis (Young and McNeilly, 2010). In addition, TICs' mitochondria have been found to play a crucial role in steroidogenesis as they convert cholesterol to pregnenolone (Miller, 2013). They also determine the net steroidogenic capacity of TICs thereby serving as chronic regulators of steroidogenesis (Miller, 2013). Theca cell mitochondrial damage will therefore affect steroidogenesis with subsequent reproductive dysfunction.

We investigated the effects of a wide range of AP concentrations (0.1, 1, 10 and 100µM) on intracellular ATP content. These concentrations correspond to therapeutic, hyper-therapeutic and toxic APs levels seen 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 µM) for CPZ, 0.1-0.7 mg/l (0.3–2µM) for CLZ, 0.006-0.24 mg/l (0.016-0.64 µM) for HAL and 0.003-0.12 mg/l (0.007–0.28 µM) for RIS (Winek et al., 2001). On the other hand, overdose serum levels were reported as 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). Higher concentrations were used to accelerate the effects of the APs on the TIC of the rats' ovaries.

The present study showed that APs decreased the intracellular contents of ATP in rat TICs. CPZ was the most toxic with the lowest IC_{50} followed by CLZ, whereas, HAL and RIS had less cytotoxic effects. This finding is not consistent with the general believe that typical APs are more cytotoxic than the atypical ones. However, recent research has revealed HAL, which is a typical AP to be less cytotoxic than CLZ metabolite (desmethyle CLZ), which is an atypical AP (Donard et al., 2003). Other studies however showed HAL to have a more potent neurotoxic due to its metabolite (Subramanyam et al., 1991; Burkhardt et al., 1993, Raudenska et al., 2013).

The present data on TICs' ATP are in agreement with several previous in-vivo and in vitro-studies investigating non-reproductive cells. Vairetti, et al. (1999) reported significantly decreased ATP contents in the cerebellum, striatum and cortex of HAL-treated rats. Heiser, et al. (2007) also found significantly decreased ATP levels in SH-SY5Y and in U-973 cell lines after treatment with HAL. On the other hand, CLZ and olanzapine showed no effect on ATP levels of these cell lines. Furthermore, our group has previously reported that CPZ, HAL, CLZ and RIS significantly decreased ATP production in brain microvascular cells in concentrations and exposure durations dependent manner (Elmorsy and Smith, 2015).

Our data on the effects of APs on TICs mitochondrial OCR are in agreement with the previous studies investigating the effect of APs on mitochondrial OCRs of non-reproductive cells. Nagads (1992) reported that CPZ in 0.1 mM increased sperms OCRs, but CPZ in 0.75mM decreased sperms OCRs. Modica-Napolitano et al. (2003) reported that that CPZ at a concentration of 100 µM increased OCR (4-5 folds) in isolated mitochondria from non-reproductive cells. On the other hand, they showed ClZ, RIS, QTN and OLZ to have no effect on OCRs. Ishikawa et al. (2006)

showed that HAL in a low concentration (10µg/mL) decreased OCRs of neuronal and myocardial cell lines.

Regarding Rh-123 assay, our results are similar to previous studies investigating the effects of APs on MMP in non-reproductive cells. Several APs (CPZ, HAL, RIS and CLZ) have been shown to decrease the mitochondrial trans-membrane potential (Neustadt and Pieczenik, 2008, Elmorsy and Smith, 2015)**.** Furthermore, Donohoe et al., (2006) showed APs to have an affect on the Calcium-calmodulin system, which is known to affect the mitochondrial membrane potential and ATP synthesis**.**

The present data regarding the effect of APs on TICs mitochondrial complexes are in agreement with other published articles on non-reproductive cells. An in-vitro study by Burkhardt et al. (1993) found that APs inhibited mitochondrial complex I. In addition, other studies found that injecting mice with different APs (HAL, CLZ and RIS) led to inhibition of complex I activity in different parts of the brain after a variable length of time ***(***Balijepalli et al., 2001; Karry et al,. 2004). Furthermore, Casademont et al. (2007) reported that HAL, RIS and CLZ could inhibit mitochondrial complex I.

It is clear from our data that all tested APs increased lactate production of TICs. This could be explained by the inhibitory effects of the APs on mitochondrial complexes and membrane potential resulting in inhibition of oxidative phosphorylation of glucose. This in turn will result in shift of the cells to glycolysis to maintain their energy requirements, thereby increasing the production of lactate. Our results are in agreement with in-vivo human studies. Glavina et al. (2011) reported that APs increased serum lactate level in patients receiving treatment with HAL and olanzapine (atypical AP). The study has also showed correlation between serum lactate levels and incidence of AP-induced extra-pyramidal side effects.

Our observations on the effects of APs on theca cell mitochondrial bioenergetics are important for several reasons. Firstly, the potency of the AP in compromising mitochondrial functions seems to correlate with the likelihood and severity of its side effects in humans. This is evidenced by the greater potency of typical APs (on mitochondrial functions), which are also known to cause greater side effects in humans compared to the typical ones. This suggests that the methods used in this study could be used to test the degree of toxicity of any newly developed APs. Secondly, the consistency of our data with previous in-vivo and in-vitro studies examining the effects of APs on mitochondrial function suggest that the mechanisms of oxidative phosphorylation are "generic" to

mammalian mitochondria. Thirdly, our results suggest that a respiratory substrate (such as hydroxybutyrate) or vitamin (such as coenzyme Q) could potentially be used clinically to overcome the inhibitory effects of APs on mitochondrial complexes, thereby potentially reducing the side effects and improving their tolerability. However, this hypothesis will need to be further investigated in future research. Finally, these data also suggest a role for serum lactate measurement as an early clinical biomarker for safety screening of the newly discovered APs.

In conclusion, APs seem to inhibit theca cell mitochondrial bioenergetics as well as steroidogenesis. These data support the hypothesis that the damaging effect of APs on TICs mitochondria could be the mechanism of APs-induced reproductive toxicity. However, the link between APs-induced mitochondrial and steroidogenic dysfunctions remains to be investigated.

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Figures legends:

Figure 1: The effects of APs on ATP content in TICs: (A) Intracellular TICs ATP content expressed as a percentage of the vehicle control. Data presented as means ± S.E.M. Solid lines have been fitted to the log (inhibitor) versus response-variable slope equation. B) estimated IC_{50}s of the four APs. IC_{50}s were estimated from the best fit of data with 95% confidence intervals. Data shown as mean ± 95% confidence interval.

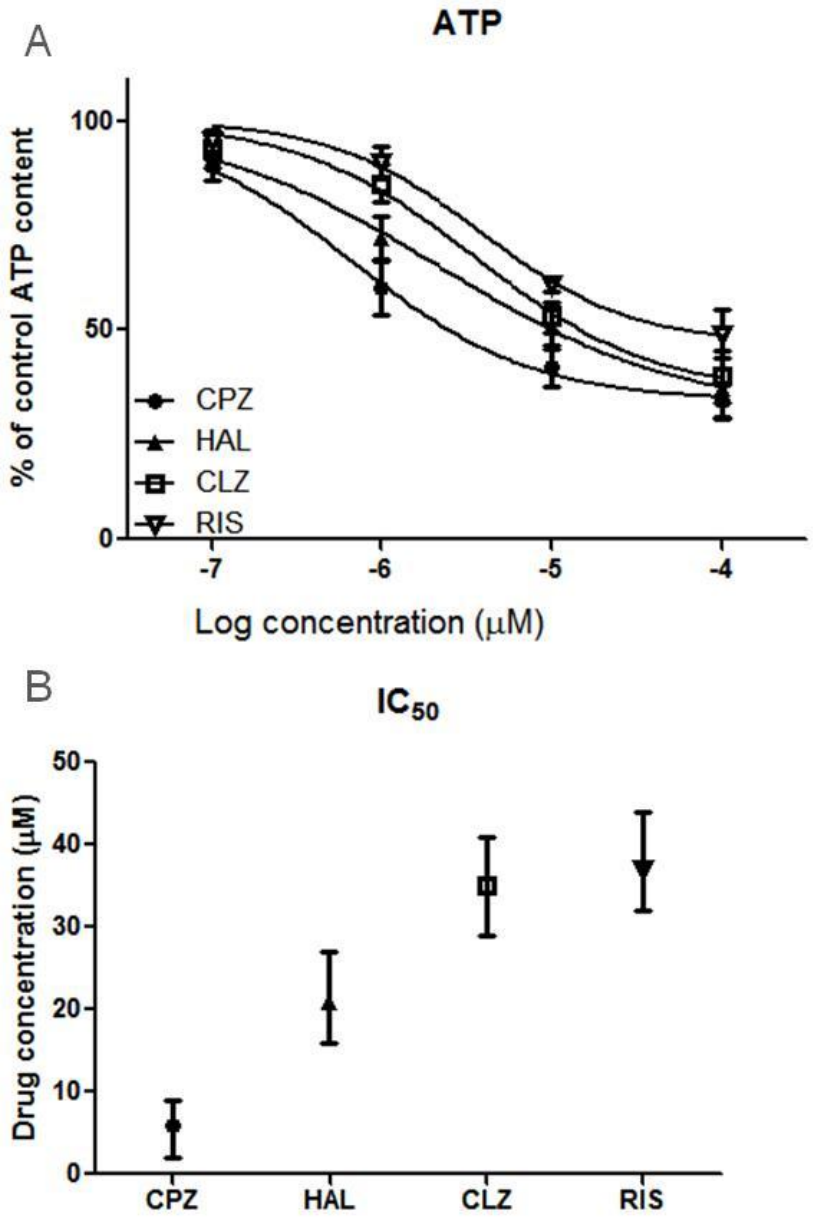
CPZ = chlorpromazine; HAL = Haloperidol; CLZ = Clozapine; RIS = risperidone

Figure 2: The effect of APs on oxygen consumption rates (OCRs) and mitochondrial membrane potential (MPP) of TICs. Data shown as mean ± SD. P-values showed in the graph were calculated by Dunn's post-test. (* indicates 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 APs in their IC_{50} and 1μM on TIC mitochondrial complexes I and III and on lactate production. Data expressed as a percentage of vehicle control. Readings for complexes activities of the treated samples was compared statistically with the vehicle samples. P-values showed in the graph were calculated by Dunn's post-test. (* indicates p-value <0.05, ** indicate p-value <0.01, and *** indicate $p < 0.001$ when compared with vehicle control treated group).

Figure 4: The effect of 24-hr incubation with APs on TICs steroidogenesis. A) the effect of APs in their IC_{50} on TICs progesterone secretion. B) the effect of the APs at 1 μM on TICs progesterone secretion. C) the effect of APs used at the IC_{50} on TICs androstenedione secretion. D) the effect of APs used at 1 μM on TICs androstenedione secretion. P-values showed in the graph were calculated by Dunn's post-test.* indicates p-value <0.05, ** indicate p-value <0.01, and *** indicate $p < 0.001$ compared to vehicle control treated group

Figure 1:



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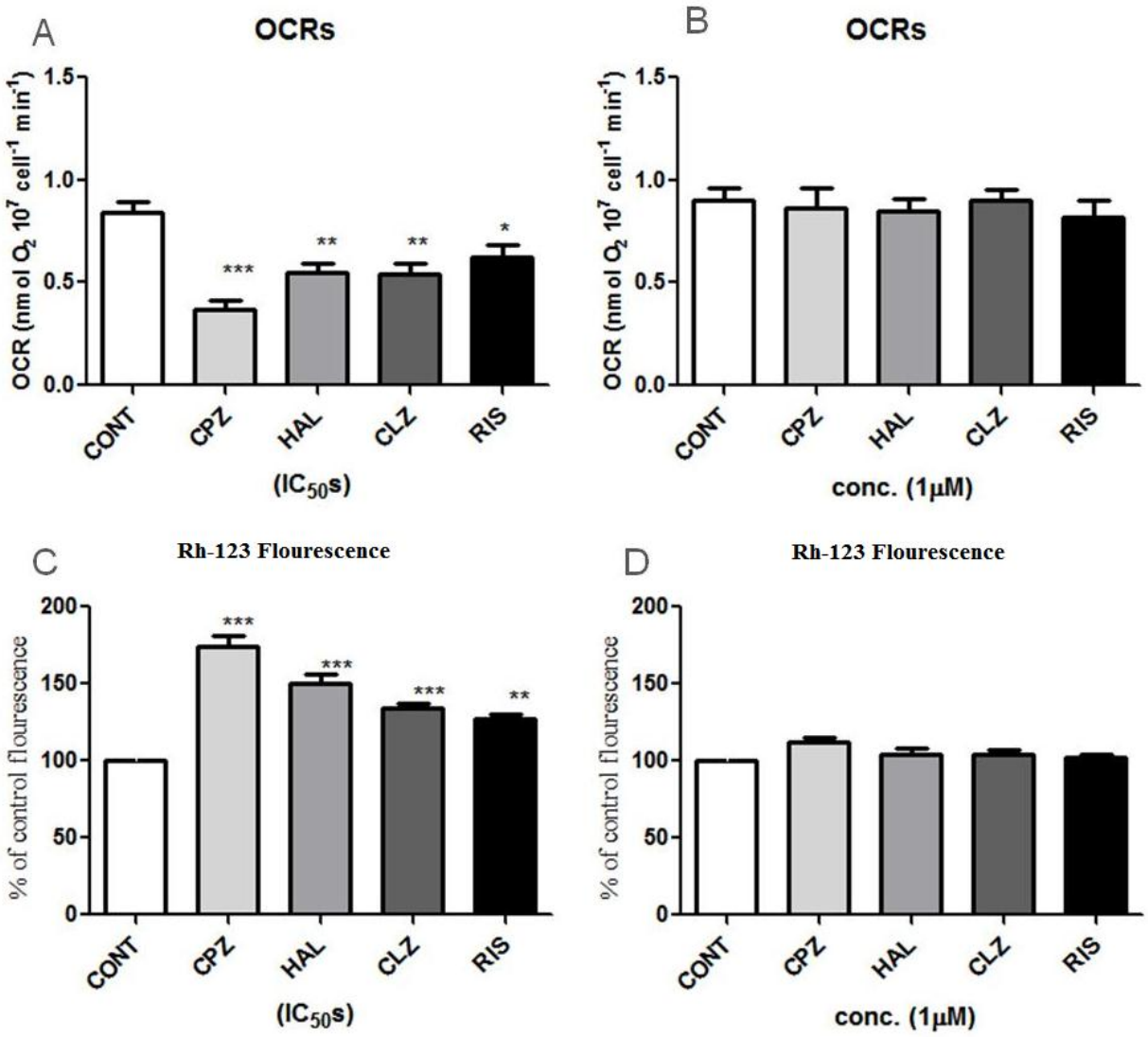

Figure 3:

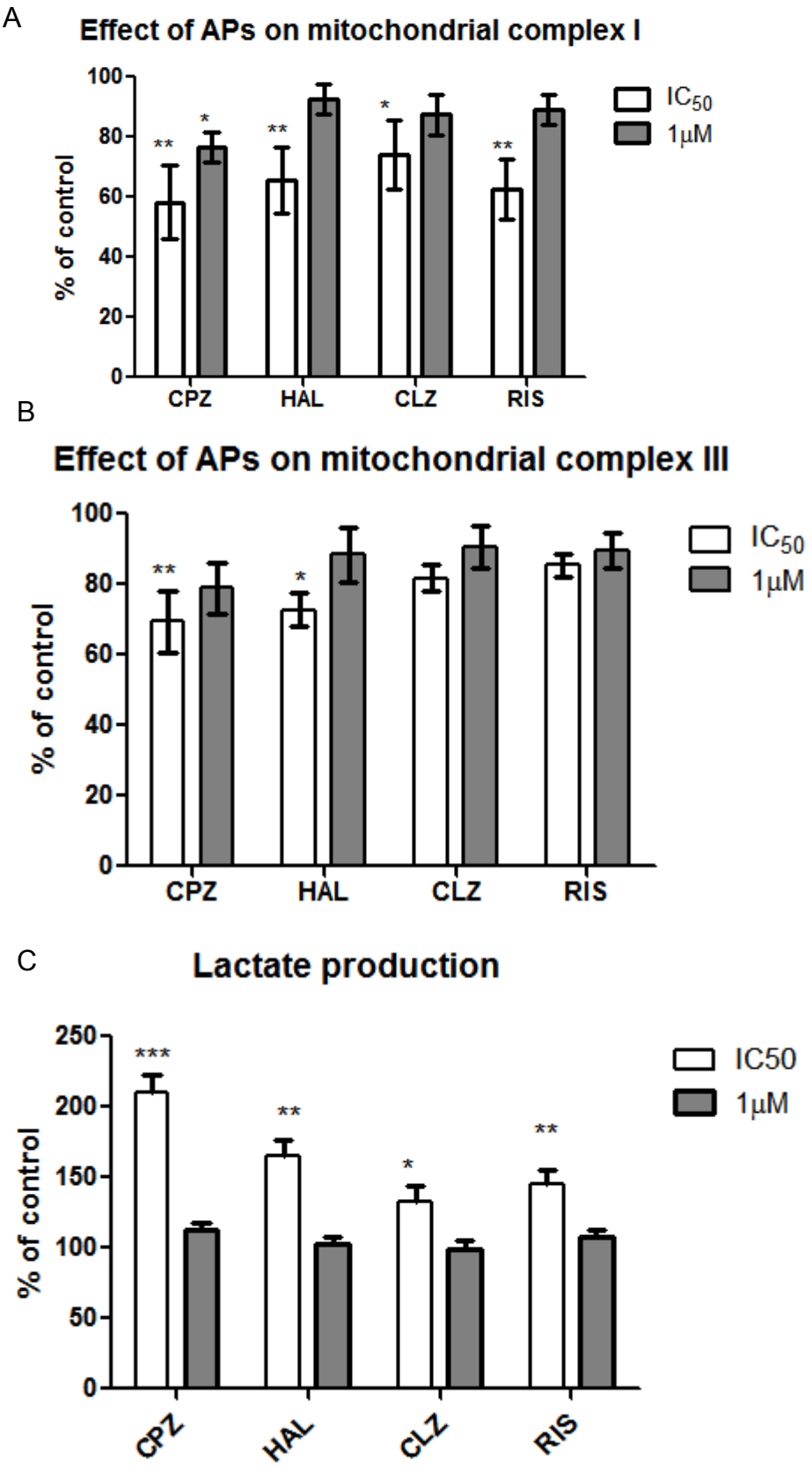

Figure 4: