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The role of oxidative stress in antipsychotics induced ovarian toxicity

Ekramy Elmorsy[a], Ayat Al-Ghafari[b], Amal Misbah Aggour[c], Raheela Khan[d], Saad Amer[d,*]

[a] Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura University, Egypt
[b] Biochemistry Department, Faculty of Science, King Abdulaziz University (KAU), Jeddah, Saudi Arabia
[c] Clinical Pathology Specialist, Ministry of Health, Egypt.
[d] Division of Medical Sciences & Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital Centre, Uttoxeter Road, Derby 22 DE22 3DT, United Kingdom

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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 μM 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 (5 mM) or quercetin (50 mM)) 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.

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; Parikha 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).

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

* Corresponding author.
E-mail address: saad.amer@nottingham.ac.uk (S. Amer).

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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)]. Theses 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 th 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 μL/ovary of M199 medium with 0.35 mg/mL collagenase type IA, 10 μg/mL DNase, and 10 mg/mL BSA at 37 °C for 30 min. After digestion, the tissue was centrifuged at 1000 rpm for 4 min 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_2 atmosphere at 37 °C.

2.4. Antipsychotics cytotoxicity by MTT

Following the manufacturer's protocol, TICs were seeded (1×10^4 cells per well) in 96-well plastic plates. After reaching confluence, cells were incubated for 4, 24 and 48 h in the presence of the test APs at concentrations of 0.1, 1, 10 and 100 μM 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 (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 IC_{50} of each AP (CPZ, 12 μM; HAL, 58 μM; RIS' 160 μM; CLZ, 44 μM) for 2 h. Following the manufacturer's protocol, cells were harvested and centrifuged at 1000 rpm for 5 min and lysed in 50 μL of chilled lysis buffer on ice for 10 mins. Cells were then centrifuged for 10 mins at 4 °C. The supernatant (50 μL) was added to an equal volume of 2 × reaction/dithiothreitol (DTT) buffer supplemented with the supplied caspase-3, -8 or -9 substrates and incubated at 37 °C for 2 h. 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 (ROSs) 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 IC_{50}s and 1 μM concentrations for 24-h 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 μM concentration. After 24 h, 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 min 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 (Armstrong and Browne, 1994). Briefly, TICs were treated with APs at their IC_{50}s and 1 μM concentrations for 24-h. 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 1000 g for 10 min. Then 700 μL of the supernatant was added to an equal volume of TBA (182 mg/25 ml) and heated to 100 °C for 15 min. The samples were then cooled and absorbance read at 535 nm (Ottolenghi, 1959).

2.9. Effect of antioxidants on APs-induced cytotoxicity

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

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 $P < 0.05$.

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