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Dual effect of insulin resistance and cadmium on human granulosa cells - *In vitro* study

Muskaan Belani [a], Preeti Shah [b], Manish Banker [b], Sarita Gupta [a,*]

[a] Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390 002, Gujarat, India.
[b] Nova IVI Fertility, Behind Xavier's Ladies Hostel, 108, Swastik Society Rd., Navrangpura, Ahmedabad 390009, Gujarat, India.

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ABSTRACT

Combined exposure of cadmium (Cd) and insulin resistance (IR) might be responsible for subfertility. In the present study, we investigated the effects of Cd *in vitro* in IR human granulosa cells. Isolated human granulosa cells from control and polycystic ovary syndrome (PCOS) follicular fluid samples were confirmed for IR by decrease in protein expression of insulin receptor-β. Control and IR human granulosa cells were then incubated with or without 32 μM Cd. The combined effect of IR with 32 μM Cd in granulosa cells demonstrated significant decrease in expression of StAR, CYP11A1, CYP19A1, 17β-HSD, 3β-HSD, FSH-R and LH-R. Decrease was also observed in progesterone and estradiol concentrations as compared to control. Additionally, increase in protein expression of cleaved PARP-F2, active caspase-3 and a positive staining for Annexin V and PI indicated apoptosis as the mode of increased cell death ultimately leading to decreased steroidogenesis, as observed through the combined exposure. Taken together the results suggest decrease in steroidogenesis ultimately leading to abnormal development of the follicle thus compromising fertility at the level of preconception.

1. Introduction

Reproductive disorders are important health issues. Many couples desire children but cannot achieve pregnancy through natural means. The causative factors of infertility may be identified in either of the partner, however, the cause of subfertility remains unknown in about 50% of these cases. It is now believed that a combination of environmental and endocrine factors may be responsible for the same.

In our previous studies we have discussed the effect of both cadmium and insulin resistance in isolation and combination as cited from various animal studies with respect to HPO axis and the results clearly demonstrate that Cd and IR together mediate more deleterious effects at different levels of reproduction affecting granulosa cell receptors, steroidogenesis, higher rate of apoptosis and dysregulation from hypothalamus in the form of decreased GnRH and gonadotropins (Belani Muskaan et al., 2014). We further wanted to study the combined effect of IR and Cd in *in vitro* condition in human model for which we chose human luteinized granulosa cells.

The presence of insulin receptor (INSR-β) in human luteinized granulosa cells (hLGC's) identifies ovary as a target of insulin activity (Poretsky et al., 1999). Alterations in insulin functioning due to sedentary life style and diet rich in carbohydrate leads to IR which is associated with abnormalities leading to infertility. Amongst several endocrine factors, IR is more prevalent and is present in 50–70% of PCOS women contributing to its pathogenesis (Mukherjee and Maitra, 2010). Increase in the fatty acid content during IR affects granulosa cells and developmental competence of oocyte possibly by influencing its lipid metabolism ultimately leading to poor ovulations, poor pregnancy outcomes and frequent miscarriages (Jakubowicz and Sharma, 2007). *In vitro* experiments with hLGC's from anovulatory PCOS subjects have demonstrated increased steroid accumulation with physiologically high levels of insulin accompanied by gonadotropins (Willis et al., 1996). On the contrary, resistance has been observed to insulin dependent glucose metabolism in human granulosa-luteal cells from anovulatory women with polycystic ovaries indicating inhibition of the metabolic activities (Rice et al., 2003). Cd is one of the reproductive toxicants exposed usually as a result of waste from human activities in the environment (Frydman et al., 2010). Its long half-life in vivo facilitates its bioaccumulation and results in bio magnification in the food chains, thereby exposing females through diet (Satarug et al., 2011). Cadmium is known to induce oxidative damage and apoptosis in cultured granulosa cells from chicken ovarian follicles (Jia et al., 2011). Exposure of Cd during their developmental age decreases the expression of StAR in granulosa cells of *F*1 generation rats (Pillai et al., 2010). Decrease in gene expression of CYP19A1 due to Cd exposure has been established in carp ovarian follicle (D DSaM, 2013). Presence of Cd has been identified

* Corresponding author at: Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390002, Gujarat, India.
E-mail addresses: muskaanbelani@gmail.com (M. Belani), preeti.shah@novaivifertility.com (P. Shah), manish.banker@novaivifertility.com (M. Banker), saritagupta9@gmail.com, sglmescrl@gmail.com (S. Gupta).

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in follicular fluid as well as oocytes of female smokers undergoing *in vitro* fertilization (IVF) therapy leading to reduction in fertility and fecundity (Zenzes et al., 1995; Thompson and Bannigan, 2008; Jackson et al., 2011). Studies have demonstrated increased accumulation of Cd in the ovary with an increase in age thus leading to failure of progression of oocyte development and ovulation (Frydman et al., 2010). *In vitro* administration of Cd to cultured human ovarian granulosa cells decreased preovulatory LH surge and progesterone secretion (Paksy et al., 1997). Cd accumulation in gonads of foetus decreases the number of germ cells and in the embryos it leads to degeneration, apoptosis and breakdown in cell adhesion thus inhibiting its progression to the blastocyst stage (Frydman et al., 2010).

Nowadays the risk of infertility is enhanced by a variety of environmental as well as lifestyle factors. Owing to this there has been an increase in the process of *in vitro* fertilization for the couples willing to have a child. hLGC's obtained from IVF patients after controlled stimulation and retrieved from the follicles during aspiration of the ovulated egg represent a homogeneous population of luteinized cells. Several such layers of cumulus and mural granulosa cells are generally discarded at the time of IVF. These granulosa luteal cells being the most abundant cell type inside the follicle undergo substantial differentiation, interact with oocytes, mediate the effect of gonadotropins on the follicular maturation and are easily accessible for studying the overall quality of the follicles in response to gonadotropins (Albertini, 2004). Hence these discarded granulosa luteal cells are used for extensive research purpose (Tripathi et al., 2013).

In the present paper, based on our prior work, we made an effort to understand *in vitro* effect of combined exposure of heavy metal Cd and IR in human luteinized granulosa cells (hLGC's) isolated from follicular fluid of PCOS and non-PCOS patients undergoing IVF. PCOS patients were classified as IR and NIR based on a novel molecular marker that is insulin receptor-β (INSR-β) expression on granulosa cells. We investigated protein expression of INSR-β in granulosa cells and then classified them as IR and NIR. We then exposed control and IR hLGC's to 32 μM Cd that has been used to define environmental, occupational and smoking risk factors in female reproductive life span (Paksy et al., 1997; Varga et al., 1993). The study is novel as there are no results for effect of Cd on naturally IR human luteinized granulosa cells mimicking today's scenario. Thus in the present study the combined effect of IR and Cd was evaluated on granulosa cell death parameters along with other physiological, biochemical and molecular parameters such as mRNA and protein expression of StAR for cholesterol transport and CYP11A1, 3β-HSD, CYP19A1 and 17β-HSD responsible for steroid synthesis.

2. Materials and methods

2.1. Hormones and reagents

Histopaque, Hyaluronidase, DMEM/F12, penstrep, amphotericin and trypan blue were from Sigma Chemical Co. (USA). Human estradiol and progesterone ELISA kits were procured from Diametra. Rabbit polyclonal antibody against INSR-β, CYP19A1 and β-actin was purchased from Cell Signaling and Goat polyclonal antibody against CYP11A1 was purchased from Santa Cruz. Rabbit polyclonal antibodies against 3β-HSD, 17β-HSD were generous gift from Dr. Vann Luu-The (CHUL Research Center and Laval University, Canada) and StAR from Dr. Douglas M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University, Lubbock, Texas, USA).

2.2. Human follicular fluid collection

Human follicular fluid samples were collected after informed consent from patients undergoing IVF/ ICSI over the course of 32 months at Nova Pulse IVI Fertility, Ahmedabad, India from 2012 August to 2015 April. All the controls and patients were non-smokers (according to hospital data) and underwent controlled ovarian hyperstimulation (COH) using flexible antagonist protocol. Recombinant FSH &/or urinary human menopausal gonadotropin (hMG) was started from 2nd day of period followed by the antagonist-cetrorelix acetate. Final oocyte maturity was triggered with recombinant human chorionic gonadotropin (hCG) or gonadotropin releasing hormone (GnRH) agonist. Follicles were aspirated by transvaginal ultrasound retrieval after 35 h of trigger injection. Follicular fluid was sent in embryology laboratory for oocyte identification & oocytes were separated out for IVF/ICSI. All the controls and patients received a GnRH analog (GnRH-a) in combination with FSH or human menopausal gonadotropin (hMG), followed by administration of human chorionic gonadotropin (hCG). The follicular fluid devoid of oocyte was collected for the experiments on the day of oocyte retrieval.

Inclusion criteria: The diagnosis included donors and PCOS with an age ranging from 20 to 40 years.

Exclusion criteria: Patients with endometriosis and poor ovarian response were excluded from the study.

The study was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The M. S. University of Baroda, Vadodara (Ethical Approval Number FS/IECHR/BC/SG2).

2.3. Human granulosa-luteal cell isolation

DMEM/F12 with 10% FBS and Penicillin-G/Streptomycin (100 IU/ml/100 mg/ml) was used as the basal medium for human luteal granulosa cell (hLGC) preparation and culture. Granulosa cells from follicular aspirates of individual patients $n = 30$ control and $n = 39$ PCOS were isolated using the protocol in the literature (Földesi et al., 1998). The follicular aspirates were centrifuged at 300 *g*, room temperature for harvesting the cells. The harvested cells were resuspended in plain DMEM/F12 media and layered on Histopaque to form a gradient followed by centrifugation at 400 *g* at room temperature to remove red blood cells and white blood cells. The middle layer of cells containing LGCs was collected and suspended in 10 ml volume of DMEM/F12 medium and washed thrice with DMEM/F12 by a further 5 min centrifugation. The hLGCs pellet was then incubated with 0.1% hyaluronidase in DMEM/F12 medium without FBS for 30 min at 37 °C with constant shaking and gentle repeated pipetting to obtain single cell suspension. The enzymatic reaction was stopped by addition of DMEM/F12 with FBS followed by centrifugation at 300 *g* at room temperature. The cell pellet was finally suspended in 1 ml of DMEM/F12 with FBS. The viable cell count by trypan blue dye exclusion was observed to be 90%. 0.5×10^6 cells from each patient were aliquoted for protein expression of INSR-β. Rest of the cells were cryopreserved for further use.

2.4. Immunocytochemistry

Isolated cells from follicular fluid samples were characterized by immunocytochemistry for granulosa cells. Cells were grown on glass slides in plastic tissue culture dishes. After their adherence, the cells were fixed with ice chilled methanol or 4% paraformaldehyde at 4 °C for 10 min followed by 2 washes with PBS. The cells were permeabilized with 0.1% Triton X-100 for 3–4 min at 4 °C followed by 2 washes with PBS. Incubation was then done with blocking buffer (0.5% BSA + 0.5% FBS in PBS) for 40–45 min at RT. The cells were then incubated overnight with primary antibody (CYP19A1, 17β-HSD and 3β-HSD) at a dilution of 1:100 at 4 °C. After 5 washes with washing buffer (PBS + 0.1% Tween 20) the cells were incubated in secondary antibody (goat anti-rabbit IgG-FITC) at a dilution of 1:250 for 1 h at R.T. After 5 washes with washing buffer nuclear staining was done with 100 ng/ml DAPI for 5 min. The cells were washed with PBS and then glass slide was mounted on a slide in mounting medium and then observed under Zeiss Laser scanning confocal microscope-710. The excitation/emission spectrum for DAPI complexes was 358 nm/461 nm. The excitation/