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Melatonin protects against clomiphene citrate-induced generation of hydrogen peroxide and morphological apoptotic changes in rat eggs

Anima Tripathi [a], Karuppanan V. PremKumar [a], Ashutosh N. Pandey [a], Sabana Khatun [b], Surabhi Kirti Mishra [b], Tulsidas G. Shrivastav [b], Shail K. Chaube [a,*]

[a] Cell Physiology Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India
[b] Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Baba Gang Nath Marg, Munirka, New Delhi 110067, India

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ABSTRACT

The present study was aimed to determine whether clomiphene citrate-induces generation of hydrogen peroxide in ovary, if so, whether melatonin could scavenge hydrogen peroxide and protect against clomiphene citrate-induced morphological apoptotic changes in rat eggs. For this purpose, forty five sexually immature female rats were given single intramuscular injection of 10 IU pregnant mare's serum gonadotropin for 48 h followed by single injections of 10 IU human chorionic gonadotropin and clomiphene citrate (10 mg/kg bw) with or without melatonin (20 mg/kg bw) for 16 h. The histology of ovary, ovulation rate, hydrogen peroxide concentration and catalase activity in ovary and morphological changes in ovulated eggs were analyzed. Co-administration of clomiphene citrate along with human chorionic gonadotropin significantly increased hydrogen peroxide concentration and inhibited catalase activity in ovary, inhibited ovulation rate and induced egg apoptosis. Supplementation of melatonin reduced hydrogen peroxide concentration and increased catalase activity in the ovary, delayed meiotic cell cycle progression in follicular oocytes as well as in ovulated eggs since extrusion of first polar body was still in progress even after ovulation and protected against clomiphene citrate-induced egg apoptosis. These results clearly suggest that the melatonin reduces oxidative stress by scavenging hydrogen peroxide produced in the ovary after clomiphene citrate treatment, slows down meiotic cell cycle progression in eggs and protects against clomiphene citrate-induced apoptosis in rat eggs.

1. Introduction

Free radicals are generated as byproducts of normal cellular metabolism and serve as key signal molecules in various physiological and pathological processes (Agarwal et al., 2005; Fujii et al., 2005). Ovary is a metabolically active organ that generates reactive oxygen species on an extraordinary scale during follicular development and ovulation and their effects are neutralized by intricate array of antioxidants (Agarwal et al., 2005; Fujii et al., 2005; Sugino, 2005). Generation of a tonic level of reactive oxygen species is beneficial for meiotic progression in mammalian oocytes (Chaube et al., 2008; Tripathi et al., 2009, 2010). On the other hand, overproduction of reactive oxygen species in the follicular fluid or depletion of antioxidant system leads to oxidative stress (Agarwal et al., 2005). The oxidative stress reduces egg quality, fertilization and pregnancy rates in mouse and human (Tamura et al., 2008).

Ovulatory dysfunction is one of the most common causes of reproductive failure in sub-fertile and infertile women. The clomiphene citrate, a nonsteroidal triphenylethylene compound, is a first line of medicine used for the induction of ovulation in anovulatory women worldwide. In spite of high ovulation induction with the use of clomiphene citrate, the pregnancy rate is much lower (Mitwally and Casper, 2002). Such a discrepancy is believed to be due to the peripheral anti-estrogenic effect of clomiphene citrate, particularly at the level of ovary, endometrium and cervical mucus (Marut and Hodgen, 1982; Mitwally and Casper, 2002). The possible mechanism by which clomiphene citrate exerts its anti-estrogenic effect at the level of ovary is poorly understood. Using rat as an animal model, we reported that the clomiphene citrate induces granulosa cell apoptosis and reduces estradiol synthesis (Chaube et al., 2005a). Reduced estradiol level in ovary leads to poor development and maturation of oocytes and induce apoptosis after clomiphene citrate treatment (Chaube et al., 2005a, 2006). Clomiphene citrate induces oxidative stress and apoptosis in somatic cell lines (Hayon et al., 1999; Shao et al., 2009). However, it remains unclear whether clomiphene citrate-induced adverse effects at the level of ovary are associated with the production of reactive oxygen species.

Melatonin (a naturally occurring potent antioxidant) scavenges free radicals (Tan et al., 2007) on one hand and induces antioxidant enzymes such as superoxide dismutase (Liu and Ng, 2000; Ozturk

* Corresponding author. Tel.: +91 542 6702507; fax: +91 542 2368174.
E-mail address: shailchaubey@gmail.com (S.K. Chaube).

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et al., 2000) and glutathion peroxidase activities on the other hand to prevent oxidative damage (Reiter et al., 2001, 2009). Exogenous supplementation of melatonin has been reported to decrease ovarian weight (Wurtman et al., 1963), inhibit steroidogenesis, folliculogenesis, oocyte maturation capacity (Adriaens et al., 2006; Hemadi et al., 2009; Shi et al., 2009), ovulation (Longenecker and Gallo, 1971; Tamura and Kogo, 1989; Ying and Greep, 1973), protect eggs from free radical damage, improve fertilization rate (Adriaens et al., 2009; Berlinguer et al., 2009; Feng and Zhang, 2005; Kang et al., 2009; Tamura et al., 2008; Tsiligianni et al., 2009; Vazquez et al., 2010) and induce embryo viability (Forcada et al., 2006). However, it remains unclear whether melatonin can reduce oxidative stress and protect against clomiphene citrate-induced morphological apoptotic changes in eggs. Hence, the present study was designed to find out whether clomiphene citrate induces generation of reactive oxygen species in ovary, if so, whether melatonin can protect against clomiphene citrate induced oxidative stress. Since clomiphene citrate does not induce ovulation in animal model, immature rats were subjected to superovulation induction protocol and then possible effects of clomiphene citrate with or without melatonin on ovary weight, ovulation rate, hydrogen peroxide concentration and catalase activity in the ovary, morphological apoptotic changes and DNA fragmentation in ovulated eggs were analyzed.

2. Materials and methods

2.1. Chemicals and animal maintenance

All chemicals used in the present study were purchased from Sigma Chemical Co. St. Louis, MO, USA, unless stated otherwise. Sexually immature female rats (24–25 days old; 50 ± 5 gm body weight; bw) of Charles-Foster strain were separated from existing colony of departmental animal facility and maintained in normal husbandry conditions with food and water ad libitum. All procedures confirmed to the stipulations of the Departmental Animal Ethical Committee of the University, Varanasi-221005 and followed the guidelines for the care and use of laboratory animals (NIH Publication).

2.2. Effects of melatonin on clomiphene citrate-induced changes in ovary weight, ovulation rate, histology of ovary, hydrogen peroxide concentration in ovary

In the first series, experiments were conducted to find out the effect of melatonin on clomiphene citrate-induced changes in ovary weight and its histology, ovulation rate, and hydrogen peroxide concentration in ovary. For this purpose, 15 rats were divided into 5 groups of 3 rats each. The first group was injected with 0.9% normal saline (vehicle control), while second group was subjected to superovulation induction protocol (i.e. 10 IU pregnant mare's serum gonadotropin for 48 h followed by 10 IU human chorionic gonadotropin for 16 h). The third group was first injected with 10 IU pregnant mare's serum gonadotropin for 48 h followed by co-administration of 10 IU human chorionic gonadotropin along with 20 mg/kg bw dose of melatonin for 16 h. The fourth group was first injected with 10 IU pregnant mare's serum gonadotropin for 48 h followed by co-administration of 10 IU human chorionic gonadotropin along with 10 mg/kg bw dose of clomiphene citrate for 16 h. The 10 mg/kg bw dose of clomiphene citrate has been reported to inhibit gonadotropin-induced ovulation almost by 50% and induced egg apoptosis (Chaube et al., 2005a, 2006). The fifth group was treated first with 10 IU pregnant mare's serum gonadotropin for 48 h followed by co-administration of 10 IU human chorionic gonadotropin, 10 mg/kg bw dose of clomiphene citrate and 20 mg/kg bw dose of melatonin. The 20 mg/kg bw dose of melatonin has been reported to prevent oxidative stress damage caused by phenylketonuria in rat (Martinez-Cruz et al., 2002). After 16 h, rats were euthanized, ovaries along with oviducts were removed in a 35 mm tissue culture dishes containing 2 ml of pre-warmed culture medium-199, pH 7.2, containing 0.035% sodium bicarbonate, 10% fetal bovine serum and 100 µl of Gentamicin/Penicillin/Streptomycin solution in 100 ml culture media. Weight of ovary was taken and then one ovary from each animal was used for histology and another ovary was used to prepare ovarian lysate for the analysis of hydrogen peroxide concentration in the ovary.

2.3. Histology of ovary

For histology, one ovary from each control and various treatment group animals were removed and placed in buffered formaldehyde (3.7% in Phosphate Buffer Saline (PBS); pH 7.4) for 24 h, embedded in paraffin by routine method, serially sectioned at 5 µm thickness and stained in haematoxylin/eosin for microscopic observation. Experiment was repeated three times and representative photographs at 100× and 400× magnification are shown in the result section.

2.4. Quantitative estimation of hydrogen peroxide concentration in ovary

In the second series, experiments were conducted using 15 rats to find out the exogenous supplementation of melatonin on hydrogen peroxide concentration and catalase activity in ovary. For this purpose, similar treatment protocol was followed as described for the first series of experiments. At the end of treatment protocol, one ovary from each control and various treatment groups were homogenized in 2 ml of phosphate buffer saline (50 mM; pH 7.4). The homogenate was centrifuged at 10,000 × *g* at 4 °C for 30 min and supernatant was used immediately for the quantitative estimation of hydrogen peroxide using hydrogen peroxide calorimetric assay kit (R&D Systems, MN) as per company manual protocol. In brief, all reagents working standards and samples were brought to room temperature before use. The 50 µl of sample diluent was added to the blank wells and 50 µl of hydrogen peroxide standards or samples to remaining wells in duplicate. Thereafter, 100 µl hydrogen peroxide color reagent was added to each well and the plate was incubated for 30 min at room temperature after tapping the side of the plate gently for 10 s. At the end of the incubation period, the optical density (OD) was determined using a microplate reader set to 550 nm. The samples were run in triplicate and all samples were run in one assay to avoid inter-assay variation and intra-assay variation was 3%.

2.5. Measurement of catalase activity in ovary homogenates

Another ovary from second series of experiments was homogenized in 2 ml cold lysis buffer (5 mM Tris, 20 mM Ethyl diamine tetraacetic acid (EDTA), 0.5% TritonX-100, pH 8). The homogenate was centrifuged at 10 000 × *g* at 4 °C for 15 min and clear supernatant was collected and kept at -20 °C until assay. The catalase activity (One unit of catalase is the amount of catalase decomposes 1.0 µM of H_2O_2 min^{-1} at pH 4.5 at 25 °C) was analyzed using catalase activity assay kit (Cat. No. K773-100) purchased from BioVision, CA, USA. The catalase activity was measured as per company manual instruction. At the end of the incubation period, the OD was determined using a microplate reader set to 570 nm. The samples were run in triplicate and all samples were run in one assay to avoid inter-assay variation and intra-assay variation was 1%.

2.6. Effect of melatonin on clomiphene citrate-induced morphological changes in eggs

In the third series, experiments were conducted using 15 rats to find out whether melatonin can protect against clomiphene citrate-induced morphological apoptotic features in rat eggs. For this