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Original research article

Roscovitine inhibits extrusion of second polar body and induces apoptosis in rat eggs cultured *in vitro*

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ABSTRACT

Background: Inhibition of cyclin-dependent kinases (Cdks) may result in meiotic cell cycle arrest and apoptosis in rat eggs *in vitro*. We aimed to find out whether roscovitine, a Cdk inhibitor, inhibits extrusion of second polar body (II PB) and induced egg apoptosis *in vitro*.
Methods: The metaphase-II (M-II) arrested eggs were collected from oviduct and exposed to various concentrations of roscovitine for 3 h *in vitro*. The morphological changes, phosphorylation status of Cdk1, cyclin B1 level, hydrogen peroxide (H_2O_2), p53, Bax, Bcl2 and cytochrome c expressions, caspase-3 activity and DNA fragmentation were analyzed.
Results: We showed that the lower concentrations of roscovitine significantly reduced Thr-161 phosphorylated Cdk1 level and inhibited extrusion of II PB. The higher concentrations of roscovitine significantly reduced Thr-161 phosphorylated Cdk1 level but total Cdk as well as cyclin B1 levels remained high. Higher concentrations of roscovitine increased H_2O_2 level and expressions of p53, Bax and cytochrome c in treated eggs. The increased proapoptotic factors induced capsase-3 activity and thereby DNA fragmentation that finally resulted in cytoplasmic fragmentation, a morphological apoptotic feature.
Conclusion: Our data suggest that roscovitine inhibited II PB extrusion possibly by reducing Thr-161 phosphorylated Cdk1 level and induced apoptosis through mitochondria-caspase-mediated apoptotic pathway in rat eggs cultured *in vitro*.

Introduction

Meiotic cell cycle in mammalian oocytes/eggs is a dynamic process that involves several stop/go channels. Freshly ovulated eggs are arrested at metaphase-M-II (M-II) stage until fertilizing spermatozoa triggers an exit from M-II arrest [1,2]. Interestingly, rat eggs do not wait for fertilization, extrude second polar body (IIPB) and undergo spontaneous exit from M-II arrest [3–5]. However, factor(s) that drive spontaneous exit from M-II arrest remain poorly understood in rat. Nevertheless, a spontaneous exit from M-II arrest limits animal cloning through somatic cell nuclear transfer (SCNT) program and directly affects assisted reproductive technologies' (ARTs) outcome [3,6]. Hence, the molecular mechanism underlying M-II arrest is of great importance in terms of increasing number as well as the quality of eggs available for various ARTs programs.

Meiotic cell cycle is regulated by maturation promoting factor (MPF) [2,7]. High MPF activity is required for the maintenance of M-II arrest, while low MPF activity triggers exit from M-II arrest [8,9]. The MPF inactivation does not solely depend on cyclin B1 degradation [8]. The dephosphorylation at Thr-161 residues of cyclin-dependent kinase 1 (Cdk1) makes MPF inactive leading to resumption of meiosis [10,11], while Thr-161 phosphorylation activates MPF and maintains M-II arrest [8,9]. Based on these studies, we propose that the increased accumulation of Thr-161 phosphorylated Cdk1 may trigger the maintenance of M-II arrest, while decrease in its level may induce spontaneous exit from M-II arrest in rat eggs cultured *in vitro*. However, there is no evidence to support this possibility. To analyze the impact of phosphorylation of Cdk1 in regulation of either M-II or exit from M-II arrest, roscovitine has been used in the present study. Roscovitine is a purine analog that competes with adenosine triphosphate (ATP) for binding to the active site of CDKs [12]. Roscovitine is reported to induce cell cycle arrest [13] and apoptosis in various somatic cell types [14,15].

The decrease of Thr-161 phosphorylation of Cdk1 or increase of Thr-14/Tyr-15 phosphorylation of Cdk1 in ovulated aging eggs

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may not stay longer. The energy utilization by an egg under *in vitro* culture conditions results in the depletion of cellular ATP that may induce generation of reactive oxygen species (ROS) and initiate egg apoptosis. Hence, we propose that high levels of both Thr-161 phosphorylated Cdk1 as well as cyclin B1 levels may promote meiotic cell cycle arrest at M-II stage, while reduction in their levels may trigger spontaneous resumption of meiosis from M-II arrest in rat eggs. Further, sustained low level of Thr-161 phosphorylated Cdk1 in aging eggs may lead to generation of ROS. The increased level of ROS may initiate pro-apoptotic signals to induce egg apoptosis. Therefore, the present study aimed to find out whether roscovitine could reduce the level of Thr-161 phosphorylated Cdk1 and induce generation of H_2O_2. If yes, whether increased level of H_2O_2 could inhibit extrusion of IIPB and induce apoptosis through p53-dependent mitochondria-mediated pathway in rat eggs cultured *in vitro*.

Materials and methods

Chemicals and preparation of culture medium

All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and antibodies from Santa Cruz Biotechnology, CA, USA unless stated otherwise. The culture medium (M-199; HiMedia Laboratories, Mumbai, India) was prepared as per company manual protocol and the pH was adjusted to 7.2 and the osmolarity of the culture medium was found to be 290 mOsmol. The culture medium was supplemented with sodium bicarbonate (0.035%, w/v), penicillin (100 IU/ml) and streptomycin (100 mg/ml).

Experimental animals

The sexually immature female rats (22–24 days old) of Charles-Foster (CF) strain were housed in air-conditioned, light controlled rooms, with food and water *ad libitum*. These female rats were subjected to superovulation induction protocol (20 IU pregnant mare's serum gonadotropin for 48 h followed by 20 IU human chorionic gonadotropin for 14 h) to get large number of ovulated eggs. All procedures were in conformation to the stipulations of the Animal Ethical Committee, Faculty of Science, Banaras Hindu University, Varanasi-221005, India.

Collection and culture of M-II arrested eggs

Ovulated cumulus-enclosed eggs were isolated in pre-warmed culture medium under a dissecting microscope (Nikon, Model C-DS; Tokyo, Japan) by puncturing oviduct using 26-gauge needle attached to a 1 ml syringe. All ovulated cumulus-enclosed eggs were picked up using microtubing (inner diameter 2 mm) attached with glass micropipette (inner diameter, 100 micrometer; Clay Adams, NJ, USA) and transferred to culture medium containing 0.01% hyaluronidase at 37 °C. After 3 min of treatment, denuded eggs were removed and washed three times with fresh culture medium and used for *in vitro* studies.

In vitro effects of roscovitine on morphological changes in eggs

Roscovitine (10 mM) was initially dissolved in dimethylsulfoxide (DMSO) and then diluted with culture medium to get final working concentrations. A group of eggs (10–12 eggs per group) were transferred to culture medium containing various concentrations of roscovitine (0.0, 12.5, 25, 50, 100, 200 μM) and then cultured for 3 h in CO_2 Incubator (Model; Galaxy 170 R, New Brunswick, Eppendorf AG, Hamburg, Germany). The similar concentrations of roscovitine have been reported to inhibit Cdk1 activity in rat eggs cultured *in vitro* [16]. Since DMSO was used as a solvent in the roscovitine stock solution, an equivalent dilution of the highest concentration (0.01% DMSO) was used in the control group. At the end of incubation period, eggs were removed, washed and transferred on to a grooved slide and then examined for morphological changes using a Phase-Contrast Microscope (Nikon, Eclipse; E600, Tokyo, Japan) at 400× magnification.

Analysis of phosphorylation status of Cdk1 and cyclin B1 level

The phosphorylation status of Cdk1 and cyclin B1 levels was analyzed by immunofluorescence microscopy using their specific antibodies. The anti-PSTAIR antibody recognizes total Cdk1 level. Hence, we used anti-PSTAIR antibody. The anti-pThr-161 Cdk1 antibody (polyclonal antibody raised against a short amino acid sequence containing phosphorylated Thr-161 of Cdk1) was used to recognize stimulatory phosphorylation of Cdk1. The anti-cyclin B1 antibody (a polyclonal antibody raised against amino acids 1–433) representing full length cyclin B1 was used to detect cyclin B1 level. For this purpose, control eggs that were either arrested at M-II stage or underwent spontaneous exit from M-II arrest and roscovitine-treated eggs were prefixed with 3.7% buffered formaldehyde and then used for the analysis of phosphorylation status of Cdk1 and cyclin B1 levels following our published protocol [17].

Quantitative analysis of H_2O_2 concentrations

The intracellular H_2O_2 concentration was analyzed using H_2O_2 assay kit purchased from BioVision, CA, USA. The egg lysate collected from control eggs (M-II arrested and roscovitine-treated groups (100 eggs from each group) was transferred to a microcentrifuge tube containing 100 μl of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8) for 1 h on ice for lysis. The freshly prepared lysates were centrifuged at 10,000 × *g* at 4 °C for 15 min and clear supernatant was immediately used for the quantitative estimation of H_2O_2 concentrations by colorimetric assay as per company manual protocols. The optical density (OD) was determined using a microplate reader (Micro Scan MS5608A, ECIL, Hyderabad, India) set at 560 nm for H_2O_2. All samples were run in one assay to avoid inter-assay variation and intra-assay variation for H_2O_2 was 1.8%.

Detection of p53, Bax, Bcl2 and cytochrome c expressions

The p53, Bax, Bcl2 and cytochrome c expressions were analyzed using their highly specific antibodies purchased from Santa Cruz Biotechnology, CA, USA. The control eggs that were arrested at M-II stage and roscovitine-treated eggs prefixed with 3.7% buffered formaldehyde were then permeabilized with triton X-100 (0.01% in PBS) for 10 min at 37 °C. The nonspecific sites were blocked using blocking buffer (2.5% PBS–BSA solution) at 37 °C for 30 min and then exposed to 100 μl of their respective primary antibodies (1:500 dilution in blocking buffer) at 37 °C for 2 h. After 5 washes with pre-warmed PBS, slides were exposed to 100 μl of FITC-labeled secondary antibody for 1 h at 37 °C in humidified chamber. After 1 h of incubation, slides were washed 5 times with pre-warmed PBS, mounted with fluorescence mounting medium and then observed under fluorescence microscope (Model, Ni-U, Nikon, Tokyo, Japan) at 520 nm at 400× magnification.

Quantitative analysis of intracellular cytochrome c concentration

The cytochrome c concentration in egg lysates was analyzed following our published protocol [18] using cytochrome c ELISA kit purchased from R&D Systems, MN, USA. The egg lysates were