Biomedicine & Pharmacotherapy 84 (2016) 680–686

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Original article

S-nitroso-*N*-acetyl penicillamine inhibits spontaneous exit from metaphase-II arrest in rat eggs cultured *in vitro*

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ARTICLE INFO

Article history:
Received 12 August 2016
Received in revised form 14 September 2016
Accepted 15 September 2016

Keywords:
Cyclin-dependent kinase 1
Cyclin B1
MPF
Spontaneous exit from metaphase-II arrest
Reproductive outcome

ABSTRACT

Background: Present study was designed to investigate the *in vitro* effects of nitric oxide (NO) donor such as *S*-nitroso-*N*-acetyl penicillamine (SNAP) on spontaneous exit from metaphase-II arrest (SEM-II) in rat eggs cultured *in vitro*.
Methods: Ovulated eggs were denuded and then exposed to various concentrations (0.0, 0.01, 0.1 and 1.0 mM) of SNAP for 3 h under *in vitro* culture conditions. The percentage of SEM-II, specific and total phosphorylated cyclin-dependent kinase-1 (Cdk1), cyclin B1 and anaphase promoting complex/cyclosome (APC/C) levels as well as Cdk1 activity were analyzed.
Results: The SEM-II was associated with a decrease of Thr-161 phosphorylated Cdk1 as well as cyclin B1 levels and increase of Thr-14/Tyr-15 phosphorylated Cdk1, APC/C levels and Cdk1 activity in aged eggs cultured *in vitro*. On the other hand, SNAP treatment prevented a decrease of Thr-161 phosphorylated Cdk1 as well as cyclin B1 levels and increase of Thr-14/Tyr-15 phosphorylated Cdk1, Cdk1 activity that finally prevented SEM-II in a concentration-dependent manner. However, APC/C level was not affected by SNAP during the course of treatment *in vitro*.
Conclusions: Present data suggest that SNAP prevented SEM-II possibly by increasing high level of NO and thereby maturation promoting factor (MPF) stabilization in rat eggs cultured *in vitro*. Hence, SNAP could be used to prevent SEM-II that reduces reproductive outcome in several mammalian species.

1. Introduction

In mammals, ovulated eggs are physiologically arrested at metaphase-II (M-II) stage of meiotic cell cycle [1]. These eggs wait for spermatozoa, which triggers exit from M-II arrest and completion of cell cycle [1]. However, in the absence of fertilizing spermatozoa, these eggs undergo postovulatory aging-mediated spontaneous exit from M-II arrest (hereafter, SEM-II) followed by incomplete extrusion of second polar body (PB-II), a first morphological criteria for abortive spontaneous egg activation (SEA) [2,3]. This is a pathological condition observed in various mammalian species [4]. The abortive SEA is an emerging problem that affects egg quality and reduces reproductive outcome in mammals [5].

Signal molecules regulate meiotic cell cycle at M-II stage in rat eggs [6]. Evidences suggest that nitric oxide (NO) modulates physiology of ovulated egg by inducing meiotic cell cycle resumption, arrest and apoptosis [7–10]. The underlying mechanism for NO-mediated cell cycle regulation at M-II stage remains obscure. Few studies indicate that increase in NO level trigger meiotic resumption from diplotene arrest in mouse [11–13], rat [10], porcine [14] and murine [15] oocytes. However, other reports suggest the decrease of NO level during meiotic resumption from diplotene arrest in mouse [16], rat [9,17] and cattle [18,19] oocytes. Similar dissensions exist for meiotic resumption from M-II arrest. Goud et al. [20] reported that the SNAP (*S*-nitroso-*N*-acetyl penicillamine), a NO donor prevents meiotic resumption from M-II arrest in mouse aged eggs [20]. Further, increased NO level has been reported during SEM-II in rat eggs cultured *in vitro* [21]. These studies suggest the biphasic roles of NO during meiotic cell cycle regulation in mammalian eggs. The biphasic roles of NO are supported by the observation that low concentration of NO (0.01 mM) induces meiotic resumption, while high concentration (0.5 mM) maintained meiotic arrest in bovine diplotene arrested oocytes [22].

NO donor has been used to prevent spontaneous exit from diplotene arrest in rat [10], cattle [19], canine [23] and porcine [14] oocytes. SNAP prevent spontaneous exit from diplotene arrest in rat oocytes [10]. Our previous study suggest that NO maintains phosphorylated Thr-161 of Cdk1 and cyclin B1 levels and reduces

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http://dx.doi.org/10.1016/j.biopha.2016.09.059
0753-3322/

phosphorylated Thr-14/Tyr-15 Cdk1 level, which prevents MPF destabilization and thereby spontaneous resumption from diplotene arrest [10]. However, it remains unclear whether SNAP could prevent SEM-II. If so, whether maturation promoting factor (MPF) destabilization is involved during meiotic resumption from M-II arrest? Therefore, present study was designed to investigate the effects of SNAP on extrusion of PB-II, total and specific phosphorylated Cdk1, Cdk1 activity, anaphase promoting complex/cyclosome (APC/C) level and cyclin B1 levels in rat eggs cultured *in vitro*.

2. Materials and methods

2.1. Chemicals and culture medium

All chemicals were procured from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The culture medium (M-199, AT014A, HiMedia Laboratories, Mumbai, India) was prepared using company manual protocol and the pH of was adjusted to 7.2 ± 0.10, while osmolarity was detected 285 ± 5 mOsmol. To prevent microbial growth during *in vitro* culture, 1 μL/mL of antibiotic solution (A007, HiMedia Laboratories, Mumbai, India) was mixed in the culture medium.

2.2. Superovulation induction and collection of M-II arrested eggs

Female rats (Charles-Foster strain) of 22 to 25 days old (body weight 45 ± 5 gm) were transferred in temperature and light-controlled room having food and water *ad libitum*. Rats were given single dose of intramuscular injection of 20 IU pregnant mare serum gonadotropin (PMSG) for 48 h followed by 20 IU human chorionic gonadotropin (hCG) for 14 h. Thereafter, ovary along with oviduct were removed and kept in pre-warmed culture medium. The cumulus-oocyte complexes (COCs) were collected from ampulla and then denuded by exposing in 0.01% hyaluronidase in medium M-199 at 37° C for 3 min following manual pipetting. Eggs were washed with fresh medium and observed for their morphological status under microscope (Eclipse E200 series, Nikon). M-II arrested eggs (12–14) were exposed to various concentrations (0.0, 0.01, 0.1 and 1.0 mM) of SNAP for 3 h in CO_2 incubator (Galaxy 170R; 37 °C temperature, 5% CO_2 and 100% humidity) as 3 h time period was enough to observe the *in vitro* effects of SNAP on SEM-II [10]. The milimolar concentrations of SNAP (0.5 mM) have already been reported to maintain meiotic arrest at diplotene [22]. Previous studies suggest that 1 mM SNAP inhibited first polar body extrusion in mouse oocytes and did not exert any toxic effects under *in vitro* culture conditions even after 3 h of treatment [16,24]. Hence, in the present study we used 1 mM concentration of SNAP. After 3 h, eggs were removed from *in vitro* culture conditions and then used for the analyses of morphological changes using light microscope (Eclipse E200 series, Nikon). To confirm the observations, three independent experiments were conducted. The Institutional Animal Ethical Committee of the University approved this project (vide letter No. Dean/11-12/CAEC/266).

2.3. Analyses of total and specific phosphorylation status of Cdk1, cyclin B1 and APC/C levels

The total and specific phosphorylated Cdk1, cyclin B1 and APC/C levels were analyzed following our previous published protocol [6] using specific antibodies anti-p-Cdc2 p34 (Thr-161) rabbit polyclonal antibody raised against a short amino acid sequence containing pThr-161 of Cdc2 p-34 (sc-12341), anti-p-Cdc2 p34 (Thr-14/Tyr-15) rabbit polyclonal antibody raised against a short amino acid sequence containing Thr-14 and Tyr-15 phosphorylated cdc2 p34 of human origin (sc-12340), anti-cdc2 p34 (PSTAIRE) rabbit polyclonal antibody raised against a peptide mapping epitope mapping within the conserved PSTAIRE domain of cdc2 p34 of human origin (sc-53), anti-cyclin B1 (H-433) rabbit polyclonal antibody raised against amino acids 1-433 representing full length cyclin B1 (sc-752) and anti-APC/C (C-20) rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of APC of human origin (sc-896) procured from Santa Cruz Biotechnology, Inc. CA, USA. A group of eggs (12–14) were collected from control and 1 mM SNAP-treated group and fixed in 4% buffered formaldehyde. Slides were then used for the detection of total and specific phosphorylation status of Cdk1, cyclin B1 and APC/C levels. ß-Actin immunofluorescent intensity was also analyzed as control in parallel to confirm that the equal conditions were used during immunofluorescence analyses of aforesaid parameters using anti-actin (C-2) mouse monoclonal antibody specific for an epitope mapping between amino acids 350–375 at the C-terminus of Actin of human origin (sc-8432; Santa Cruz Biotechnology, Inc., CA). To confirm the results three independent experiments were conducted and 36–42 eggs were subjected for corrected total cell fluorescence (CTCF) analysis. Minimum three areas of an egg cytoplasm were selected and 36–42 eggs were used for CTCF analyses as previous published method [25] using Image J Software (version 1.44 from National Institutes of Health, USA).

2.4. Analysis of Cdk1 activity

Egg lysate was prepared by transferring 50 eggs from each group (control and 1 mM SNAP-treated) to a microcentrifuge tube containing 50 μL of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% TritonX-100, pH 8). The microcentrifuge tubes were then kept for lysis on ice for 1 h. Lysate was then centrifuged for 15 min ($10{,}000 \times g$ at 4 °C). After centrifugation, supernatant was used for the analysis of Cdk1 kinase activity using MESACUP Cdc2/Cdk1 kinase assay kit (MBL, Nagoya, Japan) following the company manual protocol. The optical density (OD) was noted using ELISA plate reader (ECIL, Hyderabad, India) set at 492 nm within 10 min. Samples collected from three independent experiments were run in one assay to avoid inter-assay and intra-assay variation was 1.7%.

2.5. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) of three independent experiments. The percentage data were first converted using arcsine square-root transformation and then analyzed either by Student's *t*-test or One-way ANOVA using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL).

3. Results

3.1. SNAP prevented SEM-II

Fig. 1 shows freshly ovulated M-II arrested egg exhibiting first polar body (PB-I) (Fig. 1A, red arrow). The SEM-II was observed in control eggs cultured for 3 h as evidenced by the initiation of extrusion of PB-II ($39.20 \pm 5.80\%$; black arrow; Fig. 1B). SNAP prevented SEM-II in a concentration-dependent manner in treated eggs (One-way ANOVA, $F = 14.351$, $p < 0.01$; Fig. 1D) as evidenced by absence of extrusion of PB-II (Fig. 1C) and only PB-I is seen (Fig. 1C, red arrow). The 1 mM SNAP showed a maximum inhibition as only <10% of eggs underwent SEM-II.

3.2. SNAP prevented decrease in Thr-161 phosphorylated Cdk1 level

Fig. 2 shows changes in the immunofluorescence intensity of Thr-161 phosphorylated Cdk1 in aged eggs cultured *in vitro*. A