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Dibutyryl-cAMP Alters the Expression of Signalling Molecules, Leading to a Metaphase-I Arrest in Rat Oocytes Cultured *In Vitro*

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Abstract

One of the most important intracellular signalling molecules, cyclic adenosine monophosphate (cAMP), acts as a second messenger in the activation of gonadotrophins. In the meiotic cell cycle, the function of the cAMP analogue dibutyryl cAMP (db-cAMP) is not well understood beyond the metaphase-I (M-I) stage. In this section of the investigation, we test whether db-cAMP may induce a transient arrest of meiosis at the M-I stage. In cultured rat oocytes, we modify kinases, signal molecules, and cell cycle regulators. We harvested M-I-arrested cumulus oocyte complexes (COCs) from rats' ovaries after superovulation. These oocytes were grown in fresh complete medium with db-cAMP at 0.125, 0.25, 0.5, and 1mM. Oocytes were treated with db-cAMP, and their morphology, meiotic phases, phosphorylation of cyclin-dependent kinase 1 (Cdk1) and cyclinB1, levels of cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), intracellular reactive oxygen species (ROS), Calcium Ca^{2+}, mitochondrial membrane potential, and apoptotic status were all examined. In our *in vitro* investigation, 1 mM db-cAMP dramatically lowered Thr-14/Tyr-15 pCdk1, causing meiotic arrest in the M-I stage with competent oocytes for up to 12 hours. when db-cAMP treatment, intra-oocyte cyclic nucleotides increased, causing meiotic arrest. Control oocytes resumed meiosis when ROS and Ca^{2+} increased. Due of damaged mitochondria, long-term db-cAMP treatment causes apoptosis in oocytes. We also study db-cAMP concentrations and periods that decrease spontaneous meiosis resumption after M-I arrest. This study introduces an *in vitro* approach for meiotic arrest with equally dispersed mitochondria during M-I stage. This may increase oocyte competency in ART practices.

Keywords: Metaphase-I arrest; db-cAMP; Signal molecules; Oocyte quality; Mitochondrial membrane potential

Abbreviations: COCS: Cumulus Oocyte Complexes; ROS: Reactive Oxygen Species; cAMP: Cyclic Adenosine Monophosphate; cGMP: Cyclic Guanosine Monophosphate; MAPK: Mitogen-Activated Protein Kinase; MPF: Maturation-Promoting Factor

Introduction

Among the many exciting aspects and qualities that make the rat an interesting model for investigating meiotic cell cycle control is the fact that, in *in vitro* culture environments, the oocyte does not go beyond the metaphase-I stage (M-I) [1,2]. In mammals, the voyage from metaphase-I (M-I) to metaphase-II (M-II) is crucial because the egg extrudes the first polar body (PB-I) and becomes haploid gamete. PB-I extrusion and meiotic cell cycle progression from M-I to M-II are poorly understood. Therefore, it is critical for both fundamental research and ART to understand how mammalian oocytes undergo meiotic maturation. Meiotic arrest and resumption occur in oocytes via a series of chemical cascades that are regulated by a variety of signalling molecules produced by follicular cells [3,4]. The gonadotropins produced by the pituitary gland also play an important role in the cascade of events that ends in meiotic resumption. Several protein kinases (enzymes) play a crucial role in keeping mammalian oocytes in a state of meiotic arrest. Key protein kinases include protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) in sustaining meiotic cell cycle progression in mammalian oocytes [5].

To communicate with oocytes, adenylate cyclase uses gonadotrophins to produce the second messenger 3',5'-cyclic adenosine monophosphate (cAMP) [6-8]. Rat granulosa cells and oocytes contain a differentiation factor termed cAMP, which governs the progression of the meiotic cell cycle by maintaining the meiotic arrest [3,9,10]. During the diplotene and M-I phases,

meiosis is arrested, but when cAMP and 3′,5′-cyclic guanosine monophosphate (cGMP) concentrations drop, meiosis resumes [11]. Reactive oxygen species (ROS) generation is linked to decreased levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) following spontaneous meiotic restart after meiotic arrest in oocytes cultured *in vitro* [12]. It's well knowledge that reactive oxygen species (ROS) function as signalling molecules in many different types of cells, including mammalian germ cells. Mild increases in ROS aid in the progression of the meiotic cell cycle during *in vitro* culture [13].

An increase in cAMP levels activates cAMP-dependent protein kinase A, which in turn activates the maturation-promoting factor (MPF) [6]. Oocytes are unable to enter the M-I arrest phase of meiosis due to this. MPF regulates oocyte meiosis in all mammals [14,15]. CDK1, a component of the MPF, is essential for meiotic resumption [16]. Meiotic resumption in M-I arrested oocytes occurs when the MPF complex is functional. Stabilisation of MPF requires cyclin B1 contact with Cdk1, phosphorylation at the Thr161 residue, and dephosphorylation at the Thr-14/Tyr-15 of Cdk1 [17]. Dephosphorylation of Thr161 and phosphorylation of Cdk1's Thr-14/Tyr-15 residues turn MPF unstable, and its breakdown through ubiquitin-mediated proteolysis triggers exit from metaphase-I (EM-I) [18,19].

Meiotic competence allows an egg to develop into a viable organism, thus it's crucial. Meiotically competent oocytes have the potential to improve ART success rates in most mammalian species [20]. Oocytes may restart meiosis on their own [21] if they are separated from their follicular environment. Findings suggest that FSH improves oocyte development via a cAMP-mediated pathway while initially delaying nuclear maturation [22-24]. Dibutyryl cAMP sodium salt (db-cAMP), a membrane-permeable cAMP analogue, and 3-isobutyl-1-methylxanthine (IBMX), a type 3 phosphodiesterase inhibitor, have both been used in *in vitro* maturation (IVM) to increase cAMP levels, delay meiosis, and subsequently regulate nuclear and cytoplasmic oocyte development [6, 25, 26].

We hypothesise that *in vitro* cultured oocytes would be less capable of maturing if their cAMP levels were suddenly lowered. Oocyte morphology, mitochondrial membrane potential, and early apoptosis were also analysed as a function of db-cAMP delivery during *in vitro* growth. Oocytes in the M-I stage were essentially stopped at the arbitrary exit for up to 12 hours, while still being alive and having equally dispersed mitochondria. There is a lack of information on the method and mechanism of action in M-I rat oocytes. This study examined the impact of db-cAMP on spontaneous meiotic restart from the M-I stage in rat oocytes during *in vitro* culture at different time hours to better understand the mechanism behind db-cAMP treatment on oocyte maturation at different culture durations.

Materials and Methods

Chemicals and culture media

We purchased antibiotics and culture media from HiMedia. With the exception of the compounds specifically named, all others were acquired from the Sigma Chemical Co. located in St. Louis, Missouri. The pH of the M-199 culture media was adjusted to 7.2 ± 0.05 by putting in sodium bicarbonate (0.035% w/v) per the manual instructions. After that, we added glutamate, penicillin, and streptomycin (Cat. no. A007, HiMedia) to the culture mix to inhibit further microbial growth.

Dibutyryl Cyclic AMP (db-cAMP) working concentration preparation

Dimethyl sulfoxide (DMSO, 0.1%) was used to dissolve the sodium salt of db-cAMP in order to generate a 1 mM stock solution. The stock solution was diluted using the media to attain the working concentrations (0, 0.125, 0.25, 0.5, and 1 mM) for *in vitro* investigations. The working concentrations were prepared for 5 minutes at 37°C before use.

Animals, oocytes Collection and Culture

Charles-Foster strain female rats of sexually immature age (22-24 days old; 45 ± 5 g body weight) were housed and maintained with ad libitum access to food and water per conventional husbandry practises. Subcutaneous injections of 20 IU of pregnant mare's serum gonadotropin (PMSG) were given to rats for 48 hours, and then 20 IU of human chorionic gonadotrophin (hCG) were given to them 10 hours later (Superovulation protocol). The ovaries were removed from the rats that were killed through cervical dislocation and placed in petri plates with preheated media.

Under a stereomicroscope (SMZ800N, Nikon, Tokyo, Japan), the ovary was perforated with a 26-gauge needle connected to a 1 mL tuberculin syringe, and the ovary and fallopian tube were removed and deposited in the pre-warmed M-199 medium in a 35 mm Petri dish. Oocytes in the M-I arrest state were characterised morphologically due to the absence of GV and nucleus in the cytoplasm. Microtubing was linked to disposable glass micropipettes, which were used to collect the M-I arrested oocytes. After a brief incubation at 37°C, they were transferred to fresh culture medium containing 0.01% hyaluronidase. Oocytes that had been M-I arrested were used for *in vitro* tests after being washed three times.

Evaluation of oocyte meiotic status

With the use of Hoechst-33342 staining, we were able to assess an oocyte's meiotic state, as verified by its chromosomal status. After two washes in phosphate-buffered saline (PBS), 6-8 oocytes were incubated with 10 µg/ml Hoechst 33342 for 5 minutes at 37 °C. For chromosomal analysis, we used a fluorescence microscope

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(Eclipse Ni, Nikon, Tokyo, Japan) set to 350 nm, and for oocyte morphology, we used a phase contrast microscope (Eclipse E200, Nikon, Tokyo, Japan). Meiotic stage was verified by three independent tests.

The effect of db-cAMP on spontaneous meiotic resumption from M-I arrested oocytes

A group of (10-14) oocytes were placed in petri dishes with various concentrations of db-cAMP (0, 0.125, 0.25, 0.5, and 1 mM), with DMSO serving as the control. The petri dishes followed by placing in a CO_2 incubator (Galaxy 170R, Eppendorf, Hamburg, Germany) set at 37 °C and were placed for incubation for up to 12-24 hours. After incubation for varying amounts of time, morphological changes in the oocytes were analysed with a phase contrast microscope. At least three sets of experiments were performed to ensure the accuracy of the results.

Immunocytochemistry for cAMP analysis

The concentration of cAMP was evaluated by employing a monoclonal antibody. For this objective, oocytes (n=5-6) from the control and db-cAMP treatment groups are fixed with 4% buffered paraformaldehyde and then air-dried. Following a three-step PBS washing procedure, slides were treated with 100 µl of Triton X-100 (0.01% in PBS) at room temperature for 5 minutes. After being washed twice or thrice with PBS, the slides were then treated with sodium citrate solution (0.01 M) at 37°C for 10 mints. After a second PBS wash, the slides were incubated with a blocking buffer (2.5% PBS-BSA solution) at 37°C for 30 minutes. Next, slides were treated with 100 µl of diluted (1:500 dilution in blocking solution) primary antibody (cAMP, mouse monoclonal, sc-73761) after a PBS wash. The intensity of fluorescence was measured at 465 nm using a fluorescence microscope. Oocytes were subjected to corrected total cell fluorescence (CTCF) analysis to verify the findings, and at least three independent sets of tests were conducted. Simply said, cell-to-background integrated fluorescence (CTCF) is the cell's total fluorescence.

Evaluation of MPF evel

Our method for analysing the phosphorylation of Cdk1 and cyclin B1 levels in db-cAMP-treated oocytes was described in [17], and it included the use of extremely specific antibodies from Santa Cruz Biotechnology (Dallas, TX, USA). Thr-14/Tyr-15 p-Cdc2p34 rabbit polyclonal antibody (sc-12340) and Thr-161 p-Cdc2p34 rabbit polyclonal antibody (sc-12341) were both generated against sequences containing Thr-14 and Tyr-15, respectively. p-34Cdc2p34 (PSTAIRE) rabbit polyclonal antibody (sc-53) was generated against the conserved PSTAIRE domain of Cdc2. Following incubation, slides were rinsed thrice with PBS, and then subjected to 100µl of specific anti-rabbit fluorescein isothiocyanate (FITC)-labelled (sc-3839) secondary antibody for detecting Thr-14/Tyr-15, Thr-161, as well as total phosphorylated Cdk1 and cyclin B1 levels, and anti-mouse TRITC-labelled (sc-3796) secondary antibody for detection of β-actin at 37°C for 1 hour (1:1000 dilutions in blocking buffer). After incubating the slides for an hr, the fluorescence intensity was evaluated using a fluorescent microscope at 465 nm (FITC) and 540 nm (TRITC) wavelengths, respectively. The slides were washed three times in PBS. To correlate the data, we show you example photos from each of the three independent runs of the experiment, where 4-5 denuded oocytes were subjected to CTCF analysis using Image J software (version 1.44 from National Institutes of Health, Bethesda, USA).

Quantitative assessment of cAMP and cGMP Levels

Total arrest in the M-I stage was seen in the treatment group for as long as 12 hours, but in the control group, cyclic nucleotide levels were only measured in the control and 1 mM db-cAMP-treated groups. Eight to ten oocytes were taken from each group and deposited in lysis buffer (20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton-X 100; pH = 7.2) for quantitative analysis. All the samples, standards, and reagents were prepared following the procedures outlined in the relevant business handbook. The results section displays the cAMP and cGMP concentrations in terms of pmol/mg protein. R&D Systems Inc. ELISA kit for cAMP and cGMP analysis (Cat. No. KGE002B for cAMP and KGE003 for cGMP, respectively) from the United States

Measurement of total ROS level

Following our previously published technique [17, 27], the total ROS level was assessed using 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA). For this, 15 min at 37°C in a CO_2 incubator were spent exposing 10-12 oocytes from each control and treated group (12 and 24 hours) of 1 mM db-cAMP to H2DCFDA (10 µM). Oocytes were rinsed thrice with PBS before being subjected to a fluorescence microscope analysis of DCF fluorescence at 485 nm excitation and 520 nm emissions. Three independent experiments were performed in order to verify the findings, and CTCF analysis was carried out using ImageJ software (version 1.44; National Institutes of Health, Bethesda, USA).

Fluo-3 AM-Based Intracellular Ca^{2+} Analysis.

Following a methodology that had already been described [20], the intracellular Ca^{2+} level was examined in the control and treatment groups. In a nutshell, culture media containing 50 µM Fluo-3 AM were exposed to 10-12 oocytes from the control and 1 mM db–cAMP treated groups for 12 and 24 hours, respectively, at 37 °C in a CO_2 incubator. Oocytes were then taken out and thoroughly cleaned with PBS three times before Fluo-3 fluorescence was measured at 488 nm excitation and 520 nm emission under a fluorescent microscope (Nikon, Eclipse; E-80i, Japan). Using the Image J Software (version 1.44 from the National Institutes of Health, Bethesda, USA), the CTCF of oocytes from three different experiments were determined.

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Monitoring of mitochondrial membrane potential (ΔΨ) of db-cAMP treated oocytes

The viability of the cell was further evaluated for up to 24 hours with 1 mM of db-cAMP among all the doses. Oocytes were stained with JC-1, a reporter dye for the inner mitochondrial membrane potential, as directed by the manufacturer. In brief, treated oocytes were incubated with JC-1 for 20 minutes after being produced to a final concentration of 1 M in dilution buffer. An inverted fluorescence microscope (EVOS FL, Life Technologies) was used to observe the stained oocytes. By measuring the total fluorescence of the whole oocyte, fluorescence analysis was conducted. The CTCF approach was used to normalise the mean value for each fluorescence to the measuring area [28]. Individual oocyte values from three treatments were expressed as total red CTCF [29].

Staining With Acridine Orange and Propidium Iodide to Identify Apoptotic Cells.

Acridine orange (AO) and propidium iodide (PI) staining confirmed apoptotic profiles as a consequence of morphological alterations in the db-cAMP-treated oocytes at different time points. Briefly, oocytes from each group that had been treated with db-cAMP were washed in PBS and then stained for 10 minutes with an AO/PI mix (1 µg/ml in PBS). Fluorescence was observed using an inverted fluorescence microscope (EVOS FL, Life Technologies) in three separate investigations [28].

Statistical analysis

The data were collected from three separate studies, and the results were reported as the mean ± standard error of mean (S.E.M) Before doing statistical analysis, all percentage data were transformed using the arcsine square root. The chi-square ($\chi 2$) testing was used to compare the rates of meiotic resumption between the control and db-cAMP-treated groups (Table 1). SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, U.S.A.), was used to perform a one-way ANOVA (P 0.05), followed by the Bonferroni test on a subset of the data, and the student's t-test on the remaining data. A substantial change from the control group or the treatment group is indicated by a "*" or "***," respectively. $P < 0.05$ and $P < 0.001$were used as criteria for statistical significance.

Results

Oocytes undergo morphological changes during *in vitro* maturation

As shown in (Figure 1), oocytes collected after treatment with 20 IU PMSG and 20 IU hCG exhibited M-I arrest, as shown by the lack of GV (blue arrow; Figure 1A). Oocytes in the control group revealed the disappearance of GV and the existence of PB-I to demonstrate M-II arrest (yellow arrow; Figure 1B) after 5-6 hours of *in vitro* culture. Hoechst-33342 staining further validated their meiotic status, which included phases such as M-I arrest (db-cAMP treated group) (Figure 1, A1) and M-II arrest (Figure1, B1).

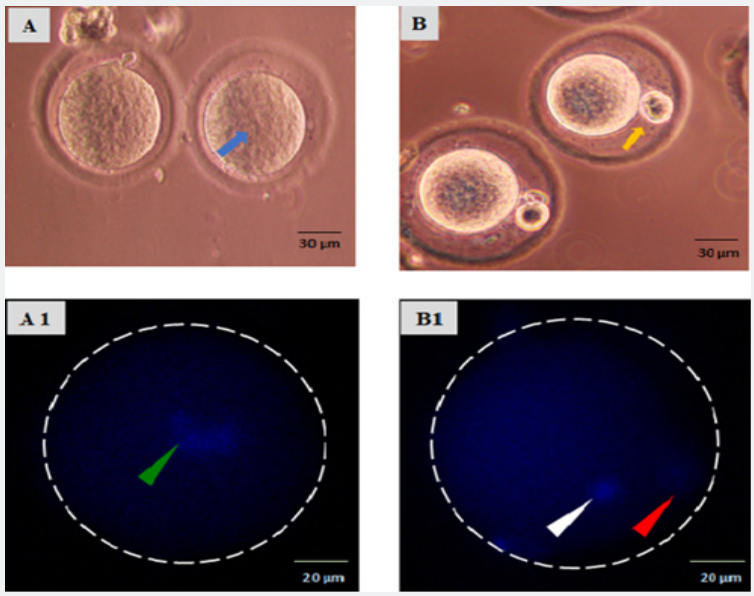

Figure 1: Representative photos of db-cAMP-treated rat oocytes' morphological alterations and meiotic phases during *in vitro* culture. (A). Oocytes at metaphase-I arrest, showing absence of germinal vesicle (GV) by blue arrow (B). Oocytes at metaphase-II arrest showing first polar body (PB-I) by yellow arrow. The formation of metaphase plate chromosomes (green arrow, A1) shows M-I arrested oocyte, while M-II arrested oocyte is confirmed by the presence of haploid set of chromosomes in oocyte cytoplasm (white arrow, B1) and another set in PB-I (red arrow, B1). Bar = 30 µm (upper panel photographs); Bar = 20 µm (lower panel photographs).

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During *in vitro* Culture, db-cAMP inhibited spontaneous meiotic resumption in a concentration-dependent manner

As shown in (Figure 2), The germinal vesicle (red arrow, Figure 2A) is absent in M-I oocytes. Oocytes in the control group spontaneously exited M-I arrest and had PB-I (76.32 ± 5.802%; blue arrow; Figure 2B) after 12 hours of *in vitro* culture. Maximum inhibition of meiotic resumption was noticed in 1 mM concentration of db-cAMP (1 mM) up to 12 hours, with proper oocyte morphology observed till 18 hours (5.31 ± 0.82%), and demonstrating oocytes at complete M-I arrest evidenced by the absence of germinal vesicle (black arrow; Figure 2F), whereas in 0.5 mM, 0.25 mM, and 0.125mM concentration of db-cAMP shows spontaneous meiotic resumption from M-I arrest evidenced by exit from M-I arrest (EM-I) (green arrow; Figure 2E) and presence of PB-I (yellow arrow; Figure 2D and 2C). The addition of db-cAMP during *in vitro* culture of M-I arrested oocytes at 12 hours significantly reduced spontaneous meiotic restart (One way ANOVA, F = 51.278, $p < 0.001$; Figure 2G) in a concentration-dependent manner.

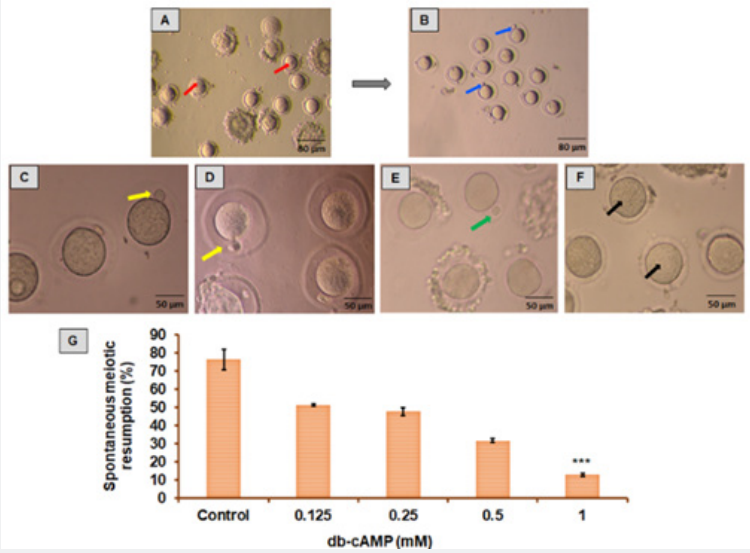

Figure 2: Representative photos of the effects of varying db-cAMP concentrations on oocytes grown for different intervals of time. Oocytes collected at M-I stage as control and possesses no germinal vesicle (red arrow; Figure 2A) (upper panel). After 12 hours of *in vitro* culture, control group showing M-II arrested oocytes and possesses PB-I (blue arrow; Figure 2B) (upper panel). Treatment of db-cAMP (1 mM) for 12 hours inhibited complete resumption showing oocytes at M-I arrest showing absence of germinal vesicle (black arrow; Figure 2F) (lower panel) whereas in 0.5 mM, 0.250 mM and in 0.125 mM concentration of db-cAMP shows spontaneous meiotic resumption from M-I arrest evidenced by exit from M-I arrest (EM-I) (green arrow; Figure 2E) and presence of PB-I (yellow arrow; Figure 2D and 2C) (lower panel). (G) The presence of db-cAMP inhibited spontaneous meiotic resumption in a concentration-dependent manner. Data are mean ± S.E.M of three independent experiments and analyzed by one-way ANOVA). Bar = 80 μm (upper panel photographs); Bar = 50 μm (lower panel photographs).

The results represent the mean standard error of the mean across three investigations and were analysed using a one-way ANOVA. Bar = 80 m (images in top panel); Bar = 50 m (pictures in bottom panel). Oocyte resumption after *in vitro* culture was 77.11% in the control group and 7.69% in the db-cAMP (1 mM) treated group. In contrast, the 1 mM db-cAMP treated group showed statistical significance when compared to the control group using the $\chi 2$ test ($\chi 2$ calculated 4.33 > $\chi 2$ critical 3.84; $p < 0.05$). Therefore, there is a notable difference between the 1 mM of db-cAMP and the control.

Camp immunofluorescence intensity increased in db-cAMP-treated oocytes

As shown in (Figure 3), Compared to the control (Figure 3A) and another db-cAMP treated group in which cAMP expression decreased as the concentration of db-cAMP decreased (Figure 3C-E), and where meiotic resumption was also observed, the 1 mM db-cAMP treated group showed a significant ($p < 0.05$) increase in cAMP expression (one-way ANOVA, F = 32.15; $p < 0.05$). The images from the light microscope (Figure 3a-e) in the top panel are shown in the bottom panel. Figure 3F shows that the CTCF analysis confirms these results in more depth.

In The db-cAMP-treated Group, MPF Stabilisation Leads to Meiotic Arrest

Oocytes treated with 0.5 mM db-cAMP exhibited a considerable rise ($p<0.001$) in the immunofluorescence intensity of Thr-14/Tyr-15 phosphorylated Cdk1 level, but oocytes treated with 1 mM db-cAMP remained entirely in M-I arrest until 12 hours of *in vitro* growth (Figure 4A1; A2). Oocytes treated with 1 mM of db-cAMP for 5 hours show a decrease in Thr-14/Tyr-15 phosphorylated Cdk1 level, while oocytes treated with different concentrations

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of db-cAMP for 12 hours maintained MPF stabilisation, resulting in meiotic arrest at the M-I stage (Figure 4 C1-C3). Oocytes treated with varying amounts of db-cAMP for 12 hours showed no difference in the total phosphorylated level (PSTAIRE) of Cdk1 (Figure 4 E1-E3). Meiotic arrest during the M-I stage is caused by an increase in cyclin B1 levels, as shown by the dramatic rise in cyclin B1 levels in oocytes treated with 1 mM db-cAMP (Figure 4G1) compared to 0.25 mM db-cAMP treated oocytes (Figure 4G3). Meiotic arrest at the M-I stage is induced by 1 mM db-cAMP in oocytes, and competent oocytes remain in arrest for up to 12 hours of *in vitro* culture, as shown by CTCF analysis (Figures. 4B, 4D, 4F, and 4H).

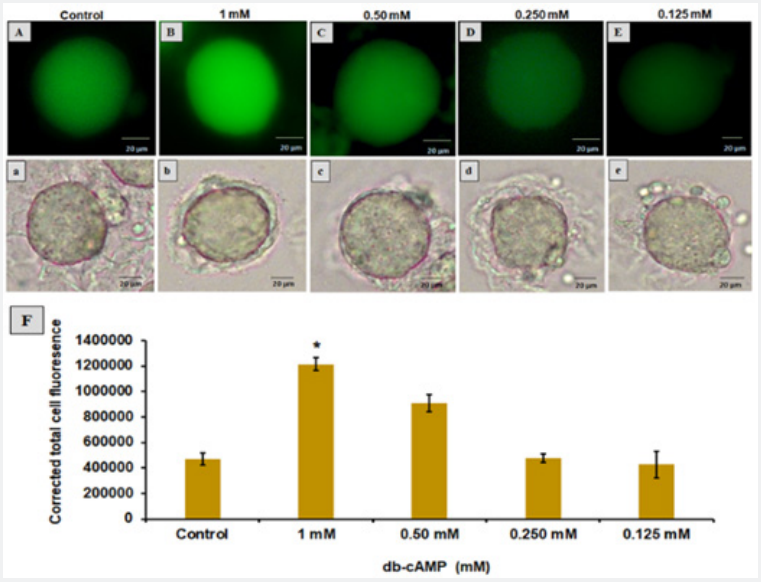

Figure 3: Photographs of db-cAMP-treated oocytes incubated for 12 hours *in vitro* demonstrating cAMP expression variations. Compared to control (A) and other db-cAMP-treated groups (C, D, and E), 1 mM db-cAMP-treated oocytes were completely arrested in M-I stage (B) and showed meiotic resumption (C, D, and E). The bottom panel displays light microscope photos (a-e) of the above panel (A-E). CTCF analysis reveals that cAMP expression decreased concentration-dependently throughout *in vitro* growth (F). Data are mean ± S.E.M. of three separate experiments analysed by one-way ANOVA and Bonferroni post-hoc analysis, *p<0.05, substantially elevated (1 mM db-cAMP treated group over control). Bar = 20 µm.

Meiotic Arrest Occurs When Intraoocyte Cyclic Nucleotides Rise

As shown in (Figure 5), oocytes arrested at the M-I stage and treated with 1 mM of db-cAMP show a significant ($p < 0.05$) of cAMP (4.23 ± 0.57 pmol /oocyte) and cGMP (3.485 ± 0.15 pmol /oocyte) levels when in contrast to the control group of cAMP (2.28 ± 0.17 pmol/oocyte; Figure 5) and cGMP (2.67 ± 0.52 pmol/oocyte; Figure 5). When M-I arrested oocytes were treated with 1 mM db-cAMP for 12 hours *in vitro*, no spontaneous meiotic restart was seen in comparison to the control group (Figure 5). Three independent analyses confirmed these results.

Variations in the levels of total ROS

A comparison of the total ROS level in control, 12-hours, and 24-hours groups of oocytes that were treated with db-cAMP is shown in (Figure 6). This comparison was carried out using the fluorescent dye H2DCFDA. When compared to the control group, the ROS level in the oocytes that had been treated with 1 mM db-cAMP showed a substantial ($p < 0.05$) drop when examined by the Figure 6A2 diagram. The CTCF analysis conducted on these oocytes using the ImageJ programme has provided support for our previous results (Figure 6B). Apoptosis may be triggered by high quantities of reactive oxygen species (Figure 6A3). However, minor increases in ROS may operate as a signal molecule for the control group's meiotic restart (Figure 6A1) in the experiment.

A Change in Intracellular Ca^{2+} Level

Oocytes treated with 1 mM db-cAMP and grown for up to 12 hours stayed in the M-I arrested stage (Fig. 7A2), as contrasted to the control group (Figure 7A1; M-II stage), as indicated in (Figure 7). This was statistically significant ($p < 0.05$). After 24 hours of treatment with 1mM db-cAMP, a significantly higher amount of free intracellular Ca^{2+} was detected inside the deformed cytoplasm condition (1.92 times, Figure. 7A3) as compared to the control group. Our results are supported by the CTCF analysis of three separate trials performed in Image J (Version 1.3) (Figure 7B).

Long-Term db-cAMP Exposure Decreased Oocyte Mitochondrial Activity

Red fluorescence is expressed more strongly by active mitochondria due to the increased accumulation of JC-1 dye in

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mitochondria, whereas green fluorescence is expressed by less active mitochondria [30]. Figure 8A shows that compared to db-cAMP (1 mM) treated oocytes cultured for 24 hours, db-cAMP (1 mM) treated oocytes treated for up to 12 hours dramatically enhanced mitochondrial activity. However, compared to a 12-hours culture of 1 mM db-cAMP and the control group, a prolonged culture in 1 mM db-cAMP treated oocytes drastically decreased mitochondrial activity (ΔΨ), as seen by a low red/green fluorescence ratio (lower panel).

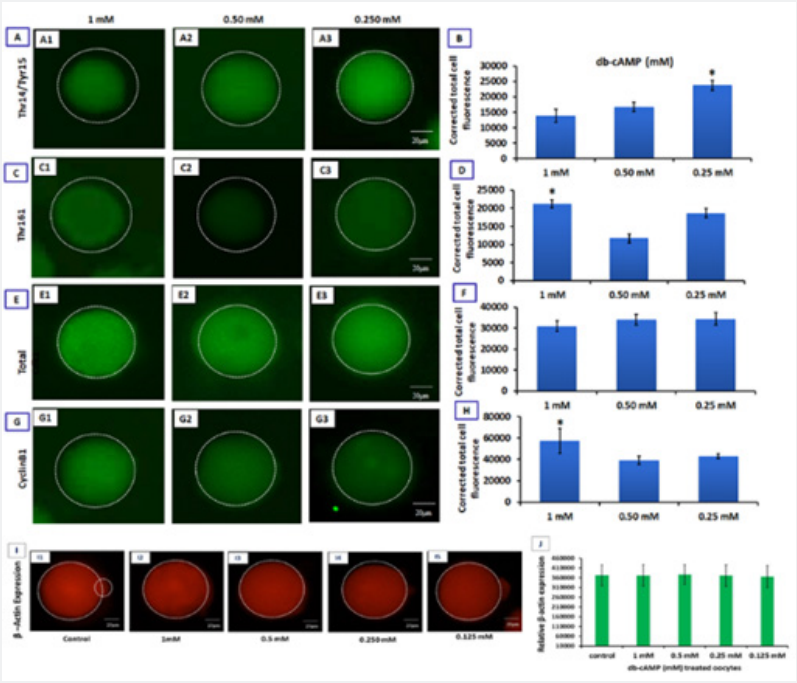

Figure 4: Representative photographs showing the immunofluorescence intensity of total as well as specific phosphorylation status of Cdk1 and cyclin B1 level in oocytes treated with different concentration of db-cAMP (mM) for 12 hours of *in vitro* culture condition. Oocytes treated with 0.5 mM of db-cAMP showed a significant increase ($p < 0.001$) in immunofluorescence intensity of Thr-14/Tyr-15 phosphorylated Cdk1. In oocytes treated with 0.250 mM of db-cAMP, meiotic resumption (47.53 ± 2.182%) was observed from M-I arrest in rat oocytes collected after super ovulation induction (Figure 4A3). Oocytes that experienced meiotic restart after M-I arrest were treated with 0.250 mM of db-cAMP (Figure 4C3), which decreased Thr-161 phosphorylated Cdk1 by $p<0.001$. Cdk1's total phosphorylated level (PSTAIRE) did not alter in oocytes treated with varied db-cAMP concentrations (Figure 4E1-E3). However, a significant increase in cyclin B1 level was observed in 1 mM db-cAMP-treated oocytes (Figure 4G1), resulting in meiotic arrest in M-I stage (47.53 ± 2.182%) (Figure 4G3). During *in vitro* culture, db-cAMP (mM) did not affect β-actin expression in oocytes (Figure 4 I1-I3). These findings were supported by Image J's CTCF analysis (Figure 4B, 4D, 4F, 4H, and 4J). Student's t-test, "*" $p < 0.001$, was used to analyse three separate experiments' mean ± S.E.M. Bar = 20 μm.

Acridine Orange/Propidium Iodide Staining Detects Apoptosis in db-cAMP-Treated Oocytes

Oocytes treated with 1 mM db-cAMP and grown for 24 hours as opposed to 12 hours in the 1 mM treatment and control groups showed signs of apoptosis. We carried out AO/PI staining to confirm apoptosis due to morphological alterations in respected group. Results showed that the 24 hours culture group of db-cAMP treatment enhanced apoptosis in oocytes as shown by an increase in the intensity of red fluorescence for PI in comparison to control and 12 hours group oocytes (Figure 9; bottom panel). As shown in (Figure 9), the oocytes exposed to 1 mM db-cAMP for 12 hours showed AO green rather than PI red. However, this suggests proper oocyte morphology and integrity for 12-18 hours. According to our research, a 1 mM db-cAMP treatment can prolong the M-I arrest for 12 to 18 hours while maintaining proper oocyte shape, compared to a 24-hours culture, which revealed oocytes with deformed morphology and membrane blebbing [31]. This extended db-cAMP (1 mM) culture of oocytes made them unfit for fertilization and IVF.

Discussion

Crosstalk between several signal molecules is crucial in modifying mammalian oocyte physiology [32]. These signal molecules are produced by the granulosa cells surrounding the oocyte or by the oocyte itself [33,34]. Changes in these signal molecule levels may determine whether an oocyte experiences meiotic arrest or resumption as the meiotic cell cycle progresses [14, 35]. Major signal molecules needed for the meiotic cell cycle

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progression in mammalian oocytes include cAMP, cGMP, ROS, and Ca^{2+} [36]. In oocytes, the meiotic cell cycle may be affected by alterations in the signal molecules that regulate the MPF stabilization and destabilization and Cdk1 activity [32, 37].

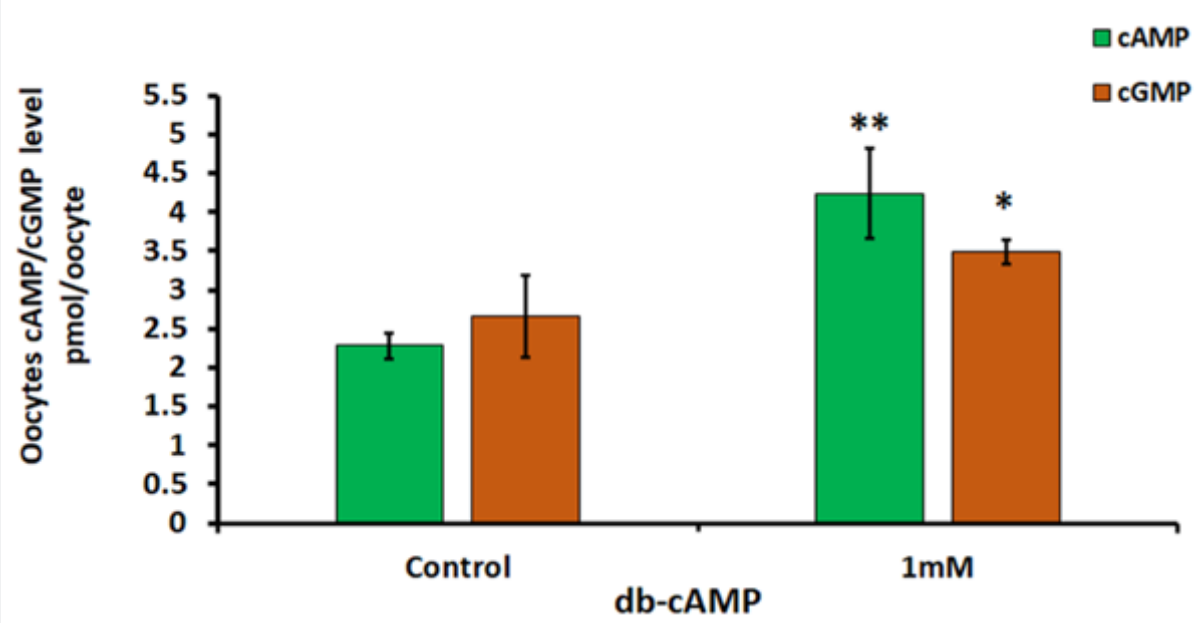

Figure 5: Effect of db-cAMP on the cyclic nucleotides present in intraoocyte during the M-I arrested stage in comparison to the control. A significant increase of cAMP as well as cGMP levels were observed in M-I arrested oocytes treated with 1 mM of db-cAMP that remains at complete arrest at M-I stage after 12 hours *in vitro* culture as compared to oocytes in control group. Data are mean ± S.E.M of three independent experiments and analysed by Student's t-test, * Significant ($p < 0.05$) difference as compared to oocytes in control group.

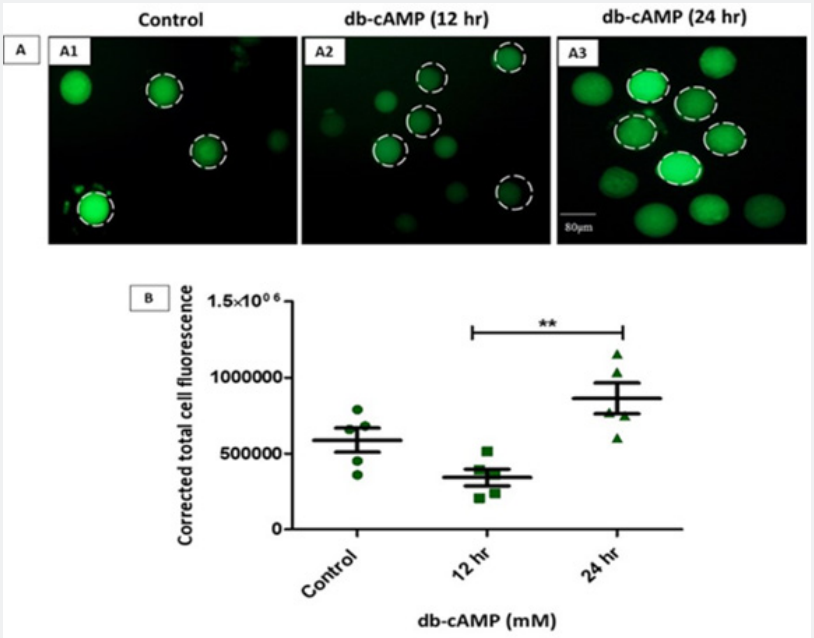

Figure 6: Fluorescence intensity of ROS in control, db-cAMP-treated oocytes at 12 and 24 hours. A significant ($P < 0.05$) decrease of total ROS level was observed in the db-cAMP (1 mM) treated oocytes as compared to control (Figure 6A2). The CTCF analysis of these oocytes using ImageJ software further confirms our findings (Figure 6B). High level of ROS can lead to apoptosis (Figure 6A3), whereas moderate increase in ROS level can act as a signal molecule in meiotic resumption in control group (Figure 6A1). Values are expressed as mean ± S.E.M of three independent experiments and data were analysed by the student's t-test. *$p < 0.05$. Bar=80 µm.

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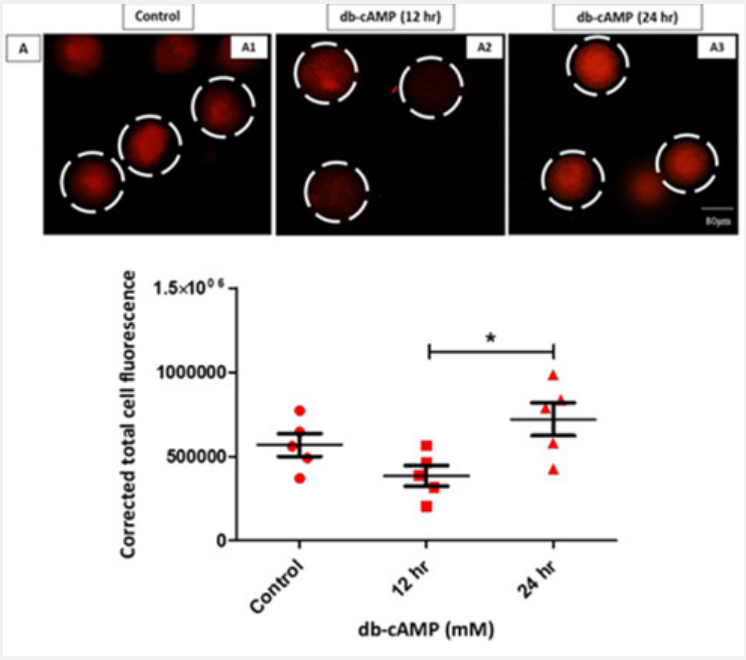

Figure7: Representative photograph showing fluorescence intensity of Fluo-3 in control and db-cAMP (1 mM) treated group. Meiotic arrest at M-I stage (7A2) was associated with decrease in intracellular Ca^{2+} as evidenced by decreased fluorescence intensity as compared to oocytes at M-II stage in control group (7A1) after 12 hours of *in vitro* culture in rat oocytes. Bar = 80 µm. (B) The CTCF analysis of fluorescence intensity of Fluo-3 in db-cAMP treated and control group as shown in Figure 7B. Values are expressed as mean ± S.E.M of three independent experiments. Data were analyzed by Student's t-test. "*" denotes significant ($p < 0.05$) increase as compared to control oocytes.

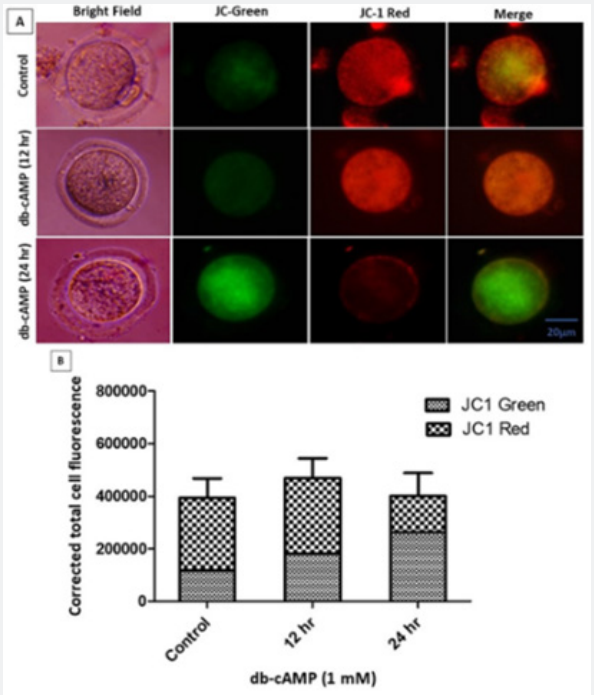

Figure 8: Representative photograph showing mitochondrial membrane potential in control group and oocytes treated with 1 mM db-cAMP after 12 hours and 24 hours. A significant increase in mitochondrial activity in oocyte as evidenced by the high ratio of red/green florescence in 1 mM of db-cAMP treated group (12 hours) as compared to 24 hours treatment group. Data was presented as mean ± standard error of the mean (SEM) of three independent experiments.

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Table 1: Effect of db-cAMP (mM) on oocytes during in vitro culture.

In-vitro culture (Concentration)	Oocytes at 0 hours (n)	Oocytes after 12 hours (n)	% Resumption from M-I arrested oocytes
db-cAMP (Control)	13	10	77.11[b]
db-cAMP (1 mM)	13	1	7.69[a]

"n" represents the total number of oocytes taken during the *in vitro* culture. ab, values with different superscript characters last column indicate a statistically significant difference ($P < 0.05$). Data were analysed using the χ^2 test and were obtained by performing 3 replicates. db-cAMP, dibutyryl cAMP; M-I, Metaphase-I.

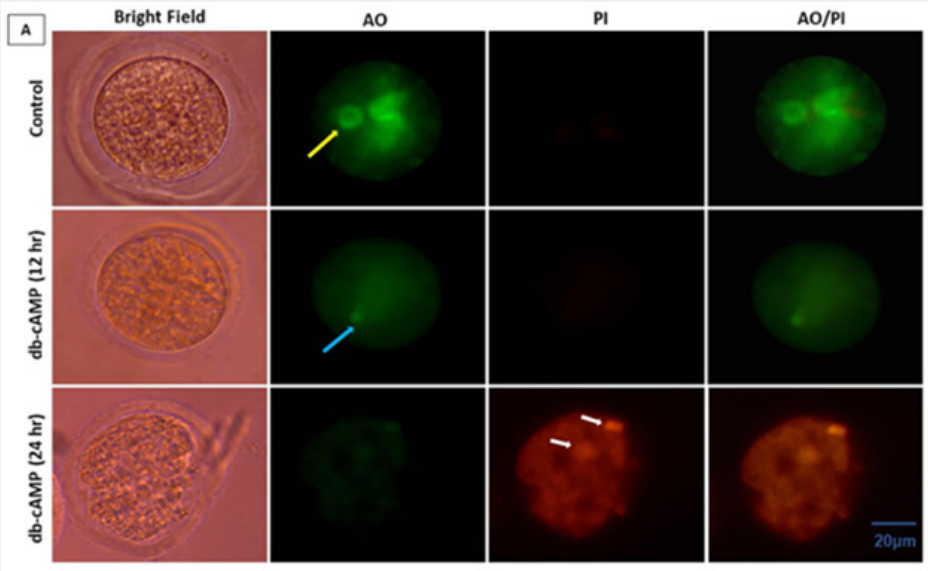

Figure 9 : Representative photograph showing acridine orange/PI staining for analysis of cell viability after db-cAMP treatment in control, 12 hours and 24 hours group. Prolonged culture in db-cAMP induced apoptotic morphological changes in oocyte and extrusion of PB-I also observed but with distorted morphology (24 hours group; white arrow) as evidenced by increased PI staining and reduced AO staining compared to control and 12 hours group (yellow and blue arrow). Three independent experiments were conducted to confirm the results. Bar represents 20 µm.

Alterations in the concentration of a variety of signal molecules may have a direct or indirect effect on MPF stabilisation or destabilisation [37-39]. Our data demonstrate that stabilisation of MPF may be maintained and phosphorylation of Thr-14 and Tyr-15 in oocytes treated with 1 mM db-cAMP for 12 hours. Oocytes cultured with 1 mM db-cAMP for 12 hours showed a substantial increase in the level of Thr-161 of Cdk1 compared to oocytes cultured with other doses of db-cAMP, resulting in meiotic arrest in the M-I stage. Similarly, oocytes treated with 1 mM db-cAMP for 12 hours showed a significant decrease in cyclin B1 level when compared with 0.25 mM db-cAMP treated oocytes; this indicates the decrease in the level of cyclin B1, which causes a meiotic arrest and keeps M-I arrest in the 1 mM db-cAMP treated group [40, 41]. Oocytes must complete the transition from the M-I to M-II stage of meiosis in order to release PB-I and become the correct gamete [42, 43]. Several physiological variables influence oocyte meiosis as the oocyte undergoes this trip. Ensuring meiotic arrest and meiotic resumption by adjusting the amount of an MPF [40], cAMP is one of the essential physiological components of oocyte meiosis that governs meiotic competence of oocytes. The oocyte protein phosphatase (MPF) activity is regulated by cAMP-dependent protein kinase A (PKA) [37].

cAMP in oocytes controls meiotic arrest [9]. Several investigations show that high intraoocyte cAMP levels maintain meiotic arrest in mature oocytes, whereas low levels resume meiosis [41]. Increasing the dose of db-cAMP kept the oocyte in total arrest for up to 12 hours at 1 mM (5.31 ± 0.82%) in multiple *in vitro* tests. Compared to 1 mM db-cAMP-treated oocytes, control oocytes display meiotic resumption (76.32 ± 5.802%) and PB-I at the M-II stage. These groups (0.5, 0.25, and 0.125 mM) showed meiotic restart and reduced cAMP expression as db-cAMP concentration fell. db-cAMP increases cAMP and cGMP

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levels in cultured oocytes, improving nuclear and cytoplasmic maturation and slowing ageing [6,9]. Thus, 1 mM db-cAMP may reduce spontaneous meiotic resumption, oocyte ageing, and oocyte growth in diverse ART programmes. Thus, 1 mM db-cAMP increased intraoocyte cyclic nucleotides, causing a complete meiotic halt. Reduced intraoocyte cAMP and cGMP levels in control oocytes promote cAMP-phosphodiesterase 3A (PDE 3A) [44, 45]. A decrease in intraoocyte cAMP may destabilise MPF and cause meiotic resumption after M-I arrest and PB-I extrusion. A modest increase in ROS stimulates the meiotic cell cycle in vitro, while high amounts alter mitochondrial potential and induce apoptosis [46].

Our results showed that 1 mM db-cAMP treatment during oocyte maturation resulted in higher levels of intracellular reactive oxygen species (ROS) and Ca^{2+} in comparison with the control group. Previous research, however, has shown the importance of a modest rise in ROS and Ca^{2+} during the maturation of mammalian oocytes [24,47]. However, our findings support that increasing the culture duration of an oocyte in db-cAMP may result in a rise in reactive oxygen species (ROS) and Ca^{2+} levels [46, 48], suggesting that an oocyte cultivated in 1 mM of db-cAMP may be fertile if not incubated for more than 12 hours. If the concentration and culture duration are raised beyond 12 hours, mitochondrial damage may occur in the oocytes. In spite of this, we have conducted experiments showing that db-cAMP treatment may keep cells in meiotic arrest for 12-18 hours.

Conclusion

In conclusion, one of the biggest obstacles to *in vitro* embryo development is improving reproductive outcomes during oocyte maturation. When the oocyte is removed from the antral follicle for *in vitro* maturation (IVM), cAMP levels inside the oocyte decrease, and meiosis restarts on its own owing to a lack of inhibitory chemicals in the follicle. Increased cAMP concentrations prior to IVM have been shown to enhance oocyte competence and, by extension, embryonic development in a number of species. Our results show that mammalian oocytes can be maintained in meiotic arrest (M-I stage) for up to 12 hours following treatment with 1 mM db-cAMP without compromising their viability, mitochondrial distribution, or developmental competence during *in vitro* culture. Since db-cAMP plays a critical function in regulating many oocyte development factors during oocyte handling in *in vitro* fertilisation, greater investigation into this route is warranted.

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Ethical Approval

All procedures confirmed to the stipulations of the Departmental Animal Ethical Committee of Banaras Hindu University, Varanasi-221005 and followed the guidelines for the care and use of laboratory animals (NIH Publication). All studies were in confirmation to the terms of Institutional Animal Ethical Committee (Ref. No.- BHU/DoZ/IAEC/2018-19/013) of the university.

Author's Contributions

Alka Sharma: Conceptualization, Methodology, Software, Data curation, Writing - original draft, Visualization, Investigation. Pawan K Dubey and Anima Tripathi: Coordinated the experiments, Writing - review & editing and approved the final manuscript.

Declarations

The authors declare that they have no conflicts of interest.

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