

Supplementation of Freezing Media With Cyclic Adenosine Monophosphate Analog and Isobutylmethylxanthine on Sperm Quality



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ABSTRACT

Background: This study aimed to explore whether the addition of a cyclic adenosine monophosphate (cAMP) analog and isobutylmethylxanthine (IBMX) in freezing media improved sperm quality and what role cAMP has in this recovery.

Materials and Methods: The research study on each semen sample was cryopreserved into four groups: fresh semen sample, as a control group, freezing medium+2.5 mM cAMP analog and 0.2 mM IBMX, freezing medium+12.5 mM cAMP analog and 0.2 mM IBMX, and freezing medium+25 mM cAMP analog and 0.2 mM IBMX. Sperm parameters after post-thaw were analyzed according to WHO instruction (2010). Viability, acrosome reaction, and DNA damage levels of the samples were evaluated.

Results: Our results indicated that the effective concentrations of 12.5 and 25 mM cAMP analog and 0.2 mM IBMX significantly improved the total motility, progressive motility, and viability of the frozen-thawed ($P<0.05$). However, non-progressive motility and immotile were significantly reduced in the 12.5 and 25 mM cAMP analogs and 0.2 mM IBMX groups after thawing ($P<0.05$). During freezing the spermatozoa, the high concentration of the cAMP analog increased acrosome reaction after thawing in the 25 mM and 0.2 mM IBMX treated samples ($P<0.05$). DNA fragmentation in 25 mM cAMP analog and 0.2 mM (IBMX) supplementation was significantly lower compared to the other groups ($P<0.05$).

Conclusion: Our findings revealed that in vitro cAMP analog and IBMX supplementation in freezing media play an important role in preventing cryodamage by maintaining the sperm functional parameters.

1. Introduction

The cryopreservation of human sperm is an essential process used for different purposes such as protection of sperm from chemotherapy patients [1]. How-

ever, cryopreservation has adverse effects on sperm fertilization ability by decreasing the sperm motility, viability, membrane integrity and structure, and activity of acrosome [2, 3]. Cryopreservation often does not maintain the viability of live sperm as much as fresh sperm. Therefore, energy supplementation is important

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to support the structure and function of frozen-thawed spermatozoa for successful freezing [4].

Sperm may access eggs by stimulating the production of metabolic energy in the form of Adenosine Triphosphate (ATP) for motility [5]. However, sperm motility is one of the vital parameters for successful fertilization [6]. In Vitro Fertilization (IVF) using frozen sperm is very limited due to the low success of embryo competence and pregnancy rate [7]. Thus, the development of optimal protocols and antioxidant treatment for sperm cryopreservation controls the stressful effect of cryopreservation procedures [8]. Several signaling pathways are involved in having a role in sperm motility. ATP, calcium, and cyclic adenosine monophosphate (cAMP) have received considerable attention as potential primary regulators of sperm motility in several species of animals [9, 10]. A cAMP analog inhibits cAMP phosphodiesterase, thereby increasing the intracellular cAMP concentration and tyrosine-phosphorylation at the tail level [4, 11].

Besides, a cAMP analog activates protein kinase A to induce protein phosphorylation and mediates the influx of calcium into sperm through CatSper calcium ion channels [12]. For sperm motility, cAMP may raise mitochondrial calcium levels, thereby activating calcium-dependent dehydrogenases involved in the Krebs cycle and ATP delivery required [13]. Furthermore, cAMP has an essential role in the acrosome reaction messenger system [14]. Treatments that enhance intracellular cAMP concentrations often cause an increase in sperm motility and often increase motility as well as the acrosome reaction and ultimately increase the fertilization rates [15, 16].

The purpose of this study was to develop an optimized freezing condition by the addition of a cAMP analog and IBMX on the quality of sperm cells of infertile men with asthenozoospermia during cryopreservation.

2. Materials and Methods

Collection of samples and analysis

This study was performed on asthenozoospermia men referred to ACECR Infertility Research Center, Qom, Iran. In this regard, 50 asthenozoospermia men between the ages of 20 and 35 years were selected. Semen samples were taken after 2 to 7 days of sexual abstinence. Samples from each patient were collected in a sterile tube after liquefaction at room temperature; all semen samples were examined for sperm analysis

according to the World Health Organization guidelines (Asthenozoospermia=sperm motility <40% or <32% progression in a semen sample). The sperm parameters were evaluated regarding their percentage of Total Motility (TM), percentage of Progressive Motility (PM), non-progressive motility (non-PM), immotility, percentage of normal sperm morphology.

Pre-freeze by cAMP analog and IBMX

Following routine semen analysis, each semen sample was split into four groups: fresh semen sample (n=25) as a control group, freezing medium+2.5mM cAMP analog and 0.2 mM IBMX (n=25), freezing medium+12.5 mM cAMP analog and 0.2 mM IBMX (n=25), and freezing medium+25 mM cAMP analog and 0.2 mM IBMX (n=25).

The protocol of sperm cryopreservation

Semen samples were divided into four separate tubes according to the total sperm concentration and then the same volume of sperm cryopreservation solution (Vitrolife, Sweden), and sperm suspension was added to each tube. During the freezing process, the samples were placed in liquid nitrogen vapor at 180°C (15-30 cm above liquid nitrogen) for 20-30 minutes and then transferred to a liquid nitrogen tank and stored for two weeks. (All parameters were recently evaluated in the control group) Then, the cryotubes containing semen samples were placed at room temperature for 5 minutes to melt the samples. In the next step, the samples were incubated at 37°C for 20 minutes. The sperm cryopreservation solution was removed by centrifugation at 1000 rpm for 5 min, and afterward analog cAMP (dbcAMP: N6,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate; Sigma) and IBMX (3-isobutyl-1-methylxanthine; Sigma) was added into each sample according to a protocol.

Assessment of sperm parameters

Assessment of sperm parameters was performed according to the guidelines of the World Health Organization (2010) [17]. In this regard, to evaluate sperm motility, first, 10 µL of each semen sample was placed on a microscope slide and covered with a cover glass, and then with a light microscope (magnification ×400), sperm motility was evaluated in several microscopic fields. Sperm motility is defined by three terms: progressive, non-progressive, and immobile. Papanicolaou staining was used to evaluate the normal morphology of sperm. Eosin-Nigrosin staining was used to evaluate sperm viability. In this staining, 20 µL of sperm suspen-

sion of each group was mixed with 20 μ l of the eosin-nigrosin solution, and then smear was prepared and using a light microscope at $\times 400$ magnification, the sperm viability was assessed. In this staining, alive sperm remain colorless, while the nucleus of dead sperm turns red. At least 200 sperms were examined in each evaluation.

Assessment of DNA fragmentation

The tunnel technique was used to evaluate cell death. Briefly, the slide was first fixed with paraformaldehyde and all steps were performed according to the instructions in the kit (Promega, DeadEnd Fluorometric TUNEL System instructions for use of product G3250). For this purpose, after the staining process, 500 sperms per slide were examined under a fluorescence microscope (BX51, Olympus, Japan) at $\times 100$ magnification. In this staining, the sperms that turned red were healthy and the sperms that turned green were positive tunnels [18, 19].

Assessment of sperm acrosome reaction

Acrosome reaction staining was performed according to Cross et al.'s method [20]. In summary, first, after sample collection, a sample was prepared (2×10^6) and then placed at room temperature to dry. The prepared extensions were immersed in methanol for 30 seconds. Then they were washed twice with distilled water and dried at room temperature. Next, the fixed slide was placed in a dark room for 50 minutes with 50 μ l FITC-PSA (100 μ g/mL) and then washed with distilled water. In the next step, the slide was fixed with 2% formaldehyde for 15 minutes at room temperature in the dark and washed twice with distilled water, and the laminate was placed on it. A fluorescent microscope (BX51, Olympus, Japan) at $\times 1000$ magnification was used to examine the

acrosome reaction. For this purpose, about 200 cells per slide were counted and the number of sperms that had undergone an acrosome reaction was calculated.

Statistical analysis

In the present study, SPSS v. 21 was used and the data were expressed as Mean \pm SD. For comparison of the cAMP analog and IBMX in all the groups following the incubation, t-test was performed according to normality analysis. The statistical significance was set at $P < 0.05$.

3. Results

Effect of the cAMP analog and IBMX on sperm parameters

The results of the present study showed that the rate of sperm motility and progressive motility in the supplement group of sperm cryopreservation medium with cAMP analog 0.125, 25 mM, and 0.2 mM IBMX increased significantly compared to other concentrations of cAMP and IBMX analogs ($P < 0.05$). On the other hand, when the samples were supplemented with 12.5 or 25 mM cAMP analog and IBMX 0.2 mM, the mean percentage of non-progressive motility and immobilized sperm decreased ($P < 0.05$). In the present study, no significant difference in normal sperm morphology was observed between the groups ($P < 0.05$) (Table 1). Also in this study, it was found that the percentage of sperm viability in the analog groups of cAMP 12.5 and 25 mM and IBMX samples 0.2 mM compared to other groups was significantly increased ($P < 0.05$) (Table 1).

Table 1. Effects of the cAMP analog and IBMX on the total motility, progressive, and non-progressive motility, normal morphology, and viability of the samples in different groups

Sperm Parameters	Mean \pm SD				P
	Control group	2.5 mM and 0.2 mM of IBMX	12.5 mM and 0.2 mM of IBMX	25 mM and 0.2 mM of IBMX	
Total motility	24 \pm 1.16	26.66 \pm 2.22	36.66 \pm 2.22*	38.42 \pm 2.60*	<0.05
Progressive motility	12.00 \pm 1.01	13.01 \pm 1.12	18.22 \pm 0.83*	20.60 \pm 0.77*	<0.05
Non-progressive motility	25.41 \pm 1.11	24.55 \pm 1.33	20.11 \pm 0.91*	19.10 \pm 0.76*	<0.05
Immotile	63.10 \pm 2.11	61.11 \pm 2.41	55.10 \pm 1.11*	53.00 \pm 0.71*	<0.05
Normal morphology	3.01 \pm 0.23	4.01 \pm 0.33	4.04 \pm 0.41	4.01 \pm 0.23	>0.05
Viability	36 \pm 1.19	34 \pm 1.02	24 \pm 1.33*	28 \pm 1.82*	<0.05

The difference between the groups were considered significant ($P < 0.05$).

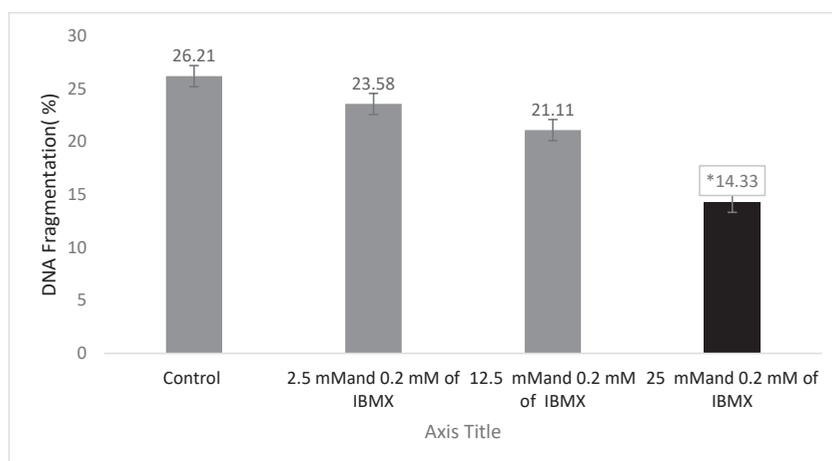


Figure 1. Effects of cAMP analog and IBMX on DNA fragmentation after sperm thawing (Mean \pm SD)

*Significant difference compared to other groups ($P < 0.05$).



Effect of the cAMP analog and IBMX on the percentage of DNA fragmentation

Data on sperm DNA damage after freezing are well illustrated in Figures 1 and 2. In this study, the mean percentage of DNA fragmentation in the cAMP 25 Mm and IBMX 0.2 Mm groups significantly reduced compared to that in the other groups ($P < 0.05$). On the other hand, a comparison of other groups showed that high doses of cAMP analog significantly prevent DNA damage.

Effect of the cAMP analog and IBMX on the acrosome reaction

The results of the rate of acrosome reaction between different groups are presented in Table 2. In the analog group cAMP 12.5 and 25 mM and IBMX 0.2 mM, the rate of acrosome reaction was significantly increased compared to the control group. The results showed that in a significantly higher percentage of sperm with intact acrosome with high supplementation of cAMP and IBMX analogs compared to low concentrations of cAMP and IBMX analogs and the control group ($P < 0.05$).

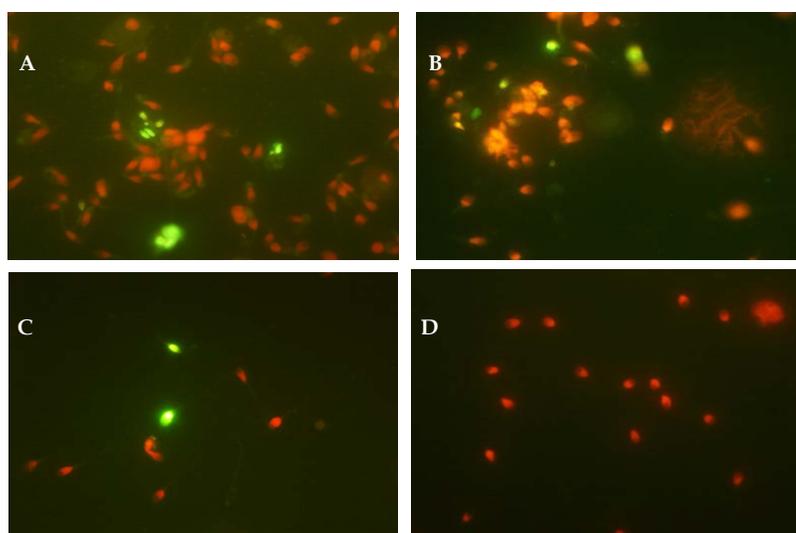


Figure 2. A-D: DNA fragmentation shown by TUNEL

Green-staining sperm have DNA fragmentation, while red-staining sperm have intact DNA; A: DNA fragmentation in the control group; B: incubation with 2.5 mM cAMP analog and 0.2 mM of IBMX; C: incubation with 2.5 Mm cAMP analog and 12.5 mM of IBMX; and D: incubation with 25 Mm cAMP analog and 0.2 mM of IBMX. The percentage of DNA damage significantly decreased by 25 Mm cAMP analog and 0.2 mM of IBMX compared to the other groups.



Table 2. The effect of the cAMP analog and IBMX treatment on sperm acrosome reaction in different groups (mean±SD)

cAMP analog and IBMX	Mean±SD				P
	Control group	2.5 mM cAMP analog and 0.2 mM of IBMX	12.5 mM cAMP analog and 0.2 mM of IBMX	25 mM cAMP analog and 0.2 mM of IBMX	
Normal acrosome reaction	11±1.82	13±2.02	22±2.94 *	26±3.82*	P<0.05

* Significant difference compared to the control group (P<0.05).



4. Discussion

Our results indicated that supplementation of the cAMP analog (12.5 and 25 mM) and IBMX protected post-thaw progressive motility, viability, and acrosome reaction compared with the control samples. Based on our findings, 25mM cAMP analog and the 0.2 mM IBMX decreased DNA fragmentation compared to those in other groups. During cryopreservation, physical and chemical stresses led to changes in the sperm membrane lipid component, sperm viability and motility, and acrosome status [21]. As previously studied, protection of sperm functions and DNA integrity is critical for success in ART (Assessment Reproductive Technique) and pregnancy [22].

Cyclic AMP has been reported to be essential for sperm physiological events, including capacitation, changes in the motility pattern known as hyperactivation, and to development of the ability of acrosome reaction [23]. Therefore, the presence of cyclic AMP due to the induction of this process and the formation of intracellular tyrosine protein phosphorylation cascades will increase sperm motility and acrosome reaction [23, 24]. Activating cAMP signal transduction pathways improves sperm motility by increasing the calcium level [24].

In our study, cAMP supplementation to the freezing medium prevented a decrease in motility observed in the groups incubated with the 12.5 and 25 mM cAMP freezing medium. The results of the cAMP analog and IBMX effects on sperm motility are consistent with results obtained in previous studies [9, 25]. Moreover, cAMP analog and IBMX supplementation led to an improvement in DNA damage in groups treated with the high concentration of the cAMP analog. Furthermore, *in vitro* incubation of the spermatozoa in a simple culture medium by pro-survival factors prevented the spermatozoa from apoptosis and DNA fragmentation [26]. This finding is consistent with previous studies that showed cAMP level could decrease DNA damage in the prepared sperm [27]. This study suggested that cAMP level regulated the sperm mitochondrial function and DNA fragmentation. However, as far as we know, no reports have described the effect of cAMP analogs and IBMX on spermatozoa during cryopreservation. This is the first evidence to

determine if supplementing cAMP can affect the DNA integrity of spermatozoa survived from cryo -damage.

The cAMP and its subunits can regulate a variety of capacitation-induced signaling pathways, including 1) remodeling lipid of the membrane [28], 2) hyperpolarizing the sperm plasma membrane [29], 3) increasing pH of sperm cells [30], 4) increasing intracellular Ca⁺⁺ [31], 5) and increasing protein tyrosine phosphorylation [32]. However one of the main aspects of sperm capacitation is to prepare the sperm to undergo a related physiological exogenous reaction known as acrosome reaction [33]. Our result indicated that the high concentration of the cAMP analog and IBMX improved the percentage of acrosome-reacted sperm cells during the freezing process. Extracellular ATP and cAMP treatment have a controversial effect on sperm capacity. In the current study, cAMP analog and IBMX supplementation increased the acrosome reaction of cryopreserved spermatozoa in the 12.5 and 25 mM groups compared to the control group without the cAMP analog and IBMX treatment. Mechanisms through which cAMP influences acrosome reaction will be explored in spermatozoa in the future [34].

5. Conclusion

Sperm incubation with cAMP analog and IBMX supplementation post-thaw improved the sperm parameters. The high concentration of the cAMP analog and IBMX protected spermatozoa from a decrease in motility. This study suggests that the cryoprotective effect of cAMP analogs and IBMX may lead to successful freezing of spermatozoa.

Ethical Considerations

Compliance with ethical guidelines

The trial design was approved by ACECR biomedical research Ethics Committee (IR, ACECR.JDM. REC.1397.001)). All patients were informed about the study and they provided written consent form.

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Authors' contribution

Conceptualization: HamidPiroozmaneshi; Formal analysis: Rahil Jannatifar; Investigation, Methodology, Writing—original draft, and Writing - review & editing: Hamid Piroozmanesh, Rahil Jannatifar; Supervision: LilaNaserpoor; All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declared no conflict of interest.

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