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Research Article

Effect of the EGTA (Ethylene Glycol Tetraacetic Acid) Supplementation in the Freezing Extender on Quality of Cryopreserved Bull Sperm

Contents

The aim of this study was to evaluate the effect of the inclusion of divalent ion chelating agent EGTA (ethylene glycol tetraacetic acid) in the freezing extender on quality of cryopreserved bull sperm. The freezing extender (egg yolk Tris glycerol) was supplemented with 0 (non-supplemented), 1, 5, 10 and 15 mM EGTA. Bull sperm EGTA improved post thaw sperm quality but in different parameters according to the concentration of EGTA, 5 mM improved the acrosomal state and 1 mM the motility. Our results suggest the EGTA supplementation in the freezing extender of bull sperm might improve the post-thawing sperm quality.

Introduction

The cryopreservation process causes severe damage to sperm. After insemination, the sperm have to survive for long time in the female reproductive tract and colonize the oviduct in order to fertilize an oocyte. But about 50% of the population of the sperm does not survive the cryopreservation process and the survivors remain with sublethal dysfunctions [1], which reduce the longevity of sperm and their fertilizing ability.

This means that there are still many weaknesses in the sperm cryopreservation process that should be improved, and the composition of the freezing extenders is one of the most important factors. The basic ingredients of current sperm freezing extenders are the same as those used 35 years ago, although in recent years several authors have investigated the incorporation of new compounds to the freezing extender, like low-density lipoproteins (LDL) [2,3], different non-permeating sugars [4,5], antioxidants [6,7] showing that it is possible to improve the post-thaw sperm quality modifying the actual cryopreservation extenders. Among one of these new compounds that could improve the quality of frozen semen is the ethylene glycol tetraacetic acid (EGTA). This compound is a chelating agent which is able to capture divalent metal ions as Mg^{2+}, Cu^{2+}, and Ca^{2+} among others, and in addition may limit their movement across the plasma membrane [7]. The chelating agents are routinely added to conservation extender solutions of boar semen such as Modena or BTS solution [8,9], and recently in the thawing extenders [10], to block the action of divalent metal ions, like calcium ion which is a mediator of sperm capacitation and the acrosome reaction [11]. But little information about the effects of the chelating agents in the bull sperm and in the cryopreservation process is available. Few studies have investigated the supplementation of the freezing media with chelating agents and they showed that these may improve the post-thaw sperm quality in some species like human, ram or bear [12-15]. Although the fact of how many of them may preserve semen quality is not completely established. Moreover, the inclusion of EGTA as chelating agent in the bull sperm freezing extender has never been tested. Therefore, the aim of this study was to evaluate the effect of the EGTA (ethylene glycol tetraacetic acid) supplementation in the bull freezing extender, determining their effect on post-thaw sperm quality.

Material and Methods

Reagents and media

Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO, USA), with purified water (18 MΩcm; Automatic GR Wasserlab, Spain). The extender used for bull sperm freezing was egg yolk Tris glycerol extender (ETG) (25 mM Tris, 8 mM citric acid, 7 mM glucose, 20% [v/v] egg yolk and 14% [v/v] glycerol; 1650 ± 5 mOsm kg^{-1}, and pH 7.2).

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Bull semen collection and freezing protocol

Bull ejaculate sperm samples were collected from three sexually mature Charolais bulls once a week for five consecutive weeks by electroejaculation method [16,17]. The ejaculates were pooled before cryopreservation (N =5). Bulls were housed at a commercial farm, fed a diet providing 100% of their nutritional needs, and provided water ad libitum.

Sperm were cryopreserved using the straw freezing procedure described by Anzar et al. 2011 [18]. After collected sperm, non-centrifuged semen was diluted to 60 x 10^6 sperm/mL in ETG extender. The diluted semen was cooled to 4 ºC in 2 h, packaged in 0.5 ml PVC-French straws and frozen in a programmable freezer [19], as follows: from +4 ºC to -12 ºC at the rate of -4 ºC/min, from -12 ºC to -40 ºC at the rate of -40 ºC/min, and from -40 ºC to -140 ºC at the rate of -50 ºC/min, and plunged into liquid nitrogen (LN_2). The straws remained in the LN_2 tank for at least two weeks before thawing. Thawing of straws was done in a circulating water bath at 37 ºC for 30 seconds. Thawed sperm samples were incubated in a water bath at 37 ºC to 150 minutes.

Sperm assessments

Sperm quality was determined by assessing for motility, acrosomal status, and viability (plasma membrane integrity) pre-freezing and at 30 and 150 minutes after thawing.

Sperm motility was objectively evaluated using a computer-assisted sperm analysis system (Integrated Semen Analysis System, ISAS® Proiser, Valencia, Spain) following the procedure described by Cremades et al. [20].

For each evaluation, a 2 µL aliquot of sperm sample was placed in a pre-warmed (37ºC) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and three fields were analyzed at 37 ºC, assessing a minimum of 100 sperm per sample. The proportion of total motile sperm (% TMS) and progressive motility sperm (% PMS), were determined. The kinematic parameters measured for each spermatozoon included the curvilinear velocity (VCL, µm/s), the straight-line velocity (VSL, µm/s), the average path velocity (VAP, µm/s), the percentage of linearity (LIN, %) (ratio between VSL and VCL), the percentage of straightness (STR, %) (ratio between VSL and VAP), oscillation index (WOB, ratio between VAP and VCL), mean amplitude of lateral head displacement (ALH, µm) and means of the beat cross frequency (BCF, Hz). For more detailed descriptions of these parameters [21].

The acrosome morphology was evaluated by phase contrast microscopy at 1000x. Samples were fixed in 2% glutaraldehyde and a minimum of 200 acrosomes per sample were examined. The damage to the acrosome cap for bull sperm was classified by the scoring system reported by Saacke and Marshall [22]. Only the percentage of sperm with normal acrosomal ridge (NAR) was considered in the results.

Sperm plasma membrane integrity was assessed by dual fluorescent probes, SYBR-14 and propidium iodide, PI (L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands), as was described by Garner and Johnson [23]. The samples were evaluated by means of fluorescence microscope (Nikon Eclipse E400, Tokyo, Japan) equipped with Nikon G-2A filter with excitation/barrier filter of 510/590, which allowed dual excitation of blue and green for SYBR-14 and PI, respectively. The sperm cells with undamaged membrane were stained for SYBR-14 resulting in a bright green fluorescence, while PI only stained red the nuclei of the damaged membrane sperm. A minimum of 300 cells per slide were examined in random fields of each sample. Only the percentage of sperm with intact plasmatic membrane (SIPM) was considered in the results (SYBR-14 positive and PI negative).

Experimental design

The effect of the EGTA supplementation was evaluated with different concentrations of EGTA in the freezing extender. In a preliminary study (data not published), the results showed that the bull sperm is able to incorporate a higher amount of EGTA. For this reason, the ETG extender was supplemented with 0 (non-supplemented), 1, 5, 10 and 15 mM.

The sperm quality of cryopreserved bull sperm were analyzed as previously described.

Statistical analysis

Statistical analyses were performed using SAS system version 9.1 (SAS Institute Inc., Cary, North Caroline, USA). The MIXED model analysis of variance (ANOVA) procedure was used to evaluate the effects of the EGTA inclusion in the freezing extender and their effect on sperm motility, plasma membrane integrity, and acrosomal morphology in both species. The statistical model included the fixed effects of the recollection week. When ANOVA revealed a significant effect, values were compared using the Tukey–Kramer test and were considered to be significant when P was less than 0.05. Results are presented as least-squares means ± SEM.

Results

The previous results between treatments before freezing showed no significant differences in any of the seminal quality parameters analyzed, with the next mean values between treatments being: SIPM (70.3%), TMS (65.7%) and NAR (68.7%).

The EGTA supplementation with a concentration of 1 mM improved the acrosome state (NAR) only at 30 min, but with a higher concentration of 5 mM EGTA the NAR was higher than non-supplemented sample for both time incubations (Table 1). Greater concentrations than 5 mM did not show a detrimental effect on the acrosome state. With respect to the percentage of SIPM, the EGTA supplementation did not improve the viability and showed a detrimental from 10 mM EGTA at 30 minutes of incubation (Table 1). On the quality of sperm motility, the EGTA supplementation at 1 mM showed a higher percentage of PMS at 150 min than non-supplemented sample (Table 1). In kinematic parameters after cryopreservation the observed results are similar, with significantly higher values in the

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treatment of 1 mM, and worsening with amounts above 10 mM (Table 2).

Discussion

Our study investigated the effect of EGTA supplementation in the bull sperm freezing extender on the post-thawing sperm quality. The results showed an improvement bull sperm in the NAR percentage with the treatment of 5 mM and the treatment with 1 mM was significantly different from control in PMS percentage and kinematics parameters after cryopreservation.

The improvement of post-thaw sperm quality has also been observed by other authors in different species when the freezing extender was supplemented with a chelating agent (12-15). Determining that, the chelating agent incorporation in the freezing extender has a beneficial effect on post-thaw sperm quality. But how the chelating agents are able to protect during the cryopreservation process is not fully established.

It is known the main changes suffered during cooling and freezing of sperm are changes in membrane fluidity [24], readily the sperm undergo an acrosome reaction [25-27], and an increase in the intracellular ion concentrations is observed [28]. This ion concentration increase is due to membrane rearrangements and consequent lipid packing faults [29], which can cause a loss of the effectiveness of the

Table 1: Effect of the EGTA (ethylene glycol tetraacetic acid) supplementation at different concentrations in the freezing media (egg yolk Tris glycerol) on bull sperm quality after cryopreservation at two incubation times (30 and 150 minutes).

	Freezing Treatments (mM EGTA)					
Variables 30 and 150 min	A (control)	B (1mM)	C (5mM)	D (10 mM)	E (15 mM)	SEM
% SIPM-30	27.2^{ab}	30.7^{a}	27^{b}	22.5^{c}	21.1^{c}	1.21
% SIPM-150	22.5^{ab}	26^{a}	24.7^{ab}	22^{ab}	19.6^{b}	1.7
% NAR-30	22.6^{a}	28.4^{b}	30.7^{b}	23.9^{a}	21.1^{a}	1.32
% NAR-150	17.7^{ac}	21.87^{ab}	25.6^{b}	20.4^{ab}	17.9^{ab}	1.76
% TMS-30	25^{abc}	25.8^{ab}	28^{a}	20.1^{bc}	18.9^{c}	2.19
% TMS-150	14.6^{ab}	20.7^{a}	16.9^{ab}	12.6^{b}	11.3^{b}	2.14
% PMS-30	19.4^{ab}	19.9^{ab}	21.6^{a}	16.1^{ab}	14.2^{b}	1.97
% PMS-150	10.6^{ac}	16.8^{b}	14.2^{ab}	11^{abc}	7.8^{c}	1.94

Values showed for each variable represent least-squares means with its corresponding standard error of the mean (SEM) in the SEM column
Values within the same row with different letters (a, b, c) differed significantly (P<0.05).
SIPM: spermatozoa with intact plasmatic membrane; NAR: normal acrosomal ridge; TMS: total motile spermatozoa; PMS: progressive motility spermatozoa.

Table 2: Effect of the EGTA (ethylene glycol tetraacetic acid) supplementation at different concentrations in the freezing media (egg yolk Tris glycerol) on bull sperm kinematics parameters after cryopreservation at two incubation times (30 and 150 minutes).

	Freezing Treatments (mM EGTA)					
Variables 30 and 150 min	A (control)	B (1mM)	C (5mM)	D (10 mM)	E (15 mM)	SEM
VCL-30	88.9^{a}	92.5^{a}	90.4^{a}	74.2^{b}	75.3^{b}	4.26
VCL-150	72^{ab}	83^{a}	77.4^{a}	82.2^{a}	59.1^{b}	5.82
VSL-30	66.9	65.9	64.3	52	54.5	4.96
VSL-150	46.3^{ad}	59.2^{b}	58.1^{ab}	62.2^{b}	41.6^{c}	4.02
VAP-30	75.8^{ab}	79.6^{a}	76.9^{ab}	64.2^{b}	66.1^{ab}	5
VAP-150	53.9^{ac}	67^{ab}	65.5^{ab}	68.8^{b}	47.3^{c}	4.48
LIN-30	75.8	71.8	71.2	69.2	72.5	3.69
LIN-150	65.1^{a}	72.4^{b}	75.8^{b}	75.6^{b}	70.4^{ab}	2.13
STR-30	88.2	83.3	83.4	79.8	82.7	2.97
STR-150	85.7^{a}	88.5^{ab}	88.8^{ab}	90.6^{b}	87.8^{ab}	1.34
WOB-30	85.7	86.3	85.1	86.2	87.8	2.49
WOB-150	75.8^{a}	81.5^{ab}	85.3^{b}	83.4^{b}	80.1^{ab}	1.92
ALH-30	2.42^{ab}	2.52^{a}	2.32^{abc}	2.05^{bc}	2^{c}	0.13
ALH-150	2.72^{a}	2.65^{a}	2.3^{ab}	2.45^{ab}	2.075^{b}	0.18
BCF-30	9.12	9	8.9	8.72	8.3	0.48
BCF-150	8.57	9.1	8.9	9	8.82	0.62

Values showed for each variable represent least-squares means with its corresponding standard error of the mean (SEM) in the SEM columnValues within the same row with different letters (a, b, c) differed significantly (P<0.05).
VCL indicates curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, oscillation index; ALH, mean amplitude of lateral head displacement; and BCF, beat cross frequency.

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plasma membrane as a barrier, resulting in an increase in membrane permeability and thus influx of extracellular ions such as Ca^{2+} and Mg^{2+}. This redistribution causes some cations to be unbalanced, increasing the amount of some of them in the intracellular sperm and others in the external environment [30-32]. These unbalanced cations were negatively correlated with fertility, contributing to high percentage of the variation in the fertility of cryopreserved sperm [33,34]. Moreover, some authors have shown that high concentrations of cations such as calcium or magnesium in the environment provided significantly less protection to the cells during freezing and thawing [35]. And even can affect the activity of the acrosin [36] and consequently the acrosome reaction. Also some authors have documented for the sperm of some species that during the freezing process there is an increase of intracellular calcium concentration [30,37-39]. This increase may cause perturbations of the plasma membrane [40], because there may have a detrimental effect in the acrosome state [41,42], and a higher concentration in the external medium may decrease the motility and viability of sperm [42,43]. Therefore, the beneficial effect of inclusion of EGTA during freezing may be determined by their ability to chelate divalent ions such as Ca^{2+} and Mg^{2+}, especially the calcium ion [44], preventing or diminishing the unbalanced cations that occurs during freezing. And especially in the acrosome protection, where it is known that the calcium increase triggered acrosome reaction [45-47], as can be observed in the results of the NAR percentage and motility parameters of this study.

In this experiment bull sperm quality results are lower than the expect results. This decrease in sperm quality may have been due to the method of semen collection. The method of electroejaculation has been shown to cause a loss of post-thaw sperm quality compared with other methods such as collection using artificial vagina [48]. But our experiment was not possible to use another method different from the electroejaculation because the animals used had not been trained to use an artificial vagina. Even so, the effect of electroejaculation occurred in all treatments and therefore the differences obtained between them are due to the effect of EGTA and no manipulation of the ejaculates.

In conclusion, the EGTA supplementation in the freezing extender can improve the quality of cryopreserved bull sperm. But it would be necessary to carry out further experiments to determine the most suitable EGTA concentration between 1 and 5 mM and the evolution of divalent cations, especially calcium, when EGTA are present or not, during the cryopreservation process.

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