Comparison Between The Effect of Ethylene Glycol and Glycerol in The Freezability of Buffalo-Bull Spermatozoa in Tris or Milk Egg-Yolk Extenders

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ABSTRACT

The experiment was conducted to evaluate the possibility of replacing ethylene glycol instead of glycerol during cryopreservation of buffalo-bull semen. Semen of eight buffalo-bulls was diluted with egg-yolk Tris and Milk extenders with ethylene glycol or glycerol. Motility, liveability, integrity of acrosome and plasma membrane as well as sperm abnormalities were assessed before processing and after thawing. Ethylene glycol give a better results than glycerol when freezing buffalo-bull semen in Tris or Milk egg yolk extenders.

INTRODUCTION

It is generally accepted that about 50% of sperms are damaged during cryopreservation *(1)*. Intracellular ice crystallization during cryopreservation is the main cause of damage to the cells *(2)*. Therefore, the composition of any extender and the suitable cryoprotectant agents are important factors for successful semen cryopreservation *(3, 4)*.

Since the discovery of glycerol *(5)*, it has been used extensively as a cryoprotectant agent for many types of cells, including mammalian sperm *(6)*. However, glycerol has osmotic and toxic effects on the plasma membrane and metabolism of cryopreserved cells *(3)*. It was responsible for the disorganization of sperm plasma membrane *(7, 8)* and reducing motility and fertilizing ability *(9)*. Higher concentrations of glycerol lead to cell death *(10)*. Glycerol has a contraceptive effect for pigs, chickens and turkeys sperms *(7)*.

Ethylene glycol has been applied to replace glycerol in many species. It has better cryoprotectant effects than glycerol in human *(11)* and bovine spermatozoa *(12)*. It penetrates sperm membranes of different species faster than glycerol resulting in lower hydraulic conductivity and osmotic stress to which cells are exposed during cooling and freezing *(11, 13, 14)*. Apparently, ethylene glycol has fewer detrimental effects on spermatozoa, providing a better protective effect to the acrosome than glycerol *(15)*. Ethylene glycol could be another option for the cryopreservation of buffalo spermatozoa *(15)*. It is used as a substitute for glycerol and the preliminary results suggest that ethylene glycol may be used for freezing bubaline spermatozoa *(16, 17)*.

There is a need to study the less toxic cryoprotectant agent and its significant contribution in improving the quality of frozen–thawed buffalo spermatozoa. Therefore, the aim of this study was to compare the effect of ethylene glycol versus glycerol post-thaw motility, livability, integrity of acrosome and plasma membrane as well as abnormality using two types of egg yolk containing diluents.

MATERIALS AND METHODS

1.Preparation of extenders

Tris-citric acid-fructose-egg yolk and Milk-egg yolk diluents were prepared according to ***Davis, et al. (18)*** and ***Vishwanath and Shannon (19)***, respectively. Centrifugation is carried to both extenders at 3310 g for 20 minutes *(20, 21)*. Each extender was divided into two parts supplied by glycerol (7%) *(22)* or ethylene glycol (5%) *(17)*. Tris-Yolk-Glycerol (TYG), Tris-Yolk-Ethylene Glycol (TYEG), Milk-Yolk-Glycerol (MYG) and Milk-Yolk-Ethylene Glycol (MYEG) were finally prepared.

2.Semen collection and initial evaluation

Eight buffalo-bulls aged 4-8 years belonging to the Buffalo Semen Freezing Center, General Organization for Veterinary Services, Ministry of Agriculture, Abbasia, Cairo, Egypt are the source of the semen tried. The buffalo-bulls are clinically proved to be in good general health condition, free from any general and genital diseases as well as have acceptable libido and good quality semen characteristics.

Two successive ejaculates were collected using an artificial vagina at weekly intervals. Semen samples were initially assessed for volume, sperm motility and sperm cell concentration. Ejaculates fulfilling minimum standard of sperm motility and sperm cell concentration were processed for freezing *(23)*. The ejaculates were pooled in order to eliminate the bull effect.

3.Semen processing

Semen was diluted gradually at 37°C in a single step with Tris-Yolk-Glycerol (TYG), Tris-Yolk-Ethylene Glycol (TYEG), Milk-Yolk-Glycerol (MYG) or Milk-Yolk-Ethylene Glycol (MYEG) extenders. Dilution rate is calculated to obtain at least 80×10^6 motile spermatozoa/ml. Extended semen was cooled slowly (approximately for 2 hrs) to 4°C and equilibrated for 4 hrs *(17)*. Semen was packed into mini (0.25 ml capacity) polyvinyl French straws. After equilibrium periods, the straws were placed horizontally on a rack and frozen in liquid nitrogen vapor (-120°C) for 9 min *(24)* after which they were immersed and stored into liquid nitrogen at -196°C.

4.Post-thaw sperm functional assays

The frozen straws were thawed at 37°C/ half minute *(22)*. The post thaw sperm functional assays include sperm motility, viability, integrity of acrosome and plasma membrane as well as sperm abnormalities.

Sperm motility percentage was assessed using microscope set at magnification of 400 and equipped with a heating plate (37°C) *(21)*. The viability, acrosomal pictures as well as sperm abnormalities were evaluated using Trypan Blue Giemsa stain *(25)*.

Plasma membrane integrity (PMI) of spermatozoa was assessed using the hypo-osmotic swelling (HOS) assay *(26)*. HOS solution was prepared by dissolving 0.735 g Sodium Citrate and 1.351 g Fructose in 100 ml distilled H_2O (osmotic pressure ~190 mOsm kg^{-1}), according to World Health Organization (1992) manual. The HOS assay was performed by mixing 50 µl of the semen samples to 500 µl of the prewarmed (37°C) HOS solution and incubated at 37°C for 30–45 min. After incubation, a drop of semen sample was examined under phase-contrast microscope (X400). Two hundred spermatozoa were counted for their swelling, characterized by coiled tail, indicating intact plasma membrane.

5.Statistical analysis:

All data are subjected to one way analysis of variance (ANOVA). Treated means are compared by the least significant difference test (LSD) at 5% level of probability.

RESULTS

In this study, the results revealed that, usage of ethylene glycol as a cryoprotectant for buffalo bull semen improve its quality significantly after thawing (high viability %, acrosomal integrity, plasma membrane integrity and low abnormality) except sperm motility % than usage of glycerol. The same improvement appeared in Tris and milk egg yolk diluents. The Tris egg yolk diluents improve quality of frozen semen after thawing (high sperm motility %, viability %, acrosomal integrity, plasma membrane integrity and low abnormality) than the milk egg yolk diluents with both cryoprotectants (Table 1 and Fig. 1).

Table 1. Effect of ethylene glycol versus glycerol for freezing of Buffalo bull semen on post-thaw sperm functional assays using Tris or milk egg yolk containing diluents.

Diluents	Motility %	Livability %	Abnormality %	Intact acrosome %	Intact plasma membrane %
Initial	a 72.14±1.47	a 83.14±1.30	d 14.43±0.92	a 89.29±0.92	a 91.29±0.84
TYG	b 50.25±0.85	b 61.75±0.85	bc 26.50±0.52	c 65.60±1.01	c 68.50±0.91
TYEG	c 45.25±0.77	b 61.15±0.73	c 24.85 ±0.41	b 69.10±0.81	b 71.75±0.72
MYG	c 45.50±0.80	c 56.60±0.76	a 29.40±0.71	d 61.10±0.74	d 63.45±0.81
MYEG	d 42.25±0.68	c 55.95±0.56	b 27.30±0.75	c 64.10±0.61	c 66.45±0.78

Means carrying different superscript letters are significantly varied at probability < 0.05
(TYG)= Tris-Yolk-Glycerol, (TYEG)=Tris-Yolk-Ethylene Glycol, (MYG)= Milk-Yolk-Glycerol (MYEG) = Milk-Yolk-Ethylene Glycol

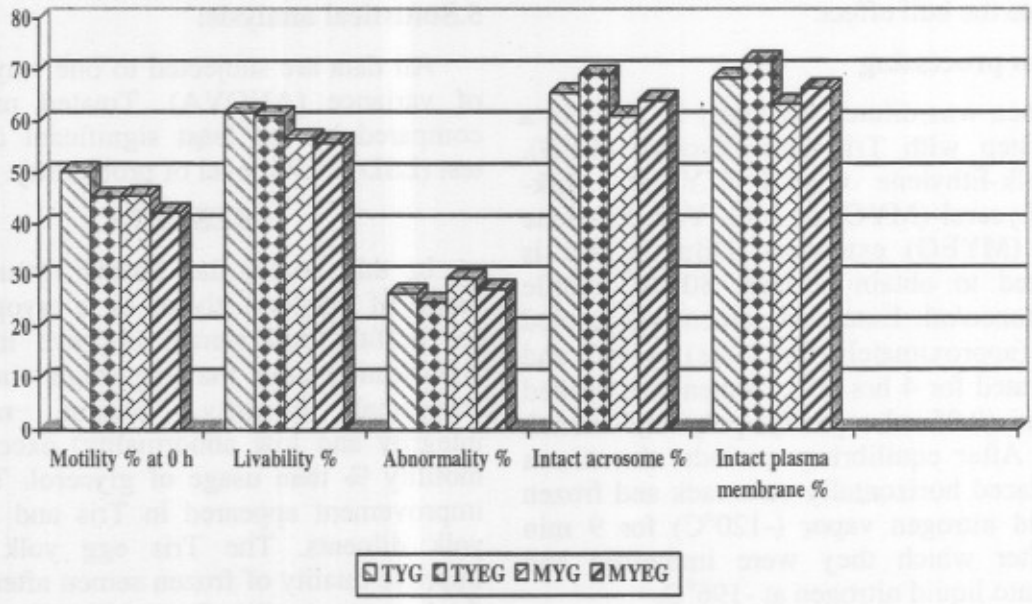

Fig. 1. The effect of ethylene glycol versus glycerol for freezing of Buffalo bull semen on Post-thaw sperm functional assays using Tris or Milk egg yolk containing diluents.

DISCUSSION

Our results demonstrated that ethylene glycol significantly improves buffalo semen quality at thawing; all the parameters that were considered are resulted higher with ethylene glycol than with glycerol except motility.

Our results disagreed with that obtained by ***Rohilla, et al. (17)*** who reported that 6.8% glycerol resulted in higher post thawing live spermatozoa and intact acrosomes than semen extended with 5% ethylene glycol. But in agreement with their results of post-thaw sperm motility and sperm abnormalities which

were less in 5% ethylene glycol than in 6.8% glycerol.

On the other hand, our results were in agreement with ***Moraes, et al. (27)*** who concluded that ethylene glycol is efficient for freezing ram sperm, allowing post thaw motility similar to glycerol but with high number of intact acrosome. While, the results of ***Brisola et al. (28)*** showed no difference between ethylene glycol and glycerol for acrosome status and ram sperm motility. The sperm cells that were preserved with ethylene glycol showed more integrity of the plasmatic, nuclear and mitochondrial membranes. From the viewpoint of cell membrane integrity, they concluded that ethylene glycol gives higher protection to the sperm cell than glycerol.

With bull semen, ethylene glycol resulted in higher post-thaw motility when compared with glycerol or DMSO. This may be because of a reduction of the osmotic lesions ***(12)***. The possibility that ethylene glycol could cause less osmotic lesions had already been suggested for stallion spermatozoa ***(15)***. When used to cryopreserve stallion semen, ethylene glycol had results similar to those of glycerol, and successfully replaced it when used in the same or lower concentrations ***(29, 30).***

During freezing, cells undergo changes in volume which can be lethal if beyond their osmotic tolerance limits ***(31)***. Ice initially nucleates in the extracellular space and causes water to leave the cell; if the cooling rate is too fast not enough water is lost, resulting in intracellular ice formation and in cell death because of membrane damage. If the cooling rate is too slow the cells may shrink excessively and may be exposed to high solute concentration for too long ***(32)***. The addition of cryoprotectants modifies water permeability, lowering the hydraulic conductivity and thus limiting volumetric excursion and osmotic stress. Cryoprotectants that penetrate into the cells are maximally effective after having reached across-membrane equilibrium ***(13)***. An optimal cryoprotectant permeates the cell rapidly, with low temperature dependence and has low toxicity ***(33)***.

The permeability of cells to different cryoprotectants is different and ethylene glycol showed the highest permeability in human ***(13)***, bull ***(12)***, boar ***(31)*** and mouse ***(14)*** spermatozoa. However, permeability to cryoprotectants is different among species, since it depends on the structure and composition of the membrane ***(13)***.

Glycerol, besides its cryoprotective properties, can induce alterations in the organization and viscosity of the sperm cytoplasm and in the permeability and stability of the plasma membrane through disruption of phospholipid and protein structural organization ***(7, 34, 35)***. These phenomena can cause potential deleterious effects on the sperm fertilizing ability.

The chemical structures of ethylene glycol and glycerol are quite similar, both having the same ratio of carbon atoms and C/OH hydroxyl groups, an indicator of the molecule lipophilia/ hydrophilia ***(36)***. However, ethylene glycol has a smaller molecular weight (62.07) than glycerol (92.10), a characteristic that may result in lower toxicity and higher permeability to cells ***(37)*** which allows it to penetrate and leave the cell faster than other cryoprotectants ***(38)***, resulting in lower hydraulic conductivity and then in a reduction in the osmotic stress to which cells are exposed during cooling and freezing ***(13)***. Thus, ethylene glycol could minimize the detrimental effects of the dehydration and rehydration during the freezing/thawing processes. Consequently, ethylene glycol is widely used for embryo freezing in various mammalian species ***(37, 39)***, as well as freezing of ovarian tissue ***(40, 41)***. In fact, the addition and removal of ethylene glycol seemed to minimize alterations of cellular volume of bovine spermatozoa, when compared with using DMSO or glycerol ***(12)***. Similarly, equine sperm had a higher osmotic tolerance to quick addition and removal of ethylene glycol, than to glycerol, DMSO or propylene-glycol ***(15)***. However, permeability of the sperm cell membrane to either ethylene glycol or glycerol, and the consequent osmotic-induced alterations of cellular

volume, may also be species-specific. In fact, glycerol penetrates dog and swine sperm cells quickly *(42,43)*. The effects of using ethylene glycol in frozen semen varied among species.

The use of ethylene glycol for cryopreservation of stallion *(29, 30, 44)* or ram *(27,45)* semen can provide similar or better results than those obtained with glycerol. Ethylene glycol and glycerol as cryoprotectant for dog semen are contradictory ethylene glycol resulting in similar or better preservation in some studies *(38, 46, 47)* and giving inferior results in another *(48)*.

Watson (49) hypothesized that the changes in temperature occurring during freezing–thawing of spermatozoa produce modifications in sperm-membranes that are similar to the changes occurring during capacitation. Capacitated sperm displays hyperactivated motility, which has a characteristic pattern, with a less linear and more vigorous movement than uncapacitated spermatozoa.

During cooling the architecture of the plasma membrane undergoes modifications and there is a redistribution of membrane proteins that are excluded from the phospoholipid regions when phospholipids change from fluid to gel phase *(42,50)*. These membrane changes have been shown to impair the functionality of calcium ion channels, resulting in rising intracellular Ca2+ concentration, which is the same mechanism observed during capacitation and acrosome reaction *(42,50)*. Capacitation is a process of membrane destabilization that occurs in the female genital tract and enables the spermatozoon to display hyperactivated motility and to undergo acrosome reaction, and eventually leads to cell death *(33)*. So, a higher percentage of spermatozoa in a capacitated like condition may represent a detrimental aspect for post-thaw semen survival. A possibility is that less altered membranes maintain the Ca2+ pump working and Ca2+ concentration is kept under the critical threshold which triggers acrosome reaction and subsequently cell death; another hypothesis is that spermatozoa may revert their capacitated status in the absence of a specific stimulus which induces acrosome reaction, thus living longer *(42)*.

Sperm volume response to osmotic shock is regulated by the activity of potassium channels and is minimised by the presence of an intact cytoskeleton. Ethylene glycol might affect the functionality of potassium channels, their activation mechanisms or the fluxes of ions and organic osmolytes *(51)*.

On conclusion ethylene glycol could significantly improve freezability of buffalo bull sperms than glycerol.

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الملخص العربي

مقارنة بين تأثير الإثيلين جلايكول و الجليسيرول في القدرة التجميديه للحيوانات المنوية لثيران الجاموس مع مخففات التريس أو الحليب مع صفار البيض

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قسم التوليد و التناسل و التلقيح الاصطناعي- كلية الطب البيطري- جامعة الزقازيق

أجريت هذه التجربة لتحديد إمكانية استعاضة الجليسيرول بالإثيلين جلايكول عند تجميد السائل المنوي لثيران الجاموس. تم تقسيم السائل المنوي المختلط و المجمع من ثمانية ثيران جاموس الى أربعة نماذج ، وجمد في مخفف التريس أو الحليب المضاف اليه صفار البيض في وجود الجليسيرول أو الإثيلين جلايكول. و قد تم تقييم السائل المنوي بعد الاساله بعدة طرق كقياس نسبة الحركة الفرديه للحيوانات المنوية ، و الحيوانات المنوية الحيه ،وكذاك المشوهه و الغير مشوهه ، و سلامة الاكروزوم ، وكذلك غشاء البلازما. لوحظ تحسن خصائص السائل المنوي لثيران الجاموس المسال بعد التجميد باستخدام الإثيلين جلايكول اكثر من الجليسيرول باستخدام مخففات التريس أو الحليب مع صفار البيض.