The Role of Honey Supplementation to Cryopreservation Solution on Human Sperm Parameters and DNA Integrity during Cryopreservation

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Abstract

Background:

Semen cryopreservation is a useful tool for preserving fertility for men involved in ART program who have been diagnosed with cancer and will undergo chemotherapy, radiotherapy or testicular surgery.

Objective:

The objective of this study to evaluate the effect of honey bee supplemented to cryoprotectant medium on post-thaw sperm DNA integrity ,morphology and round cells.

Subjects ,Materials and Methods:

Thirty semen samples were collected from 30 infertile patients. After assessment of semen analysis, semen samples were divided into 3 aliquots (0.7ml for each sample) and mixed with 1 ml of cryopreservation solution (G1,control) alone, enriched with 5% honey bee(G2) with 10% honey bee (G3) for cryopreservation. Current study used the acridine orange test to investigate the effect of honey bee in two concentration (5%, 10%) on the integrity of sperm chromatin structure pre- and post cryopreservation. Cryopreservation was done at -196 C^{o} in liquid nitrogen and thawing was performed after six months. Direct swim up technique was used for *in vitro* sperm preparation post- thawing, sperm parameters were assessed and data were statistically analyzed pre- and post- thawing.

Results:

The results show there was a significant($P<0.05$) increase in the percentage of morphologically normal sperm for all groups post-thawing, particularly for G3 group that was significantly ($P<0.05$) increased as compared to G1 and G2 groups, In contrast, non-significant differences ($P>0.05$) were observed between G1 and G2 groups. Sperm DNA fragmentation percentage (%) was significantly($P<0.05$) increased in G1 post thawing without any additives as compared to pre-cryopreservation , where as in G2 and G3 sperm DNA fragmentation were increased but not significantly as post-cryopreservation groups. Also the results show that there were no significant differences between G2 and G3 whereas G3 gives better result as compared to G1 with significant differences. Round cells counts for all groups of post cryopreservation were significantly($P>0.05$) decreased as compared to pre-cryopreservation. Non significant ($P>0.05$) differences in the round cells counts among all the groups of post -thawing in spite of G3 group has the lowest mean.

Conclusions:

From these results we concluded that supplementation of 10% of honey bee to the freezing cryoprotectant medium gives best protection to the morphology and DNA integrity of human sperm.

Key words: DNA fragmentation, Honey bee, Spermatozoa, Cryopreservation, Cryoprotectant, Human sperm morphology.

Introduction:

The procedure that makes it possible to stabilize the cells at cryogenic temperatures is called cryopreservation, also known as an applied aspect of cryobiology or the study of life at low temperatures. Cryopreservation of human semen is the most commonly accepted method of preserving reproductive capacity. Semen can be collected and banked for use in assisted reproductive techniques(ART) [1,2,3] especially in cases where the patient elects to undergo vasectomy for contraception[1] or, most importantly, when a patient is diagnosed with cancer and the treatment may render him infertile [4].Compared with other cell types, spermatozoa seem to be less sensitive to cryopreservation damage because of the high fluidity of the membrane and the low water content (about50%). Despite this, cryopreservation may lead to deleterious changes of sperm structure and function [5,6]. It was largely reported that several damaging processes could occur during freezing-thawing of human spermatozoa, such as thermal shock with formation of intracellular and extracellular ice crystals, cellular dehydration, and osmotic shock [7].The primary cause of cellular damage during cryopreservation is the formation of intracellular or extracellular ice crystals. During the freezing process, the cooling rate plays an important role in determining the extent of cryo injury to the spermatozoa [8].While the effects of cryopreservation on the fertilization capacity, motility, morphology, and viability of spermatozoa are well documented, still open is the question of the possible alteration of sperm DNA integrity after freezing-thawing procedures. There is no agreement in the literature neither on whether cryopreservation induces DNA damage nor on the amount of damage. In some studies, authors have reported significant alterations of sperm DNA integrity after cryopreservation/thawing [6,8], whereas other studies have expressed a different opinion[9,10] .Cryoprotectants are low-molecular weight and highly permeable chemicals used to protect spermatozoa from freeze damage by ice crystallization. Cryoprotectants act by decreasing the freezing point of a substance, reducing the amount of salts and solutes present in the liquid phase of the sample and by decreasing ice formation within the spermatozoa [11].Glycerol and egg yolk extenders are amongst the first to be used for freezing semen [12,13,14] and today many extenders use glycerol as the major cryoprotectant[15].Honey bees are a sweet food made by bees using nectar from flowers. The variety produced by honey bees (the genus, *Apis*) is the one most commonly referred to and is the type of honey collected by beekeepers. Flavors of honey vary based on the nectar source, and various types and grades of honey are available [16,17].As with all nutritive sweeteners, honey is mostly sugars and contains only trace amounts of vitamins or minerals. Honey also contains tiny amounts of several compounds thought to function as antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin.The specific composition of any batch of honey depends on the flowers available to the bees that produced the honey [18] .So the main aim of this study was to investigate the effects of honey bee supplementation to cryoprotectant medium on sperm parameters post-thawing.

Subjects, Materials and Methods:

Subjects:

Thirty normal semen samples were obtained from the male partner of infertile couples attending to the Infertility Clinic of Higher Institute for Infertility Diagnosis and Assisted Reproductive Technologies/ AL-Nahrain University. The mean age of fertile men was 31±1.06 years. The standard seminal fluid analysis involving macroscopic and microscopic examination were performed. Macroscopic parameters were semen appearance, volume, liquefaction time, viscosity and pH. After semen liquefaction at 37ċ, sperm morphology, round cell, DNA integrity, was evaluated according to the last edition of WHO criteria [19]. Same sperm parameters were also assessed post – thawing.

The experimental design of this study includes that each semen sample was divided into 3 equal parts: First cryovial tube G1 containing 0.7ml semen mixed with 1ml cryoprotectant medium (Ferti-Pro, Belgium); Second cryovial tube G2 containing semen + cryoprotectant medium supplemented with 5% of pure honey bee, third cryovial tube G3 containing semen+ cryoprotectant medium supplemented with 10% of pure honey bee. Usually the cryoprotectants are added in an equal volume of semen in a drop wise manner, gently mixed at room temperature, and then placed at 37 °C for 10–15 minutes to allow for proper equilibration between the cells and the medium. It is necessary that the medium interacts with the cells. Indeed, the effectiveness of cryoprotecting substances is also a function of the time of interaction between the cryoprotectants and the cells [20]. Each vial was labeled with code number refer to the patient, and date of cryopreservation .Each vial was labeled with code number refer to the patient, and date of cryopreservation.

Each mixture including G1, G2, G3 were exposed to liquid nitrogen vapor for 10-12 min then store in liquid nitrogen tank. After 6 months of cryostorage, semen samples were thawed by place cryovial containing above mixture at room temperature for 3-5 min then added 1 ml of diluents and mix with above mixture then centrifugation 1000 rpm for 5 min to remove supernatant and add 1 cm of of diluents and incubate for 30 min[21]. The fresh and thawed aliquots were also assessed by light and fluorescence microscopy (after Acridine Orange staining, AO).

Acridine orange test was functioned to assess spermatozoa with fragmented DNA as described by Tejada *et al.*[22].Each semen sample was smeared pre-cryopreservation first and post-thawing for

each(G1, G2 and G3) onto a pre-cleaned glass slide and allowed to air dry for 20minutes. The smear was then fixed overnight in Carnoy's solution (methanol:acetic acid = 3:1), which was daily prepared. After fixation, the slide was allowed to dry for a few minutes before staining. The acridine orange staining solution was prepared as follows: 10ml of 1% AO in distilled water was added to a mixture composed of 40 ml of 0.1M citric acid and 2.5 ml of 0.3M Na2HPO.7H2O, pH 2.5.The smear was stained for 5 minutes, and gently rinsed and mounted with distilled water. Each slide was interpreted on the same day by a fluorescence microscope using a 490-nm excitation filter and a530 nm barrier filter. Two hundred cells were counted on each slide with the duration no longer than 40 seconds by the same investigator. Spermatozoa with normal DNA content exhibited green fluorescence of the heads, while those with abnormal DNA content expressed a spectrum varying from yellow green to red. As shown in figure (1) below.

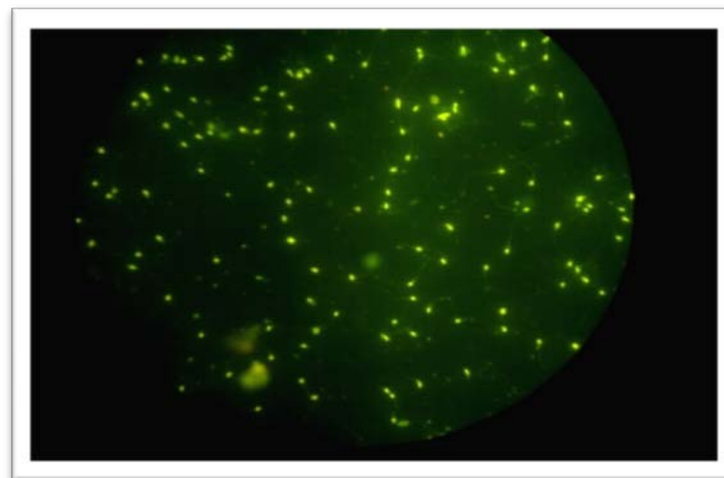

Figure (1): This figure shows Spermatozoa with normal DNA content exhibited green fluorescence of the heads, while those with abnormal DNA content expressed a spectrum varying from yellow green to red.

Sperm parameters were evaluated statistically according to the data obtained pre-freeze and post-thawing. The data were statistically analyzed using Statistical Package for Social Sciences (SPSS; Version 14,Chicago,USA). Sperm parameters of sperm morphology , round cell ,DNA integrity were analyze using one way ANOVA and complete randomized design (CRD).Differences among mean of groups were computed using the Dancan multiple ranges test [23]. A P value <0.05 was considered statistically significant.

Results:

In table(1), the percentage of DFI pre- and post-cryopreservation were presented.Non significant differences (p>0.05) were assessed between pre-cryopreservative group and post-cryopreservative (G2 and G3)groups. Mean while the percentage of DFI for G1 group was significantly increased(P<0.05) as compared to pre-cryopreservation group and G3 group post-cryopresevation. However, no significant difference (p>0.05) in the percentage of DFI were noticed between G1 and G2 groups post-cryopreservation. Significant increased (P<0.05) in the percentage of morphologically normal sperm for all groups pos-thawing (Table 1).Post-thawing, morphologically normal sperm (%) for G3 group was significantly increased (P<0.05) as compared to G1 and G2 groups. In contrast, non significant differences(P>0.05) were observed between G1 and G2 groups. Round cell counts for all groups of post cryopreservation were significantly reduced (P<0.05) as compared to pre-cryopreservation group (table 1).From same table, non significant differences (P<0.05) in the round cell counts among the groups of post -thawing in spite of G3 group has the lowest mean.

Table 1: Effect of honey bee supplementation to cryopreservation solution on percentages of, DNA fragmentation, morphologically normal sperm and round cell pre and post cryopreservation.

Sperm parameters	Pre-cryopreservation	Post-cryopreservation		
		G1:Control	G2:CS+5%H	G3: CS+10%H
DNA Fragmentation	22.10 b ±9.65	34.20a ±10.90	25.60ab ±11.91	24.06b ±12.20
Morphologically Normal Sperm	39.33 b ±11.87	51.47a ±11.02	45.67 ab ±12.15	63.60 b ±12.78
Round cells	7.20 a ±7.89	1.47 b ±2.89	1.31 b ±2.30	0.37 b ±1.24

Data are mean+ S.E.M.; similar letters mean no significant differences; Different letters mean significant differences; CS: Cryoprotactants Supplement H: Honey.

Discussion:

Glycerol remains to be one of the most favored cryoprotectant(CPA), It is penetrating cryoprotectant, acting as a solvent and readily taken up by spermatozoa, entering the cell within one minute of addition to the surrounding medium [16].Its presence, both intra- and extra cellularly, acts to lower the freezing point of the medium to a temperature much lower than that of water. This in turn reduces the proportion of the medium which is frozen at any one time, reducing the effect of low temperature on solute concentrations and hence on osmotic pressure differences[24].It also provides channels of unfrozen medium, between ice crystals, in which spermatozoa may exist while at low temperatures. A further effect of glycerol may be a salt buffering action [15]. In this study, honey bee has been used with cryoprotectant medium at two concentrations.Honey has a glass transition between -42 and -51 °C (-44 and -60 °F). Below this temperature, honey enters a glassy state and will become an amorphous solid (noncrystalline) [25, 26].

According to the National Honey Board[27].Honey bee is a mixture of sugars and other compounds. The question of the possible alteration of sperm DNA integrity after freezing-thawing procedures. There is no agreement in the literature neither on whether cryopreservation induces DNA damage nor on the amount of damage. Authors reported significant alterations of sperm DNA integrity after cryopreservation/thawing [8,9]. According to Marlea Di Santo *et al* 2012[6] though further investigations the real influence of cryopreservation on sperm DNA integrity and the impact of the use of cryopreserved spermatozoa on the reproductive outcome, technical measures should be applied to provide maximum protection to the male gametes, appropriate use of cryoprotectants before sperm selection technologies after cryopreservation seems to have the greatest impact on preventing DNA fragmentation.Thus improving sperm cryosurvival rates.

Donnelly and colleagues [28]investigated precryopreservation and post cryopreservation DNA integrity of both semen and prepared sperm samples (density gradient centrifugation or direct swimup) in 50 men. They reported that freezing sperm in seminal plasma improves post thaw DNA integrity: sperm-frozen unprepared in seminal fluid seems to be more resistant to freezing damage than frozen prepared sperm; further improvement can be achieved by preparing sperm and freezing after readdition of seminal plasma. This may be due to the presence of abundant antioxidants in

seminal plasma. This result agree with a recent study.

Petyim S. *et al* [29] evaluate the cryo damage effects on human sperm characteristics, especially on sperm DNA integrity, after 6 months of freezing comparing between using liquid nitrogen vapour (LNV) and using computerized program freezer (CPF). Post-thawed sperm motility was greater after CPF than after LNV .No significant difference was observed in the percentage of morphologically normal sperm comparing the two freezing methods. The study demonstrated a post-thawed decrease in sperm DNA integrity as well as other sperm characteristics after freezing in both methods .In case of 6 months of cryostorage, therefore, we recommend the computerized program freezer as a preference for sperm cryopreservation.

According to Chohan KR *et al* [30]concluded that staining of cells with acridine orange (AO) has been widely accepted as a predictor of DNA damage in many cell types. Because of variability of protocols used in previous studies, the AO staining technique has not been widely accepted as a screening test to predict DNA damage in human sperm. This study also elucidated the effects of cryopreservation on sperm DNA. In conclusion, Carnoy's fixative provides a better predictive value for DNA damage to sperm using AO staining. Additionally, cryopreservation increased damage to the sperm DNA. This result disagreement with the study results.

The study performed by Rasheed [31] concluded that the morphologically normal sperm (%) was highly significantly increased after thawing and sperm preparation than before preparation. These results agreement with our results .Also there was no studies show the effect of cryopreservation on the round cells in semen samples.

Conclusion:

From these results we concluded that supplementation of 10% of honey bee to the freezing cryoprotectant medium gives best protection to the morphology and DNA integrity of human sperm.

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