

Honey Supplementation to Semen-Freezing Medium Improves Human Sperm Parameters Post-Thawing

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Abstract

Objective: To evaluate the effect of honey supplemented to cryoprotectant medium on post-thaw sperm motility, concentration, morphology and agglutination.

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) and mixed with 1 ml of cryopreservation solution (G1, control) alone, or enriched with 5% honey (G2) or with 10% honey (G3) for cryopreservation. Cryopreservation was done at -196°C 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: Results appeared that the percentage of sperm motility for G1 and G2 groups were significantly reduced ($P<0.05$) post-thawing when compared to pre-cryopreservation. However, there was no significant difference in the total motility (%) of the post-thaw sperm between the G1 and G2 groups. While there was significant increased ($P<0.05$) in the percentage of normal sperm morphology for G1 and G3 groups post-thawing. Post-thawing normal sperm morphology (%) 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. Significant reduction ($P<0.05$) was seen in the sperm concentration for all groups post-thawing as compared to pre-cryopreservation groups. After thawing the results reveal significant reduction ($P<0.05$) in the sperm agglutination (%) for G3 group as compared to G1 and G2 groups.

Conclusion: The results of this study indicated that the supplementation of honey (10%) to cryoprotectant solution results in enhancement of sperm quality post-thawing.

Keywords: Honey bee, Spermatozoa, Cryopreservation, Cryoprotectant

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.

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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) especially in cases where the patient elects to undergo vasectomy for contraception or, most importantly, when a patient is diagnosed with cancer and the treatment may render him infertile (1-4).

Semen can be used successfully indefinitely after cryopreservation. For human sperm, the longest

reported successful storage is 21 years. Cryopreservation of human spermatozoa introduced in the 1960's has overcome many space and time limitations and now forms integral part of assisted reproduction technologies (ARTs) (5).

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 (6). The addition of a cryoprotectant into the semen sample is needed in order to protect spermatozoa from cold shock. Based on the composition and dynamics of the sperm membranes, some substances such as lipids, fatty acids and proteins have been incorporated to the semen with the goal of decreasing sperm damages related to cryopreservation. Glycerol and egg yolk extenders are amongst the first to be used for freezing semen and today many extenders use glycerol as the major cryoprotectant (7- 10).

Honey is 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 (11, 12). 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 (13).

Honey has attractive chemical properties for baking, and a distinctive flavor that leads some people to prefer it over sugar and other sweeteners (14). Most microorganisms do not grow in honey because of its low water activity of 0.6. The physical properties of honey vary, depending on water content, the type of flora used to produce it (pasturage), temperature, and the proportion of the specific sugars it contains. Fresh honey is a supersaturated liquid, containing more sugar than the water can typically dissolve at ambient temperatures. At room temperature, honey is a super cooled liquid, in which the glucose will precipitate into solid granules. This forms a semisolid solution of precipitated sugars in a

solution of sugars and other ingredients (15). So the main aim of this study was to investigate the effects of honey supplementation to cryoprotectant solution on sperm parameters post-thawing.

Materials and methods

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 was performed. Macroscopic parameters were semen appearance, volume, liquefaction time, Viscosity and pH. After semen liquefaction at 37°C, sperm motility, concentration, agglutination and morphology was evaluated according to WHO criteria (16). Same sperm parameters were also assessed post-thawing.

The experimental design of this study include that each semen sample was divided into 3 equal parts: First cryovial tube G1 containing 0.7 ml semen mixed with 1ml cryoprotectant medium (Ferti-Pro, Belgium); Second cryovial tube G2 containing semen + cryoprotectant medium supplemented with 5% of pure honey, third cryovial tube G3 containing semen+ cryoprotectant medium supplemented with 10% of pure honey. Then the mixture was left for 10 min. at room temperature for equilibration. Each vial was labeled with code number refer to the patient, and date of cryopreservation.

Each mixture including G1, G2 and G3 was 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 culture medium and mix with above mixture then centrifugation 1000 rpm for 5 min to remove supernatant and add 1 cm of culture medium and incubate for 30 min.

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 were analyzed using one way ANOVA and complete randomized design (CRD). Differences among mean of groups were computed using the Duncan multiple ranges test (17). A *p* value <0.05 was considered

statistically significant.

Results

The results of sperm motility and activity were presented in table 1. Post-thawing, the percentage of sperm motility for G1 and G2 groups were significantly reduced ($p<0.05$) when compared to pre-cryopreservation. However, non significant differences ($p>0.05$) were observed for sperm motility between G1 and G2 groups. While significant reduction ($p<0.05$) in the sperm motility (%) for G1 and G2 groups was noticed as compared to the G3 group. The percentage of progressive sperm motility for pre-cryopreservation was significantly increased ($p<0.05$) as compared to post-thawing groups G1 and G2.

Post-thawing, there are non significant differences in the percentages of non progressive motility and immotile sperm were evaluated between G1 and G2 groups, while G3 group was significantly increased ($p<0.05$) as compared to G1 and G2 groups. Non significant differences ($p>0.05$) was assessed for

percentage of immotile sperm between G1 and G2 groups. In contrast, percentages of progressive and non progressive sperm motility for G3 group were significantly reduced ($p<0.05$) as compared to G1 group. Conversely, non significant difference ($p>0.05$) was reported in the percentage of non progressive motility between pre-cryopreservation group and G3 group.

Results of the present study appeared significant reduction ($p<0.05$) in the sperm concentration post-thawing as compared to pre-cryopreservation groups as shown in table 2. Significant increment ($p<0.05$) in the percentage of sperm agglutination for pre-cryopreservation group was seen as compared to post-thawing groups. Post-thawing, non significant differences ($p>0.05$) were assessed between G1 and G2 groups. In contrast, significant reduction ($p<0.05$) in the sperm agglutination (%) for G3 group as compared to G1 and G2 groups was seen (Table 2).

As compared to pre-cryopreservation, increment ($p<0.05$) was seen in the percentage of normal sperm morphology for all groups post-thawing. Post-thawing, normal sperm morphology (%) for G3 group

Table 1: Effect of honey supplementation to cryopreservation solution on percentages of sperm motility and activity pre and post-cryopreservation

Sperm parameters	Pre- cryopreservation	Post- thawing		
		G1:Control	G2:CS+ 5% ^H	G3: CS+ 10% ^H
Sperm motility (%)	60.02 a + 7.09	34.50 b + 9.64	38.01 b + 7.68	52.82 a + 6.78
Progressive sperm motility (%)	27.26 a + 5.95	13.17 c + 6.89	18.86 b + 4.80	24.23 a + 6.34
Non progressive sperm motility (%)	32.91a + 2.29	21.33 b + 7.06	19.47 b + 3.54	28.59 c + 3.66
Immotive sperm (%)	39.83 a + 7.09	65.50 b + 9.64	61.67 b + 7.87	47.18 c + 2.39

Data are mean \pm S.E.M.; Similar letters mean non significant differences; Different letters mean significant differences; CS: Cryostorage H: Honey

Table 2: Effect of honey supplementation to cryopreservation solution on sperm concentration, agglutination and morphology pre- and post-cryopreservation

Sperm parameters	Pre- cryopreservation	Post-thawing		
		G1: Control	G2: CS+ 5% ^H	G3: CS+ 10% ^H
Concentration (m/ml)	55.50 a \pm 17.68	39.00b \pm 14.34	33.53 bc \pm 12.83	27.00 c \pm 10.22
Agglutination (%)	7.00 a \pm 4.47	3.80 b \pm 2.72	3.00 b \pm 2.49	1.67c \pm 2.39
Normal morphology (%)	39.33 c \pm 11.87	51.47b \pm 11.02	48.67 b \pm 12.15	63.60 a \pm 12.78

Data are mean \pm S.E.M.; Similar letters mean non significant differences; Different letters mean significant differences; CS: Cryostorage H: Honey

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 (Table 2).

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 (18). Its presence, both intra- and extracellularly, 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 (19). 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 (10).

According to Batellier et al and Varner et al, there are a large variety of extenders combining various components (sugars, electrolytes, buffers, egg yolk, milk and milk products), have been proposed and used for extending sperm. Milk and milk-based extenders are known to be practical and efficient in protecting spermatozoa of various species (20, 21).

In this study, honey has been used with cryoprotectant medium at two concentrations. However, normally honey exists below its melting point, and it is a super cooled liquid. At very low temperatures, honey will not freeze solid. Instead, as the temperatures become colder, the viscosity of honey increases. Like mostviscous liquids, the honey will become thick and condense with decreasing temperature. While appearing or even feeling solid, it will continue to flow at very slow rates. 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 amorphous solid (noncrystalline) (22, 23). 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(24).

According to the National Honey Board Honey is a mixture of sugars and other compounds (14). With respect to carbohydrates, honey is mainly fructose (about 38.5%) and glucose (about 31.0%), making it similar to the synthetically produced inverted sugar syrup, which is approximately 48% fructose, 47% glucose, and 5% sucrose. The remaining carbohydrates of honey include maltose, and other complex carbohydrates. Therefore, honey may be protect sperm during cryopreservation, as well as enhances sperm parameters post-thawing.

However, in the present study, sperm concentration as well as the sperm qualities, decreased significantly ($P<0.05$) after the six-month freeze-thawing procedure with liquid nitrogen vapor. Our results agreement with Thitikan and Somboon (25).

According to Januskauskas and Zillinskas and Ruiz-Pesini et al sperm motility induced by cryopreservation is believed to be mainly associated with mitochondrial damage in human spermatozoa, mitochondrial enzymatic activities were shown to be correlated with spermatozoal motility (26, 27). These studies agreement with our results which reveal there was significant decrease of sperm motility post-thawing.

Also the study performed by Hu Jm explained the effect of sucrose on sperm motility after adding cryoprotectant containing sucrose to the semen sample then the motility of the post-thaw human sperm was compared before and after cryopreservation and concluded decreased sperm motility after cryopreservation, and the sucrose is a feasible sperm cryoprotectant (28).

Our results show significant increased ($P<0.05$) in the percentage of normal sperm morphology for all groups post-thawing. According to Axnér they concluded that the percentage of morphologically normal epididymal spermatozoa is not correlated with cryopreservation induced sperm damage using the described freezing protocol (29). The study performed by Rasheed explains the effect of cryopreservation on the normal morphology and have been concluded that the normal sperm morphology (%) was highly significantly increased after thawing and sperm preparation than before preparation (30). These results are in agreement with present results.

Conclusion

From the results of this study it concluded that supplementation of honey bee (10%) to cryoprotectant solution show enhancement sperm parameter post-thawing.

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