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EFFECT OF HONEY BEE AND COQ10 SUPPLEMENTED TO CRYOPRESERVATION SOLUTION ON FREEZE-THAWED HUMAN SPERM PARAMETERS

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ABSTRACT

Background: Cryopreservation techniques highly developed field of Assisted Reproductive Technologies (ARTs) to help infertile couples to overcome infertility. Coenzyme Q10 is a vitamin – like substance is present in most eukaryotic cell, and produced in the mitochondria primarily. **Objective:** To study the effects of honey bee supplemented to freezing solution and COQ10 supplemented to thawing solution on sperm parameters during cryopreservation and cycle of human spermatozoa. **Materials and Methods:** One hundred and eight males were participated in this study. The mean age for all males included in this study was (29.94 ± 0.65) years and duration of infertility was 2-14 years. Semen samples were collected and seminal fluid analysis was done according to WHO (2010). Each semen sample was divided into three aliquots. The first group contain 1mL from semen sample enriched with 0.7 mL of cryopreservation solution only, while the other two groups contain low concentration 5% HB and high concentration 10% HB supplied to cryopreservation solution. **Results:** There was significant reduction ($P<0.05$) in the sperm concentration, significant improvement ($P<0.05$) in the percentages of sperm motility and morphologically normal sperm when compared to control group. Present study appeared that the direct swim-up technique resulted in significantly ($P<0.05$) better results for sperm parameters than the swim-up technique from all types of ejaculates classified according to male infertility factors. The study proved that there was significant ($P<0.05$) improvement in sperm parameters. **Conclusion:** Combination of 10%

HB and 10□M COQ10 obtained best results for human sperm parameters post-thawing compared to other groups. Best technique in this study direct swim-up technique compared with centrifuge technique.

KEYWARDS: cryopreservation, honey bee, Coenzyme Q10.

INTRODUCTION

Cryopreservation is widely used in many assisted conception units to preserve male fertility, for example before cytotoxic chemotherapy, radiotherapy or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction.[1] Freezing and thawing involve damage to the plasma membrane and acrosome of human spermatozoa as evidenced by significant ultra-structural changes demonstrated by electron microscopy.[2] This damage is exacerbated during the cryopreservation process by a decrease in the lipid content of the membranes, which contain a high proportion of polyunsaturated fatty acids; the decrease in lipid content indicates lipid peroxidation in the presence of reactive oxygen species.[3] Honey is a sweet food made by bees using nectar from flowers.[4] 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.[5] Coenzyme Q10 (Co Q 10) is essentially a vitamin or vitamin-like substance. CoQ10 likewise is found in small amounts in a wide variety of foods and is synthesized in all tissues.[6] The biosynthesis of CoQ10 from the amino acid tyrosine is a multistage process requiring at least eight vitamins and several trace elements. Coenzymes are cofactors upon which the comparatively large and complex enzymes absolutely depend for their function.[7] CoQ10 is a component of the mitochondrial respiratory chain, play role both in energy metabolism and as antioxidants for cell membranes and lipoproteins.[8,9]

Objective: To study the effects of honey bee supplemented to freezing solution and COQ10 supplemented to thawing solution on sperm parameters during cryopreservation and thawing cycle of human spermatozoa.

MATERIALS AND METHOD: This study was conducted in the High Institute of Infertility diagnosis. The period of study was from November- 2014 to June- 2015. One hundred and eight men were included in the present study, the mean age years 29.94 ± 0.65 were ranged between 18-44 years.

The duration of infertility ranged from 2 to 14 years. The analyses were done according to the World Health Organization standard criteria (2010). The container must be labeled with the following information, name, age, abstinence period and time of sample collection. The specimens were placed in an incubator at 37 ºC for 30 minutes to allow liquefaction. Freezing was made by using Sperm Freeze TM kit. Sperm Freeze is a 15% glycerol based cryoprotectant in HEPES buffer. It contains 0.4 % human serum albumin (HIV and Hepatitis negative). In the present study added the honey bee to cryoprotactant (G2: low concentration honey bee 5%) and (G3: high concentration honey bee 10%). Then mixing the honey bee with the cryoprotactant. Semen sample divided into three group G1: contain cryoprotactant only, G2: contain cryoprotactant and 5% HB and G3: contain cryoprotactant and 10% HB.

Stock solution A was prepared by dissolving 0.8633 gm. in 10 ml of DMSO. Then, stock solution B prepared by take 0.1ml from Stock solution A diluted with 0.9 ml of SMART medium. Low concentration of treated group (G2; 5µm) was prepared by adding 0.05 ml of stock solution B to 0.95 ml of SMART medium. However, addition 0.10 ml of stock solution B to 0.90 ml of SMART medium to prepare high concentration group (G3; 10 µm). After liquefaction of semen sample, 1mL of semen was mixed with 0.7mL of cryoprotactant.[10]

THE RESULTS

The effect of different concentrations of honey bee supplied to cryopreservation solution using direct technique assessment post- thawing as shown in table (1). Best results for sperm concentration post thawing were appeared when using 10% of honey bee supplied to all group of control group and both concentrations of COQ10 groups in the present study. Within control group (GA), significant increment ($P<0.05$) in the sperm concentration was assessed when using 10% HB (G3) as compared to control (G1) and 5% HB (G2). Similarly, significant increase ($P<0.05$) in the sperm concentration was noticed for 5% HB (G2) as compared to control group (G1). From same table, non-significant differences ($P>0.05$) in the sperm concentration were observed between 5%HB and 10%HB for 5µM.COQ10 (G2) and 10 µM.COQ10 (G3). In contrast , significant reduction ($P<0.05$) in the sperm concentration was obtained for control group (G1) as compared to 5%HB and 10%HB for GB and GC.

The percentage of progressive sperm motility post- thawing appeared significant reduction ($P<0.05$) for control group (G1) as compared to other treated groups (G2; 5%HB

and G3; 10%) for all groups of control group (GA) and COQ10-treated group (GB and GC). However, non-significant differences (P>0.05) in the progressive sperm motility (%) were assessed between 5% HB and 10%HB for GA and GB and GC. While for same sperm parameter, non-significant differences (P>0.05) were assessed for control group (G1) as compared G2 and G3 for 10 µM.COQ10 group (GC).

Progressive sperm motility grade A (%) appeared best results post thawing when using 10% of HB supplied to both concentrations of COQ10 (GB and GC) in the present study except for GA(control) produced best result when using 5% HB. Within control group (GA), significant increment (P<0.05) was noticed in the progressive sperm motility grade A when using 5% HB and 10% HB as compared to G1 (control). In the same parameter, non-significant differences (P>0.05) were observed between 5% HB and 10% HB for GA and GB 5µM.COQ10 except to GC 10 µM.COQ10 was significant reduction (P<0.05).

The percentage of progressive sperm motility grade A and no progressive sperm motility post- thawing appeared significant reduction (P<0.05) for control group (G1) as compared to other two treated groups (G2and G3) with HB (5% and 10%) for all groups of control group (GA) and COQ10-treated group (GB and GC). However, non-significant differences (P>0.05) in the progressive sperm motility grade A (%) were assessed between5% HB and 10% HB for GA and GB. While for same sperm parameters, significant reduction (P<0.05) was assessed for 5% HB as compared to G3 for GC 10 µM.COQ10.

The percentage of progressive sperm motility grade B post- thawing, Best results were appeared when using 10% HB for GA and GB but in the GC the best result appeared when using 5% HB in the present study. in the control group and both concentrations of COQ10 groups, Significant increment (P<0.05) in the sperm motility grade B was assessed for control group as compared to 5% HB and 10% HB for control group GA, GB 5µM.COQ10 and GC 10µM.COQ10. Non-significant differences (P>0.05) when compared between 5% and 10% HB for all groups.

The immotile sperm (%) after thawing. Best result when using 10% HB supplied to all groups of control group and both concentrations of COQ10 group in the present study. in the control group (GA) and 5µM.COQ10 (GB) and 10 µM.COQ10 (GC), significant increase (P<0.05) was assessed when control group (G1) as compared to 5% and 10% HB for

all groups control(GA) and (GB) and (GC). In the same sperm parameter, non-significant differences (P>0.05) when compared between 5% and 10% HB for all group control group (GA) and (5µM.COQ10; GB) and (10 µM.COQ10; GC).

The percentage of normal sperm morphology post- thawing appeared significant reduction (P<0.05) for control group (G1) as compared to other treated groups (G2 and G3) with HB (5% and 10%) for all groups of control group (GA) and COQ10-treated group (GB and GC). However, significant increase (P<0.05) when G1 (control) as compared to 5% and 10% HB for all all groups GA (control) and (5µM.COQ10; GB) and (10 µM.COQ10; GC). Similarly, significant increase (P<0.05) in the normal sperm morphology when compared between 5% HB (G2) and 10% (G3) for GA and GC groups. Non-significant differences (P>0.05) when compared between 5% and 10% HB for (5µM.COQ10; GB) group. Non-significant differences (P>0.05) in the percentage of sperm agglutination and round cell post- thawing for control group and both treated groups (5% and 10% HB) for GA group (control group). Similar results were achieved for all groups of GB (5µM.COQ10) and GC (10µM.COQ10).

Concentrations of honey bee and COQ10 supplemented to freezing and thawing solution as respectively for centrifuge technique present in the table (2). The percentage of sperm concentration and progressive sperm motility grade B after thawing. Best results for sperm concentration were appeared when using 10% supplied to all group of control group and both concentration of COQ10 group in the present study. In the control group (GA) and (5µM.COQ10; GB) and (10µM.COQ10; GC), significant increase (P<0.05) when control group as compared to 5% and 10% HB. Non-significant differences (P>0.05) when compared between 5% and 10% HB for all groups (control; GA) (5µM.COQ10; GB) and (10µM.COQ10; GC).

The percentage of progressive sperm motility (A+B) and non-progressive sperm motility appeared significant reduction (P<0.05) for control group (G1) as compared to other treated groups (G2 and G3) with HB (5% and 10%) for all groups of control group (GA) and COQ10- treated group (GB and GC). However, non-significant differences (P>0.05) in the progressive and non-progressive sperm motility (%) were assessed between 5% and 10% HB for GB and GC. While for same sperm parameters, significant reduction (P<0.05) was assessed for 5% HB as compared to G3 for control group (GA).

The progressive sperm motility grade A(%) appeared significant reduction (P<0.05) for control group (G1) as compared to other treated groups (G2 and G3) supplied with 5% HB and 10% HB for all groups of control group(G1) and COQ10-treated group (GB) and GC). Also significant reduction (P<0.05) was assessed for control (G1) as compared to 5% HB and 10% HB for all groups GA , GB and GC. In the same parameter significant reduction when compared between 5% and 10% HB for GA and GB. Non-significant differences (P>0.05) when compared between 5% and 10% for GC.

The immotile sperm (%) after thawing. Best results for this parameter when using 10% HB supplied to all group of control group and both concentrations of COQ10 group in the present study. Within control group (GA), significant increment (P<0.05) in the immotile sperm was assessed when using 10 HB (G3) as compared to control group (G1) and 5% HB (G2). In the same parameter significant increase when G1 as compared to 5% and 10% HB for (5µM.COQ10; GB) and (10µM.COQ10;GC). Non-significant differences (P>0.05) when compared between 5% and 10% HB for GB and GC.

The percentage of normal sperm morphology post- thawing appeared significant reduction (P<0.05) for control group (G1) as compared to other treated group (G2 and G3) with HB(5% and 10%) for all group (GA) and COQ10-treated group (GB and GC). Within control group (GA), significant increase (P<0.05) when using 10% HB as compared to 5% HB for control group (GA) and (10µM.COQ10; GC) group. However, non-significant differences (P>0.05) when compared between two concentrations of HB 5% and 10% for (5µM.COQ10; GB) group.

Sperm agglutination (%) and round cell count appeared non-significant differences (P>0.05) among all control and treated groups (5% HB and 10% HB supplied to cryopreservation solution) for all major groups in controls (GA) and treated groups (5µM and 10 µM COQ10 enriched to thawing solution).

DISCUSSION

In this study, two concentrations of honey bee (HB) has been supplied to cryoprotectant solution. 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 most viscous liquids, the honey will become thick and condense with decreasing temperature.[11] 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 an amorphous solid (non-crystalline).[12] 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^{0}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 cryoprotectant substances is also a function of the time of interaction between the cryoprotectants and the cells.[10] According to our knowledge, this work as second research used HB for sperm cryopreservation, however, the first original article used HB as supplement to cryopreservation solution was done by Fakhrildin and Al-Saadi.[13] In general, best results for sperm parameters post-thawing were achieved using 10% HB than 5% HB in the current work. One and the most important factor is the composition of HB which caused improvement in the sperm parameters post-thawing. 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.[15]

In the current study, superior results for human sperm parameters post-thawing when using 10 □M CoQ10 than using 5 □M CoQ10. The main and principal factor is the direct and/indirect roles of CoQ10 on sperm plasma membrane, metabolism and physiology. Coenzyme Q10 is a non-enzymatic antioxidants that the related to low density lipoproteins and protects against peroxidative damage.[16] It is an energy promoting agent and enhances sperm motility.[17]

Two sperm assessment techniques including direct swim-up and centrifugation swim-up techniques were applied. Therefore, different results in the sperm parameters were achieved. In this study, it is assumed that one of the major deleterious effects of freezing/thawing is the formation of reactive oxygen species.[18] Although there are several studies suggesting that seminal plasma has a protective role during the freezing processes, there are also papers reporting advantages of swim-up or other separation methods.[19][20]

Centrifugation swim-up technique for sperm parameter assessment, used in the present study, decreased sperm concentration and activity as a result of ROS release and consequently causes oxidative stress.[21] ROS production during cryopreservation may be is the main cause of cellular damage.[22] Normally, a balance is maintained between the amount of ROS produced and that scavenged. Sperm damage appears when this equilibrium is disturbed. A shift in the levels of ROS towards pro-oxidants in semen can induce an oxidative stress on spermatozoa, in order to scavenge ROS and reduce their destructive action under normal physiological conditions, and seminal plasma.[21]

In the current study, results of the semen cryopreservation were significantly decreased in the means of sperm concentration, sperm motility (%) and progressive sperm motility (%) post-thawing processing compared to pre-cryopreservation. These results may can be attributed to the cryopreservation process itself, since it causes change in osmotic pressure that leads to altered in ion concentration show the impact on mitochondrial activity then caused failure in energy production and finally caused decreased in motility. While there was significant increase ($P<0.05$) in the mean of immotile sperm percentage after cryopreservation. This result agreement with other studies done by Rahana.[23] and Rasheed.[24] Post-thawing sperm motility and progressive activity are routinely only about 50% of pre-freeze values as mentioned by other studies Nijis[25] and Anger.[26] The quantitative motility assessments showed reductions in progressive motility (25-75%).[19] A recent study appeared that the addition COQ10 caused significant changes on sperm progression in each separation time. The mechanism by which COQ10 significant affected the sperm progression is still to be determined. The possible explanation is the involvement of the regulation of energy production as it is concentrated within the mitochondrial. Furthermore, coenzyme Q10 is an energy promoting agent, and reduces super oxideanion.[27] Cryopreservation increases the percentage of dead or membrane damaged sperm. The process of cryopreservation is known to cause more production of reactive oxygen species, as antioxidant defenses are reduced in the process. Hence, addition of antioxidants may have beneficial effects on spermatozoa function during the cryopreservation.[28] In this study, among control and treated groups post-thawing appeared that the sperm concentration as well as other sperm parameters were decreased significantly ($P<0.05$) after the six month freeze-thawing procedure with liquid nitrogen. Present results agreement withnThitikan and SomboonL.[15] According to Januskauskas and Zillinskas and RuizPesini et al, sperm deformity 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.[29, 30] In present study results showed significant increased (P<0.05) in the percentage of normal sperm morphology for all groups post-thawing. According to Axner they concluded that the percentage of morphologically normal epididymal spermatozoa is not correlated with cryopreservation induced sperm damage using the described freezing protocol.[31] These results are in agreement with present results. This functional impairment is due to the structural damage in the flagella caused by alteration in permeability and membrane fluidity.[32] Furthermore, the percentage of normal sperm morphology was significant decreased (P<0.05) compared with before cryopreservation. This result goes with that of other studies.[33] [34] Further damage has been reported by Dan Yu and Luke Simon as reduction in intact acrosomal caps.[35]

CONCLUSIONS

Generally, 10% of HB supplemented to cryopreservation solution proved better results of sperm parameters than 5% of HB for cryopreserved- thawed human sperm. Combination of 10% HB and 10□M COQ10 obtained best results for human sperm parameters post-thawing.

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