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Original Research Article

Assessment of Post-Thaw *In-Vitro* Quality of Male Wistar Rat Spermatozoa Preserved in Diluent with Natural Honey as Supplement

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ABSTRACT

There is always the need for extension of the lifespan of biological specimens like sperm cells, stem cells and egg cells for purposes of research and experimentation. However, availability and affordability of standard cell freezing reagents and extenders especially in developing countries has been very challenging. This study therefore investigates the short-term effect of natural honey supplementation on *in-vitro* sperm quality following freeze – thaw cycles of Wistar rat spermatozoa in preservation diluent with the aim of comparing efficacy of natural honey supplementation with standard cell freezing reagent. Three variations of honey supplemented diluent; – 2.5%, 5% and 10% concentration were compared with a commercially available standard cell freezing reagent, pZerve. Some sperm parameters – sperm count, sperm motility, sperm viability and morphological defects were assessed for one week following four freeze-thawing cycles on days 1, 3, 5 and 7 after one week of freezing. The results revealed that 10% honey supplementation in preservation diluent showed no significant differences when compared to the standard solution with 7 days of freeze-thawing cycles. However, sperm concentration, motility, viability and morphological defects were significantly altered in diluents supplemented with 2.5% and 5% natural honey following successive freeze-thawing cycles. In conclusion, 10% natural honey supplementation in sperm preservation diluent was capable of retaining sperm quality within 7 days following freeze-thawing cycles.

Keywords: Honey, Spermatozoa, Diluent, Sperm parameters, Cryopreservation.

Introduction

Semen cryopreservation has many biotechnological applications. It can be used to solve problems of infertility, life threatening diseases, preservation of semen and DNA from endangered species and conservation of biodiversity.[1] Undeniably, researchers in areas of reproductive biology and other related fields concerned with sperm cells preservation are faced with the several challenges when carrying out their study. This has become worrisome and ultimately affects scientific experimentation and advancement in these areas especially in developing countries. [1] Understandably, there is always the need for extension of the lifespan of biological specimens like sperm cells, stem cells, and egg cells (ovum) for hours, days, weeks, months or years, so that researchers can work at convenience following specified experimental design and using globally accepted protocol. [2] Sperm cells extracted from epididymis of experimental animals are not only exposed to environmental insults but will not last long because after some hours, most or all of the cells will be death. As a consequence, experimental protocol meant to take longer period of time will suffer setbacks and so researchers are bound within time limits or left to the frustrating experience of having to start the process all over.[2] Commercially available cryopreservation media like pZerve, Cryosofreeze, Coolcell SV10, cell banker, Bam banker, Cryostor and other conventional cryopreservation solution are expensive, unavailable and stand the risk of being adulterated. More so, procuring them in developing countries like Nigeria, is an enormous challenge that will sometimes require importation of these products which can take several months before delivery. It has become expedient for researchers, especially in the area of reproductive biology to use natural products to formulate diluent that can serve the purpose of diluents and extenders for sperm cell preservation. [1]

Several trials have been carried out by researchers with a view to finding possible locally available natural products that can be used as supplement in cryopreservation solutions. Some natural supplements like honey, sugarcane and tomatoes paste have been tested as supplement in cryopreservation reagent formulation.[2]

Natural honey is mainly composed of a complex mixture of carbohydrates, proteins, enzymes (invertase, glucose oxidase, catalase, phosphatases, amino acids, organic acids (gluconic acid, acetic acid), lipids, vitamins (ascorbic acid, niacin, pyridoxine), volatile chemicals, phenolic acids, flavonoids and minerals.[3] Toxicity is a major limitation to successful spermatozoa cryopreservation and it depends on type of preservation media, concentration, temperature and exposure time and number of freeze-thawing cycles.[4] This study therefore, attempts to investigate sperm quality cryopreserved in formulations supplemented with graded concentrations of natural honey following freeze-thawing cycles.

Materials and Methods

Experimental Animals: Twenty (20) adult male Wistar rats (five months old, average weight 180-210 g), obtained from the Department of Pharmacology, University of Calabar, Nigeria were used for this research. The rats were kept in clean cages and divided into groups designated A, B, C, D with eight rats in each group. The rats were kept for two weeks to allow for acclimatization in the animal house, Faculty of Basic Medical Sciences, University of Calabar, and allowed unrestricted access to commercially available chow (livestock feed) and water.

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Ethical Clearance
Ethical clearance number FBMS/EC/21-056 was obtained from the Ethical Committee, Faculty of Basic Medical Sciences, University of Calabar, Nigeria.

Preparation of cryopreservation solutions
Egg Yolk Extender: Chicken eggs used for this study were three days old as at the time of use. The eggs were washed with soap and water and then sterilized by rinsing in 100% alcohol. The shells were carefully cracked and the yolk transferred into a petri dish containing a filter paper. A syringe was used to puncture the yolk membrane and aspirate its content. The aspirated yolk was centrifuged for 15 minutes and the supernatant collected and stored at 4^0C for further use. [5]

Preparation of DYSG Solution: DYSG (Dimethyl sulphoxide + Yolk + Sodium Citrate + Glycerol) was prepared as follows.
Dimethyl sulphoxide (DMSO) (5 mL) was measured and mixed with 2.5g of Sodium Citrate and then dissolved in 100 mL of distilled water to form Solution A. 20 mL of egg yolk extender (20%) and 7 mL of Glycerol (7%) was measured and used to substitute 27ml of Solution A, forming Solution B.

Natural honey supplements: Natural honey was obtained from a natural source at a cattle ranch in Obudu Local Government Area of Cross River State, Nigeria. It was filtered and stored for further use. Solution B was put into four (4) glass tubes containing 10ml per tube. 2.5%, 5% and 10% (v/v) graded concentration of honey supplement was obtained by substituting 0.25 mL, 0.5 mL, and 1 mL of Solution B with natural honey in three (3) of the glass tubes respectively.

Collection of samples
At termination, the animals were sacrificed and scrotal incisions were made to expose the testes and epididymis. The epididymis was dissected and extraneous tissues trimmed. The caudal epididymis was minced with anatomical scissors into a glass beaker and 19 drops of diluting solution added to dilute the semen in a proportion of 1:20. A sample from this homogenate was used for semen analysis of fresh samples.

Experimental design
The design for this research is shown in Table 1
Equilibration: Prior to freezing, an equilibration period of 30 mins was observed at 4^0C to allow permeating cryoprotectants to penetrate the sperm cells.

Sperm Freezing and Thawing: A recommended freezing and thawing protocol was adopted.[6] Before freezing, PH of samples were kept within limits of 7.2 and 7.7 using buffer. Freezing was done with Liebherr Sup-zero Ultra-cold Freezer D430 (Austria) at sub-zero temperature range of -15^0C, -18, -21, -25 to -62^0C for first one week (7 days)[6]. First thawing was done on the first day of week 2 by placing the sample plastic container on 37^0C water bath for 2 minutes [6] Subsequent cycles of freezing and thawing followed on days 3, 5 and 7 of the second week.

Table 1: Experimental design

Group	Designation	Cryopreservation Media
A	Control	Standard Cryopreservation solution (pZerve)
B	Low Grade Honey supplement	DYSG + 2.5% Honey supplement
C	Medium Grade Honey supplement	DYSG + 5.0% Honey supplement
D	High Grade Honey supplement	DYSG + 10 % Honey supplement

DYSG (Dimethyl sulphoxide + Yolk + Sodium Citrate + Glycerol)

Determination of sperm count
Sperm count was conducted using Neubauer's improved haemocytometer. Ruled chamber of haemocytometer was filled with sample using Pasteur pipette. After three minutes, estimation of sperm count was evaluated in the area of 5 square millimetre examined under a contrast, high powered light microscope (Olympus microscope Bx43, Germany) at x400 magnification. Images were viewed using Computer Assisted Semen Analysis (CASA).
The final count for each sample was obtained by dividing the total count by 0.1(depth of chamber), dividing the value by 5mm (surface area counted) then multiplied by 1000 (1000mm^3 per ml) and finally multiplied by 20 (dilution factor). The final value represented the sperm count for each sample.

Determination of sperm motility
After the sperm harvest, sperm motility was determined by putting one drop of well mixed homogenate on a clean slide with the help of a pipette and covered with a 20x20 mm cover slip. The specimen was viewed under the microscope (Olympus microscope Bx43, Germany) using X40 objective and the condenser iris closed sufficiently to give a good contrast. Motility estimation was assessed from three random fields in each sample and mean of the three estimates taken to represent the percentage final value. Motility rating above was done based on 2010 WHO grading.

Examination of sperm morphology
One drop each, of nigrosin stain (eosin) and sperm homogenate was placed on same slide using a pipette. The edge of another slide was used to smear back and forth a few times to mix sperm and the stain on a clean slide. The specimen was allowed to dry on a warming plate and then viewed under microscope (Olympus microscope Bx43, Germany) at x400 magnification. The parameters used to assess sperm morphological abnormalities were total defects, expressed in percentages (%) of morphological normal sperm. [7,8]

Statistical analysis
Data obtained from this research was recorded and analysed using ANOVA - one way analysis of variance with SPSS program (version 20). Post-hoc test was carried out using Fischer's Least Significant Difference (LSD). Probability level of $P<0.05$ was considered significant.

Results and Discussion

Tables 2, 3, 4 and 5 show the values of sperm concentration (million/L), sperm motility (%), sperm viability (%) and sperm morphological defects (%) respectively.
As shown in Figures 1, 2 and 3, a graphical representation of sperm concentration, motility and viability respectively, there was a remarkable difference between the initial and final (day 7) values of specimens with 2.5% and 5% natural honey supplementation revealed by a downward course of the line when compared to the standard reagent (pZerve). However, values obtained from diluent specimens with 10% natural honey did not show remarkable changes between initial and final values in comparison with standard solution.
Figure 4 revealed that final values of sperm morphological defects were remarkably higher than initial values in specimens with 2.5% and 5% honey concentration and this was not the case with 10% concentration. The line course for 10% honey supplementation and standard pZerve solution were almost running parallel indicating that there was no significant variation between initial and final values of sperm morphological defects.
In this research, we investigated the effect of variable concentration of natural honey supplementation on sperm preservation diluent of Wistar rat spermatozoa during freeze-thaw cycles. This study has shown that natural honey supplementation improved sperm quality in a concentration dependent manner. 10% natural honey supplementation resulted in significantly higher values of sperm count, sperm viability and sperm motility when compared to diluents with 2.5% and 5% natural honey supplementation.

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Table 2: figures of sperm concentration (million/ml) in different experimental groups

Sperm Concentration (million/ml)						
Cryoprotectant		**Incubation Period**				
Diluent Conc.		**Initial**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Standard Solution (pZerve)		28.65±64	25.91±11	25.31±45	24.77±66	24.09±12
Experimental Solution	2.5 %	27.85±16	21.54±31	15.66±07	14.90±76	10.07±14
(Honey Conc.)	5%	28.67±54	26.13±07	23.66±04	20.09±05	16.10±60
	10%	27.56±41	26.44±54	25.21±16	22.67±03	21.55±32

Table 3: figures of sperm motility (%) in different experimental groups

Sperm Motility (%)						
Cryoprotectant		**Incubation Period**				
Diluent Conc.		**Initial**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Standard Solution (Pzerve)		84.44±11	84.12±07	83.55±16	82.23±44	81.70±12
Experimental Solution	2.5 %	85.87±15	66.32±05	62.11±32	50.66±33	35.89±31
(Honey Conc.)	5%	85.90±29	70.54±44	65.12±10	64.02±01	53.11±22
	10%	83.54±50	80.19±19	79.88±33	79.45±22	78.73±19

Table 4: figures of sperm viability (%) in different experimental groups

Sperm Viability (%)						
Cryoprotectant		**Incubation Period**				
Diluent Conc.		**Initial**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Standard Solution (Pzerve)		84.87±18	84.56±07	83.12±01	82.86±20	81.67±03
Experimental Solution	**2.5 %**	87.43±11	80.05±11	65.11±19	53.44±09	41.32±11
(Honey Conc.)	**5%**	85.29±01	81.09±12	70.03±03	65.65±02	59.22±12
	10%	86.63±44	83.44±10	81.36±03	78.44±01	75.12±02

Table 5: figures of sperm morphological defect (%) in different experimental groups

Sperm Morphological Defects (%)						
Cryoprotectant		**Incubation Period**				
Diluent Conc.		**Initial**	**Day 1**	**Day 3**	**Day 5**	**Day 7**
Standard Solution (Pzerve)		4.34±10	4.92±01	5.51±14	5.23±14	6.60±14
Experimental Solution	2.5 %	4.47±16	9.32±04	15.31±32	23.06±63	42.19±11
(Honey Conc.)	5%	5.90±19	7.54±19	10.12±10	14.02±01	23.11±22
	10%	4.54±51	6.19±12	7.88±33	9.45±20	9.73±10

Researchers have demonstrated that honey supplementation to freezing extenders improved sperm viability index and maintained membrane and acrosome integrities after Arab stallion sperm cryopreservation. [9] Sperm motility has been shown to be driven by cellular energy produced by spirally arranged mitochondria within the middle piece of the sperm cell. Change of states during the freeze-thaw cycle is capable of initiating a cascade of degenerative reaction that may interrupt cellular energy generation thereby affecting motility in spermatozoa. This finding is supported by El-Badry *et al*., (2014) [10] and El-Sheshtawy *et al*., (2014) [11], who reported that honey supplementation in cooling and freezing extenders preserved spermatozoa motility in ram, cattle and goat semen. 2.5% and 5% honey concentration may not have been sufficient enough to prevent alteration in sperm quality but natural honey supplementation at 10% concentration preserved sperm motility as values were not significantly different when compared to the standard reagent pZerve.

Again, Fructose content in honey has been found to be responsible for maintaining sperm quality as it is regarded as potent energy sources for cellular energy. [12]

Change of states during freeze-thaw cycles may have been responsible for causing direct physical damage to sperm cell thereby depleting sperm count. However, natural honey has been shown to possess the property of an amorphous solid and will not freeze at sub-zero temperature instead its viscosity increases at lower temperatures without forming crystals. Ogretmen *et al.,* (2014) and Kantor *et al.,* (1999) [13,14] have also demonstrated that natural honey can attain sub-zero temperature without forming crystals.

Sperm count and viability was remarkably maintained in specimens supplemented with 10% natural honey as revealed in this study. This may be connected with the bioactive and nutritional components of natural honey such as enzymes, antioxidants, vitamins and organic aids present in trace concentration.

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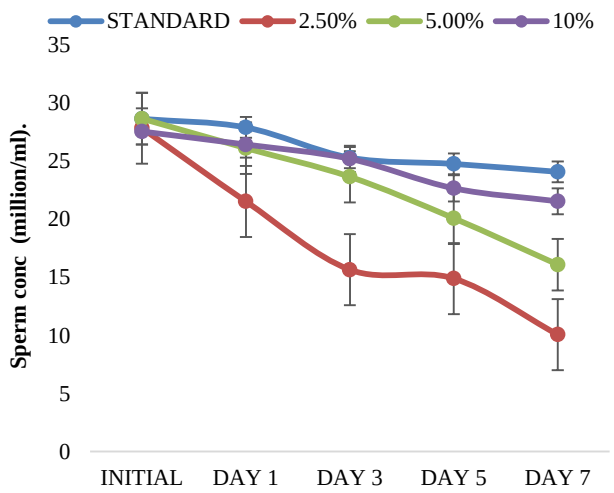

Figure 1: Graphical presentation of sperm count of experimental groups following freeze-thaw cycles in 7 days

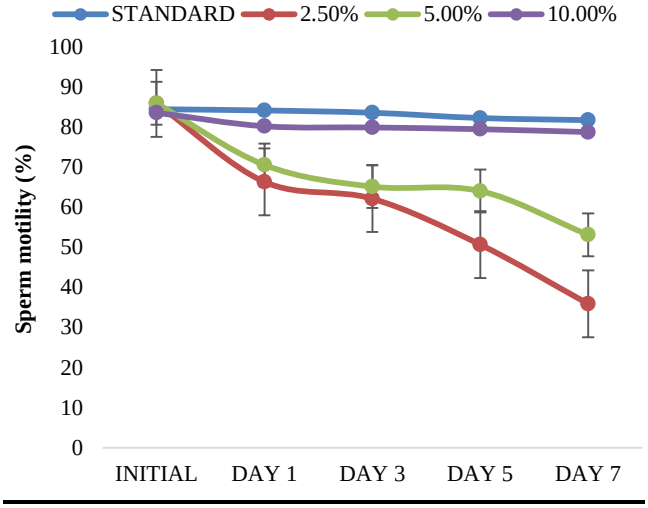

Figure 2: Graphical presentation of sperm motility of experimental groups following freeze-thaw cycles in 7 days

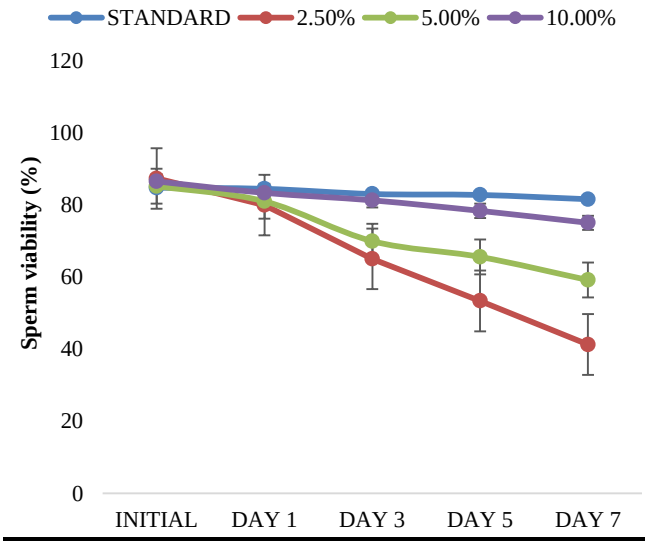

Figure 3: Graphical presentation of sperm viability in experimental groups following freeze-thaw cycles in 7 days

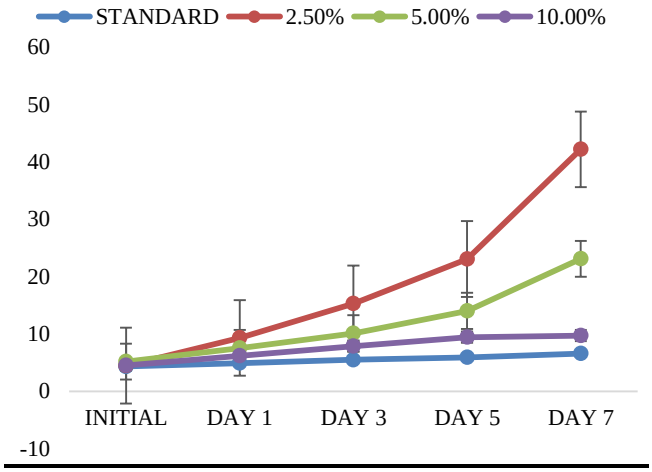

Figure 4: Graphical presentation of sperm morphological defect in experimental groups following freeze-thaw cycles in 7 days

This is consistent with the work of Bogdanov *et al*., (2008)[15] and Jerez-Ebensperger *et al.*, (2015) [16]. The lower values of morphological defects recorded in diluents with 10% natural honey supplementation may be attributed to flavonoid content in honey which has the potentials to inhibit DNA damage [12] thereby preserving sperm morphology. This finding is consistent with reports by Olayen *et al.*, (2011); Fakhrldin *et al.*, (2014); Jerez *et al.*, (2015). [17,18,19]

Conclusion

In conclusion, this study has demonstrated that 10% natural honey supplementation in sperm preservation diluent prevented freeze-thaw cycle damages on sperm quality. The observed preservation of sperm quality was concentration dependent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

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