Effect of Low-Level Laser Irradiation Versus Room Temperature on Cryopreserved Sperm Motility and DNA Integrity as Modes of Thawing

تاثير استخدام الليزر كطريقة لاذابة النطف البشرية المجمدة مقابل الاذابة بدرجة حرارة الغرفة على سلامة الاحماض النووية والحركة

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Abstract

During cryopreservation, cells and tissue undergo dramatic transformation in chemical and physical characteristics. Thawing process is also an important step since the cryoinjury is not limited to the freezing process but may also occur during the thawing process. Laser by its photo-stimulation effect can improve the resistance to cooled storage of some human sperm and this will lead to improvement in the quality of frozen semen and in the potential of sperm fertilization. The objective of this study is to use laser as a method of thawing and its effect on human sperm motility and DNA integrity versus the thawing through room temperature. This prospective study carried on 70 cryopreserved semen samples. Each sample was prepared by centrifugation procedure, and divided into 2 parts, freezed and thawed by laser irradiation till melting for one part and by room temperature for other. The DNA integrity and sperm motility were assessed before vitrification and after the two methods of thawing. The results of cryopreserved semen samples showed that laser irradiation thawing has significantly increased sperm motility as well as a significant decreased DNA fragmentation (P< 0.05) versus room temperature thawing. Conclusion(s): Laser irradiation thawing of post freezing sperm improves post thaw motility and DNA integrity.

Key wards: cryopreservation, laser irradiation, DNA integrity, sperm motility.

الملخص

ان التجميد قد يؤدي الى تحولات مؤثرة في الخواص الكيميائية والفيزيائية للخلايا والانسجة المجمدة، فضلا عن طرق الاذابة التي تعد من الخطوات المهمة والتي من الممكن ان تؤثر عليها ان اضرار الحفظ عن طريق التجميد لا تقتصر على اضرار التجميد فقط بل من الممكن ان تحدث اثناء الاذابة ايضا. ان تاثير التحفيز الضوئي لليزر من الممكن ان يحسن المقاومة لتاثير الحفظ عن طريق التجميد للنطف وهذا قد يؤدي الى تحسين جودة النطف المجمدة وبالنتيجة قد يؤدي الى تحسين القابيلة الاخصابية لها. الغرض من الدراسة تاثير الليزر كطريقة لاذابة النطف البشرية المجمدة وتاثيرها على سلامة الاحماض النووية والحركة مقابل الاذابة بدرجة حرارة الغرفة. هذه الدراسة اجريت على 70 نموذج من النطف البشرية وبعد تحضيرالنموذج بطريقة الطرد المركزي قسمت النماذج الى قسمين، جمدت، واذيب كل جزء من كل نموذج اما بالليزر اوبدرجة حرارة الغرفة . درس تاثير التجميد والاذابة بالليزر وبدرجة حرارة الغرفة على سلامة الاحماض النووية حركة النطف. النتائج اظهرت ان الاذابة عن طريق الليزر لها تاثير معنوي (P<0.05) في تقليل تحطيم الاحماض النووية مع زيادة في الحركة الفعالة للنطف عند المقارنة مع الاذابة بدرجة حرارة الغرفة. الاستنتاجات: ان الاذابة بالليزر من الممكن ان تحافظ على سلامة الاحماض النووية والحركة النشطة للنطف.

الكلمات الدالة: التجميد بطريقة التزجيج ، الاذابة بالليزر، سلامة الاحماض النووية، حركة النطف.

Introduction

Laser is a device that emits light through a process of optical amplification based on the stimulated emission of electromagnetic radiation. The term "laser" originated as an acronym for "light amplification by stimulated emission of radiation"[1]. As it is well known, the cryopreservation process induces morphological changes in sperm and damages the plasma, acrosome, and mitochondria membrane. These

changes are sufficient to adversely affect the fertilizing capacity of the sperm, reduction in motility, in a reduced capacity of spermatozoa to pass through the cervix and to decrease of viability in the female reproductive tract [2]. Laser irradiation of spermatozoa can increase sperm motility as well as velocity [3]. In addition, laser irradiation by its photo-stimulation effect has been reported to improve the resistance to cooled storage of some sperm species [4] and this will lead to improvement in the quality of frozen semen, as well as it can improves the potential of sperm fertilization [5]. Cryopreservation is the procedure that makes it possible to stabilize the cells at cryogenic temperatures. Many advances in the cryopreservation technology have led to the development of methods that allow for low-temperature maintenance of a variety of cell types including male and female gametes, small multicellular organisms, and even more complex organisms such as embryos [6]. During cryopreservation, cells and tissue undergo dramatic transformation in chemical and physical characteristics as the temperature drops from +37 to -196°C. The cells can lose up to 95% of their intracellular water. The concentration of solutes increases considerably, triggering the possibility of osmotic shock. Moreover, potential intracellular ice crystallization and mechanical deformation by extracellular ice may cause significant injury leading to cell death. If cells survive freezing, they might sustain additional damage during the thawing process due to osmotic shock, uncontrollable swelling and ice re-crystallization [7, 8]. It is known that temperatures between 5 °C and 15 °C maintain the DNA intact for longer periods of time than higher temperatures like 20 or 37 °C [9]. Sperm chromatin structure and DNA integrity are known to be altered or damaged during cryopreservation [10] were the cryopreservation process and thawing can reduce the ability of sperm chromatin to decondense and increase abnormal chromatin condensation in human sperm [11], in addition, cryopreservation could alter the quality of the spermatozoon's nucleus [12, 13], induces DNA denaturation [14], increases sperm DNA fragmentation [15] and it causes liquid phase transition changes and increased lipid peroxidation leading to irreversible rupturing of plasma and nuclear membranes and disturbance of cellular organelles [16]. The objective of this study is to compare the use of laser as a method of thawing and its effect on human sperm motility and DNA integrity versus room temperature thawing.

Materials and Methods

A prospective study was carried out on the 70 human semen samples at the laboratories of Biotechnology Research Center, AL-Nahrain University, Baghdad and Kamal AL Samuray Hospital, from January 2016 till December 2016. Each sample was prepared by centrifugation technique as designed by and divided into 2 parts, freezed by vitrification. One part was thawed by laser irradiation till melting and the other by room temperature, DNA integrity and sperm motility were assessed after preparation, before vitrification and after thawing by the two methods of thawing. Twenty samples were analyzed by comet assay test and 50 samples by Acridine orange test .The mean age of patients was 32.33±5.96 years. They were not alcoholic, not smokers, and there was no history of drug intake. The samples were obtained from men after a minimum 3-7 days of abstinence and ejaculated into a clean, wide-mouthed container. After liquefaction for 30 minutes at 37 C° incubator, routine semen analysis was performed manually according to WHO [17] to assess sperm concentration, sperm motility, sperm agglutination, sperm morphology, [17]. Semen samples were with normal parameters were selected for experimental use. Samples were prepared by centrifugation method, adding 5ml flushing medium to the native semen sample and mixing. Centrifugation for 5minutes at 3500 RPM was carried out. The supernatant was removed, and a swim-up was performed, by adding flushing medium and incubating for 30 minutes.

Freezing protocol

This was conducted using Sperm Freeze Medium (FertiPro, Belgium) for freezing human sperm. The medium was left for few minutes in room temperature before use to avoid cold shock. The duration of the freezing was for 24 hours. One ml of prepared sperm (prepared by using flushing media (FertiPro, Belgium) was mixed with 0.7ml of Sperm Freeze medium (adding the medium drop by drop to the post wash sample (taking about 3 or 4 minutes to prevent osmotic shock to the sperms with constant shaking to

ensure thorough mixing of the two. The mixture was left for 10 minutes at room temperature for equilibration. The sample/medium mixture was put into the freezing cryo tube, freezed vertically for 15 minutes, just above the level of the liquid nitrogen and stored in liquid nitrogen .

Thawing

After cryopreservation for 1 day, the samples were divided and thawed by two methods, at room temperature between 20-25 minutes, by the laser for 25 minutes as well. The laser light source used was (He–Ne) device laser model (VLM -635-03-LPT), with a dimension of Ø 0.7 x 21 mm, the operating voltage is 4.5 VDC, the average output power of < 1mW, the wave length at beak emission 630-645 nm, the operating temperature 20 °C -30 °C (at room temperature) with a beam spot of 6 mm in diameter, and it was of a continuous type Figure (1). The laser source was put at 1 cm distance from the cryotube. When the thawing was complete, the samples were mixed thoroughly, added to an equal amount of HEPES containing media at room temperature (at least 3ml per 0.5ml semen) and mixed thoroughly, centrifuged for 5 minutes at 3500 RBM. The Supernatant was discarded and the pellet was re-suspended in one ml of suitable insemination medium (Flushing medium). The suspension was left for incubation at 37 C for 20 min. The supernatant was aspirated and analyzed by light microscope for motility and DNA Integrity assessment by AOT test and comet assay .

DNA Integrity assessment

Acridine orange test (AOT) [18] A sperm smear was prepared and let it dry, Fixation was conducted overnight in menthol-glacial acetic acid (3:1) at room temperature. Slides were removed from the fixative and allowed to dry for few minutes before staining with Acridine orange (0.19 mg/mL, pH 2.5) for 5 minutes at room temperature . Staining solution was prepared daily from stock solution consisting of 1 mg AO 1/1000 mL of distilled water and stored in dark at 4 °C. To prepare the staining solution, 10 mL of stock solution was added to 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M Na_2HPO4, 7 H_2O.After staining, slides were gently rinse in a stream of distilled water, sealed under a coverslip, and viewed by a fluorescence microscopy. Sperm cell heads with good DNA integrity had green fluorescence, and those with diminished DNA integrity had orange - red staining. Samples were scored within 1 hour after staining. Counting fifty sperm were count, and scoring the percentage of green, yellow –red stain sperm.

Comet Assay

Is a single cell gel electrophoresis assay (SCGE) for simple evaluation of cellular DNA damage in live cell. Individual cells were mixed with molten agarose before application to the comet slides, these embedded cells were then treated with lysis buffer and alkaline solution, which relaxes and denaturant the DNA. The samples were electrophoresed in a horizontal chamber to separate intact DNA from damaged fragments, samples were dried, Stained with a DNA dyes and Visualized by fluorescence microscope. Under fluorescence fields, damage cellular DNA (containing cleavage and strand breaks) will migrate further than intact DNA, producing a classic comet tail shape under the microscope. Extent of DNA damage is usually visually estimated by comet tail measurement. Fifty randomly selected sperm were counted per sample to quantify the comet cell. The score was calculated from the ratio of (L/W) comet to determine the Comet Index (CI). Scores ranging from 1.2 to 2 were considered of low DNA damage (LD), from 2.1 to 3 of medium DNA damage (MD), and up to 3 of high DNA damage (HD) [19].

Statistical analysis

Statistical package for social science version 20 (SPSS Inc., Chicago, IL, USA) was used for both data entry and data analysis. Continuous variable presented as mean ± SD. Independent sample t test was used to test the significance of association of variables. P-value of < 0.05 was considered significant.

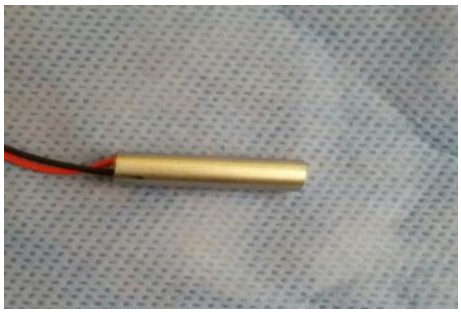

Fig. (1): Laser device

Results and Discussion

Sperm motility:

Table (1)**,** showed the effect of freezing and the effect of two methods of thawing (laser and room temperature) on sperm motility .In regard of Rapid progressive motility (RP) The mean % of RP before freezing was 37.66% ± 4.98, the decrement in percentage of RP motility of sperm by laser thawing was 28.54 % ± 4.29, while by room temperature thawing was 10.54 % ± 2.37. The difference between before and after freezing was significant ($P < 0.05$) as well as the differences between the two methods of thawing ($P< 0.05$). For the Non progressive motility (NP) The mean percentage of NP motility before freezing was 21.33 % ± 6.49, the increment in percentage of NP motility of sperm by laser was to 38.00 % ± 6.58, and at room temperature thawing was to 45.53 % ± 10.9. The differences between before and after freezing was significant ($P<0.05$) while there was no significant differences between the two methods of thawing ($P > 0.5$), and the Immotility (IM): The mean percentage of IM before freezing was 41.00 % ± 7.62. There was a decrement in percentage of IM of sperm by laser method of thawing to 33.43 %± 8.04,while thawing by room temp. caused an increase percentage to 44.67% ± 8.52. The difference between two methods of thawing was significant ($P < 0.05$). The differences between before and after freezing was significant ($P<0.05$) only by laser thawing while it was not significant ($P>0.05$) by room temp. thawing.

Table (1): Sperm motility of prepared sperm before freezing and by two methods of thawing, room temperature, and laser.

Groups	RP		NP		IM	
	Before	After	Before	After	Before	After
	A,a	A,b	A,a	A,b	A,a	A,a
Thawing room temp.	37.66± 4.98	10.56± 2.37	21.33± 6.49	45.53± 10.9	41.00± 7.62	44.67± 8.52
	A,a	B,b	A,a	A,b	A,a	B,b
Thawing Laser	37.66± 4.98	28.54± 4.29	21.33± 6.49	38.00± 6.58	41.00± 7.62	33.43± 8.04
P-Value		0.00114		0.00312		0.00117
		P<0.05 sig.		P<0.05 sig.		P<0.05 sig.

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DNA Fragmentation

Acridine orange test (AOT):

Table (2) showed the percentage of DNA fragmentation of the sperm before and after two methods of thawing. Figure (2) illustrated the image of AOT. The percentage of DNA fragmentation before freezing was 18.22± 4.56%. There was an increment of DNA fragmentation by laser to 23.24 ± 5.44 %, while by room temperature to 41.93 ± 8.79%. The differences were significant ($P < 0.05$).

Table (2): Acridine Orange test for DNA fragmentation % of sperm before freezing and by two methods of thawing, room temperature and laser

Groups of AOT	DNA Fragmentation % (mean+SD)	
	Before freezing	After freezing
	A,a	B,b
Thawing room temp.	18.22 ± 4.56	41.9 ± 38.79
	A,a	C,b
Thawing Laser	18.22 ± 4.56	23.24 ± 5.44
P-Value		0.00032
		P<0.05 sig.

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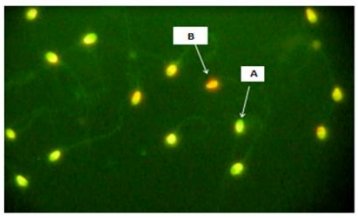

Fig(2): A normal green AOT, B abnormal red AOT

Table (3) showed the results of Comet assay [no damage (ND), low damage (LD), medium damage (MD), high damage (HD)] before freezing and after thawing by the two methods of thawing at room temperature and by laser. Figure (3) showed the image of Comet assay. ND results revealed that, the least percentage of DNA damage occurred by laser thawing. The percentage of ND before freezing was 68.27± 7.58, by laser thawing it decreased to 51.63± 9.52, and by room temp. thawing it decreased to 37.58 ± 5.70, while for LD results of the percentage of low DNA damage before freezing was 19.46± 4.33% and by laser it increased to 33.38 ± 4.72 %, while by room temp. it increased to 27.41± 4.22, and for HD results of the % of high DNA damage before freezing was 4.11± 1.49 %. The lowest increased in % was by laser thawing reaching to 7.47 ± 2.17 %, while by room temp. thawing it increased to 23.64 ±4.37%. In these three degrees of Comet assay (ND, LD, HD) there were a sig. differences between before and after freezing as well as between the two methods of thawing (P<0.05). MD results of the percentage of medium DNA damage before freezing was 8.15 +3.26 %. By laser thawing it decreased to 7.52± 2.08 while by room temp. it increased to 17.37± 2.79%. The difference between before and after freezing was significant (P< 0.05) for room temp. only but not for laser thawing (P>0.05) while it was significant (P<0.05) between the two methods of thawing.

Table (3): Percentage of Comet of sperm before freezing and by two methods of thawing, room temperature, and laser.

Groups	No damage		Low damage		Medium damage		High damage	
	Before	After	Before	After	Before	After	Before	After
Thawing room temp	A,a 68.27± 7.58	A,b 37.58±5.70	A,a 19.46± 4.33	A,b 27.41± 4.22	A,a 8.15± 3.26	A,b 17.37± 2.79	A,a 4.11±1.49	A,b 23.64±4.38
Thawing Laser	A,a 68.27± 7.58	B,b 51.63+9.52	A,a 19.46± 4.33	B,b 33.38± 4.72	A,a 8.1± 53.26	B,a 7.52± 2.08	A,a 4.111±.49	B,b 7.47± 2.17
P-Value		0.00162 P<0.05 significant		0.0121 P<0.05 significant		0.00279 P<0.05 significant		0.00840 P<0.05 significant

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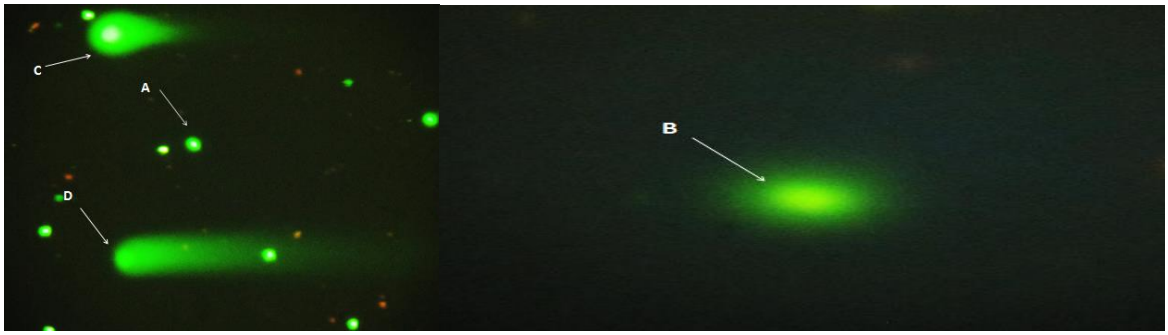

Fig. (3): Scoring categories for comet assay A: Normal, B: Low DNA Damage (LD); C: Medium DNA Damage (MD); D: High DNA Damage (HD)

Although cryopreservation of sperm was feasible, the recovery rate of motile sperm after thawing can vary widely. Factors influencing sperm recovery include rate of freezing and thawing as well as choice and concentration of cryoprotectants [20]. Additionally, the quality of thawed sperm can be improved by concentrating the ejaculates prior to freezing [21].The results of DNA fragmentation either by AOT or Comet assay and that of motility provide evidence that thawing by laser irradiation till melting results in less DNA fragmentation and improvement of motility of the freezed -thawed sperm than that at room temperature. These results are similar to those in the literature, which shows that laser irradiation leads to an improvement in thawed semen quality parameters, Dobrin *et. al.* [5] showed that irradiation with a He-Ne laser of thawed ram sperm leads to an increase of the motility, viability and functional integrity of the sperm membrane. Similar results were obtained on turkey semen in which laser irradiation resulted in an increase of the quality parameters after thawing [22], Ocana- Quero *et. al.* demonstrated an increase in acrosome reaction of bull semen and a decrease mortality of spermatozoa after irradiation[23]. Wenbin *et. al.* found that the laser irradiation leads to an increased sperm fructose fermentation, respiration, the absorption of $Ca^{+2,}$ thereby increasing the motility and time survival of the buck spermatozoa [24] [25]. Corral - Baqués *et. al.* reported that the irradiation of dog sperm with laser improves the speed and progressive motility of sperm [26]. According to the studies of Zan-Bar *et.al.* laser effects are mediated through reactive oxygen species. Although high levels of reactive oxygen species can lead to cell death (by ATP depletion and lipid peroxidation), low level of reactive oxygen species may play an important role in the activation of many cellular processes[27,28].

Conclusion

From these results it was concluded that using the laser as a method of thawing may exert beneficial effects in the preservation and improvement of sperm motility and DNA integrity. More studies are recommended to find out fertilization point of the sperm.

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