

Relationship between The Addition of Glutathione to Sperm Freezing Medium and Cryosurvival Rate of Sperm Motility and Viability in Asthenozoospermic Patients

العلاقة بين إضافة الكلوتاثاين الى وسط تجميد النطف ومعدل الحركة والعيوشية النطفية بعد النجاة من التجميد في مرضى وهن النطف

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الخلاصة

الهدف: تهدف الدراسة إلى تحديد العلاقة بين إضافة الكلوتاثاين الى وسط تجميد النطف ومعدل الحركة والعيوشية النطفية بعد النجاة من التجميد في مرضى وهن النطف.

المنهجية: تم الحصول على تسعة وخمسين عينة من السائل المنوي من مرضى وهن النطف من الشريك الذكر للأزواج العقيم الذين يراجعون مركز الخصوبة في مدينة الصدر الطبية التعليمية في النجف وأمدت الدراسة من المدة (١ تشرين الاول ٢٠١٦ حتى ١ اذار ٢٠١٧). تم تقسيم كل عينة من السائل المنوي إلى أربعة أجزاء متساوية ، حجم كل واحدة ٠,٥ مل: الجزء الأول (قبل التنشيط)، الجزء الثاني (بعد التنشيط)، الجزء الثالث (جمد باستخدام وسط تجميد النطف لمدة شهر واحد)، والجزء الرابع (جمد باستخدام وسط تجميد النطف مضافا اليه الكلوتاثاين لمدة شهر واحد). تركيز النطف، حركة النطف التقدمية ، و المظهر الطبيعي للنطف و العيوشية تم حسابها في كل جزء، ومعدل الحركة والعيوشية بعد النجاة من التجميد، تم حسابها في الجزء الثالث والرابع. تم إجراء التحليل الإحصائي للدراسة باستخدام اختبار t المستقل واختبار t المقارن لتقييم الفروق بين المجموعتين.

النتائج: بعد تنشيط النطف باستخدام تقنية السباحة ، أظهرت نتائج الدراسة زيادة معنوية ($p < 0.01$) في حركة النطف التقدمية، و المظهر الطبيعي للنطف و عيوشية النطف، في حين كان هناك إنخفاض معنوي ($p < 0.01$) في تركيز النطف. أما معدل الحركة والعيوشية بعد النجاة من التجميد إنخفضت بشكل معنوي ($p < 0.01$) بعد عمليات التجميد والإذابة ، بينما بينت النتائج تحسنا معنويا ($p < 0.01$) في معدل الحركة والعيوشية النطفية بعد النجاة من التجميد بعد إضافة الكلوتاثاين الى وسط تجميد النطف .

الاستنتاج: يمكن أن نستنتج من هذه الدراسة أن إضافة الكلوتاثاين إلى وسط تجميد النطف حسن ($p < 0.01$) معدل الحركة والعيوشية النطفية بعد النجاة من التجميد خلال عمليات التجميد والإذابة .
التوصيات : أوصت الدراسة بإضافة ٥٠ مايكرو مول من الكلوتاثاين إلى وسط تجميد النطف بتركيز ١ ميلي مولار.

ABSTRACT

Objective: This study aimed to determine the relationship between the addition of Glutathione to sperm freezing medium and cryosurvival rate of sperm motility and viability in asthenozoospermic patients.

Methodology: fifty nine semen samples of asthenozoospermic patients were obtained from the male partner of infertile couples attending the fertility center of al-sadder medical teaching city in Al-Najaf during the period 1st October 2016 until 1st March 2017. Each semen sample was divided into four equal parts, volume of each part is 0.5 ml: first part (before activation), second part (after activation), third part (cryopreservation with sperm freezing medium for one month), and fourth part (cryopreservation with sperm freezing medium plus glutathione for one month) .

Sperm concentration , sperm progressive motility, sperm viability and sperm normal morphology were counted in first two parts, cryosurvival rate of sperm motility, viability was counted in third and fourth parts. Statistical analysis of the study performed by using Independent t-test and paired test to assess the differences between two groups .

Results: After sperm activation by using the swim up technique ,the study results showed significant increase ($P < 0.01$) of progressive sperm motility, normal sperm morphology and sperm viability, while there were a significant decrease ($P < 0.01$) of sperm concentration. cryosurvival rate of sperm motility and viability were significant decrease ($p < 0.01$) after cryopreservation and thawing processes while the results were revealed significant improvement in cryosurvival rate of sperm motility, and viability ($p < 0.01$) after the addition of glutathione to the sperm freezing medium.

Conclusion: it can conclude from this study that addition of glutathione to sperm freeze medium improves. ($p < 0.01$) cryosurvival rate of sperm motility and viability during cryopreservation and thawing processes .

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Recommendations: the study recommended to addition 50 microliter of glutathione to sperm freezing media in concentration 1 mM .

Keyword: Glutathione, sperm freezing medium, cryosurvival rate, sperm motility, viability, asthenozoospermic .

Introduction

Male Infertility is generally defined as the defect in Semen Parameters and its quality⁽¹⁾. asthenozoospermia is one type of Male Infertility which can be defined as a condition in which the percentage of progressive sperm motility is less than 50% or the percentage of sperm with rapid progressive motility is less than 25% within one hour after ejaculation ⁽²⁾.

In some cases for patients including asthenozoospermia, such as chemotherapy, radiotherapy, bladder neck surgery, vasectomy, and testicular biopsy, the cryopreservation process is very important ⁽³⁾.

Cryopreservation process means the keep of the biological materials with temperature less than zero such as -80 or -196 and this process causes the stop in cellular diffusion and decrease in temperature energy of the chemical reactions, It is aimed to the maintenance on viability and functional activity in cells within certain period⁽⁴⁾.

Cryopreservation process includes addition of cryoprotectants substances, immersion of mixture in liquid nitrogen with temperature - 196 C, thawing process by water bath , centrifugation, and the addition of activation medium ⁽⁵⁾. Each step of cryopreservation influences negatively on the structure of plasma membrane of sperm, sperm motility, fertilization, and premature nuclear decondensation ⁽⁶⁾. Cryopreservation process leads to incidence a significant decrease of enzymatic and non-enzymatic antioxidants and increase of reactive oxygen species levels inside the medium⁽⁷⁾.

Glutathione (L- -glut amyl-L-cysteinylglycine; GSH) is the most abundant non-protein thiol in mammalian cells, being present in concentrations of 0.5-10 mmol/L. Cellular GSH plays a key role in many biological processes, including the synthesis of proteins and DNA and the transport of amino acids, but notably, it plays a key role in protecting cells against oxidation: the sulfhydryl (SH) group is a strong nucleophile, and confers protection against damage by oxidants, electrophiles and free radicals⁽⁸⁾.

Objective:

This study aimed to determine the relationship between the addition of Glutathione to sperm freezing medium and cryosurvival rate of sperm motility and viability in asthenozoospermic patients.

Methodology:

Semen collection: This study was carried out in the laboratory of sperm freezing in Fertility Center Of Al-Sadder Medical Teaching City In Al-Najaf Province – Iraq from October 1st 2016 to March 1st 2017 .

59 Asthenozoospermic patients were selected for this study who enrolled in fertility center for treatment or entry the In Vitro Fertilization program (IVF). They had 25 -35 years old age. The semen samples having less than 2 ml were excluded.

Each semen sample was divided into four equal parts, volume of each part is 0.5 ml: first part (before activation, raw semen), second part (after activation by swim up from pellet), third part (cryopreservation with sperm freeze medium for one month), and fourth part (cryopreservation with sperm freezing medium plus 50 microliter of (1 mM/L) glutathione for one month. sperm concentration, sperm progressive motility, sperm viability and sperm normal morphology were counted in first two parts, cryosurvival rate of sperm motility, viability was counted in third and fourth parts.

Cryosurvival rate of sperm motility, viability was assessed according to the following formula:

Percentage of cryosurvival = $\frac{\text{Post thaw sperm motility}}{\text{Pre freeze sperm motility}} \times 100^{(9)}$

Seminal fluid analysis: Fresh semen samples were collected after 3-5 days of abstinence by masturbation directly into container made of disposable glass or plastic, in a private and quite room adjacent to the semen analysis laboratory under oral instruction⁽¹⁰⁾.

The specimens were placed in an incubator (Binder –Germany) at 37°C for 30 minutes up to 60 minutes for liquefaction. Sperm concentration, sperm motility, and sperm morphology were assessed according to WHO guidelines (1999).

Sperm preparation by swim up from pellet.

0.5ml of semen specimen was transferred from a plastic cup to a sterile 15 ml conical. The specimen then mixed with IVF medium (fertipro -Belgium) (1-2ml) by using a sterile pasture pipette, the tubes were centrifuged at 3000 rpm for 10 minutes, Carefully discard the supernatant and resuspend the pellet in 0.5 ml of IVF medium, Incubate the tubes for one hour for sperm swim-up in vertical rack in a 37°C incubator 5 % CO₂ ⁽¹¹⁾.

Preparation of Glutathione:

Glutathione was prepared by dissolved 0.03 g powder of glutathione in a certain volume of distilled water, and the volume Complete to the 100 mL of distilled water, which is equivalent to 1 mM, Sperm freezing medium was contain 50 microliter of glutathione⁽¹²⁾.

The cryopreservation of specimens:

0.25 microliter of prepared specimen (by swim up from pellet) was transferred to 1.8 ml cryovial (Thermo scientific, Denmark), then sperm freeze medium (contains 50 microliter from Glutathione or not) was added to prepared specimen in ratio (0.7: 1) slowly with mixing. The mixture was exposure to liquid nitrogen vapor with 20 cm of height upon the level of liquid nitrogen inside the freezing tank for 10 minutes The cryovial which contain the mixture was plunged into liquid nitrogen (- 196 C) for one month⁽¹³⁾.

The thawing of specimens:

The cryovials were removed from liquid nitrogen and placing them in path water for 5 minutes, then the whole mixture was diluted with 1-2 ml of IVF medium and transferred into a test tube, centrifuged for 10 min at 3000 rpm .The pellet then mixed with 0.25 ml of IVF medium ⁽¹⁴⁾.

STATISTICAL ANALYSIS

Statistical analysis in our study done by used SPSS (Statistical Package for Social Science; Version (17) program. Independent t-test and paired test were used to assess the differences between two groups. Results were reported as (mean \pm SD) unless otherwise indicated ($P < 0.01$), was considered statistically significant⁽¹⁵⁾.

Results:

Table (1) sperm parameters before and after activation by swim up from pellet in asthenozoospermic patients

Sperm parameters	Before activation n = 59	After activation n = 59	Significance (p - value)
Sperm concentration million / ml	58 . 45 \pm 11. 76 ^a	12 . 85 \pm 3.24 ^b	0.001 HS
sperm progressive motility %	35.5 \pm 6 ^a	59.5 \pm 7 ^b	0.001 HS
sperm viability %	58 \pm 6 ^a	78 \pm 7.5 ^b	0.001 HS
sperm normal morphology%	37.70 \pm 5.9 ^a	57.85 \pm 7.5 ^b	0.001 HS

Values were expressed as (mean + SD).

a , b Within compare of each group, numbers with different letter refers to a significantly different ($P < 0.01$) from each other.

Table (1) showing a significant decrease ($p < 0.01$) in sperm concentration after activation by swim up from pellet and IVF medium compared with before activation, While the activation process improve sperm progressive motility, sperm viability and sperm normal morphology, there were a significant increase ($p < 0.01$) compared with before activation .

Table (2) sperm parameters after activation and after cryopreservation and thawing in asthenozoospermic patients

Sperm parameters	After activation n = 59	After cryopreservation and thawing n = 59	Significance (p - value)
Sperm concentration million / ml	12 . 85 ± 3.24 ^a	11.75± 3.15 ^a	NS
sperm progressive motility %	59 .5 ± 7 ^a	26.25± 5. 5 ^b	0.001 HS
sperm viability %	78 ±7.5 ^a	31.75±5.7 ^b	0.001 HS
sperm normal morphology%	57.85 ± 7.5 ^a	56.45 ± 7.1 ^a	NS

Values were expressed as (mean + SD).

a , b Within compare of each group, numbers with different letter refers to a significantly different (P < 0.01) from each other.

NS = Not significance.

Table 2 showing a negative effect of cryopreservation and thawing processes on sperm parameters compared with that before cryopreservation and thawing processes (After activation), The results showing a significant decrease (p < 0.01) in sperm progressive motility, sperm viability ,while there was no significant effect (p < 0.01) on sperm concentration and normal sperm morphology .

Table (3) cryosurvival rate of sperm motility and viability After cryopreservation for one month and thawing with and without adding GSH to the IVF medium in asthenozoospermic patients

Sperm parameters	cryopreservation and thawing without GSH n = 59	cryopreservation and thawing with GSH n = 59	Significance (p - value)
cryosurvival rate of sperm motility	44. 3±10.98 ^a	70.0±12.82 ^b	0.001 HS
cryosurvival rate of sperm viability	40.60 ±5.93 ^a	59.11 ±7.26 ^b	0.001 HS

Values were expressed as (mean + SD).

a, b Within compare of each group, numbers with different letter refers to a significantly different (P < 0.01) from each other.

(Table 3) showing the positive role of addition glutathione as antioxidant to the IVF medium , improvement of cryosurvival rate of sperm motility and cryosurvival rate of sperm viability which have a significant increase (p <0.01) compared with the result without addition of the glutathione to the IVF medium ,

Discussion

In our study, swim up from pellet and IVF medium technique was used to prepare semen samples of asthenozoospermic patients, the study result showed a significant increase ($p < 0.01$) of progressive motile sperm and sperm normal morphology and sperm viability after activation compared with before activation, the reason is probably that the good movement and normal sperm can reach to the top of the tube where weak sperm and white blood cells remain at the pellet. Sperm, which has good speed is able to swim to the top and the resistance of gravity⁽¹⁶⁾.

The increase in progressive motile sperm is may bedue to the exclusion of white blood cells, phagocytes and non- motile sperm, and the ability of sperm to take advantage of the nutrients and inorganic ions found in the culture layer during the incubation period in energy production in the mitochondria, the adenosine triphosphate (ATP), which is a source of energy for sperm motility by converting it into the adenosine di phosphate (ADP) and the transfer of energy from the mitochondria to the tail of the sperm to increase sperm movement⁽¹⁷⁾.

The other study⁽¹⁸⁾ mentioned that sperms which have normal morphology able to reach to upper part while the sperms which have abnormal morphology are stay in the bottom of tube.

The results of our study showed a significant increase in the percentage of sperm viability ($p < 0.01$), compared to their pre-activation values. This may due to the fact that the activation samples were examined in the upper part of the culture containing the live and motile sperm⁽¹⁹⁾.

The study results showed that sperm cryopreservation had a negative influence on sperm parameters especially the progressive sperm motility and normal morphology, this may due to that the freezing of sperm and the addition of the freezing media lead to decline in the movement of sperm and change the structure and morphology of sperm⁽²⁰⁾. This may be due to the fact that sperm cryopreservation involves a number of processes, such as the process of dilution of the medium to be frozen with the addition of chemicals with low partial weights known as cryoprotectants Substances for the decreasethe freeze damage, as well as the process of immersion of the mixture in nitrogen liquid with temperature 196-, and thawing the samples, which in turn include a number of processes of warming using water bath and the process of separating the freezingsamples by centrifugation and add the activation media and others⁽⁵⁾. Each of these processes is thought to induce negative effects that may cause damage in both the structure and function of the sperm⁽¹⁴⁾.

It has been found that there are some negative effects generated by the use of sperm Cryoprotectants Substances, which affect the structure and function of the cell, including the toxic effects of those substances that result from being chemicals that do not exist in the composition of the cell in the natural state, and the risk of these toxic effects increased when it used at high concentration and at a temperature less than zero centigrade⁽²¹⁾. The plasma membrane of sperm contains high quantity of lipids in it structure. The plasma membrane of the sperm contains high quantity from gel or fluid

lipids. When these lipids are fluids, the plasma membrane of sperm becomes functional. Two factors affect the change of plasma membrane permeability are the ratio of the phospholipid to the cholesterol and the degree of temperature (24).

The freezing and thawing processes cause of unrevised changes of the ability of plasma membrane to passage of the ions, solvent and materials from and into the cells that lead to decrease of functional ability of sperm, these changes called cold shock (25). The effect of the cryopreservation on mitochondria, plasma membrane and the formation of ice crystal outside cell lead to decline the sperm motility and sperm normal morphology (26).

The cryopreservation process cause of increase the levels of reactive oxygen species (ROS) and decrease levels of antioxidants, therefore this study was aimed to prevent the harmful effect of cryopreservation on sperm parameters by addition of glutathione as antioxidant to sperm freezing medium, The results of this study (Table 3) showed a significant improvement of progressive motile sperm after the addition of (1 mM) glutathione to sperm freezing medium.

The results of the study showed that the addition of glutathione Sperm freezing media was significantly improved in both the cryosurvival rate of sperm motility and viability, The results of our study were agreed with other study (25) which showed that the addition of glutathione to the thawing medium resulted in a significant improvement in the sperm parameters included the motility, viability as well as membrane integrity and reduction in the percentage of chromatin damage and DNA, This may be due to the fact that glutathione is a non-enzymatic antioxidant with an effective effect in the resist against free radicals which capable of influencing cells and glutathione is one of major regulator of the scavenging of reactive oxygen species (26). The reason for the improved movement may be explained by the ability of glutathione to decompose the Hydrogen peroxide H_2O_2 glutathione reduce the level of steady-state stability of Hydrogen peroxide and peroxides in the membrane during the thawing process, leading to both improvement the percentage of the progressive motile sperm and the percentage of sperm viability (27).

Another study (28) find that the glutathione surface thiol group has a role in maintaining the sperm motility and that the freezing process affect the reduction of this structure, which affects the sperm motility, so the addition of glutathione may lead to an increase in the percentage of cells containing the group of thiol, which may lead to increased sperm motility.

The results showed a significant increase in the percentage of sperm viability, which may explain the ability of glutathione to scavenging the molecules of reactive oxygen species, and thus reduce the severity of the damage generated by the plasma membranes (12).

Conclusion

The present study revealed the positive role of glutathione as antioxidant in the minimize damage which result from freezing and thawing processes, which lead to a significant increase in the sperm cryosurvival motility rate and sperm cryosurvival viability rate.

Recommendation

The study recommended to addition 50 microliter of glutathione to sperm freezing media in concentration 1 mM .

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