Advanced Stimulatory Method for Activation of Sperm Function Parameters by using TAD Glutathione in Iraqi Infertile Men

Azher S. Hindal, Hayder A.L.Mossa, Muayad S.Abood
High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al- Nahrain University, Baghdad- IRAQ.

Abstract:

Background: One of the major concerns among the male reproductive health is the inability to achieve parenthood even after regular unprotected intercourse. The efficiency of the sperm selection techniques is expressed as the concentration of spermatozoa with normal motility. Discontinuous density gradient centrifugation (DGC) method widely used over the last decade due to effectiveness and reproducibility in recovering most motile sperm, which separates sperm based on their motility, size, and density differential. Glutathione act as an amino acid donor during spermatogenesis. If the levels of glutathione are too low during spermatogenesis, the number of mature and morphologically normal spermatozoa generated will be decreased.

Objectives: The aim of this study was to assess the beneficial role of TAD 600mg Glutathione combined with DGC technique for activation of human sperm of asthenozoospermic, oligozoospermic and normozoospermic subjects compared with the DGC technique alone.

Patients, Materials and Methods: Sixty males were involved in this study, divided into three groups, (twenty asthenozoospermic, Twenty oligozoospermic and twenty normozoospermic subjects)during their attendance to the Infertility Clinic at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies; Al- Nahrain University. Semen samples were obtained, and seminal fluid analysis was assessed. Each semen sample was divided into three parts. The first part prepared as *in vitro* sperm characterization before activation, the second part using density gradient centrifugation technique, while the last part prepared using density gradient centrifugation combined with TAD 600mg Glutathione.

Results: After *in vitro* sperm activation for asthenozoospermic, oligozoospermic and normozoospermic samples a significant increase was observed in the sperm function parameters including sperm concentration, motility and morphologically normal sperm percentage as compared to pre-activation using combined techniques (density gradient centrifugation and TAD 600mg Glutathione) as compared to DGC technique alone.

Conclusions: TAD 600mg Glutathione combined to the DGC technique was found to give higher significant results on sperm function parameters (sperm concentration, motility and morphology) when using a low quality of semen samples such as decreased sperm motility as compared with DGC technique alone.

Keywords: Glutathione, Density Gradient Centrifugation, normozoospermia,asthenozoospermia, oligozoospermia.

Introduction:

Assisted reproductive technologies (ART's) were advanced through the earlier decades to produce high-yielding numbers of embryos, and have been revealed the need for suitable and effective techniques of sperm treatment in the laboratory (1). Many of sperm separation or isolation methods exist to select spermatozoa. These methods include swim-up methods, density gradient centrifugation, glass wool and others have been developed to separate viable sperm from the seminal fluid for the use in ART's (2). The density gradient centrifugation technique can be modified to treat the issues of each individual specimen, and it is the method of choice for preparation of the sperm in the majority of ART's and andrology laboratories (3). Advantages of this method include: Clean part of very motile spermatozoa,
very low sperm density can be separated of spermatozoa from ejaculates and significant reduction in reactive oxygen species (4).

Glutathione is one of the antioxidant added to different semen specimen; it has a serious role in the antioxidant role of endogenous, exogenous composts (5). Glutathione a naturally occurring tri-peptide in semen play an serious role in scavenging reactive O2 intermediates and other radicals with the help of the glutathione reeducates/peroxidase cycle (6). Glutathione has different functions with very important roles in the physiological and metabolism of the cell including the protection from oxidative stress, production of protein and DNA and fertilization of gamete cell. Glutathione effect cell metabolism by detoxication and block production of free radicals in spermatozoa (7).

Patients, Materials and Methods:

In this study 60 semen samples were carried out during their attendance to the infertility clinics at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies(ART's) at Al-Nahrain University. The period of study was from August 2017 to June 2018. Semen samples were classified into subgroups according to criteria of WHO (2010) and WHO (1999) for semen analysis: Asthenozoospermic group: 20 semen samples with a sperm progressive motility <32, oligozoospermic group: 20 semen samples with a sperm progressive motility < 15%: Varicocele group : 10 semen samples and Non-Varicocele group : 10 semen samples, Normozoospermic group: 20 semen samples with normal motility.

Semen was collected from all individuals of the study in an average of 3 ml, each semen sample was divided into 3 parts as following: 1ml of semen for examination of seminal fluid analysis before activation, 1ml of semen for examination of density gradient centrifugation (DGC) after activation and 1 ml of semen for examination of DGC+TAD 600mg Glutathione after activation.

1- Discontinuous Density Gradient Centrifugation (DGC) Technique:

Density gradient centrifugation technique used for the separation of sperms has been golden technique In a lot of ART's laboratories because of its performance that are easily and quickly resulting in highly quality of sperm motility (8).

This technique is carefully done by adding 1mL of 80% of Sil-Select Plus gradient as a first layer solution in a test tube followed by 1mL of 40% of Sil- Select Plus gradient as a second layer solution, then 1 ml liquefied semen sample was added on the second layer. This test tube was carefully put in centrifuge at 3000 rpm for 15 minute. Then the supernatant was discarded and 1mL of FertiCult Flushing medium was added to the pellet and put in air incubator for 30 minutes at 37°C. A drop of 10µL was aspirated and put on a slide with cover slip and examined under the microscope at 400X objective to assess the sperm parameters.

2- Discontinuous Density Gradient Centrifugation (DGC) Technique with TAD 600mg Glutathione:

The experiment was repeated as mentioned above with the addition of 0.5ml (15.4 mg/ml) (9)TAD 600 mg Glutathione to the last step of the procedure with the FertiCult Flushing medium and placed in air incubator for 30-45 minutes at 37°C. A drop of 10µL was aspirated and placed on a slide with cover slip and examined under the microscope at 400X objective to assess the sperm parameters.

Results: Result of *in vitro* sperm activation for asthenozoospermic and oligozoospermic and normozoospermic samples using DGC and DGC with TAD 600mg Glutathione techniques.

The outcome of sperm concentration for the three groups of semen samples before-activation and after-*in vitro* sperm activation techniques.

After- *in vitro* sperm activation techniques observed a significant($P<0.05$) decrease in the sperm concentration, when using DGC and DGC with TAD 600mg Glutathione techniques as compared to before- activation evaluation, except for the Oligozoospermia group which showed non-significance when comparing the sperm concentration before and after *in vitro* activation as shown in Table (1).

There is no significant difference for sperm concentration between density gradient centrifugation technique compared to DGC with TAD 600mg of Glutathione technique as shown in Table (1) and the highest value for sperm concentration of the three groups was observed after-activation when using DGC with TAD 600mg Glutathione technique (42.0 ±1.83, 45.5 ±2.69 and 14.8 ±0.67), but it is lower than sperm concentration before activation (52.5 ± 2.16, 47.5 ± 2.65 and 14.95 ± 0.26).

Table (1): Before-and after *in vitro* sperm activation comparison in sperm concentration between groups

Treatment	Mean ± SE of conc.			LSD value
	Normozoospermia	Asthenozoospermia	Oligozoospermia	
Before activation	52.5 ± 2.16	47.5 ± 2.65	14.95 ± 0.26	7.432
DGC	38.5 ±2.05*	43.4 ±2.77*	14.75 ±0.84	5.879
DGC + TAD	42.0 ±1.83 *	45.5 ±2.69*	14.8 ±0.67	5.204
LSD	6.493*	1.574*	0.731 N.S	---

* Significant at $P<0.05$; N.S: Non-Significant ; SE: Standard Error ; Conc.: Concentration; Mill: Million ; DGC: Density Gradient Centrifugation.

Progressive sperm motility outcome after- *in vitro* activation for the three groups of samples (normozoospermic, asthenozoospermic, and oligozoospermic semen sample), compared before -activation, shows significant increase at ($P<0.05$) of progressive (A+B) sperm motility, as shown in Tables (2, 3).

Density gradient centrifugation with TAD 600mg Glutathione technique showed the highest value of sperm motility grade (A+B) progressive after-activation as compared to before-activation for the three groups, normozoospermic, asthenozoospermic, and oligozoospermic groups. DGC technique, a significant increase was recorded in progressive sperm motility (A+B), but, the lowest value for sperm motility was noticed before sperm activation, as shown in Table (2, 3).

Table (2): Before and after *in vitro* sperm activation comparison in sperm progressive motility Grade A between groups.

Treatment	Mean ± SE of A Progressive			LSD value
	Normozoospermia	Asthenozoospermia	Oligozoospermia	
Before activation	10.0 ±0.77*	0.75 ±0.41**	0.0	2.543
DGC	15.0 ±1.22**	8.75 ±1.4**	3.25 ±0.75 **	3.421
DGC + TAD	20` ±1.51**	10.25 ±1.1**	8.0 ±1.42**	3.433
LSD value	2.451	3.121*	2.601	---

* Significant at P<0.05; **Highly Significant P<0.01; SE: Standard Error; DGC: Density Gradient Centrifugation.

Table (3): Before and after *in vitro* sperm activation comparison in sperm progressive motility grade B between groups.

Treatment	Mean ± SE of B Progressive			LSD value
	Normozoospermia	Asthenozoospermia	Oligozoospermia	
Before activation	40.0 ±2.4	22.75 ±2.1*	15.0 ±2.7**	3.451
DGC	35.0 ±2.43*	35.75 ±3.37*	24.5 ±2.26*	1.769
DGC + TAD	40 ±3.46*	39.5 ±3.89*	32.75 ±2.92*	2.546
LSD value	4.713	5.123*	5.432	---

* Significant at P<0.05 ; **Highly Significant P<0.01; N.S: Non-Significant ; SE: Standard Error ; DGC: Density Gradient Centrifugation.

The result of non- progressive sperm motility grade C for the three groups were observed as shown in Table (4). Before and after activation techniques noticed a significant reduction of grade C percentage. The lowest value for non-progressive motility (grade C) was noticed after-activation when using DGC with TAD 600mg Glutathione technique as compared to before-activation.

On the other hand, non-significant reduction for the same parameters when using DGC with TAD 600mg Glutathione technique as compared with DGC technique, as shown in Table (4).

While, a significant (P<0.05) reduction was assessed for the percentages of non-progressive sperm motility (grade C).when using DGC with TAD 600mg Glutathione technique when compared before activation, as shown in Table (4).

Table (4): Before and after *in vitro* sperm activation comparison in sperm non-progressive motility grade C between groups.

Treatment	Mean ± SE of C Non-Progressive			LSD value
	Normozoospermia	Asthenozoospermia	Oligozoospermia	
Before activation	36.0 ±2.61	47.75 ±2.77*	40.25 ±2.94	3.541
DGC	37.0 ±2.84*	43.25 ±3.9	43.3 ±2.95	2.121
DGC + TAD	30.5 ±3.12*	40.25 ±3.62*	44.25 ±3.47	2.458
LSD value	0.723	3.677*	0.168 N.S	---

* Significant at P<0.05; **Highly Significant P<0.01; N.S: Non-Significant; SE: Standard Error ; DGC: Density Gradient Centrifugation.

A significant increase at (P<0.05) of the MNS for the three groups of semen samples after the activation was showed in table (5), when using DGC and DGC with TAD 600mg Glutathione techniques as compared to before - activation. The highest value for the same parameter was noticed using DGC with TAD 600mg Glutathione technique as compared to pre- activation.

On the other hand, a significant (P<0.05) increase when using DGC with TAD 600mg Glutathione technique as compared to DGC technique, as shown in Table (5) .

Table (5): Before and after *in vitro* sperm activation comparison in morphologically normal sperm between groups.

Treatment	Mean ± SE of MNS			LSD value
	Normozoospermia	Asthenozoospermia	Oligozoospermia	
Before activation	30.5 ±1.7*	29.0 ±2.37**	23.75 ±1.81*	1.761
DGC	51.5 ±1.78**	45.25 ±3.47**	34.0 ±2.5	3.874
DGC + TAD	61.25 ±1.92**	53.75 ±3.7**	34.5 ±2.54	3.988
LSD value	10.132	8.547*	1.769	---

* Significant at P<0.05 ; **Highly Significant P<0.01; N.S: Non-Significant ; SE: Standard Error; DGC: Density Gradient Centrifugation.

Discussion:

In this study, the results of sperm concentrations parameter for the three groups of Normozoospermic, Asthenozoospermic and Oligozoospermic semen samples showed a significant (P<0.05) decrease in the concentration of sperm between groups of study before *in vitro* sperm activation techniques; the sperm concentration in DGC and DGC with TAD 600mg glutathione as compared with before activation also showed a decrease in concentration between groups. This reduction occurs as a result of abnormal and dead spermatozoa exclusion during sperm preparation techniques especially in Oligozoospermic.

The typical methodology for the DGC technique included discontinuous gradients (10). Discontinuous gradient layers show clear layers between each other. The semen placed above the density media is higher in density and is then centrifuged. All cells reach the semen sediment, during this procedure.

A spermatozoon with high motility actively moves in the direction of the sedimentation gradient and can therefore penetrate the layers faster than sperm with poor motile or immotile cells. Thus, sperm cells with high motility are aggregated in the pellet at the bottom (11).

The pellet is then re-suspended with gradient media again in DGC technique with and without TAD 600mg Glutathione, so spermatozoa migrate through density of media and then from pellet to upper media, the data of this study showed increase concentration rate of sperm was in DGC with TAD 600mg Glutathione technique. These results were in contrast with other studies using DGC techniques compared with other techniques (12).

As sperm penetrations and transport through genital tract of the female required sperm motility, so it's very important parameter of sperm for successful fertilization (13).

In this study, there is a significant result of activation by DGC and DGC with TAD 600mg Glutathione for increasing sperm motility noticed with progressive motile sperm (grade A and grade B). This result due to activation of sperm with culture media, so spermatozoa of grade B become grade A. The data of this study showed the high percentage of motility was found in DGC with TAD 600mg glutathione.

The active progressive sperm motility Grades A and B are increased following activation for one hour (60 minutes) compared before activation, because activation techniques of sperm lead to excellent yields in motility of spermatozoa especially in DGC with TAD 600mg Glutathione. Therefore, these good sperm preparation techniques can improve sperm quality by selecting high percentage of motility (14).

In general, DGC technique showed results higher than other techniques as a result of saline coated silica that used for sperm preparation, and these result are in agreements with the findings of other studies that reported by the main improving role of this technique on the recovery of motile spermatozoa (15).

In this study, the best choice to prepare or select functional sperm depending on the feature of the semen samples for all the three groups of samples Asthenozoospermic, Oligozoospermic and Normozoospermic samples is DGC technique specially when combined with TAD 600mg Glutathione, in which yielded high result for sperm motility and morphology more than other techniques. This in agreement with studies that shown a significant improvement in the percentage of sperm motility with hyper activation and forward progressive movement, following activation by TAD 600mg glutathione or that's used DGC alone study has that low seminal plasma glutathione levels are associated with abnormal values for sperm concentration, semen volume, and sperm motility and morphology. Higher levels of seminal plasma glutathione corresponded with healthy, fertile men in this study (16).

Morphologically normal sperm besides the concentration and motility of sperm are good indicators of semen quality, as ability to fertilize the oocyte (17). Morphological normal sperms with low rate have been correlated with lower infertility rate in males (18).This was in contrast with the results of this study, as the MNS showed a significant increase using activation techniques especially in the DGC with TAD 600mg Glutathione. In addition to that, the components of liquid artificial medium may improve the percentage of MNS and progressive motility because it dilute and reduce the semen viscosity (16).

Grade C is known to be non-progressive, ,fertilization needs progressive motility for successful concept.so it is important to be as low as possible.

In this study Grade C showed a significant decrease after activation in the three groups of Normozoospermic, Asthenozoospermic and Oligozoospermic especially when using the DGC with TAD 600mg Glutathione technique. The aim of DGC sperm preparation techniques is to isolate the motile sperm cells from the other contents of semen as immotile sperm cells, leukocytes, epithelial cells, debris, and any other content that produce reactive oxygen species and eliminate agglutination, as a result of the anti-sperm antibodies presence resulting in limitation of sperm motility, to provide better quality of sperm regarding motility, which may lead to advanced success rates for assisted reproduction (19).

Density gradient centrifugations are more gentle techniques and widely applied in clinical practice. In assisted reproductive techniques the success rates of sperm selection are currently based on standard criteria such as motility, viability and morphology (20,21). In this study, the data showed significant results in semen samples with best recovery rates, motility and morphology and with low rate of agglutination and debris for all the three groups of samples, when using DGC with TAD 600mg glutathione.

In summary it concluded that after *in vitro* activation by two techniques DGC and DGC with TAD 600mg Glutathione techniques compared before-activation in fertile subjects and infertile ones which indicate for improvement in sperm function parameters after- *in vitro* activation.

Active sperms in semen specimens were successfully isolated using DGC and TAD 600mg Glutathione technique in infertile Asthenozoospermic and Oligozoospermic men. Activation of the isolated sperms by DGC with TAD 600mg Glutathione was significantly achieved in infertile men.

Lowest significant reduction were observed in reduction process of the non-motile, non-active and non-fertilizable sperm numbers in the outcome of using DGC with TAD 600mg Glutathione rather than other techniques since it yields better sperm concentration rather than the outcome of DGC technique.

References:

1. European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology; Calhaz-Jorge, C.(August 2016). "Assisted reproductive technology in Europe, 2012: results generated from European registers by ESHRE". Human reproduction (Oxford, England). 31 (8): 1638–1652.

2. Spalekova E, Makarevich A, and Pivko J.Effect of caffeine on parameter of ram sperm motility. Slovak J. Anim. Sci.2011;44(2):78-83.

3. Teppei T., Yasushi Y., Shinnosuke K., Takashi K., Hiroji U. & Akira I. Effect of density gradient centrifugation on reactive oxygen species in human semen. , Systems Biology in Reproductive Medicine.2017;63:3, 192-198.

4. petyim S,Choavaratana R, Suksompong S, *et al.* Outcome of sperm preparation Using Double-Gradients Technique Study in Siriraj Hospital. J Med A ssoc Thai.2009;92(7): 878-884.

5. Luberda,Z. (2005). The role of glutathione in mammalian gametes .Reprod Biol, 5: 5-17.

6. Pahune, P.P., Choudhari, A.R. and Muley, P.A. (2013) The total antioxidant power of semen and its correlation with the fertility potential of human male subjects. J Clin Diagn Res.2013;7:991–995.

7.De-Matos,D.G. and Fumus,C.C. The importance of having high glutathione (GSH) level after bovin *in vitro* maturation on embrio development effect of β-mercaptoethanol, cysteine and cysteine. Theriogenology;2000;53: 761-771.

8. Astarto N W, Tjahyadi D, and Jatnikasari S. Comparison between two-layer density gradient and three-layer density gradient technique for sperm preparation at aster fertility clinic, Dr. Hasan Sadik in General Hospital.IJIHS.2014;2(1):40-4.

9. Ghorbani M, Vatannejad A, and Khodadadi I. Protective Effects of Glutathione Supplementation Against Oxidative Stress During Cryopreservation of Human. US National Library of Medicine.2012; 37(1): 34-40.

10. Camargo L, Viana J.H, Sa W.F, Ferreira A, Ramos A, and Filho V. Factors influencing *in vitro* embryo production. Animal Production.2006; 3(1): 19-28.

11. Suarez S, and Ho H. Hyperactivated motility in sperm. Reproduction in Domestic Animals. 2003; 38(2):119-124.

12. Astarto N W, Tjahyadi D, and Jatnikasari S. Comparison between two-layer density gradient and three-layer density gradient technique for sperm preparation at aster fertility clinic, Dr. Hasan Sadikin General Hospital. International Journal of Integrated Health Sciences.2014;2(1):40-4.

13. Ali E, Al-Kawaz U, and Fakhrildin MB. A new sperm preparation technique by combination of density gradient centrifugation and glass wool filtration technique versus each one alone for infertile men with asthenozoospermia. International Journal of Advance Research.2016; 4(4):1112-1115.

14.Shams Alddin N.N, Al-Dujaily S.S, and Al-Sultani YK. *In vitro* sperm activation with pentoxifylline and L-carnitine for infertile men semen usinglayering and sedimentation techniques. Iraqi Journal of Embryos and Infertility Researches.2013;3(6): 32-37.
15.Malvezzi H, Sharma R, Agarwal A, Abuzenadah A, and Abu-Elmagd M. Sperm quality after density gradient centrifugation with three commercially available media: A controlled trial. Reproductive Biology and Endocrinology.2014; 12:121.
16. Naher, Z. *et.al.* (2011). Role of glutathione in male infertility. Bangladesh Journal of Medical Biochemistry.2011; 4(2):20-25.
17.Lundin K, Soderlund B, and Hamburger L. The relationship between sperm morphology and rates of fertilization, pregnancy and spontaneous abortion in an *in-vitro* fertilization / intracytoplasmic sperm injection programme. Human reproduction.1997; 12(12):2676-81.
18.Karabulut A, and Tekin A. Alteration in the morphology and motility of spermatozoa:relation with total sperm count. Pam Med J.2013; 6(1): 1-4.
19.Bur R, Siegberg R, Mathews C, and Flaherty S. The influence of spermatozoa morphology and the number of motile spermatozoa inseminated on the outcome of intrauterine insemination combined with mild ovarian stimulation. Fertil Steril.1996; 65(1):127-132.
20.Jayaraman, Upadhya D, Narayan PK, and Adiga SK. Sperm processing by swim-up and density gradient is effective in elimination of sperm with DNA damage. J Assist Reprod Genet.2012; 29(6): 557–563.
21. Naam A. Hamza , Mohammed O. Selman, Hayder A. L. Mossa. Comparison of Best Yield of *in vitro* Sperm activation Techniques with New technique of Caffeine Combined with Density Gradient Centrifugation in Iraqi Patients. J. Pharm. Sci. & Res.2018;10(1):36-39.