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Effect of *In-vitro*Sperm Activation Techniques and Albumin Concentrations on Human Sperm Parameters and Chromatin Structure

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Abstract:

Current study used the acridine orange test to investigate the effect of two techniques of human sperm preparation involved direct swim up technique and centrifugation swim up technique on the integrity of sperm chromatin structure, and also the supplementation of two concentrations of Human serum albumin (HSA) in the culture medium that has been used in the previously mentioned methods. Assay results demonstrated that, both techniques reduce the percentages of isolated or activated spermatozoa with fragmented DNA as compared with their percentages pre-preparation, whither this decrement was significant or not (P=0.05). Also we found that using culture medium supplemented with concentrations of HSAenhance the isolation of spermatozoa with intact DNA, and (10%)HSA was the better.Therefore, the present study concluded that neither direct swim up nor centrifugation swim up techniques with single centrifuge step (559g) for 8minutes can cause further DNA damage in the activated spermatozoa by both, also the involvement of HSA in sperm preparation media enhances sperm parameters and the isolation of spermatozoa with intact DNA in-vitro.

Key words: *DNA fragmentation, sperm activation, swim up*

Introduction:

The isolation of more spermatozoa with intact DNA can improve the likelihood of pregnancy (1). Sperm DNA damage significantly contributes to the growing number of infertility cases, and their tests should be a part of a modern andrology laboratory (2). Conventional semen analysis continues to be the only routine test to diagnose male factor infertility, although semen parameters have a limited power to predict spontaneous or assisted conception (3). It has been proposed that the use of ''invisible damaged'' spermatozoa could result in fertilization failure, impaired normal embryo development, reduced implantation or pregnancy rate, and/or even transference of damaged DNA to the new generation (4). This is particularly important in an era where advanced forms of assisted reproductive technologies (ARTs) are commonly utilized that often bypass the barriers to natural selection, also some uncertainty and worry concern the safety of utilizing DNA-damaged spermatozoa in this setting (5,6). Therefore, it is important to identify strategies and optimize the conditions by which spermatozoa can be prepared that may reduce sperm DNA damage.

Culture media and procedures for sperm washing can impair or enhance sperm function in ART(7). The recovery of higher quality spermatozoa can be improved by the use of culture medium supplemented with albumin (8). The beneficial effect of albumin on sperm motility has been well documented , further more Albumin helps in neutralize lipid peroxide-mediated damage to the sperm plasma membrane and DNA (9). Currently, one of the four major sperm DNA fragmentation tests is the acridine orange test (AOT), and the others include the Comet, TUNEL, Sperm chromatin structure assay (SCSA). However, the clinical value of sperm DNA normality detected by AOT for the prediction of ART outcomes is currently still inconclusive and requires further investigation (10). Various studies have suggested different

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or no threshold values with assorted tests for the percentage of DNA fragmentation in the ejaculated sperm above which natural conception, fertilization or embryo development and/ or clinical pregnancy rates are compromised. Current DNA fragmentation assessment methods provide very little specific information on the nature and severity of the DNA damage detected (11). Therefore, the present study was aimed to investigate the effect of two techniques for in-vitro sperm preparation parameters and chromatin structure.

Material and Methods:

Semen analyses were done for fifty three fertile men involved in this study. Twenty four semen samples were prepared using direct swim-up technique as described by Arny and Quagliarello with some modification(12).Other semen samples (No.=29) were prepared by centrifugation swim-up as described by Mahadevan and Baker with some modification(13). Hams F-12 medium (Sigma-Aldrich Inc.,USA) was used for sperm preparation and HSA (Life Global, USA) was added to prepare culture medium supplemented with 5% and 10%. Sperm parameters were assessed involving concentration, motility, and normal morphology, according to the latest issue for WHO standard criteria(14).

Acridine orange test was functioned to assess spermatozoa with fragmented DNA as described by Tejada et al. (15). Briefly, after washing with Tyrode's solution composed of a small amount of warm distilled water added to 0.24g/L (Mg Cl2), followed by adding all the components (NaCl 7.054g/L, KCl 0.439g/L, CaCl2.2H2O 0.24g/L, NaH2PO4.2H2O 0.187, NaHCo3 1.302g/L) together, then, the volume was completed to (1L) and pH was adjusted to 7.3),medium-thick smears on cleaned slides were air dried, fixed overnight in freshly prepared Carnoy'ssolution (3 parts methanol/1 part glacial acetic acid), air dried again and stained with acid AO (Sigma, Deisenhofen, Germany) solution. All slides were read the same day on a fluorescencemicroscope. Sperm heads were subdivided into those showing a green color and those with colors ranged from yellow to red as recommended (15). A total of 300 cells were counted on each slide. The ratio of colors other than green/ colors other than green + green yields the percentage of DNA fragmentation, referred to DNA fragmentation index (DFI%).

Statistical analysis:

The data were statistically analyzed using SPSS/PC version 19 software (SPSS, Chicago, USA). Sperm parameters were analyzed using one way ANOVAs complete randomized design (CRD) and expressed as mean±S.E. Differences between values of means were considered statistically significant at ($P<0.05$).

Results:

Results of the present study appeared that the most sperm parameters were significantly enhanced ($P<0.05$) post-preparation in-vitro as compared to pre-preparation (control 1) using direct swim-up technique (Table 1). Mean values of progressive motility percentages was significantly increased ($P<0.05$) in G2 group where Ham's F-12 supplemented with (5%) HSA was used as compared to G1 group that lacks to HSA(control 2) and G3group. Means of sperm total motility (%) and normal morphology (%) were also significantly increased ($P<0.05$) post- preparation as compared to (control 1).

Table (2) shows the results of the normozoospermic men prepared by centrifugation swim-up technique. Percentages of sperm progressive motility was significantly increased ($P<0.05$) post-preparation as compared to pre-preparation (control 1). However, G2 group was the better in mean of progressive motility (%) post-preparation as compared to G1 and G3. Percentages of total motility and normal morphology were significantly increased ($P<0.05$) post preparation as compared to pre-preparation (control 1). Furthermore, in G2 and G3 these percentages were increased whether significantly or not as compared to (control 2), in which total motility was significantly increased ($P<0.05$) in G2 and G3 as compared to (control2), but G3 was slight better than G2 with no significant differences($P>0.05$).

Whereas sperm normal morphology percentages were significantly increased in G2 and G3 groups as compared to (control 2), and G2 was better than G3 with non significant differences ($P>0.05$).

As a comparison between direct swim up and centrifugation swim up techniques appeared in table (3). There was no significant differences between direct swim up and centrifugation swim up for most main sperm parameters in G1, G2 and G3 groups, except to normal morphology percentage that was significantly increased in G2 for spermatozoa prepared by centrifugation swim-up technique.

Figure (1) presents the effect of direct swim-up technique on sperm parameters for sperm parameters for G1, G2 and G3 groups as compared with the analogous group that was prepared using centrifugation swim-up technique. This figure shows that DFI% of normozoospermia prepared using direct swim-up for G1 group was significantly better ($P<0.05$) than those of centrifugation swim-up technique. Andadditionof HSA concentrations in G2 and G3 groups increased the efficiency of centrifugation swim-up technique prepared spermatozoa with lower DFI% but not significantly different ($P>0.05$)from those prepared by direct swim-up technique . However, G3 in both technique was the best with non significant differences in reduce means of DFI% ($P>0.05$).

Discussion:

Sperm preparation techniques,culture media and the properties of semen sample itself can play a critical role in determining the outcomes of ART (7). With respect to sperm functional capacity, each laboratory should determine the centrifugation force and centrifugation time that are necessary to form a manageable recovery of sperm (14).

Direct swim-up technique showed improvement in the

sperm quality of selected population compared with pre-preparation (control 1) allowing for a good recovery of sperm.These results agreed also with Boomsmaet al.(16) in the efficiency of this method for preparing normozoospermia specimens.However, centrifugation swim-up technique was as effective as direct swim up technique but the latter in an easier way and this agreed withSiam(17).Generally, sperm motility increased with the use of in-vitroculture because of their aqueous nature with lower viscosity than of seminal plasma resulted in making spermatozoa move more freely (18). Ham's F-12 contains certain components like Ca2+and HCO3− which play important regulatory roles in promoting capacitation and hyperactivation according to Visconti and Kopf (19). progressive motility and sperm recovery may be increased due to the presence of bicarbonate in the sperm preparation medium as proposed by Henkel et al. (20).

Ham's F-12 contains also pyrovate and glucose as a source of energy and according to Folgero, et al.(21).These components can increase in motile spermatozoa particularly pyrovate. However, results of the present study refers to the further improvement in sperm parameters when culture medium are supplemented with Human serum albumin as a stimulator compared to control lacks (HSA). Lamirande and Gagnon (22) reported that in contrast to caffeine, which stimulated sperm motitily for less than 1 h, the effect of humanserum lasted for more than 16h.Armstronget al. (8)reviewed the beneficial effects ofalbumin and other protein on the motility and morphology of the recovered spermatozoa

Table (1): Effect of direct swim up technique and different concentrations of HSA on sperm parameters for normozospermicmen.

Sperm parameters	Direct swim up Technique			
	Pre-preparation	Post-preparation		
		G1*	G2**	G3***
Sperm concentration ×million/mL	77.750 a ±4.54	39.791 b ±4.70	45.083 b ±5.00	37.916 b ±4.23
Sperm progressive motility (٪)	41.458 c 92,٢±	67.458 b ±3.08	77.292 a ±2.83	68.083 b ±3.06
Sperm non progressive motility (٪)	25.792 a ±2.81	21.438ab ±3.01	16.500 b ±2.78	25.583 a ±2.92
Sperm immotile (٪)	32.750 a ±2.93	11.104 b ±2.09	6.625 b ±1.70	6.125 b ±1.24
Total sperm motility (٪)	67.250 b ±2.81	89.313 a ±2.11	93.792 a ±1.61	93.667 a ±1.22
Normal sperm morphology(٪)	40.583 b ±2.47	60.250 a ±3.67	66.458 a ±3.17	64.875 a ±3.59

(Mean±S.E). G1 means Ham's F-12 culture medium, ** G2 means Ham's F-12 +5% HSA, ***G3 means Ham's F-12 +10% HSA.*

Different superscripts within each row are significantly different (P<0.05), means with same superscripts within each row are not significantly different (P>0.05).No. of normozospermic men=24.

Table (2): Effect of centrifuged swim up technique and different concentrations of HSA on sperm parameters for normozospermicmen.

Sperm parameters	Centrifugation swim up technique			
	Pre-preparation	Post-preparation		
		G1	G2	G3
Sperm concentration ×million/mL	72.275 a ±5.11	44.103 b ±4.81	42.655 b ±4.03	42.517 b ±4.22
Sperm progressive motility (٪)	44.310 c ±2.82	66.966 b ±2.97	81.379 a ±2.38	69.345 b ±3.30
Sperm non progressive motility (٪)	27.379 a ±2.41	22.586 a ±2.93	10.552 b ±2.07	26.241 a ±3.52
Sperm immotile (٪)	٢٨,٥٥٢ a ١,٨١±	٩,٦٥٥ b ١,٧٧±	٨,٠٣٤ bc ٢,٠٤±	٤,٣٧٩ c ٠,٨٩±
Total sperm motility (٪)	71.655 c ±1.85	89.103 b ±1.96	91.966 ab ±2.04	95.621 a ±0.89
Normal sperm morphology(٪)	٤٢,٤٨٣ c ٢,١٠±	٦٥,٩٦٦ b ٢,٩٩±	٧٥,٥١٧ a ٢,٨٩±	٧٣,١٠٣ ab ٣,١٢±

(Mean±S.E).

Different superscripts within each row are significantly different (P<0.05), Same superscripts within each row are not significantly different (P>0.05).
No. of normozospermic men=29.

Table (3): Effect of methods of sperm preparation supplemented with different concentrations of HSA on the sperm parameters in normozoospermia group post-preparation.

Sperm parameters	Normozoospermia					
	G1		G2		G3	
	Direct swim up	Centrifugation swim up	Direct swim up	Centrifugation swim up	Direct swim up	Centrifugation swim up
Sperm concentration	39.791 a ±4.70	44.103 a ±4.81	45.083 a ±5.00	42.655 a ±4.03	37.916 a ±4.23	42.517 a ±4.22
Progressive motility (٪)	67.458 a ±3.08	66.966 a ±2.97	77.292 a ±2.83	81.379 a ±2.38	68.083 a ±3.06	69.345 a ±3.30
Non Progressive motility (%)	21.438 a ±3.01	22.586 a ±2.93	16.500 a ±2.78	10.552 b ±2.07	25.583 a ±2.92	26.241 a ±3.52
Immotile sperm (٪)	11.104 a ±2.09	9.655 a ±1.77	6.625 a ±1.70	8.034 a ±2.04	6.125 a ±1.24	4.379 a ±0.89
Total motility (%)	89.313 a ±2.11	89.103 a ±1.96	93.792 a ±1.61	91.966 a ±2.04	93.667 a ±1.22	95.621 a ±0.89
Normal morphology (٪)	60.250 a ±3.67	65.966 a ±2.99	66.458 b ±3.17	75.517 a ±2.89	64.875 a ±3.59	73.103 a ±3.12

(Mean±S.E)

*Different superscripts within two technique of each treatment group are significantly different (P<0.05).
* * Same superscripts within two technique of each treatment
group are non significantly different (P>0.05).

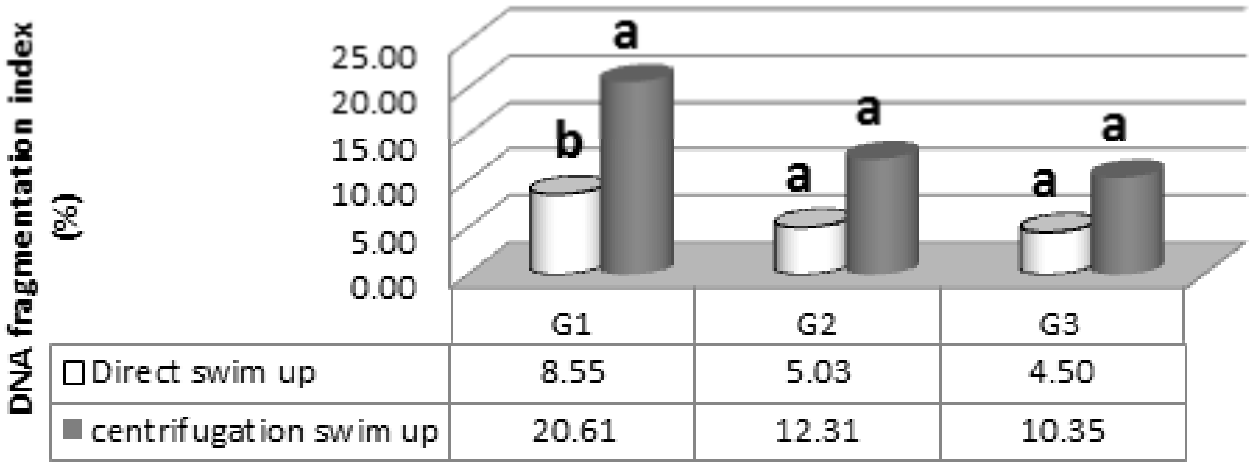

Figure (1): Effect of sperm preparation techniques supplemented with different concentrations of HSA on DFI% of normozoospermia groups post-preparation

**Different superscripts within each columns is significantly different (P<0.05).*

** * Same superscripts within each columns is non significantly different (P>0.05).*

and mentionedthe role of HSA as antioxidant. Ali et al.(23) cleared the importance of albumin to be involved in preparation medium in maintaining viscosity, membranesatiability and osmo-regulation.

Agarwal and Said (24) noticed that the overproduction of reactive oxygen species (ROS) and decreased antioxidant defense activity cause low sperm motility and viability, DNA fragmentation and protein denaturation.In general, sperm preparation techniques should also minimize the risk of ROS generation, as excessive production of these could adversely affect deoxyribo nucleic acid (DNA) integrity and sperm functions in-vitro (25).

Furthermore, Sikka (26)mentioned that high generation of ROS can be activated by sperm
processing like excessive centrifugation, cryopreservationand thawing accompanied by low scavenging and antioxidant levels in serum, seminal plasma, and/ or sperm-processing media will induce a state of oxidative stress. Particularly those procedures that need centrifuge step, considering the detrimental effect associated with this process and it's determining factor like relative centrifugation force and time (27). DNA of normal spermatozoa is less susceptible to gentle processing techniques than the DNA of abnormal or immature spermatozoa (28).

Therefore, the present study concluded that gentle processing of semen during in-vitro sperm activation with the presences of antioxidants can provide a good population of spermatozoa with intact DNA for artificial insemination, neither direct swim up nor centrifugation swim up techniques with single centrifuge step (559g) for 8minutes can cause further DNA damage in the activated spermatozoa by both, and best results of activation belongs to (5%) HSA. On the other hand 10% HSA provides best protection to the isolated spermatozoa with intact DNA.

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تأثير تقنيات تنشيط النطف وتركيز الألبومين مخبارياً على دالات (مؤشرات) النطف البشرية وبنية الصبغين

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الخلاصة :

تهدف الدراسة الحالية الى معرفة تأثير تقنيتين من تقنيات تحضير النطف البشريةوهما تقنية السباحة المباشرة(Direct swim up)وتقنية سباحة النطف المنتبذة مركزياً(Centrifugation swim-up) على بنية الصبغين للنطف وكذلك تأثير تضمين تركيزين من زلال المصل البشري في الوسط الزرعي المستخدم في التحضير بالطريقتين الأنفة الذكر , باستخدام فحص (Acridine orange).
أظهرت نتائج الفحص ان كلا الطريقتين قد خفضت من نسبة التجزؤ في دنا النطف المفصولة او المنشطة بالمقارنة مع نسبها قبل التنشيط سواء كان هذا الانخفاض معنوياً أو غير معنوي(P=0.05) ,وان تضمين تراكيز الزلال الى الوسط المستخدم في التحضير يزيد من المحافظة على دنا النطف المفصولة والأفضلية كانت للتركيز (10%) من زلال المصل البشري. لذلك نستنتج من الدراسة الحالية بأن تقنية سباحة النطف المنتبذة مركزياً التي تتضمن خطوة طرد مركزي مفردة وبقوة (559g) لمدة 8 دقائق, و تقنية السباحة المباشرة كلاهما لايسببان ضرراً إضافيا للنطف المنشطة في المختبر, و ان وجود زلال المصل البشري في وسط التحضير يعزز من مؤشرات النطف بالإضافة إلى انه يزيد من فرصة الحصول على نطف سليمة ألدنا بعد التنشيط.