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Sperm Quality after Swim Up and Density Gradient Centrifugation Sperm Preparation with Supplementation of Alpha Lipoic Acid (ALA): A Preliminary Study

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Abstract. Intra uterine insemination (IUI) as one of the treatment for infertility, persists low success rate. A factor that contributes to the unsuccessful of IUI is sperm preparation, performed through Swim-up (SU) and Density Gradient Centrifugation (DGC) methods. Furthermore, studies have shown that Alpha Lipoic Acid (ALA) is a potent antioxidant that could enhance the sperm motility and protect the DNA integrity of the sperm [1]. This study is aimed to re-evaluate the efficiency of the DGC and SU methods in selecting sperm before being transferred for IUI by the supplementation of ALA based on the sperm DNA integrity. Semen samples were obtained from 13 men from partners of women who are infertile (normozoospermia) and underwent IUI. Semen analysis based on the guideline of World Health Organization (WHO) 2010 was performed to measure the sperm motility and velocity, before and after sperm preparation. Then, samples were incubated with Alpha Lipoic Acid (ALA) in 0.625 mg (ALA 1), 1.25 mg (ALA 2) and 2.5 mg (ALA 3). The Sperm Chromatin Dispersion (SCD) test was performed to evaluate the sperm DNA Fragmentation Index (DFI). The percentage of motile sperm was higher in prepared sperm (post-DGC and post-SU) than in whole semen. Furthermore, the percentage of motile sperm was higher in post-DGC compared to post-SU. The level of DFI after the supplementation of ALA was decreased in prepared sperm compared to the whole semen. ALA was proved capable to select the better sperm quality with decreased sperm DNA fragmentation of prepared sperm in the all of DFI category.

Keywords: alpha lipoic acid, swim up, density gradient centrifugation, sperm motility, sperm DNA fragmentation index.

INTRODUCTION

Intra uterine insemination (IUI) as one of the treatment for infertility, persists low success rate. A factor that contributes to the unsuccessfulness of IUI is sperm preparation. The sperm preparation was aimed to obtain sperms with higher motility and low percentage of DNA damage. There are some methods for sperm preparation that are mostly used, namely Swim-up (SU) and Density Gradient Centrifugation (DGC) methods. Studies showed that the sperm preparation could produce a better quality of sperm samples for IUI and in vitro fertilization (IVF), and investigated which of two sperm preparation could generate better sperm quality. In SU method, the sperm cells with higher motility will migrate up into the clear culture medium, whereas in DGC method, the motile sperms will be separated from immotile sperms, leukocytes, and cellular debris [1]. Some studies reported that SU method resulted higher level of DNA fragmentation compare to DGC method [2]. This correlated with the consequence that sperm preparation techniques may induce uncontrolled production of reactive oxygen species (ROS). If the ROS production exceeded the capacity of antioxidant inside the sperm cells, it would impair the DNA integrity [3].

Furthermore, studies have shown that Alpha Lipoic Acid (ALA) is a potent antioxidant that could enhance the ability of sperm motility [4]. In addition, studies also reported that ALA enhances sperm ability to tolerate higher volumes of free radical attack, thus protecting the DNA integrity of the sperm [5]. Moreover, ALA also has proven in decreasing the sperm DNA fragmentation, related to the production of ROS [6].

This study aimed to re-evaluate the efficiency of the DGC and SU methods in selecting sperm before being transferred for IUI by the supplementation of ALA, based on the sperm DNA integrity.

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METHODS

Semen Collection and Analysis

Semen samples were obtained from 13 men from partners of women who were infertile (normozoospermia) and underwent IUI. The female partners were unable to conceive after had unprotected sexual intercourse for 24 months. The ethical clearance was approved by the institutional ethical committee. Semen analysis based on the guideline of World Health Organization (WHO) 2010 was performed to analyze the sperm motility and velocity, before and after sperm preparation [7]. Aliquots from each sample were taken for SU and DGC sperm preparation and also for sperm DNA integrity assay.

Swim-up

One mL of whole semen was washed with 1 mL Sperm Rinse medium (Vitrolife, Gothenburg, Sweden) in 15 ml Nunc conical tube (Thermo Scientific Nunc, New York, USA) and then centrifuged (Thermo Scientific Centrifuge, New York, USA) at 300xg for 10 min. After the supernatant was removed, then the pellet was transferred into the bottom of a 15 ml Nunc conical tube (Thermo Scientific Nunc, New York, USA) containing 1 mL of Sperm Rinse medium (Vitrolife, Gothenburg, Sweden). The tube positioned at an angle of 45° and kept for 45 minutes at 37°C. Next, the upper phase was tenderly suctioned and performed in further analysis.

Density Gradient Centrifugation

Two mL each of 90, 45% Sperm Grad™ medium (Vitrolife, Gothenburg, Sweden) and 1 mL of whole semen in a three-layer was prepared in 15 mL Nunc conical tube (Thermo Scientific Nunc, New York, USA), then centrifuged (Thermo Scientific Centrifuge, New York, USA) at 300xg for 20 min. After the supernatant was removed then the pellet was suspended in 1 mL Sperm Rinse medium (Vitrolife, Gothenburg, Sweden) and centrifuged (Thermo Scientific Centrifuge, New York, USA) again at 500xg for 10 min. The pellet was re-suspended in 0.5 mL Sperm Rinse medium (Vitrolife, Gothenburg, Sweden) and continue to perform further analysis.

ALA Incubation

An aliquot of ALA (Santa Cruz, California, USA) in 0.625 mg (ALA 1), 1.25 mg (ALA 2) and 2.5 mg (ALA 3) suspension was added into post-SU and post-DGC sperm sample for 1 hour at 37ºC. After that, semen analysis for sperm motility and velocity was performed before and after ALA incubation.

Sperm DNA Integrity Assay

The SpermFunc® DNAf kit (BRED Life Science Technology Inc, China) was performed to assay the sperm DNA integrity or fragmentation. The agarose in the tube was melting in a 90-100ºC water bath for 5 minutes then 25 µl of semen were added and mixed to the agarose. Then 25 µl of suspension was placed into a 0.65% agarose-coating slide and 20x20 mm cover-slip was placed to cover it. Next, the slide was then kept in a horizontal position at 4°C for 5 minutes. After the suspension had congealed, the coverslip was tenderly uncovered and the slide was nurtured with a lysis solution for 25 minutes at room temperature. Then the slide was washed with ddH_2O for 5 minutes. A series of ethanol (70, 90 and 100%) was performed for 2 minutes each for progressive dehydration. After the slide was dried, then it was stained by Wright staining for 25 minutes. After rinse-and-dry treatment, the slide was observed for the unfragmented DNA (big and medium halo) and fragmented DNA (small, no halo and degraded sperm cell) in 500 sperm cells by two observers [2]. The percentage of unfragmented and fragmented DNA was calculated as the sperm DNA fragmentation index (DFI). There are three classifications of DFI, termly (1) good (DFI 0-15%), (2) moderate (DFI >15-<30%) and (3) severe (DFI >30%) [8].

Statistical Analysis

This study was statistically analyzed using SPSS version 16th software, and statistical tests were deemed significance at $p<0.05$. A non-parametric Mann-Whitney test was demonstrated to compare between the percentage of motile sperm and sperm velocity of whole semen and prepared sperm (post SU or post DGC)

RESULTS AND DISCUSSION

The sperm quality of the selection process for IUI was measured as the percentage of motile sperm and velocity. This study showed that the percentage of motile sperm was higher in prepared sperm (post-DGC and post-SU) than in whole semen. Furthermore, the percentage of motile sperm-was higher in post-DGC compared to post-SU. (Table 1A & 1B)

TABLE 1. The sperm motility in whole semen and prepared sperm with ALA supplementation

1A.

	Whole semen	**Post-DGC**				**p-value**
		ALA (-)	ALA 1	ALA 2	ALA 3	
Percentage of motile sperm (%)	54.0±2.5	88.0±4.4	92.0±6.5	90.0±5.2	95.0±4.8	0.31
Velocity (µm/s)	23.3±1.7	29.7±1.8	30.0±2.0	30.3±1.9	31.0±1.6	0.23

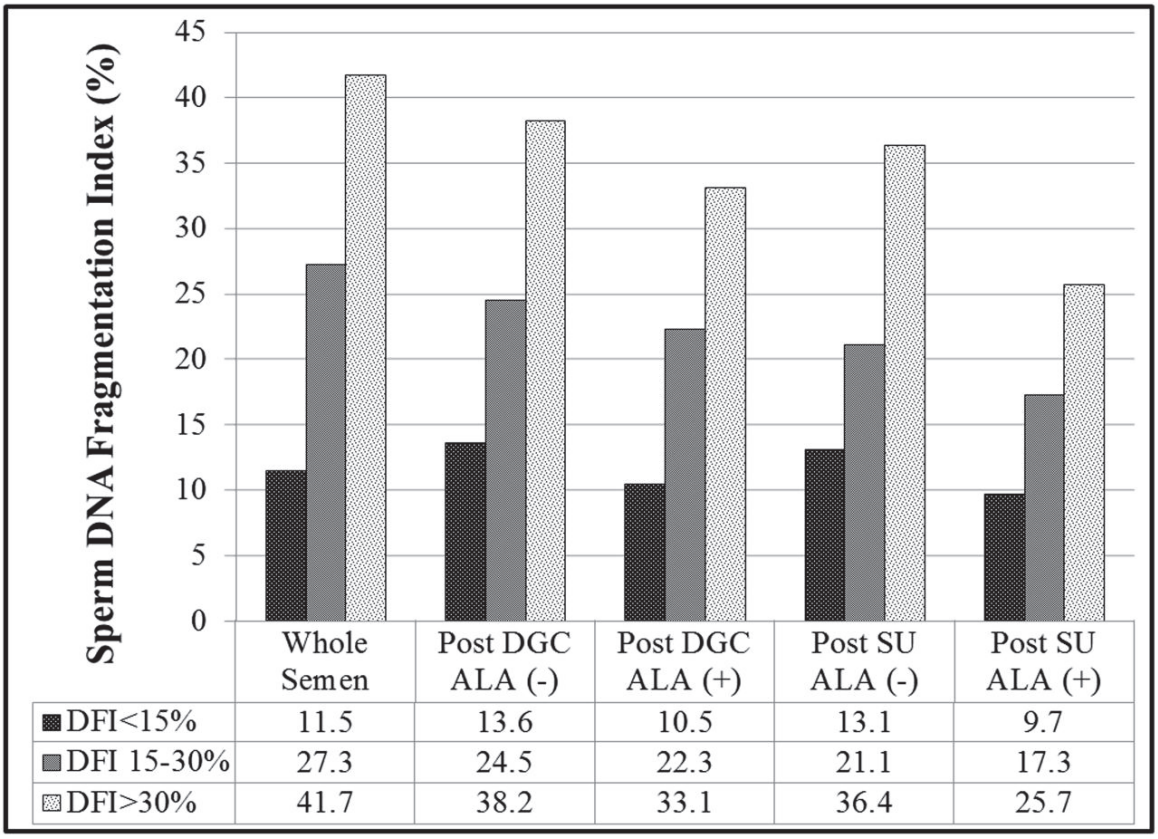
1B.

	Whole semen	**Post-SU**				**p-value**
		ALA (-)	ALA 1	ALA 2	ALA 3	
Percentage of motile sperm (%)	54.0±2.5	75.0±2.8	77.0±3.2	61.0±3.2	54.5±3.5	0.17
Velocity (µm/s)	23.3±1.7	30.1±1.4	33.3±2.1	29.8±1.4	27.9±1.6	0.19

Note: Values are mean ± SE; ALA1 = 0.625 mg, ALA2 = 1.25 mg, ALA3 = 2.5 mg.

In contrast, even though the sperm velocity in prepared sperm was higher than in whole semen, but the sperm velocity in post-SU was higher than in post-DGC. (Table 1A & 1B) It means that SU is not better than DGC method since the sperm quality parameter of normozoospermia sample before sperm preparation was different. The best method for sperm preparation depends on the quality of the sample [3]. Furthermore, the sperm quality of sperm preparation is not qualified enough for the success of IUI. It needs additional substances as an update of sperm preparation method, such as ALA. This study demonstrated that the percentage of motile sperm was higher in ALA supplementation in all concentrations than in without ALA. The percentage of motile sperm was higher in post-DGC compared to post-SU with ALA, (Table 1A & 1B) but post-DGC achieved the highest percentage of motile sperm in ALA3 as the highest concentration, (Table 1A) while post-SU achieved the highest percentage of motile sperm in ALA1 as the lowest concentration. (Table 1B) In contrast, the sperm velocity was higher in post-SU compared to post-DGC with ALA, (Table 1A & 1B) but post-DGC achieved the highest sperm velocity in ALA3 as the highest concentration, (Table 1A) while post-SU achieved the highest sperm velocity in ALA1 as the lowest concentration (Table 1B) This study demonstrated that ALA3 worked properly in DGC method, as to the contrary of ALA1 in SU method. It means that ALA supplementation worked uniquely in different concentration, since the highest concentration does not always perform the best result.

Our study is the first that compare the sperm quality of sperm preparation with the addition of ALA, based on the sperm DNA integrity. In the 'mild' DFI category, the DFI of prepared sperm was higher compared to the whole semen. (Fig.1) This finding promotes the result by Jayaraman et al. that there was an increase of sperm DNA fragmentation during the sperm preparation related to the selection process such repeated centrifugation and incubation at 37°C [3]. Furthermore, by ALA supplementation, the level of DFI was decreased in prepared sperm compared to the whole semen. (Fig.1) Figure 2 confirmed the result by showing the appearance of sperm halos in prepared sperm with and without ALA supplementation. (Fig.2) On the contrary, in the 'moderate' and 'severe' category, the DFI of prepared sperm was lower compared to the whole semen. In contrast to the 'mild' category, this finding did not support the result by Jayaraman et al. [3], but promoted the result by Lestari et al. [6]. This could be explained by the existence of more sperms with DNA damage in the 'moderate' and 'severe' category of sperm sample, than the 'mild' category. In addition, by the supplementation of ALA, the level of DFI was decreased more in prepared sperm compared to the whole semen. (Fig.1)



	Whole Semen	Post DGC ALA (-)	Post DGC ALA (+)	Post SU ALA (-)	Post SU ALA (+)
DFI<15%	11.5	13.6	10.5	13.1	9.7
DFI 15-30%	27.3	24.5	22.3	21.1	17.3
DFI>30%	41.7	38.2	33.1	36.4	25.7

FIGURE 1. The sperm DNA fragmentation index (DFI) of human sperm in whole semen, prepared sperm (post SU and post DGC), with and without ALA incubation.

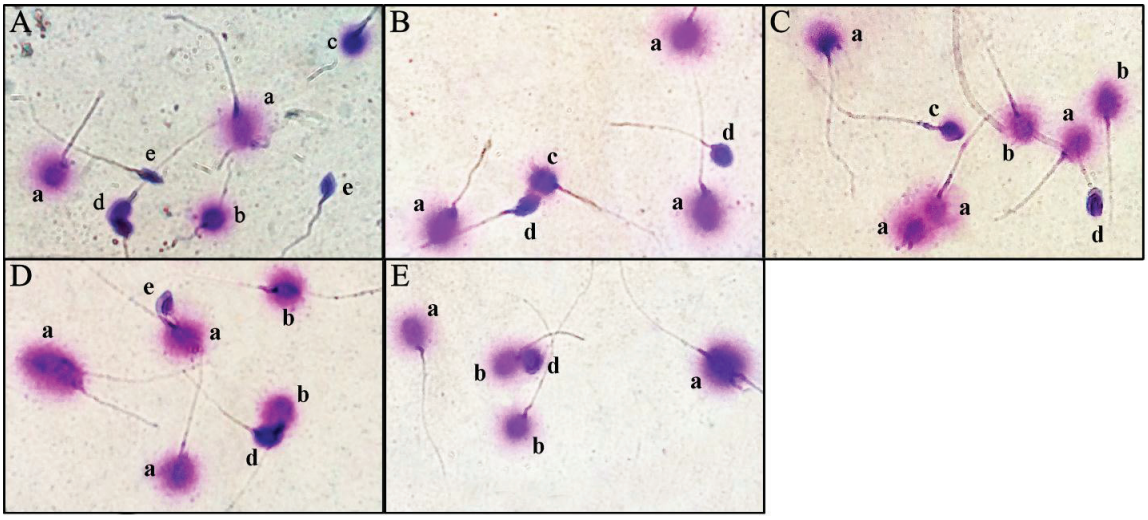


FIGURE 2. The result of sperm DNA integrity. (A) The result of the sperm DNA integrity in whole semen samples; (a) designates a sperm with a big halo; (b) designates a sperm with a medium halo; (c) designates a sperm with a small halo; (d) designates a sperm with no halo and (e) designates a degraded sperm cell. (B) The result of the sperm DNA integrity in post-DGC samples without ALA incubation. (C) The result of the sperm DNA integrity in post-DGC samples with ALA incubation. (D) The result of the sperm DNA integrity in post-SU samples without ALA incubation. (E) The result of the sperm DNA integrity in post-SU samples with ALA incubation. (C) and (E) presented more sperms with big and medium halos, than (B) and (D).

Previous studies demonstrated that the free radical or ROS could induce oxidative stress if the amount exceeded the capacity of the antioxidants inside the cell [9]. Thus, in sperm it will finally cause DNA damage or fragmentation through lipid peroxidation in sperm membrane [10]. Since the high polyunsaturated fatty acids (PUFAs) are contained in sperm membrane, the interaction between ROS and the membrane will generate lipid electrophiles which is associated with the oxidative stress leading to lipid peroxidation [11]. These circumtances could be the main cause of sperm dysfunction which affecting the membrane fluidity, thus decreasing the sperm viability and finally disrupting the sperm DNA integrity. ALA is an antioxidant that enhance the capacity of sperm in tolerating a higher volume of free radical attack. ALA has the ability to create a robust shield on the cell membrane, therefore it is maintaining the structural integrity of sperm cell membrane [12].

Beside the lipid peroxidation in sperm membrane, the sperm viability and motility also depends on energy or ATP supplied by mitochondria, which in contrast, the mitochondria itself generates the free radicals. Thus, the supplementation of ALA will be functioning in the oxidative metabolism by performing as an essential co-enzyme that would enhance the concentration of cytochrome C, thus will regulate the potential membrane of mitochondria. In addition, ALA will also react with other oxidants such as hydroxyl radicals, singlet oxygen, superoxide radicals and prevent oxidative damage. In other words, the regulation of ALA will adequate the ATP for sperm motility and improve the protection of free radical that will cause the mitochondrial dysfunction [13].

Furthermore, ALA is considered as a powerful antioxidant with the ability in regenerating other antioxidants, such as ascorbate and vitamine E from their oxidized forms [14]. Previous studies stated that vitamine E is the major chain breaking antioxidant that protects membranes from lipid peroxidation [15]. ALA also recycles and extends the metabolic lifespan of vitamine C, E and gluthatione [16]. It is assumed that ALA as an antioxidant could prevent the formation of sperm DNA fragmentation through manipulating the oxidative stress mechanism as one of the etiology of sperm DNA fragmentation. This study confirmed the finding by Shen T et al. that ALA increased the percentage of boar motile sperm after freezing [17]. In this study, the sperms with DNA damage as symbolized as DFI, particularly in the ‘moderate’ and ‘severe’ category of DFI, were selected by the sperm preparation in order to get more sperms with integrated DNA as the better sperm quality to be transferred for IUI.

CONCLUSION

In summary, this study is the first to compare the effect of sperm quality by the supplementation of ALA in sperm preparation method, based on sperm motility and DNA fragmentation. In conclusion, ALA was proved to select the better sperm quality with decreased sperm DNA fragmentation of prepared sperm in the all of DFI category.

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