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RESEARCH ARTICLE

Sperm DNA Fragmentation and Chromatin Maturation of Prepared Sperm with ALA Supplementation: The Effect of Embryo Development Quality

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Abstract

This study was aimed to determine the differences in embryo quality between spermatozoa with good or poor of DNA fragmentation index and chromatin maturation, through spermatozoa preparation with the addition of ALA. This experimental study compared the quality of embryos that fertilized by spermatozoa with DNA fragmentation and chromatin maturation, with and without ALA supplementation. In this study semen samples from 20 patients were collected, which were then prepared and examined for DNA fragmentation index (DF) and chromatin maturation (CM). Then the sample was divided into 4 groups, namely the good DF - good CM, good DF - poor CM, poor DF - good CM and poor CM - poor CM, further divided again with and without treatment (ALA supplementation). After injection of spermatozoa into the oocyte, the development of embryo was observed in each group. In the group without treatment, significant differences in embryo quality were obtained between groups of good DF - good CM with poor DF - good CM, good DF - good CM with poor DF - poor CM, good DF - poor CM with poor DF - good CM and good DF - poor CM with poor DF - poor CM ($p < 0.05$). In the treatment group, there were not significant difference in the quality of the embryo between treatment groups, namely with or without ALA, in the group with good or poor DF, and with good or poor CM ($p > 0.05$). Embryo fertilized by spermatozoa with good or poor DF and with good or poor CM, with the supplementation of ALA, the quality was not significantly better than without the supplementation of ALA.

Keywords: *Spermatozoa DNA fragmentation, Spermatozoa chromatin maturation, ALA, in vitro fertilization, Embryo quality.*

Introduction

In vitro fertilization (IVF) by Intracytoplasmic Sperm Injection (ICSI) performs as the promising management towards infertile couple. Despite the successful rate is remaining low in achieving pregnancy [1], an effort to increase the conception from paternal perspective is conducted by the modified of sperm preparation, as the prime option. Paternal factors are associated with the concentration, motility, morfology, chromatin maturation, and DNA fragmentation of spermatozoa [2,3].The sperm preparation modification which contributes to increase the quality of spermatozoa is performed such as the supplementation of hormone and antioxidant. Alpa lipoic acid (ALA) performs as a substrat which capable to regulate reactive oxidative species (ROS), restore other important antioxidants, and involved in the mitochondrial dehydrogenase reaction [4,5].Our previous study investigating the influence of ALA demonstrated the spermatozoa quality was enhance notably in motility, low DNA fragmentation index (DFI) and good chromatin maturation [6,7].

The high DFI defines as the low DNA integrity in spermatozoa, as well as the poor level of chromatin maturation contributed to IVF failure significantly [8].This study was aimed to determine differences in embryo quality between spermatozoa with good or poor DFI and chromatin maturation, through spermatozoa preparation with the addition of ALA.

Methods

Sampling

The number of semen samples taken consecutively was 20 normozoospermia underwent IVF who met with the inclusion criteria (abstinence 2-7 days, semen volume ≥ 2 mL). The written informed consent had been asked and the conducted protocol of this study was approved by the ethical clearance committee, Faculty Medicine, Universitas Indonesia (No. KET-754/UN2.F1/ETIK/PPM.00.02/2019).

Sperm Preparation and ALA Supplementation

Swim Up

An aliquot of semen (1 mL) was rinsed by *Sperm Rinse Medium*, further centrifuged (300 *g*, 10 min). Supernatant was discarded and resuspended the pellet by 1 mL *Sperm Rinse Medium*. Fixed the tube in 45° and incubated (45 min, 37°C). The upper phase of gently aspirated and further assayed [7].

Density-Gradient Centrifugation

Three layer gradient consisting 2 mL 90%, 45% *Sperm Grade Medium* and 1 mL of semen were prepared and further centrifuged (300 g, 20 min). Supernatant was discarded and the pellet was resuspended with 1 mL *Sperm Rinse Medium*, followed by the centrifugation (500 *g*, 10 min). The obtained pellet was resuspend with 0.5 mL *Sperm Rinse Medium* [7, 9].

ALA Supplementation

The post prepared semen samples was supplemented by ALA 2.5 mg and incubated (1 hour, 37°C) [7].

DNA Fragmentation Assay

DNA fragmentation was evaluated utilizing Spermfunc® DNAf kit (BRED Life Science Technology Inc., China). The conducted protocol was in line with the previous research Lestari et al., 2016. Unfragmented DNA observed as big and medium halo, while fragmented DNA observed as small and no halo. DNA fragmentation was calculated by the DNA fragmentation index (DFI), classified to good (DFI < 30%) and poor (DFI > 30%).

Chromatin Maturation Assay

DNA fragmentation was evaluated utilizing *Sperm Nuclear Chromatin Kit*. The conducted protocol was in line with the previous research [3].The imature chromatin spermatozoa were stained blue, while the mature were red-purple. The chromatin maturation analysis was classified into good (>70%) and poor (<70%).

ICSI

Spermatozoa obtained from the modified sperm preparation was added to micro doplet containing 10% Polyvinylpyrrolidone (PVP) in ICSI dish. Micro doplet was prepared consisting GMOPs medium to place the fresh oocyte. Further, micro doplet was covered by liquid paraffin. During ICSI, single oocyte was injected by a viable and normal morphology of spermatozoa.

Spermatozoa was immobilized by touching the tail with injection pipette. Oocyte with the extruded polar body placed in 6 or 12 o'clock. Spermatozoa was aspirated and penetrated to the ooplasma at 3 o'clock position. and the pipette was withdrawn slowly. The injected oocyte placed in a drop containing culture medium (Cleavage and Blast) and further incubated for three days (37°C, 5% O_2, 6% CO_2, 5% N_2) [3, 6].

Embryo Development

The fertilyzed oocyte obtained in 16-18 hours after ICSI, presented by the appearance of two pro nuclei (2PN), while the embryo cleavage evaluated on day-3 (68-72 hours after ICSI), initiated by the presence of blastomeres. The quality of embryo development was defined based on the amount of blastomeres (category A. Good: 4 cells (day-2), 7-9 cells (day-3); B. Moderate: 5-6 cells (day-2), 6 cells (day-3); C. Poor: <4 cells (day-2), <6 cells (day-3), the similarity of blastomeres (category A. Good: all blastomeres are in equal size; B. Moderate: <50% of blastomeres are in unequal size; C. Poor : >50 % of blastomeres are in unequal size) and the existence of fragmentation (category A. Good : <10 % of fragmentation;

B. Moderate: 10-25% of fragmentation; C. Poor : >25% of fragmentation) [10, 13].

Statistical Analysis

The comparison between the outcome of modified sperm preparation with ALA was measured by Wilcoxon test. The influence of modified sperm preparation, DFI, chromatin maturation of spermatozoa towards embryo development were measured by Friedman test. Data were analyzed using SPSS 16th.

Results

DNA Fragmentation Assay

The assesment of DNA fragmentation performed an overview of halo, shown as Figure 1, between group after preparation with and without ALA supplementation. Both groups demonstrated a varied of halo (large, medium, small and no halo). Yet the group with ALA supplementation presented more large halo than without ALA.

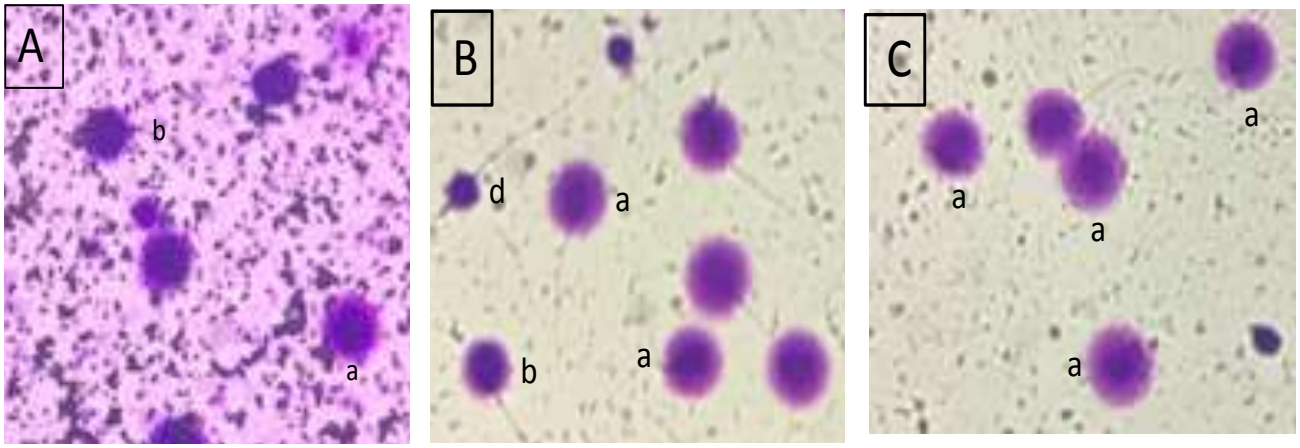

Figure 1: The results of DNA fragmentation assay (A) Before preparation; (B) After preparation, without ALA; (C) After preparation, with ALA. (a) Large halo; (b) Medium halo; (c) Small halo; (d) No halo and (e) Degraded spermatozoa

Table 2: The result of DNA fragmentation spermatozoa

		Group		*p*
DFI	**Before Preparation**	**After preparation without ALA**	**After preparation with ALA**	0.268
Good	10	12	15	
Poor	10	8	5	
Total	20	20	20	

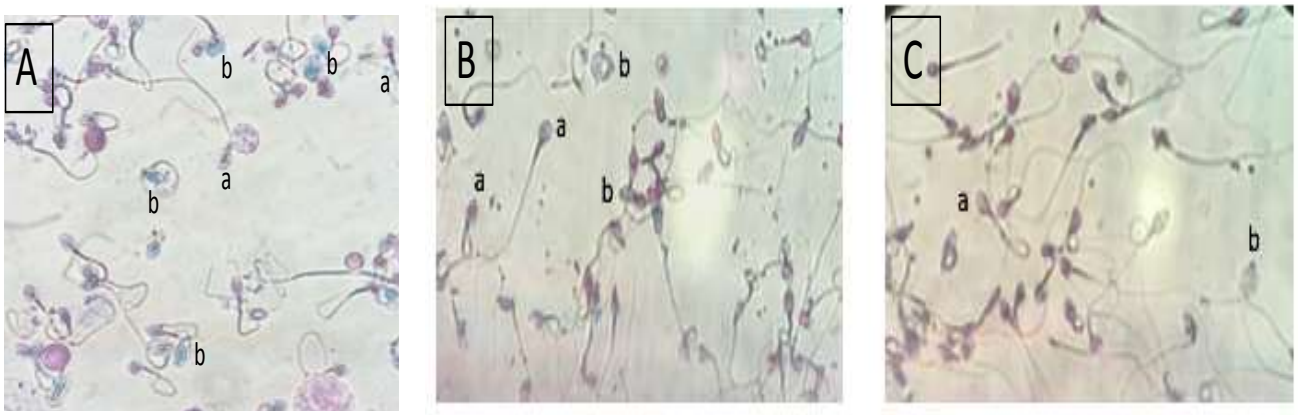

Figure 2: The result of chromatin maturation assay. (A) Before preparation; (B) After preparation, without ALA; (C) After preparation, with ALA. (a) Mature chromatin (red-purple); (b) Imature chromatin (blue).

Table 2: The result of chromatin maturation spermatozoa

	Group			*p*
Chromatin maturation	**Before Preparation**	**After preparation without ALA**	**After preparation with ALA**	0.936
Good	10	9	9	
Poor	10	11	11	
Total	20	20	20	

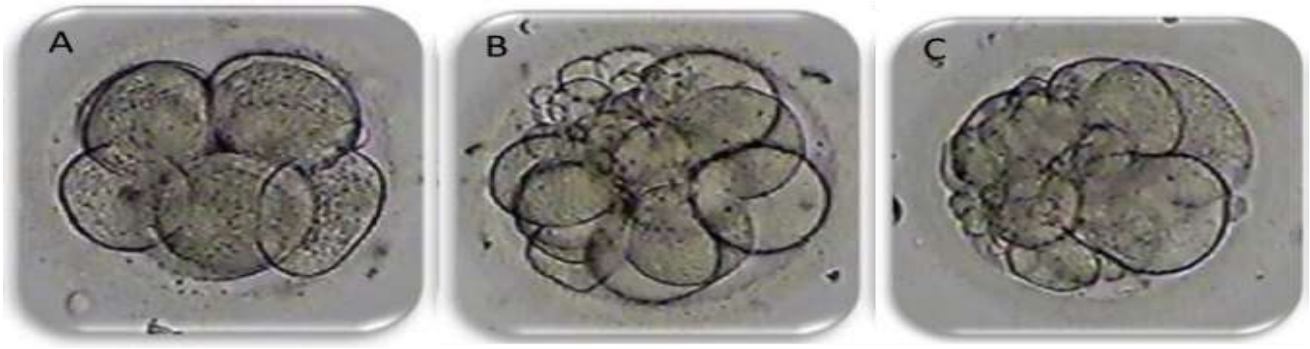

Figure 3: Embryo development (Day-3, 8 cells). (A) Good quality; (B) Medium quality, (C) Poor quality

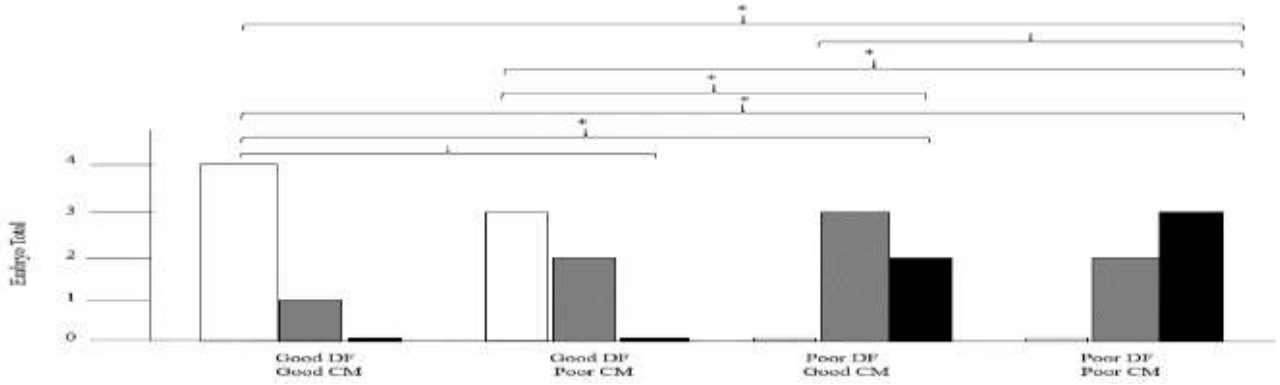

Figure 4: Comparative test between groups (without treatment) Note: White bar = Good embryo quality (A), Gray bar = Medium embryo quality (B), and Black bar = Poor embryo quality (C), DF: spematozoa DNA fragmentation, CM: spermatozoa chromatin maturation, * = sign of significance (p <0.05)

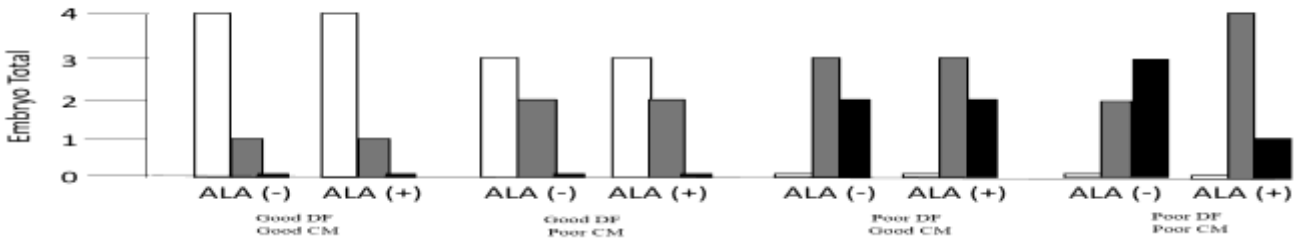

Figure 5: Comparative test between treatment groups. Description: White bar = Good embryo quality (A), Bar gray = Medium embryo quality (B), and black bar = poor embryo quality (C), DF: spermatozoa DNA fragmentation, CM: spermatozoa chromatin maturation, ALA (-) : without supplementation of ALA and ALA (+) : with ALA supplementation

DNA fragmentation with good and poor category in spermatozoa before preparation, after preparation with and without ALA supplementation presented in Table 2. The total number of sample with DNA fragmentation with good category were multiplied either after spermatozoa preparation or after ALA supplementation, despite no significant ($p = 0.268$).

Chromatin Maturation Assay

The assesment of chromatin maturation of spermatozoa presented the spermatozoa were stained by blue indicating for immature chromatin in all of the groups. Group of ALA supplementation presented more mature spermatozoa than without ALA group. There was no significant difference between chromatin maturation of spermatozoa with good and poor categories ($p = 0.936$).

Embryo Development

The quality of the embryo was presented as Day-3 embryo, consisting of 7-8 cells or less, with an equal and unequal size, with and no fragmentation, resulting in the differences quality of embryo (Figure 3).

Comparation between Groups without Treatment

There were significant differences of the quality of embryo in several groups: good DF-good CM vs poor DF-good CM; good DF-good CM vs poor DF-poor CM; good DF-poor CM vs poor DF-good CM; good DF-poor CM vs poor DF - poor CM ($p < 0.05$).

There were insignificant difference of the quality of embryo in two groups: good DF-good CM vs good DF-poor CM; poor DF-good CM vs poor DF-poor CM ($p > 0.05$). (Figure 5).

Comparation between Groups with Treatment

There was insignificant difference between embryo quality among groups, with and without ALA supplementation group, good and poor DNA fragmentation of spermatozoa, and good and poor chromatin maturation of spermatozoa (Figure 5).

Discussion

DNA Fragmentation Assay and Embryo Quality

In this research, we evaluated the functionality of spermatozoa towards the DNA fragmentation, further classified to good DNA fragmentation (DFI < 30%) and poor DNA fragmentation (DFI>30%). This findings was not in accordance to our previous research stated there was significant differences after ALA suplementation [7]. It was suggested due to the different kind of data and statistic measurement among the research.

Good quality of embryo were more presented in good DNA fragmentation than in poor DNA fragmentation group. In the same DNA fragmentation group (good DF-good DF; poor D-poor DF), the total number of the good quality of embryo was insignificant. This finding was in line with Avendano et al (2009), stated there was negative corelation between DNA fragmentation and fertility rate and embryo development. The impact of fragmented DNA was still controversial, yet the deleterious effect towards embryo development and pregancy rate was proven. Bungum et al (2004) demonstrated the the fertilization of spermatozoa (DFI > 27%) was not followed by the preganncy after IVF-ICSI [14].

Chromatin Maturation Analysis and Embryo Quality

There was no different between chromatin maturation among before and after preparation, either in ALA suplementation group or not. To the best of our knowledge, this was the first study investigating the effect of ALA supplementation to prepared spermatozoa, in maturation chromatin perspective. The different presented in the effect of ALA supplementation could select the good DNA fragmenattion after preparation, therfore in this study the supplementation of ALA could not be used in spermatozoa selection with good chromatin maturity after preparation. The good quality of embryo more presented in good and poor chromation maturation, as well the poor quality of embryo. This finding was in accordance to Zini et al., 2005 which did not find the corelation between Chromomycin A3, Acridine Orange and Toluidine Blue toward the fertilization rate after IVF-ICSI [15].

Strengthening this study by others research, Hammadeh et al, demonstrated there was no corelation between spermatozoa with abnormal chromatin and fertilization rate. The assesment of this study was performed in whole semen containing not functional spermatozoa, not survive or degeneration state with abnormal chromatin. In this study, we evaluated the chromatin maturation after preparation. Sperm preparation pulled out the abnormal spermatozoa yielding the motile and normal morphology spermatozoa. Yet the detected spermatozoa still could not representing the spermatozoa population which selected prior ICSI.

The Effect of ALA Supplementation Towards Chromatin Maturation Analysis and Embryo Quality

ALA supplementation in sperm preparation could enhance the embryo quality. Previous research showed that ROS induced the oxidative stress if the amount exceeds capacity of antioxidants in the cells, including spermatozoa [16]. DNA fragmentation could induce from the lipid peroxidation in the spermatozoa membrane [17].Due to the acidity of high polyunsaturated fat (PUFA) contained in the spermatozoa membrane, interaction between ROS and membrane leading to electopphilic lipid, involoved in oxidative stress which casued lipid peroxidation [18].

ALA enhance the spermatozoa capacity by more tolerable to free radicals. ALA maintain the integrity of membrane structure, protects from lipid peroxidation [19, 20] and regenerate another antioxidants, ascorbate and vitamin E, from oxidized form [4]. ALA also recycles and extends the shelf life of vitamin C, E metabolism and glutathione [21].

ALA demonstrated could protect rat embryos against stress oxidative [22].ALA was found could enhance the maturation rate of cloned-goat oocytes [23]. Further research performed that ALA reduced apoptosis by suppressing apoptotic activator gene [23] and triggered the activation of antioxidant genes in protecting from stress oxidative [23, 24].

Although there had been many studies investigating the effect of ALA on spermatozoa as well as animal embryos, but the results of this study indicated that the supplementation of ALA in spermatoza preparation did not significantly improved the quality of human embryos after IVF-ICSI.

The good motility and morphology of spermatozoa, followed by the the intact DNA and the mature chromatin, will be ideal factor to fertilize the oocyte yielding the optimal embryo. It is well known that the oocytes able to repair the damage of spermatozoa genetic material. Indeed, ALA had proven to improve the quality of spermatozoa after preparation, both in terms of DNA fragmentation, but maybe ALA unable to improve the quality of the embryo after IVF-ICSI. The limitation of this study was not only due to spermatozoa detected still did not reflect the spermatozoa selected for ICSI, was also limited by the quantity and the quality of oocytes obtained from various female respondents who participated IVF-ICSI program.

Conclusion

Embryos fertilized by spermatozoa with good or poor FD and with good or poor MK, with the addition of ALA, the quality is not significantly better than without the addition of ALA

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Authors' Contribution

Silvia W Lestari: First author of the manuscript, journal collection and supervisory role of the review article.

Anom Bowolaksono, Asmarinah, Aucky Hinting, Supardi journal collection, data analyze and manuscript review art.

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