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RESEARCH ARTICLE

Prolactin Supplementation in Sperm Preparation A Study of Sperm DNA Fragmentation

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Abstract

One of the infertility management is performing intra uterine insemination (IUI), yet several reports indicated that the success rate is remained low. Sperm preparation, as one of contributing factor to IUI succeeds, has a possibility in generating on sperm DNA fragmentation. Recently, prolactin (PRL) is proved in enhance the sperm motility and protect the DNA integrity of the sperm. This study aimed to evaluate the efficiency of sperm preparation, in selecting sperm prior being utilized for IUI by PRL supplementation, based on the sperm DNA fragmentation. Semen samples were gained from 10 normozoospermic male who underwent sperm preparation for IUI. Semen analysis was performed to measure the sperm motility and velocity, before and after sperm preparation based on the WHO guideline. Samples were incubated at 500 ng (PRL 1), 750 ng (PRL 2), and 1000 ng (PRL 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 than in whole semen. PRL supplementation could not increase the percentage of motile sperm in sperm preparation by DGC method, while in SU method PRL worked uniquely in different concentration, since the lowest concentration performed the best result. The level of DFI after the supplementation of PRL was decrease in prepared sperm compared to the whole semen. PRL was proved to select the better sperm quality with decreased sperm DNA fragmentation in sperm preparation, particularly in moderate and severe DFI category.

Keywords: *Density gradient centrifugation, Prolactin, Sperm DNA fragmentation, Sperm motility, swim up.*

Introduction

One of the management of infertility is intra uterine insemination (IUI), even the success rate of IUI is reportedly low, ranging approximately 10-20% [1]. Sperm preparation contributes to the success of IUI because sperm do not pass the natural stage that occurs in *in vivo* fertilization.

In in-vivo fertilization, the sperm must pass through the mucous layer of the female reproductive tract, completing the capacitance and undergoing an acrosome reaction. These stages do not occur in the IUI process, hence the development of sperm preparation techniques is essential [2, 3].

There are two methods of sperm preparation, namely swim up (SU) and density gradient centrifugation (DGC), which performed to produce high motile sperm count. The SU method uses the principle of an active self-migration of sperm capability that removes plasma semen and debris; hence the percentage of motile sperm with more normal morphology is obtained. The DGC method separates motile sperm based on sperm ability to penetrate into solution with gradient or different concentration by centrifugation, hence it will select sperm with better motility ability compared with non-progressive sperm or immotile that retained

at gradient solution [4, 5]. It is necessary to increase the success rate of IUI by increasing the sperm quality after sperm preparation. Besides the sperm motile count, sperm DNA fragmentation is also reported to be associated with the success rate of IUI. Furthermore, sperm DNA fragmentation is stated to be associated with implantation failure, success rate of in vitro fertilization (IVF) and failure of embryonic development. In addition, sperm DNA fragmentation may also result from techniques used in sperm preparation, through the formation of reactive oxygen species (ROS).

To date, several studies have investigated the role of some substrates involved in preventing sperm DNA fragmentation, associated with uncontrolled ROS activity, such as antioxidants or hormones [6, 7]. The prolactin hormone (PRL) is known to be in correlation with sperm motility, particularly being involved in sperm capacitation and acrosome reaction. [8, 9]. However, until now the other underlying mechanism at the cellular or molecular level are not clear. Therefore, this study was conducted to determine the effect of prolactin supplementation on sperm from sperm preparation of SU and DGC methods, based on sperm DNA fragmentation.

Methods

Sample Collection

Semen samples were gained from 10 normozoospermic male who underwent sperm preparation for IUI. All patients agreed to enroll in this research by signing informed consent forms and this research was approved by Fakultas Kedokteran Universitas Indonesia (FKUI) research ethical committee with the ethical number 796/UN2.F1/ETIK/2017. Semen samples were obtained by masturbation after male partners eligible for abstinence for 3 - 5 days. The ejaculated semen is collected in a sterile container, followed by the semen analysis [10].

Semen is dripped on Makler counting chamber and observed under a microscope in 100-time magnification on 100 sperm. According to WHO, normal progressive sperm motility is determined as more than or equal to 32%, whereas total sperm motility is defined as more than or equal to 40 % [10].

Semen analysis for motility and velocity was performed before and after sperm preparation. Aliquots were taken for sperm preparation and sperm DNA fragmentation assay.

Swim-up

The sperm preparation with the SU method was done by using Sperm Rinse reagent (Vitrolife, Gothenburg, Sweden). Into a 15 ml Nunc conical tube (Thermo Scientific Nunc, New York, USA), slowly inserted the reagent and semen with a 1:1 ratio, then centrifuged at 300xg for 10 minutes. (Thermo Scientific Centrifuge, New York, USA) After the supernatant was discarded, then the pellet being placed into the bottom of the tube which containing of 1 ml of Sperm Rinse reagent. The tube was tilted at 45° in the incubator at 37° Celsius for 45 minutes. Then, sample was taken on the surface of the solution in a slowly rotating motion and continued for further analysis.

Density Gradient Centrifugation

Sperm preparation by DGC method using Sperm Grade reagent .(Vitrolife, Gothenburg, Sweden) Into 15 ml tubes were inserted 2.5 ml gradient 45%, 90% and semen respectively. Next, the tube was centrifuged (Thermo Scientific Centrifuge, New York, USA) at 300xg for 20 minutes. The supernatant was discarded and the pellet was added with 2 ml of Sperm rinse reagent (Vitrolife, Gothenburg, Sweden), then homogenized and centrifuged at 300xg for 10 minutes. The supernatant was discarded, and then the pellet was added with 2 ml of Sperm Rinse reagent, then homogenized and centrifuged again at 300xg for 8 minutes. At last, the gained pellet was analyzed further.

Prolactin Incubation

The sperm samples were taken 2 µl with sperm concentration of 5 million / ml, then incubated using Prolactin Human Recombinant at concentration at 500 ng / ml (PRL1), 750 ng / ml (PRL2) and 1000 ng / ml (PRL3). Subsequently, the sample was incubated at 37°C for 30-45 minutes. After that, semen analysis for motility and velocity of sperms was performed before and after PRL incubation.

Sperm DNA Fragmentation Assay

Sperm DNA fragmentation assay was done by using Sperm chromatin dispersion (SCD) method with Spermfunc DNA f kit (BRED Life Science Technology Inc, China).

Twenty- five µl sperm samples with concentrations of 5-10 million/ ml were inserted into melted agarose gel-filled tubes, then homogenized and dripped onto agarose-coated slides. The slides are then covered with a slide cover and incubated at 2-8°C for 5 minutes. Then slide cover is slowly uncovered, and slide is placed on the shelf then drip denaturation solution for 7 minutes. Subsequently, the slides were re-dripped with a lysis solution for 25 minutes.

Then the slides were rinsed with ddH_2O for 5 minutes. Next, the samples on the slides were fixed with serial concentration of ethanol (70%, 90% and 100%), for 2 minutes per each after drying, the slides were continued with Wright staining for 20 minutes. After were rinsed with ddH_2O and dried, the slides were examined for big, medium, small and no halo (fragmented and unfragmented sperm) by two examiners.[11] Its percentage was defined as sperm DNA fragmentation index (DFI), which further classified into (1) good (DFI 0-15%), (2) moderate (DFI >15-<30%) and (3) severe (DFI >30%). [12]

Statistical Analysis

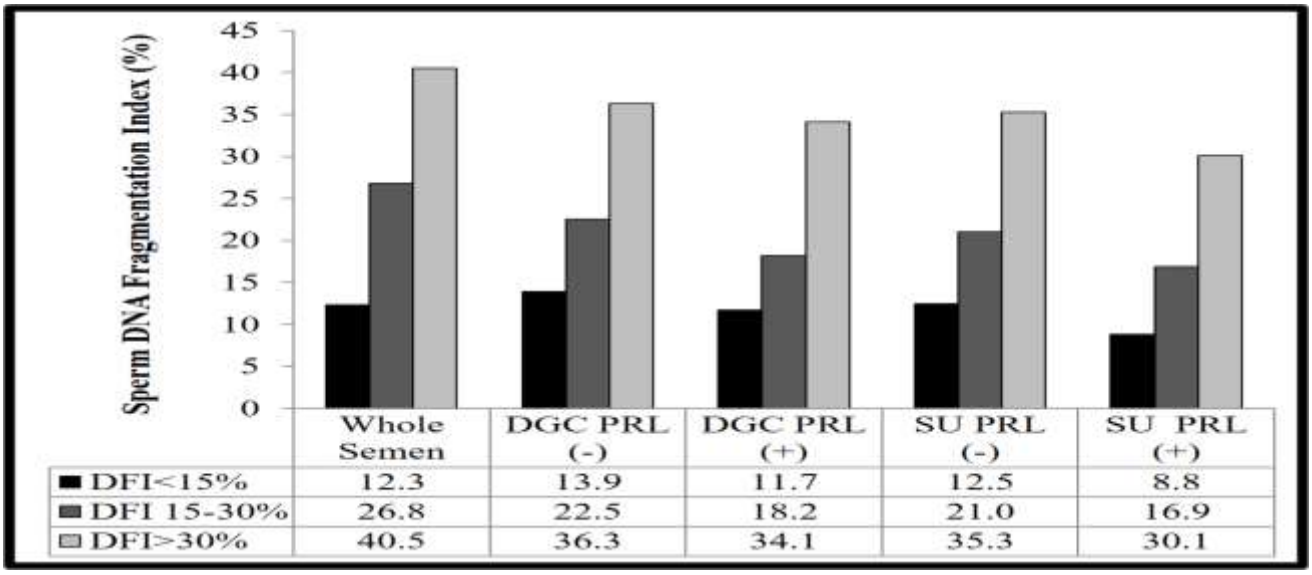
This study used the 22nd version of SPSS to perform the data analysis. The Mann-Whitney was demonstrated to compare between the percentages of motile sperms, sperm velocity and DFI of whole semen and prepared sperm (post SU or post DGC). The level of significance was set at $p<0.05$.

Results

In this study, the selection process for IUI was based on the percentage of motile sperms and velocity and the findings were shown at Table 1. This study demonstrated that the percentage of motile sperm after sperm preparation, using both SU and DGC methods, were increase compare to whole semen. Findings presented the SU methods could produce more motile sperm than the DGC. In sperm velocity, the result showed higher in sperm preparation than whole semen. In particular, the velocity of sperm preparation by DGC method was higher than in SU method.

The findings of the percentage of motile sperm from sperm preparation with PRL supplementation demonstrated that the percentage of motile sperm after DGC method was lower in all concentration, compared to without PRL supplementation. As well as the SU method, PRL2 and PRL3 performed the lower percentage compare to group without PRL supplementation. Interestingly, the percentage of motile sperm by the SU method was the highest in PRL1 as the lowest of PRL concentration among groups.

In the supplementation of PRL, this study showed that the percentage of sperm motility reached the highest value in sperm preparation by SU method at supplementation of PRL1 (500 ng / ml), while with DGC method at supplementation of PRL 2 (750 ng / ml). The findings of the sperm velocity from sperm preparation which showed the highest velocity was performed by DGC method at PRL1 (500 ng / ml) and SU method at PRL 3 (1000 ng / ml concentration), although not significant.



	Whole Semen	DGC PRL (-)	DGC PRL (+)	SU PRL (-)	SU PRL (+)
DFI<15%	12.3	13.9	11.7	12.5	8.8
DFI 15-30%	26.8	22.5	18.2	21.0	16.9
DFI>30%	40.5	36.3	34.1	35.3	30.1

Fig. 1: The sperm DNA fragmentation index (DFI) of human sperm in whole semen, prepared sperm (post SU and post DGC), with and without PRL supplementation

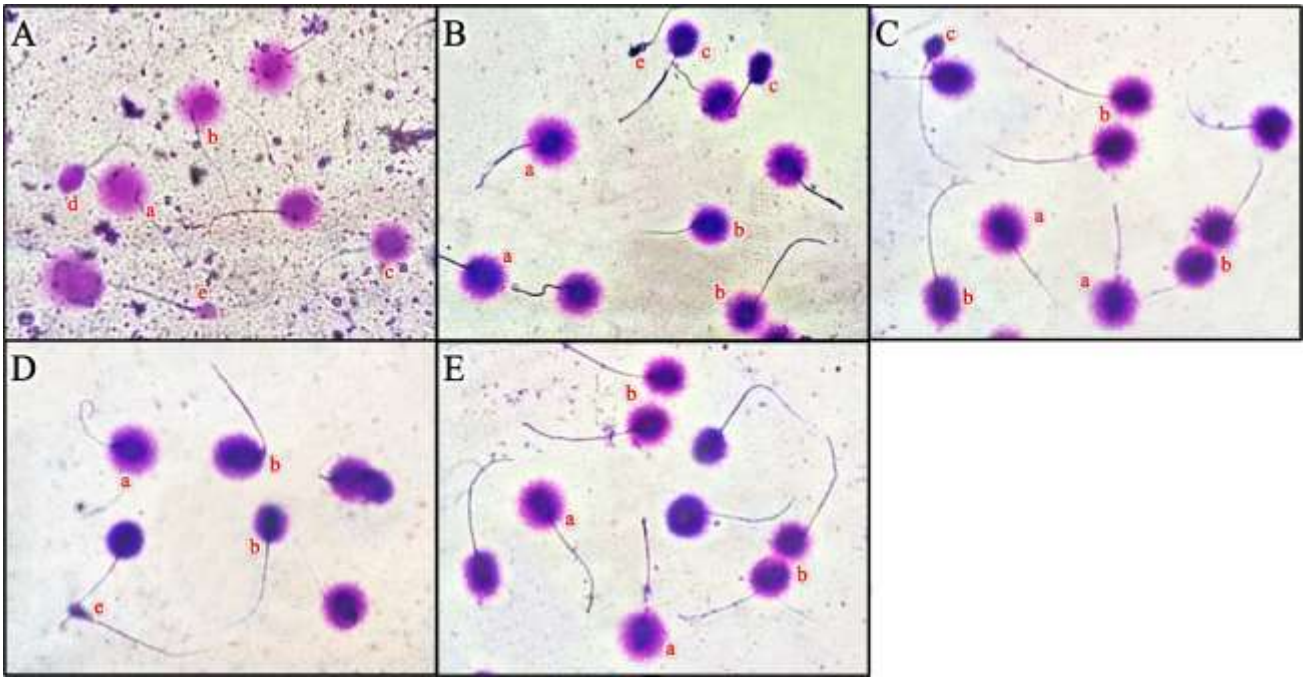


Fig. 2: The result of sperm DNA fragmentation(A) The result of the sperm DNA fragmentation in whole semen samples; (a) designates a sperm with a big halo; (b) designates a sperm with a medium halo; (c) designates a sperm with a small halo; (d) designates a sperm with no halo and (e) designates a degraded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

This study is the first conducted research regarding the comparison of the sperm quality from sperm preparation with PRL supplementation, based on the sperm DNA fragmentation (Fig. 1) at the mild category, the DFI after sperm preparation was higher than before, using both SU and DGC methods. The findings which presented in Figure 1 were confirmed by the appearance of sperm halos after sperm preparation with PRL supplementation. (Fig. 2) In contrast, in the moderate and severe category, the DFI after sperm preparation was higher than before, using the SU and DGC methods, due to the higher amount of sperm with DNA damage in these categories compared to good DFI category. (Fig. 1) Another finding in this study showed that in the treatment group after sperm preparation in SU and DG methods, which accompanied by PRL supplementation also found a decrease in DFI, particularly in moderate and severe DFI categories.

Table 1: The sperm motility in whole semen and prepared sperm with prolactin supplementation

	Whole semen	Post-DGC				Post-SU				p-value
		PRL (-)	PRL 1	PRL 2	PRL 3	PRL (-)	PRL 1	PRL 2	PRL 3	
Percentage of motile sperm	54.7±4.8	81.9±3.4	75.4±4.8	79.8±1.8	75.7±4.0	81.6±3.2	82.8±1.6	78.9±2.8	78.1±2.9	0.001[a];0.001[b]
Velocity	33.1±5.1	35.9±2.4	34.9±2.0	33.3±1.9	34.3±1.7	33.1±1.8	32.0±1.5	33.2±1.6	34.5±2.0	0.567[a];0.28[b]

Note: Values are mean ± SE; PRL1 = 500 ng, PRL2 = 750 ng, PRL3 = 1000 ng; 'a' is the p value from the comparison of sperm from whole semen and post-DGC semen; 'b' is the p value from the comparison of sperm from whole semen and post-SU semen

Discussion

The development of sperm preparation techniques is fundamental for IUI particularly in generating the numerous amounts of motile sperm. The findings of this research indicated that SU method performed in generating more motile sperm than DGC methods. This is in accordance with Volpes et al, [13] which stated that the SU method is better at producing sperm with high motility because the SU method relies on the motile sperm ability to move to the top layer of the sperm preparation reagent. In addition, the results obtained by Jameel [14] also showed an increase in pregnancy rate through the SU method that produces more motile sperm than the DGC method. The current highlight of sperm preparation research is trying to add several agents that considered could improve the quality of sperm such as hormone supplementation.

Prolactin (PRL), as one of peptide hormone, is proved in enhance the sperm motility and protect the DNA integrity of the sperm. In this study, we tried to add PRL as the supplementation of sperm preparation in order to increase the sperm quality after sperm preparation, so it will improve the success rate of IUI. In this research, the supplementation of PRL did not increase the percentage of motile sperm after sperm preparation by DGC method. The author considered that PRL supplementation did not work in DGC method.

The mechanism underlying this phenomenon is remained poorly understood. While in sperm preparation by SU method, PRL worked uniquely in different concentration, since the lowest concentration performed the best result. The result of this study is also supported by the role of PRL in semen that has been known to increase cAMP formation in sperm, stimulate of Akt phosphorylation and restrain phosphatidylinositol-3-OH kinase. Camp is an important factor in regulation of mitochondrial bioenergetic through protein phosphorylation and adenyl cyclase. In addition, PRL also plays a role in energy supply for sperm metabolism and motility [15-17].

PRL is found could prolong the human sperm motility and prevent caspase activation. The result of the DFI after sperm preparation was higher than before; using both SU and DGC methods was in accordance with a study conducted by Jayaraman et al that an increase in DFI after sperm preparation may be caused by recurrent centrifugation or incubation in the incubator. [18] At the DGC method, the presence of two gradient layers will produce motile sperm by eliminating abnormal sperm and debris [19].

This is consistent with the previous study, by Hammadeh et al, stated that the DGC method selects the good morphology of sperm which contain the better DNA integrity by suppressing the numerous amount of fragmented DNA presented in semen[20]. Compared with the DGC method, the SU method has the ability to select sperm with better core vacuolization; therefore sperm preparation using the SU method enhanced more sperm DNA integrity. [5] The previous study, by Lestari et al, reported that the SU and DGC methods were able to select sperm with lower DFI levels compared to the overall semen sample [5]. On the contrary, this finding did not promote the study conducted by Jayaraman et al [18].

PRL receptors identified on some mammalian spermatozoa, mostly on the head and sometimes on the mid piece [9, 21].Numerous researches established in order to investigate the function of PRL receptor in sperm including the study regarding the effect of PRL on human sperm metabolism and the effect of PRL on sperm capacitation. [9] According to the result of this study and other previous supported studies, it is assumed that PRL may play a role in maintaining sperm quality, particularly in sperm DNA integrity.

This may be caused by prolactin acting as a regulator in activation pathway of the apoptotic process. Therefore, the addition of PRL is presumed to have an effect on sperm DNA fragmentation. This study is consistent with a study by Pujianto et al that PRL has a prosurvival effect to sperm. [21]Nevertheless, the effect of PRL on other sperm quality parameter such as the percentage of motile sperm and velocity were different, according to the method of sperm preparation whether SU or DGC and the dose or concentration of PRL [21].

As have stated in the above, the PRL did not increase the percentage of motile in DGC method, compared to SU method. In addition, PRL increased the percentage of motile in the lowest concentration of PRL (PRL1). At last, PRL did not increase the velocity of sperm at after sperm preparation in both methods, almost in all concentration. These results indicate that maybe PRL increased the sperm quality in certain condition, such as in SU method or in dose/concentration dependent [22, 23].

In contrast, those results indicate that maybe PRL had no effect to sperm quality, such as in DGC method, or even suppressed the sperm quality, such as sperm velocity. Other studies also supported this study result, even though in different underlying mechanism. [9, 24, 25] PRL may have various metabolic action to maintain sperm motility, thus this study assumed that PRL could increase the sperm quality after sperm preparation

through maintaining the sperm DNA integrity, particularly in the more severe DFI category. [26]

Conclusion

Although more studies are needed to determine the underlying mechanism that maintain sperm DNA integrity especially in sperm preparation, this study is the first that conducted research about comparison of the sperm quality of sperm preparation with the supplementation of prolactin, based on the sperm DNA fragmentation. In conclusion, prolactin was attested to select the better sperm quality with decreased sperm DNA fragmentation in sperm preparation, particularly in more severe DFI category. It is hope that the result of this study will increase the success rate of IUI.

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