The quality of sperm post-immobilization at some parts of FH sperm using laser diodes

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Abstract. The aim of this study was to find the proper method for sperms immobilization without reducing DNA integrity and damaging the shape of bovine sperms. Sperm immobilization was carried out by double shots using laser Octax MTG with wavelength of 1.48 μm and Eye Ware 3.0 software. A total of 20 sperms were shoot at 4 points, which was the tail end, the mid-tail, the neck and the head of the sperms. The shooting of 1.48 μm laser had the highest effect on immobility reaching 100% on the head and the DNA integrity of the sperms reached up to 90%, the shape of sperms was remained normal (100%) and did not showing significant effect. The results of this study indicated that (1) the laser diode with a wavelength of 1.48 μm at the mid-tail section of the sperm was most effective for sperm immobilization; (2) the use of laser diodes with a wavelength of 1.48 μm can be used to immobilize the sperms before intracytoplasmic sperm injection (ICSI). The diode laser shooting with a wavelength of 1.48 μm at the tail was the most effective in immobilizing the sperms with an immobility of 95.00 %± 5.98 and DNA integrity of 85.00% ± 11.65.

1. Introduction

Intracytoplasmic Sperm Injection (ICSI) is an efficient assisted reproduction biotechnology method because it only requires one sperm cell and an egg cell that has matured to begin the process of forming new individuals [1]. This method was first invented by [2] to prove that the spermatozoa decondensation event and the formation of male pronuclear will not occur before spermatozoa enter the egg. In 1976, Uehara and Yamaguchi continued Hiramoto's research to test the feasibility of using the ICSI method in humans [3].

The report on ICSI's success began with the successful application of the In Vitro Fertilization (IVF) and Embryo Transfer (ET) method in couples who have difficulty getting offspring by Edwards et al. [4]. Since then, scientists have developed a variety of assisted fertilization methods such as the Thinning Zone (ZT), Drilling Zone (ZD), Sub-Zonal Insemination (SUZI) and ICSI advanced methods to provide solutions to various fertilization failures that cannot be solved using IVF methods [5].

The discovery of the ICSI method has ushered in an era of radically assisted reproductive revolution through the use of a single sperm to produce pregnancy in humans and pregnancy in animals [6]. Saili et al. [7] reported that the use of the ICSI method was successful in rabbits [8,9], mice [10], cats [11], horses [12], sheep [13], cattle [14], apes [15], pigs [16] and mice [17]. Before spermatozoa are injected
into the oocyte, every ICSI immobilization is carried out in every spermatozoa movement. This is done so that the spermatozoa are easily inserted into the injection pipette and do not make further movements in the injection pipette. Spermatozoa immobilization is generally done by pressing the spermatozoa tails to the bottom of the petri [18] or by separating the head and tail by ultra sonication [19]. Boediono [20] states that immobilization of spermatozoa in goats prior to ICSI will increase the rate of division in the early stages of embryonic development. However, this has a high risk of damaging spermatozoa. At present, there is a new technique in sperm immobilization before ICSI, namely by using diode lasers. The use of diode lasers can identify spermatozoa that are suitable for ICSI and show an increase in pregnancy success [21]. However, information about laser immobilization of sperm is still very limited.

2. Material and methods

2.1. Thawing
Semen of FH in thawing by dipping 1 straw sperm on goblet containing water with a temperature of 37°C for 30 seconds. Straw is dried with a tissue, then both ends of the straw are cut and placed in a micro-tube. Furthermore, it was incubated on a warm plate at 37°C for 10 minutes, then microscopic evaluation was performed.

2.2. Evaluation post thawing
Semen examination is carried out microscopically. Valuable cement assessment microscopic namely motility, viability, abnormality and intact plasma membrane.

2.2.1. Spermatozoa motility. Spermatozoa motility is calculated by first making a 10 μl spermatozoa spot on the object glass. Spermatozoa were then analysed using CASA with the Sperm Vision Version 3.7.5 program. with observations four times the field of view.

2.2.2. Viability of spermatozoa. Viability of spermatozoa is calculated by mixing evenly 10 μl of cement and 10 μl of Eosin 2% above the object glass. Furthermore, a review was made and observed using a microscope at 200 times magnification with Indomicro View 3.7 software. Dead spermatozoa absorb red and live colorless, at least 100 sperm cells per observation are counted to determine the percentage of live spermatozoa. spermatozoa viability percentage is calculated using the formula:

\[
A = \frac{P}{P+Q} \times 100\%
\]

\(A\) = Percentage Viability
\(P\) = Total Living Spermatozoa
\(Q\) = Total Dead Spermatozoa

2.2.3. Spermatozoa abnormalities. Spermatozoa abnormalities are calculated by mixing evenly 10 μl of cement and 10 μl of Eosin 2% above the object glass. Furthermore, a review was made and observed using a microscope at 200 times magnification with Indomicro View 3.7 software. Abnormal spermatozoa observed were severed tails, broken tails and abnormal head shape of at least 100 sperm cells per observation counted to determine the percentage of live spermatozoa. Furthermore, the preparations were made and examined using a microscope. At least count to 100 spermatozoa cells, the percentage of spermatozoa abnormalities is calculated using the formula:

\[
A = \frac{P}{P+Q} \times 100\%
\]

\(A\) = Percentage Abnormality
\(P\) = Total Abnormal Spermatozoa
\(Q\) = Total Normal Spermatozoa

2.2.4. Intact Plasma Membrane. Intact Plasma Membrane was observed by inserting a 10 μl semen sample into a HOST solution (0.179g NaCl in 100 ml of aquabides), then incubated for 1 hour at 37°C.
Spermatozoa with intact plasma membranes are marked with a circular tail and damaged sperm are characterized by a straight tail. Evaluation was carried out with a 400 times magnification microscope using Indomicro View 3.7 software, counting 100 spermatozoa cells using the formula:

\[
A = \frac{P}{P+Q} \times 100\% \\
A = \text{Intact Plasma Membrane} \\
P = \text{Total Circular Tail Spermatozoa} \\
Q = \text{Total Straight Tail Spermatozoa}
\]

2.3. Spermatozoa selection (swim up)
Spermatozoa selection aims to get proper spermatozoa with the swim up method. In the swim up method, the first step is carried out by inserting a 10 ml TALP medium, then mixing 50 µl of cement. Then centrifugation was carried out at 1800 rpm for 10 minutes and supernatant removal was carried out. The second step, the centrifugation results were put into a 1.5 ml micro-tube containing 1 ml of TALP medium. Then the third stage, allowed to stand on a warm plate for 30 minutes.

2.4. Immobilization spermatozoa use laser diode
The swim up spermatozoa were given 5 µl of Hoechst coloring in a medium containing 7.5% PVP. Next make 8 medium spots each of 5 µl (4 spots containing spermatozoa and 4 spots without spermatozoa). Prepare an inverted microscope with a diode laser and holding pipette controlled by a pneumatic micromanipulator. Sperm immobilization was carried out using a laser wavelength diode of 1.48 µm 2 times [22] with Eye Ware 3.0 software. The treatment of spermatozoa firing was carried out at 4 points, namely the tail tip of the spermatozoa, the tail body of the spermatozoa, the neck of the spermatozoa and the head of the spermatozoa each with 20 spermatozoa cells. Spermatozoa from diode laser firing are then transferred to another spot using a holding pipette for further observation.

2.5. Evaluation post-immobilization sperm
Evaluation of spermatozoa after sperm immobilization is done in a manner microscopically by observing the immobility, integrity and integrity of spermatozoa DNA. Observation of immobility and integrity of spermatozoa is observed shortly after laser shooting. Furthermore, DNA integrity observation was carried out using a Hoechst dye which was observed through a fluorescence microscope with Axio Vision Rail software. 4.8 using DAPI filters.

3. Result and discussion

3.1. Quality spermatozoa post-thawing
The quality spermatozoa post-thawing spermatozoa can be seen in Table 1.

Table 1. Quality spermatozoa post-thawing.

| Parameters                | Average       |
|---------------------------|---------------|
| Motility (%)              | 49.23 ± 1.94  |
| Viability (%)             | 73.46 ± 1.48  |
| Abnormality (%)           | 12.38 ± 3.40  |
| Intact Plasma Membrane (%)| 73.0 ± 0.71   |

In Table 1 shows the quality of post-thawing spermatozoa where motility is (49.23 ± 1.94), viability is (73.46 ± 1.48), abnormality is (12.38 ± 3.40) and plasma membrane for (73.00 ± 0.71). This data shows that the motility quality of post-thawing spermatozoa in this study is in accordance with SNI 4869-1-2017 standards for frozen cow cement products, which have spermatozoa quality showing minimum 40% spermatozoa motility [23]. Garner and Hafez [24] state that the viability of spermatozoa has a minimum of 60% to 75% of live spermatozoa. The percentage of spermatozoa life is determined by the intact plasma membrane because the spermatozoa plasma membrane serves to protect
spermatozoa organelles and electrolyte transport for spermatozoa metabolism [25]. Damaged plasma membrane can affect the physiological function and metabolism of spermatozoa, causing spermatozoa to die [26]. An intact plasma membrane has correlation with spermatozoa motility, the more intact spermatozoa plasma membrane, the more motile spermatozoa [27].

Indonesian National Standard (SNI) SNI 4869-1-2017 frozen cow sperm products require that cow cement has a morphological abnormality both primary and secondary <20% [23]. A similar statement was stated by [28] which states that a male will not have high fertility if spermatozoa are found to have an abnormality of >17%. These results show that the post-thawing spermatozoa used meet the technical feasibility standards.

3.2. Quality spermatozoa post-swim up

The quality spermatozoa post-swim up spermatozoa can be seen in Table 2.

| Parameters                           | Average    |
|--------------------------------------|------------|
| Motility (%)                         | 53.59 ± 3.52 |
| Viability (%)                        | 72.63 ± 1.56  |
| Abnormality (%)                      | 13.71 ± 3.23  |
| Intact Plasma Membrane (%)           | 72.33 ± 1.19  |

In Table 2 it can be seen the quality of spermatozoa after Swim Up has motility of (53.59 ± 3.52), viability of (72.63 ± 1.56), abnormalities of (13.71 ± 3.23) and intact plasma membrane of (72.33 ± 1.19). Spermatozoa quality after swim up shows a higher tendency compared to post thawing. This is because the swim up method makes the separation of living and motile spermatozoa separate from dead and immotile spermatozoa. Pasaribu [29] states that the increase in motility percentage of spermatozoa after the separation process with swim up is caused by the separation of dead spermatozoa from the living. Living spermatozoa can migrate out of the pellet to the surface of the medium, while the dead spermatozoa remain in the lower layer. Research data also shows an increase in abnormalities and a decrease in the intact plasma membrane percentage caused by the mechanical influence of centrifugation forces such as the friction of the surface of the spermatozoa membrane with the tube wall during the swim up process. This is consistent with the statement of Donnelly et al [30] that the separation of spermatozoa by centrifugation can induce damage to the plasma membrane of spermatozoa, resulting in increased abnormalities. Although there is a decrease in the percentage of viability, it is still in the normal level, where the spermatozoa viability is at least 60% [24]. These results show that the post-swim up spermatozoa used meet the technical feasibility standards.

3.3. Quality spermatozoa post-immobilization

The quality spermatozoa post-immobilization spermatozoa can be seen in Table 3.

| Treatments | Parameters | Immobility (%) | Integrity DNA (%) | Intact (%) |
|------------|------------|----------------|-------------------|------------|
| Head       | 100.00 ± 0.00       | 9.38 ± 4.17   | 100.00 ± 0.00    |
| Neck       | 98.75 ± 2.32        | 9.38 ± 10.5   | 100.00 ± 0.00    |
| Mid-Tail   | 95.00 ± 5.98        | 85.00 ± 11.65 | 100.00 ± 0.00    |
| End-Tail   | 86.88 ± 8.43        | 90.63 ± 6.78  | 100.00 ± 0.00    |

In Table 3 it can be seen that the quality of spermatozoa after laser diode shooting has the highest average immobility on the head (100.00 ± 0.00) not significantly different (P > 0.05) with the results of firing on the neck (98.75 ± 2.32) and tail body (95.00 ± 5.98). While the shooting at the tail end (86.88 ± 8.43) was significantly different (P < 0.05) lower than the shooting on the head, neck and tail body. DNA integrity of spermatozoa after laser diode shooting at the tip of the tail (90.63 ± 6.78) and tail body
(85.00 ± 11.65) were significantly different (P <0.05) higher compared to shooting at the neck (19.38 ± 10.5) and head (9.38 ± 4.17).

Based on the results of diode laser shooting on the head, neck and tail body produces a high degree of immobility and not significantly different, but the integrity of DNA spermatozoa from laser diode shooting results on the tail body produces high DNA integrity. Rink et al [31] stated that the shooting of a diode laser with a wavelength of 1.48 μm had a thermal effect that made the spermatozoa immobile. Furthermore Montag et al. [22], states that the thermal effect due to laser shooting makes denotation of protein and permeable of the tail membrane possible. The results of this study show that laser diode firing with a wavelength of 1.48 μm in the tail body has less negative effect on membrane damage resulting in decreased viability. The results of this study also showed that diode laser shooting at the tail end produced high DNA immobility and integrity, but the immobility was significantly lower than in the tail body.

The integrity of spermatozoa after laser diode shooting in this study there is no different value in all shooting parts with a value (100.00 ± 0.00) means that the use of diode lasers with a wavelength of 1.48 μm in all parts of shooting does not affect the integrity of the spermatozoa. These results are in line with the research of Montag et al. [22] stated that the use of diode lasers with a wavelength of 1.48 μl can maintain the integrity of spermatozoa.

4. Conclusion
It is concluded that (1) the laser diode with a wavelength of 1.48 μm at the mid-tail section of the is most effective for sperm immobilization; (2) the use of diode laser with a wavelength of 1.48 μm can be used to immobilize the sperms before intracytoplasmic sperm injection (ICSI).

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