

The Different Calcium⁺² Intensity Profile and Quality of Oocyte and Goat Sperms after Cryopreservation

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Abstract

This research aims were to study the effect of the oocyte and sperms cryopreservation of Indonesian local goat on the post-thawing quality and profile or characters of Calcium⁺² intensity in relating with their fertility capacity. A study was conducted to test the freezing method and post-thawing viability both stock cells stored in the deep freezer and liquid nitrogen (-80°C of vs -196°C). A fertility test of sperms has been conducted through in vitro of sperm quality, while the oocytes cryopreserved test was done by in vitro maturation (IVM) rate (%). The profile of Calcium 2+ was performed and analysis by Confocal Laser Scanned Microscope (CLSM). The result showed that IVM rate of goat oocyte is considered lower when cryopreserved in -80°C than in -196°C. Meanwhile, sperm is considered having a good quality in 2 methods of cryopreservation with post-thawing motility > 40 % (SNI 2014). There is an important difference between Calcium intensity of fresh and post-thawing both for oocyte and spermatozoa. Calcium +2 profiles is varied individually on the peak of intensity, but it considered expressed the same profile of each fresh and post-thawing cell. In vitro fertilization test need to be performed to complete the viability and fertility competence of these sperm and oocyte freezing stocks.

1. Introduction

The induction of Ca⁺² releases in fertilized M-II oocyte following fertilization by sperms was explained by the sperm receptor and the sperms factor theory. Sperm factor postulated as a cytoplasmic factor in the spermatozoa has been used to parthenogenetically activate mammalian oocytes. Spermatozoa release cytosolic substances into oocyte at fertilization and it induces Ca⁺² oscillations [1,2,3]. The parthenogenesis activation of oocytes is an interesting and valid tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development.

Repetitive Ca⁺² rises at fertilization are necessary to accomplish degradation of Matured Promoting Factors (MPF). Calcium oscillation in oocytes is responsible for pronuclear formation. In general, egg activation is caused by a dramatic increase in intracellular Ca⁺² concentrations common to every animal species including mammalian [4]. Mammalian oocytes at the time of fertilization occurred exhibit a series of transient increase in intracellular calcium ion (Ca⁺²) concentration which is prerequisite and sufficient for oocyte activation. Immediately after the sperm attaches to the oocyte, increase in intracellular Ca⁺² in the oocytes are firstly seen near the site of sperm-oocyte fusion.



Cytoplasmic factor in the spermatozoa have been used to activate mammalian M-II oocytes artificially. Ciptadi *et al* [5,6], reported research was being undertaken to characterize the phenotypic variation of intra cellular crude sperm extract (CSE) for improving activation rate of the M-II oocytes.

This research was attempted to study the characters of Ca^{++} intensity of goat sperm and oocytes after freezing by nonconventional method. It is well-established that calcium plays an important role in the capacitation and then fertilization. In this study, we would like to test whether goat fresh sperm have different calcium levels intensity than sperm from freezing (post thawing fertility

2. Material and Methods

2.1. *In vitro* Maturation (IVM) Preparation of Oocyte

The oocytes-cumulus cell complexes were isolated from goat antral follicles with the diameter range of 2 – 6 mm, cultured in bicarbonate-buffered medium 199 supplemented with 10 % FBS, 0.1 mg sodium pyruvate [7]. The culture was carried out in 5 % CO_2 in humidified air at 38°C for 48 h. Oocytes were completely denuded by pipetting after treated with 0.01 % hyaluronidase (Sigma). Only those with a compact, non-atretic cumulus and a homogeneous cytoplasm which were previously classified as Grades A and B [7,8], were selected. Oocytes emitting the 1st polar body were selected in this experiment.

2.2. Calcium Intensity Preparation and Analysis Sperm and Oocyte

Early sperm characters of Ca^{+2} Intensity was performed by Confocal Laser Scanning Microscope (CLSM) through Fluo-3 staining and analysis descriptively. Semen prior to freezing for research has previously been quality tested by SNI with a minimum of 70% of individual motility. Each ejaculate was cryopreserved in commercial diluter according to standard procedures. For each experiment, 0.25 ml straws (40x10 6 sperm/straw) were thawed in a 37°C water bath for 60 seconds. Freezing is done with conventional modified methods with the speed (-1oC /min) with the help of Mr. Frosty system ® and cryopreserved in – 45°C using deep freezer then immersed in liquid nitrogen (LN2) at a temperature of - 196°C, to do quality testing to post-thawing.

3. Result and Discussion

3.1. Different Calcium (Ca^{+2}) Intensity of Sperm and Oocytes

The result of this research showed that different characters of the Ca^{+2} intensity of sperm and oocytes after freezing by the modified conventional method (Figure 1). A freeze on modified simple conventional local goat semen showed good results and meet start to research and development of Artificial Insemination (AI) and its implementation [9]. The intensity of intra-cellular calcium spermatozoa with CLSM showed that there was no change in the pattern of fresh semen and results of modified conventional freezing, but different in the peak of Ca^{+2} intensity [9].

The oocytes intensity of Ca^{+2} showed different profile of each. M-II oocyte with no treatment showed strong enough intensity on the area of zona pellucid [10]. Meanwhile, the oocyte exposed with CSE, Ca^{+2} is well expressed spread out on cytoplasm. However, the responses to CSE supplementation are varied after 24 to 48 hour of culture (Fig. 1). It was assumed that if Calcium $^{+2}$

was expressed in the cytoplasm, it means that it exhibits increase in Calcium intracellular. Other researchers [2,4] mentioned that egg exhibits increase in Ca^{+2} is indications of egg activation. Several recent research results suggest that mammalian sperm factor would be introduced into the oocyte cytoplasm through sperm-egg cytoplasmic connection, initially distributing evenly throughout the cytoplasm [11].

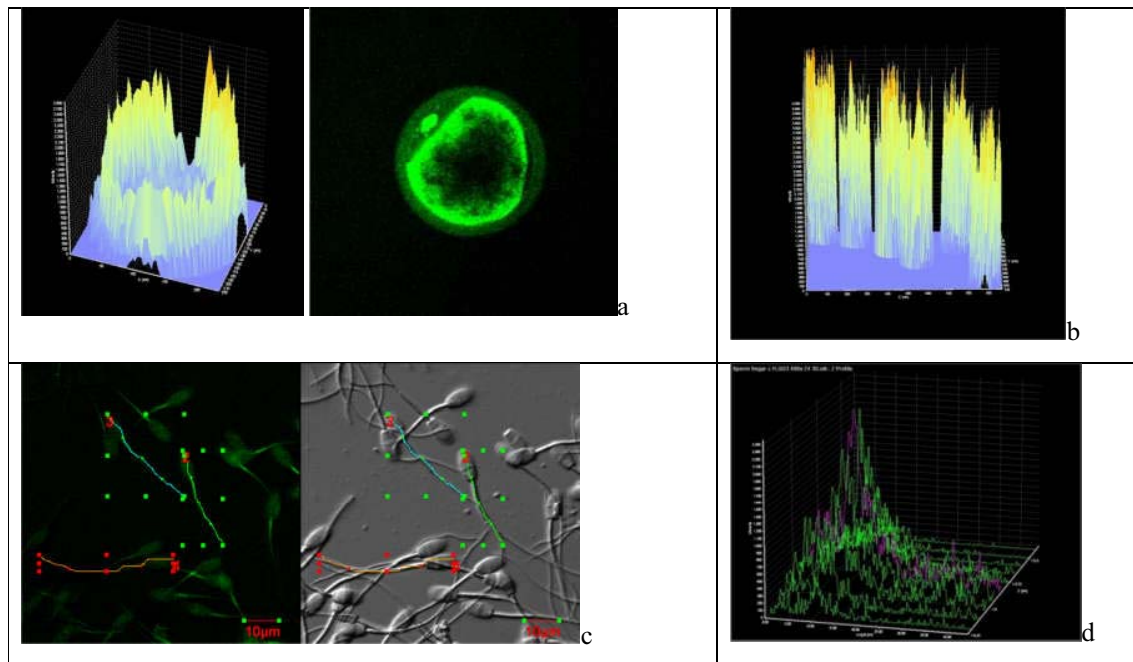


Figure 1. A comparison of Histogram profile of oocyte and sperms . A. Oocyte cultured IVM,48 hours of culture. Calcium intensity expressed mainly zona pellucida. Variation of oocyte response on different oocyte Calcium intensity [11]. B. . the intensity of intra-cellular calcium spermatozoa showed that there was no change in the pattern of fresh semen and results of modified conventional freezing, but different in the peak of Ca^{+2} intensity [9].

A number of experiments have attempted to study the molecular identity of sperm factor protein. Phospholipase C (PLC) zeta was identified as a strong candidate for the mammalian sperm factor[2]. The molecular weight of PLC zeta is approximately 74 kDa [12,13]. [14], confirmed that the most convincing evidence that PLC(z) is the mammalian sperm factor. Further work is required to clarify the component of sperm factor in this goat CSE.

3.2. The Ca^{+2} Profile of Oocyte activated (M-II)

In this research, we study the Ca^{+2} intensity of activated by crude sperm extract (CSE) to replace the sperm function. Activation of mammalian oocytes could be induced by a wide variety of chemical [15] and physical [16] stimulation that mimic the fertilization process by providing calcium signals to the MII arrested eggs by different mechanisms. The transfection method used in this research might be causing the lower result because of the improper method of activation Result of cleavage rate especially using CSE alone (2 %) was lower than achieved by [17] which resulted in approximately 30 % of oocyte cleaved using microinjection method of CSE (Table 1.). CSE may be a representation of the sperm factor complex that having a better influence in activation compared to CSE with 100 kD

proteins specific. It is necessary that a complex of unknown composition of the active factor in CSE to be determined in next research.

The confirmation of Ca^{+2} intensity using fluo-3 through Confocal Laser Scanning Microscope showed the different profile of M-II oocyte activated using CSE. After 48 h activation of the oocyte, it was observed an increase in Ca^{+2} intensity only in the outer region of M-II activated by CSE alone) in contrary the increase of Ca^{+2} intracellular intensity of the oocytes was observed at all sites of M-II activated using chemical agent and CSE. Phenotype variation of Ca^{+2} intensity was varied large among slices observed (Fig.2). In mammalian eggs, Ca_2 oscillations associated with Ca_2 waves occur at fertilization and trigger egg activation [18,19]. Different cleavage rate resulted from activated M-II goat oocytes (Table 1.)

Table 1. Cleavage rate different treatment of parthenogenetic using CSE supplementation [5]

Activation method	Selected M-II Oocytes	Cleavage rate (%)
(1). Control (M-II) oocyte	40	(0.00)
(2). CSE 2.5 ug/ml	251	(36. 33)
(3). CSE of 100 kDa protein	240	(2.00)

The increase of Ca^{+2} intensity that equal to Ca^{+2} concentrations, might lead to egg activation. Oocytes activation was induced by chemical agent supplemented with CSE, with the evidence of rise Ca^{+2} intensity. This was considered to be sufficient factor for activation. [13] suggested that if a large quantity of protein with high ability for oocyte activation was extracted from spermatozoa, it might improve the rate of oocyte activation. Repetitive Ca^{2+} release from the endoplasmic reticulum is necessary for activation of mammalian eggs. Calcium influx during Ca^{+2} oscillations induced by injection of sperm extract into mouse eggs were investigated by Mn2 quenching of intracellular Fura-2 after adding Mn2 to external medium [20]. A qualitative image analysis of Ca^{+2} intensity done by histogram and line series. [21] reported that Calcium regulation by sperm from sires of low fertility appears to be deficient because their post-thaw relative intracellular calcium level is higher than it is in bulls of good fertility. Cryopreservation is known to disrupt the sperm plasma membrane and it induces premature capacitation of a sperm subpopulation, which may be a result of the increased internal calcium levels after thawing [22]. Figure 2 showed the variation of Ca^{+2} intensity in different slice of cells by CLSM.

Simon et al., (2000) [22] reported that sperm intracellular calcium level is correlated with in vivo fertility, with access to intracellular calcium levels in frozen-thawed sperm from bulls of varying degrees of fertility. The ultra-structural changes affect the ability of cells to control calcium flux across their plasma membranes. The different profile of ca intensity has been observed between fresh and post-thawing sperms.

The result showed that different diluter is influenced on viability motility and abnormalities of goat semen. Pre-freezing qualities of viability and motilities were $88.67 \pm 4.16\%$ and $66.33 \pm 1.53\%$ respectively. Meanwhile, post-thawing qualities are considered as good as standard qualities at least more than 40% ($56.0 \pm 5.6\%$). The different diluents were influenced highly significant ($P < 0.01$). The best diluents ration is 1:4 (v/v) for both final sperms stocked. However freezing sperm conserved in -196°C is better than -45°C (i.e. motility $39.3.94\%$ vs. $56.0 \pm 5.6\%$). Meanwhile, the sperm characters of two local breed showed the important variation of Ca^{+2} intensity, with the length of region measurement of 39.06 ± 4.595 and $32.696 \pm 9.011 \mu\text{m}$ each. It was concluded that this conventional method of the freezing system was considered as a feasible method for goat semen in the reason for practical purposes of Artificial Insemination.

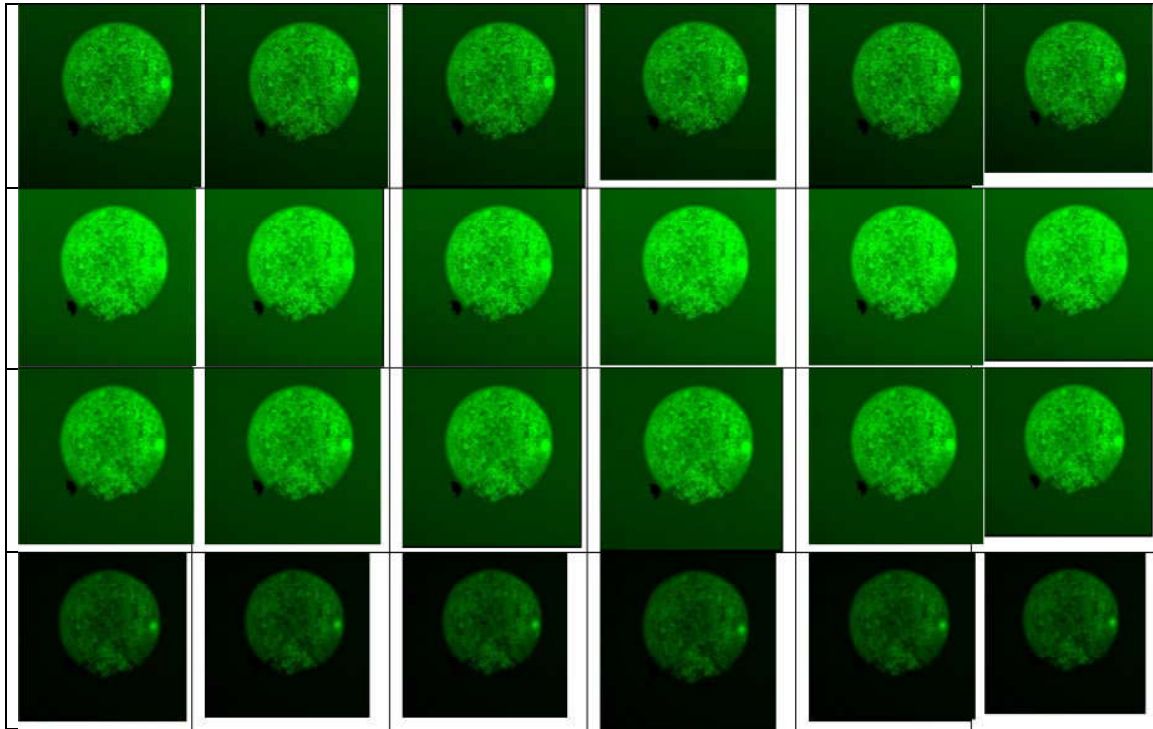


Figure 2. Different Calcium intensity base on the slice of oocyte performed by CLSM.

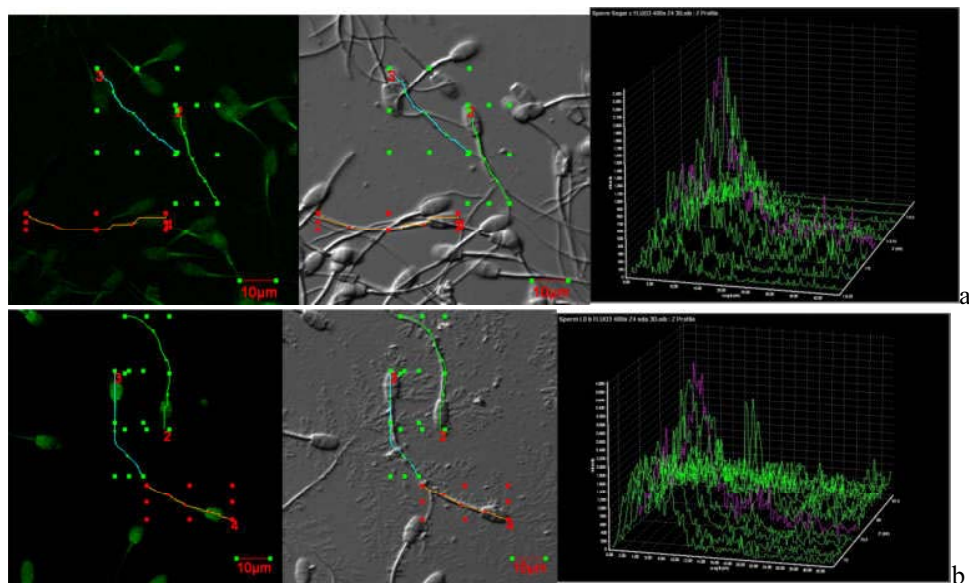


Figure 3. Different sperm Calcium Intensity: a. Fresh spermatozoa fresh and post thawing. Different profiles were observed mainly in peak of calcium intensity. Different profile of Calcium intensity of local goat sperm. It was analysed by Confocal Laser Scanning Microscope CLSM : 400, Z.4 , 3 D. [6,9]

4. Conclusion

The increase of Ca^{+2} intensity was considered to be sufficient indicator activation of M-II oocyte is occurred. The supplementation of CSE could improve the induction of M-II oocyte activation. The calcium intensity is varied more and higher in fresh sperm than freezing sperm. The sperm frozen by nonconventional could be used for the application of AI. The increase of Ca^{+2} intensity was considered to be sufficient indicator that showed activation of M-II oocyte occurs. This method needs to be enhanced using diluents and cryoprotectant more appropriate.

Acknowledgments

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