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FAKULTAS KEDOKTERAN HEWAN UNIVERSITAS AIRLANGGA
Animal Care and Use Committee (ACUC)

KETERANGAN KELAIKAN ETIK

" ETHICAL CLEARENCE "

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Dekan FKH-Unair,

Prof. Dr. Pudji Srianto, M.Kes.,Drh.
NIP. 195601051986011001

Ketua,

Dr. Nusdianto Triakoso, M.P.,Drh.
NIP. 196805051997021001

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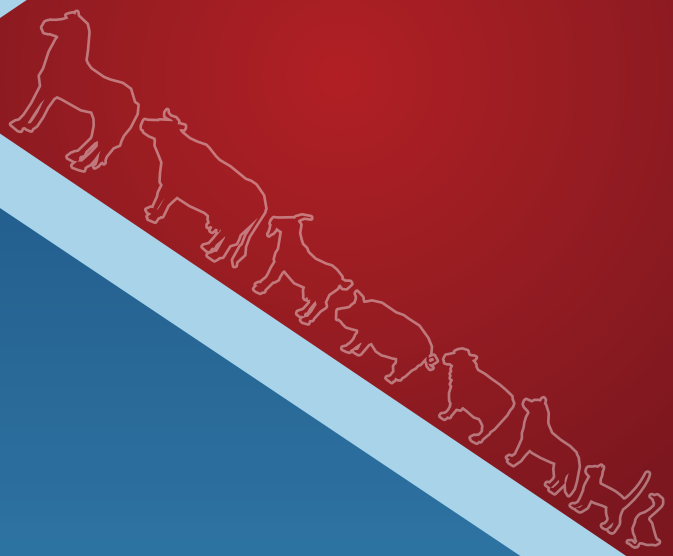

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ESER OFSET MATBAACILIK
BOSNAHERSEK CAD. ALTUNALEM YAPI KOOP. ZEMİN KAT - ERZURUM
Tel: +90 442 2334667 E-mail: eserofset25@hotmail.com

Address for Correspondence

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü 36040, Kars - TÜRKİYE
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RESEARCH ARTICLE

Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Vitrified Embryos Produced from Intra-cytoplasmic Sperm Injection (ICSI)

Widjiati WIDJIATI [1,a] Zakiyatul FAIZAH [2,b] Viski Fitri HENDRAWAN [3,c] Helly Nurul KARIMA [4,d] Choirunil CHOTIMAH [4,e] Sutiman Bambang SOEMITRO [5,f] A. A. Muhammad Nur KASMAN [6,g] Epy Muhammad LUQMAN [1,h (*)]

[1] Universitas Airlangga, Faculty of Veterinary Medicine, Department of Veterinary Science, 60115 Surabaya, INDONESIA
[2] Universitas Airlangga, Faculty of Medicine, Department of Biomedical Science, 60132 Surabaya, INDONESIA
[3] Universitas Brawijaya, Faculty of Veterinary Medicine, Department of Reproduction, 65151, Malang, INDONESIA
[4] Universitas Brawijaya, Bio-Science Central Laboratory, 65151, Malang, INDONESIA
[5] Universitas Brawijaya, Faculty of Science, Department of Biology, 65151, Malang, INDONESIA
[6] Universitas Muhammadiyah Mataram, Faculty of Health Science, 83115, Mataram, INDONESIA
ORCIDs: [a] 0000-0002-8376-1176; [b] 0000-0003-0962-9123; [c] 0000-0003-1089-0070; [d] 0000-003-2551-9303; [e] 0000-0001-6610-9306
[f] 0000-0001-8001-4338; [g] 0000-00003-2511-4920; [h] 0000-0001-7110-0939

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Abstract

This study aims to determine the intracellular calcium profile and viability of embryos produced by the Intra-Cytoplasmic Sperm Injection (ICSI) method. In this study, there were 2 groups (T1: fresh embryos, T2: embryos post vitrification). The stages of the study included medium preparation, goat oocyte collection, *in vitro* maturation of Kacang goat oocytes, fertilization using the ICSI method, and examination of the calcium (Ca^{2+}) intensity profile of fresh embryos and embryos post vitrification per unit time (sec). Measuring the intensity of Ca^{2+} using a Confocal Laser Scanning Microscope (CLSM) with time-lapse, taken at 3 points, namely point 1: edge, point 2: middle, and point 3: edge of the embryo sample. The fertilized embryos showed that the average calcium intensity of T1 was 334.62±8.60 and T2 was 408.2±13.67. The intensity of Ca^{2+} in embryos post vitrification is higher than that of in fresh embryos. The oscillation of Ca^{2+} in fresh embryos was in tune to the measurement point of 50 sec, while in embryos post vitrification the intensity from the 10^{th}, 20^{th} early sec and the 50^{th} end interval were not consistent. It can be concluded that the intensity of Ca^{2+} in embryos post vitrification is higher than that of in fresh embryos. The dynamics of Ca^{2+} in frozen embryos experiencing changes in intensity indicate a change in embryo quality due to vitrification.

Keywords: *Food production, Calcium, Embryo, Freezing, ICSI, Oscillation*

İntrasitoplazmik Sperm Enjeksiyonu (ICSI) İle Üretilen Taze Embriyoda ve Vitrifiye Embriyoda Kalsiyum (Ca^{2+}) Salınımları ve Yoğunluğu

Öz

Bu çalışma, Intra-Sitoplazmik Sperm Enjeksiyonu (ICSI) yöntemi ile üretilen embriyoların hücre içi kalsiyum profilini ve canlılığını belirlemeyi amaçlamıştır. Çalışmada 2 grup vardı (T1: taze embriyolar, T2: vitrifikasyon sonrası embriyolar). Çalışma, besiyeri hazırlama, keçi oositlerinin toplanması, Kacang keçi oositlerinin *in vitro* olgunlaştırılması, ICSI yöntemi kullanılarak embriyoların döllenmesi ve taze embriyoların ve vitrifiye embriyoların birim zamanda (sn) kalsiyum (Ca^{2+}) yoğunluk profillerinin incelenmesi aşamalarını içermiştir. Ca^{2+} yoğunluğu, time-lapse Lazer Taramalı Konfokal Mikroskobu (CLSM) kullanılarak 3 bölgede, bölge 1: kenar, bölge 2: orta ve bölge 3: embriyo numunesinin kenarı, gerçekleştirildi. Döllenen embriyolardan, T1'in ortalama kalsiyum yoğunluğu 334.62±8.60 ve T2'nin 408.2±13.67 olarak saptandı. Vitrifikasyon sonrası embriyolardaki Ca^{2+} yoğunluğu, taze embriyolardakinden daha yüksekti. Taze embriyolarda Ca^{2+} salınımlarının 50 sn'lik aralıktaki ölçümleri uyumluyken, vitrifikasyon sonrası embriyolarda 10 sn, 20 sn öncesi ve 50 sn'lik son aralıklara ait yoğunluklar uyumlu değildi. Vitrifikasyon sonrası embriyolardaki Ca^{2+} yoğunluğunun taze embriyolardakinden daha yüksek olduğu sonucuna varılabilir. Yoğunluk değişiklikleri yaşayan donmuş embriyolardaki Ca^{2+} dinamikleri, vitrifikasyon nedeniyle embriyo kalitesinde bir değişikliğe işaret eder.

Anahtar sözcükler: *Gıda üretimi, Kalsiyum, Embriyo, Dondurma, ICSI, Salınım*

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(*) Corresponding Author

Tel: +62 315 992785, Mobile: +62 812 3090594 Fax: +62 315 993015
E-mail: epy-m-l@fkh.unair.ac.id (E.M. Luqman)

Introduction

One of the assisted reproductive technologies that are now popular in tackling infertility problems is *in-vitro* fertilization (IVF). IVF is a very profitable method because besides being able to overcome infertility problems, this method can produce embryos of high quality and in large numbers. However, the drawbacks of this technology are poor oocyte quality and limited oocyte sources for *in vitro* embryo production [1].

An alternative breakthrough technology to produce embryos can be done *in vitro* using the Intra-Cytoplasmic Sperm Injection (ICSI) method. The ICSI method is a method by inserting sperm directly into the ooplasm of the metaphase II oocyte using a microscopic injector needle. This method is mostly used on humans and animal models to increase the reproducibility and productivity of livestock. The advantage of IVF and ICSI embryos is that they can be frozen using the cryopreservation method. During the freezing process, all cell metabolism stops and then return to normal when the embryo is thawed again. Drastic temperature changes in the cryopreservation of embryos cause damage to blastomere cells, sometimes causing blastomeric cell apoptosis [2,3].

In the ICSI process, oocyte activation is one of the factors that influence the success of fertilization. The oocyte activation occurs due to a complex interaction triggered by the entry of spermatozoa into the oocyte. An early indicator of oocyte activation is characterized by repeated increases in the intracellular calcium concentrations. The increase in intracellular calcium occurs due to a complex interaction triggered by the entry of spermatozoa cells into the oocyte during the fertilization process. During the fertilization process, the endoplasmic reticulum inside the oocyte releases Ca^{2+} ions as important triggers for development into embryos. Increased levels of calcium ions (Ca^{2+}) in the oocyte cytoplasm will initiate the formation of a pronucleus as a sign that the oocyte has been fertilized [4]. The most important principle of embryos cryopreservation is the removal of water from the cells (dehydration) before intracellular freezing. If dehydration does not occur, large ice crystals form in the cell and damage the cell. Reversely, if severe dehydration occurs the cell experiences membrane damage and dies [5]. Damage to the embryonic membrane during vitrification causes disruption of calcium oscillations and affects the process of embryonic development.

Calcium is important for embryonic development. If there is damage to the cell membrane, the released calcium will not re-enter the cell quickly. The dynamics of calcium uptake in cells greatly affect the quality and viability of the embryo. As a second messenger, intracellular calcium signals are capable of decoding and integrating into both chemical and physical environments. These calcium signals control cell division, differentiation, migration, and cell death. Calcium through signal transduction plays a role in the oocytes into embryos transition through the fertilization process, and in the embryo formation [6].

During the fertilization, the fusion of spermatozoa and oocytes triggers a repetitive calcium transient in the oocyte that normally responds to the initiation of embryogenesis. Calcium oscillations that occur due to the trigger by sperm-specific isozyme of phospholipase C (PLCζ) are expressed by the acrosomes of spermatozoa cells. Spermatozoa cells carrying PLCζ cleave phosphatidylinositol 4,5-bisphosphate (PIP2) in the oocyte to release InsP3 and calcium from storage in the endoplasmic reticulum. During embryogenesis, calcium is important in the process of left-right patterning and asymmetric morphogenesis, heart and vascular formation, and kidney formation. Calcium signaling is known to influence the nodal flow process from E7.5 by cilia-driven asymmetric fluid flow originating from the ventral node and transient to the middle during early somitogenesis. This process plays an important role in influencing the permeable channel polycystin-2 (PKD2) protein during the embryogenesis process [7].

It has been shown that calcium plays an important role in the formation of heart through the family of voltage-gated calcium, such as Cavβ2, NCX1, and ryanodine receptor type 2 (RyR2) which are essential for heart formation and embryonic heart function. In addition, calcium signaling plays an important role in the formation of the cardiovascular system with spontaneous calcium signaling for the first pulse transduced by PIEZO1 [8]. A previous research also reports that calcium ions as intracellular messengers play a role in kidney formation [9]. Using genetic and molecular biological approaches, it has been identified several Ca^{2+}-permeable ion channel families as important regulators of Ca^{2+} homeostasis in kidney. In addition, the role of Nuclear Factor Activated in T cells (NFAT) which has implications for signaling pathways for calcium invertebrates was also determined [10].

The dynamics of Ca^{2+} release and uptake greatly affect the cleavage process during embryonic development. If the post thawing blastomere cell death or degeneration occurs after vitrification due to temperature stress, Ca^{2+} released into the cytoplasm cannot be retracted into the cell so that it will affect the quality of the embryo. This study aims to determine the embryo's intracellular calcium profile and viability using the Intra Cytoplasmic Sperm Injection (ICSI) method among fresh embryo and embryo prior to vitrification produced.

Material and Methods

Ethical Approval

This research was conducted at the Biomedical Laboratory of the Faculty of Medicine, Universitas Airlangga, Surabaya

Indonesia. It has been ethically approved with no.1. KE.061.04.2019 issued by the Animal Care and Use Committee, Universitas Airlangga, Faculty of Veterinary Medicine Universitas Airlangga, Surabaya Indonesia.

Methods

This study used the Completely Randomized Design method and the sample used was a Kacang goat oocyte which was obtained from a slaughterhouse. The stages of this research included medium preparation, goat oocyte collection, *in vitro* maturation of Kacang goat oocytes, fertilization using the ICSI method. ICSI-produced embryos were divided into 2 groups, group T1 which was fresh embryos, and T2 which embryo with vitrification. Both of them then observed the intracellular calcium profile. Examination of the Ca^{2+} intensity profile of fresh embryos and vitrified embryos per unit time (sec). Measuring the intensity of Ca^{2+} using Confocal Laser Scanning Microscopy (CLSM) with time-lapse, taken at 3 points, namely point 1: edge, point 2: middle, and point 3: edge of the embryo sample.

- Medium Preparation

Media oocytes collection, maturation, and ICSI was prepared by making Earle's Balanced Salt Solution (EBSS) (E2888-Sigma-Aldrich) media drops with 7% Fetal Calf Serum (F7524-Sigma-Aldrich), HCG (Chorulon-Intervet), and PMSG (Folligon-Intervet) on disposable petridish (Nunc; Copenhagen, Denmark) with micropipette. The medium droplets were then covered with 2500-3000 µL mineral oil, and incubated in an incubator with 5% CO_2 at 38ºC for 22 h with 98% humidity before being used for *in vitro* maturation.

- Oocyte Collection

Kacang goat ovaries were obtained from a slaughterhouse and then brought to the laboratory in a flask containing 0.95% physiological NaCl at 37ºC (60 min). After arriving to the laboratory, the ovaries were sterilized and washed with physiological NaCl 0.95% + 100 µL gentamycin. Oocyte collection and aspiration using a syringe with an 18G needle containing 1 mL medium EBSS. The collected oocytes were inserted into a sterile petridish and then observed for the grade oocytes. Only oocytes surrounded with cumulus complexes of more than 3 layers were used for *in vitro* maturation.

- In Vitro Maturation

Oocytes with completed layers of cumulus washed 3 times with EBSS medium. Oocytes then transferred to the previously prepared maturation media and then incubated in a 5% CO_2 incubator at 38ºC for 22 h until the expansion of cumulus cells occurs. Then oocyte maturity level is examined.

After 22 h of culturation, the level of oocyte maturation was observed using a microscope with magnification of 100x and 400x to observe polar body I. The oocyte was immersed in a medium containing the hyaluronidase enzyme (HYASE-10x™, Vitrolife®) and left for 30 sec and then transferred to the culture medium [11,12].

- Spermatozoa Preparation Using Density Gradient Centrifugation

Frozen spermatozoa that have been collected and ready to be used were added with Sil-Select Plus medium (Fertipro, Beernem, Belgium) and centrifuged at 2500 rpm for 10 min, then put into a drop in the form of a well. Motile spermatozoa in small wells were used for fertilization [13].

- Fertilization Using Intra Cytoplasmic Sperm Injection (ICSI)

Oocytes that have been denuded in hyaluronidase enzyme to knock out the cumulus cells so the polar body would be visible. Good and motile spermatozoa that have been prepared are then immobilized by injuring the tail with an injector and then inserted into the injector and injected into the oocyte that has a polar body appearance. ICSI was carried out under mineral oil in 50x9 mm^2 Petri dishes (BD Falcon, Bedford, MA; Catalog No. 351006) using a Nikon Eclipse TE-2000-U microscope (Nikon, Melville, NY) equipped with manipulators (Eppendrof, Hamburg, Germany) [14-16].

- Embryo Vitrification

The ICSI embryos were exposed to a vitrified equilibration medium containing PBS + 15% ethylene glycol + 0.5 M sucrose + 15% PROH/propanediol (V2) with an exposure time of 18-20 min. The embryos were then put into M2 (Sigma, Saint-Quntin-Fallavier, France) (V3) medium for 30 sec. The embryos were then placed in a 0.25 mL transparent hemistraw, therefore exposed to nitrogen vapor for 10 sec, and placed in a liquid nitrogen container [17].

- Embryo Thawing

After being vitrified, the embryos were warmed (warming/thawing) in the air for 10 sec and then in a water bath at 35ºC for 30 sec. Embryos in hemistraw were poured and exposed to an equilibration medium containing PBS + 20% serum + 0.5 M sucrose (V4) for 2 min. Then the embryos were put into a medium containing PBS + 20% serum + 0.25M sucrose (V5) for 2 min then continued into PBS medium + 20% serum + 0.1M sucrose (V6) for 3 min [17].

- Embryo Ca^{2+} Oscillation Profile Examination

Fresh embryo and the thawed embryos were then fixed and washed with PBS 3 times for 5 min. The intensity of Ca^{2+} during activation embryo was observed by Fluo-3 staining with Confocal Laser Scanning Microscope (CLSM). Embryo were loaded with Fluo-3 AM Calcium indicator (Molecular Probes, Eugene, USA) (0.5 µg/mL in HEPES medium, at 37ºC

for 60 min). The cells were washed three times with Krebs-Ringer solution (K4002-Sigma-Aldrich) for 5 min and then incubated in Krebs-Ringer solution for 15 min to remove any non-specific staining on the cell surface. The embryo cell coverslips incubated with Fluo-3AM were placed in a special perfusion chamber of the laser scanning confocal microscope. After the perfusion apparatus was installed, the amount of injected and aspirated liquid was balanced at a speed of 2-3 mL/min; the temperature of the water bath was maintained at 37°C. The fluorescence was excited by an argon ion laser with an excitation wavelength of 506 nm and an emission wavelength of 525 nm. The cells were scanned in an XYT-plane fashion under the laser scanning confocal microscope using the Time Series program time-lapse. All of the results were analyzed using the TCS-SP2 CLSM software.

Statistical Analysis

Data were analyzed using the SPSS 21.0 software (IBM Corp., NY, USA) and first tested for normality using the Saphiro Wilk test then using T independent test with $P<0.05$ signification. If the distribution of the data abnormal, the data were verified using the Kruskall Wallis Test then intervariable using Mann Whitney U Test to find the differentiation of each group. $P\leq0.05$ was considered statistically significant.

Results

From the results of the average calcium intensity of T1 embryos treated with fresh embryos and T2 embryos post vitrification, *Table 1* data is obtained.

The mean result of fresh embryo calcium intensity was 334.62±8.60. Meanwhile, the average embryonic calcium intensity post vitrification in the T2 group was slightly higher at 408.2±13.67. The embryos in both groups were then examined for calcium intensity with a CLSM microscope per unit time. The examination of the calcium intensity of fresh embryos result was shown in *Fig. 1*.

Based on the results, it was known that the intensity of Ca^{2+} in the T1 group moves up and down as its activity goes in and out of cells freely. Changes in Ca^{2+} concentration are called changes in Ca^{2+} oscillations. At all three points, the Ca^{2+} oscillation profile shows a decreasing intensity with time. At point 1, the highest intensity is known to occur at the initial 10-sec interval (376.92) and the lowest at the 50-sec interval (279.89). At point 2, the highest Ca^{2+} intensity was at the 10th sec (335.97) and the lowest was at the 50th time (236.16). At point 3, the highest Ca^{2+} intensity was also at the 10th initial time interval (296.54) and the lowest at the 40th-sec interval (225.20).

In the T2 group, the Ca^{2+} intensity also moved up and down according to intracellular activity. At all three points, the Ca^{2+} oscillation profile shows an intensity that fluctuates over time. At point 1, the highest Ca^{2+} intensity was at the 40th-sec interval (310.77) and the lowest was at the 10th initial time interval (208.95). At point 2, the highest intensity is known to occur at the initial 10-sec interval (483.50) and the lowest at the end of the 50-sec interval (231.34). At point 3, the highest Ca^{2+} intensity was at the 20th time interval (311.77) and the lowest was at the 50th sec (112.34) *(Fig. 2)*.

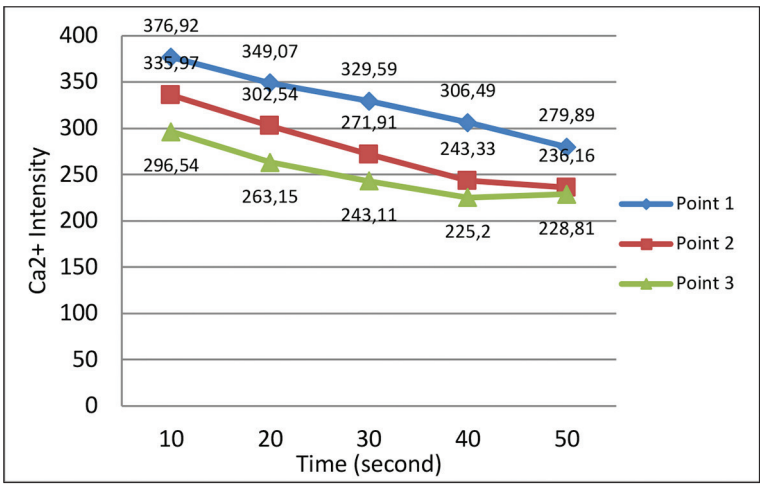
Based on the different tests using ANOVA between points against time per 10 sec, in fresh embryos, there is a tangent approach to calcium intensity at point 2 (middle) and point 3 (edge) at the end of the 50th sec between 236.16-228.81. In embryos after vitrification, contact occurs more as much as 3 times. The tangent approach of calcium intensity occurs at the interval of 20 sec between point 1 (edge)

Table 1. *Calcium Intensity Kacang goat frozen embryo post vitrification and fresh embryo*

Group	Mean±SD*
T1 (Fresh embryo)	334.62±8.60^{a}
T2 (Frozen embryo)	408.2±13.67^{b}

** Different supercript in the same column have significant difference (P<0.05)*

Fig 1. Calcium Oscillation Profile in Fresh Embryo group (T1)



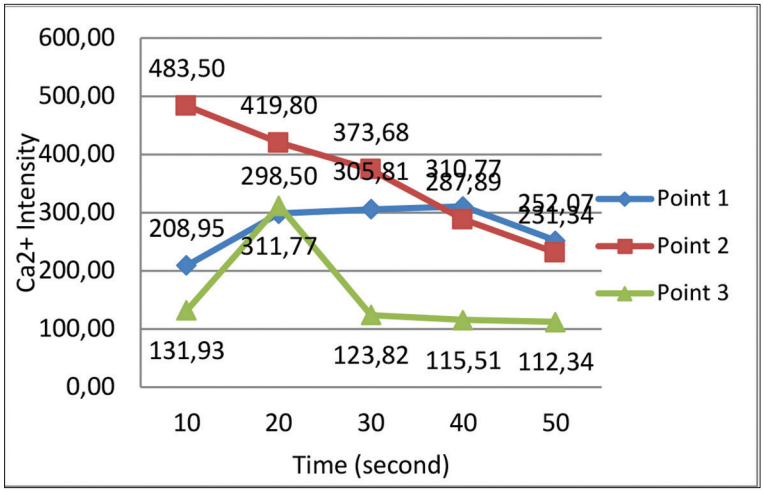


Fig 2. Calcium Oscillation Profile in Embryo post Vitrification (T2)

and point 3 (edge) between 298.50-311.77; at the 40th sec between point 1 (edge) and point 2 (middle) the range is 287.99-310.77, and the 50th sec between point 1 (edge) and point 2 (middle) of the intensity range from 231.34-252.07. Based on the LSD test, it was found that there was no significant difference between the 20th and 50th sec (p: 0.046; P<0.05) in both T1 and T2 embryos.

Discussion

The results of the average calcium intensity of fertilized embryos using the ICSI method showed that the calcium intensity of fresh embryos was 334.62±8.60. While the embryo after vitrification was slightly higher at 408.2±13.67. This is comparable to the report of Bonte et al.[18] which states that the frozen embryo recovery process during the warming process run quickly and optimally in the right medium. Cellularly in the endoplasmic reticulum, the storage of calcium ions after the warming process increases significantly compared to the fresh condition. This can be seen in the strong indicator of the SERCA pump and the calcium outlet valve. Excess release of calcium on the endoplasmic reticulum would start the process of calcium ions influx into the mitochondria to overload. The negative side if this happens also causes a high rate of cell apoptosis. Increase in intracellular calcium occurs after the clotting process followed by osmotic stress and regulatory volume decrease (RVD) due to calcium imbalance. The increase in optimal calcium is stimulated by the mitogen-activated protein kinase (MAP kinase) pathway. The extracellular-signal-regulated kinase (ERK) stimulation occurs after triggering hypoosmotic cell stress by increasing ERK phosphorylation and inhibiting RVD and keeping cells stable [18-20].

The Ca^{2+} intensity in the fresh embryo group had the characteristics of increasing and decreasing as Ca^{2+} activity entered and left the cells freely. At the three points, the Ca^{2+} oscillation profile shows the highest intensity in the first sec (10th sec) and the lowest in the last second (40-50 sec). Nikiforaki [21] reported according to their research that the characteristics of calcium oscillations in the first initiation transient were seen at the beginning of the phase with the highest amplitude of 1.1 (1-1.4) AU and initiation with the lowest amplitude of 0.6 (0.6-0.8) AU at the end of the phase. In the vitrified embryos group, the intensity of Ca^{2+} also moved up and down according to intracellular activity. The Ca^{2+} oscillation profile at points 2 and 3 of the highest intensity occurred in the initial phase time interval (10 sec and 20 sec) and the lowest at the final second phase (50 sec). While at point 1, the highest Ca^{2+} intensity was at the 40th-sec interval and the lowest was at the 10th initial time interval. Nikiforaki [21] explained in their research that the transition amplitude of calcium would significantly increase and slowly decrease after the vitrification process. In his study, frozen embryos showed an increase in the early phase of 1.8 (1.4-2.2) AU and decreased to 0.8 (0.7-0.9) AU [21].

In fresh embryos, there is a tangent approach to calcium intensity at the end of the 50th sec. In frozen embryos, the approximation of the tangent occurs at an interval of 20 sec; the 40th sec; and the 50th sec. Based on the LSD test, it was found that there was no significant difference between the initial and final sec (P: 0.046; P<0.05) both in fresh embryos and after vitrification, but in embryos post vitrification the calcium intensity points (Ca^{2+}) were not aligned and did not meet at one point at the end of the 50 sec measurement. Nikiforaki [21] explained that the source of the oocyte and the origin of its shape, both fresh and frozen *(in vitro* and *in vivo)* influences the pattern of calcium oscillations following ICSI. Direct comparison of the values of the calcium oscillation parameters results have been reported for failed fertilized.

In this study, the pattern of calcium oscillations also showed differences in the calcium oscillation model for 1 min which showed a calcium oscillation pulse 10-20 sec early and late. According to the study of Tesarik [22], calcium oscillations begin 2-12 h after ICSI and their intracellular initiation occurs every 1-5 min in the MII division of fresh

embryos. Micro intracellular oscillations occur every 20 sec and continue for 0.5-1 h. Different things were reported by Yanagida [23], who stated that calcium initiation occurred at 18.4±3.8 min after ICSI and was seen every 11 sec [21-23].

The difference in the results of each report on calcium analysis in oocytes and embryos do not depend on oocyte variability, differences in the use of spermatozoa, or the medium, but is also influenced by the use of calcium indicators. Shwann [24] who used the hydrolysis form of acetoxymethyl ester calcium reported that the indicator could diffuse into the organelles and vesicles longer during the measurement process so that the measurement time was also longer. In addition, the Fura-2 dextran indicator is also able to remain in the cytosol better so that the measurement is more accurate [24].

The pattern of calcium oscillations in vitrified embryos appears to be more likely to occur at contact and pulse oscillations. The same thing is comparable to the research of Nikiforaki [21] which showed that calcium oscillations with vitrification and warming started more slowly with a long period of time and high amplitude and low frequency. However, according to the different tests conducted in this study, it was shown that there was no significant difference in calcium intensity between fresh and vitrified embryos. This is also in accordance with the research of Kim [25], which showed that embryos vitrified using ethylene glycol and DMSO with fresh embryos do not show any significant difference in calcium oscillations after ICSI. However, this study also showed that calcium oscillations in fresh embryos appeared to be more stable when compared to the frozen embryos. Even though vitrified embryos can survive the freezing process and would be able to increase the survival rate and embryo development, sometimes there would be a slight decrease in quality during fertilization and embryo transfer when compared to fresh embryos. This is because calcium signaling affects not only oocyte activation, but is also required for the post-implantation formation [21,25-27].

In addition, embryos with a vitrification process would experience more calcium shocks or pulsations. This happens because the vitrification process would reduce and damage cortical granules and smooth endoplasmic reticulum. Disturbance in the mitochondria-endoplasmic smooth reticulum would interfere with the calcium signaling process and form wide endoplasmic reticulum vesicles and result in membrane rupture causing calcium leakage. Therefore, cryoprotectants are used as cell protective agents, such as 1,2-propanediol, ethylene glycol, and DMSO, which can induce an increase in intracellular calcium concentration. The release of calcium with cryoprotectants induces potentially oocyte activation, fertilization, and embryo formation. Calcium oscillations induce calcium entry into oocytes and embryos and affect meiosis activation and cell division [28-33].

The intensity of Ca^{2+} in embryos post vitrification is higher than in fresh embryos. The oscillations of Ca^{2+} in fresh embryos was aligned from the measurement point of 50 sec, while in embryos post vitrification the intensity from the initial 10^{th} and 20^{th}-sec intervals and the end of the 50^{th}-sec interval did not match the intensity. This is due to the dynamics of Ca^{2+} in frozen embryos experiencing changes in intensity indicating a change in embryo quality due to vitrification.

Availabity of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author.

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Conflict of Interest

The authors report no conflicts of interest. The authors are responsible for the content and writing of paper.

Author Contributions

WW, SBS and EML conceived and supervised the study. WW, ZF and VFH collected and analyzed data. WW, HNK, CC and AAMNK made laboratory measurements. HNK and CC applied the embryo Ca^{2+} oscillation profile examination of the study. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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