Kafkas Universitesi Veteriner Fakultesi Dergisi

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Inbox

Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Wed, Jul 28, 2021, 5:15 PM

to me

Dear Associated Prof. Epy Muhammad Luqman,

Thank you for submitting your manuscript entitled "Calcium (Ca2+) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection" to Kafkas Universitesi Veteriner Fakultesi Dergisi.
Your manuscript will first be evaluated by the editors and if it meets the Journal's standards, will be forwarded to referees for scientific review. You will be able to learn the stage of your manuscript in the
review process through the author center to which you will have access with your user name and password. You can use the author center for revisions and new submissions.

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Inbox

Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Wed, Jul 28, 2021, 12:31 AM

to me

Dear Associated Prof. Epy Muhammad Luqman,

Some changes are needed on your article named Calcium (Ca2+) Oscillations and Intensity in

Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection which you have sent to Kafkas Universitesi Veteriner Fakultesi Dergisi. We need you to make these changes and send your article again, before the editor evaluation.

Needed changes:
Please review the REFERENCES section. There are numerous errors in journal abbreviations and in the writing of references. It must be written according to our rules. There is \\\"Sample file\\\" in the system, you can use it as a guide.

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Kafkas Universitesi Veteriner Fakultesi Dergisi

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Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Sat, Oct 24, 2021, 9:26 PM

to me

Dear Associated Prof. Epy Muhammad Luqman,

The Editors have now assessed the reviewer response and have concluded that, in its present form, the manuscript is not yet ready for publication in the Journal.

Below you will find the relevant review comments and editorial notes. Acceptance of the paper is contingent upon effectively revising the work by taking these comments into serious consideration, and by responding or rebutting them in detail.

We ask you to submit your revision through the online system.
The site is located at http://submit.vetdergikafkas.org Please upload the file containing your revised manuscript. The rebuttal letter should be placed in “cover letter” section. Please note that you should submit your revised letter by clicking on “Submit Revision” link, not as a new manuscript.
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Kafkas Universitesi Veteriner Fakultesi Dergisi

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Inbox

Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Sat, Oct 24, 2021, 9:26 PM

to me

Dear Associated Prof. Epy Muhammad Luqman,

The Editors have now assessed the reviewer response and have concluded that, in its present form, the manuscript is not yet ready for publication in the Journal.

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Sincerely
Kafkas Universitesi Veteriner Fakultesi Dergisi
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info@vetdergikafkas.org

Rebuttal letter

October 31, 2021
Kafkas Universitesi Veteriner Fakultesi Dergisi
Editorial Office

I am submitting a revision of manuscript entitled " **Calcium (Ca2+) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection**" (ID: 26332-KVFD). I had corrected manuscript according the reviewer comments (reviewer 1: red color text (No. 1-11) and reviewer 2: green red color text (No. 12-19):

1. I have made the title in the center.
2. I have added the abbreviation of ICSI in abstract section.
3. I have improved on the conclusion section in the abstract.
4. I have replaced the first letter of each word with a capital letter and italic font in the keyword section.
5. I've added the reference on page 3 and changed the sentence cited from that reference.
6. I have replaced the last paragraph in the introduction and added it for research purposes (page 3).
7. I have added the name of the city and country in the Ethical Approval section (page 3).
8. I have changed the trademark ® to superscript font (page 4).
9. I have changed the mineral oil volume to 2500–3000 μL (page 4)
10. The Shapiro–Wilk test is a test of normality in frequentist statistics (page 5).
11. I have corrected all the reference writing according to the reviewer's suggestion (page 13).
12. I have explained the 50th end of the 50th-sec in the abstract section (page 1)
13. This sentence provides information about the mechanism of damage to the embryonic membrane when vitrification is carried out so that it can have an impact on calcium oscillations affecting the process of embryo cleavage (page 2).
14. I've added a reference to the first paragraph of page 3.
15. Ovaries taken from the abattoir for 60 minutes (page 4).
16. I've added device for ISCI in method section (page 5).
17. I have replaced the frozen Embryo by vitrification with frozen embryo in table 1 (page 6).
18. I've added the abbreviations of MAP (the mitogen-activated protein kinase) and ERK (page 8).
19. I have explained the source of the oocyte and the origin of its shape, both fresh and frozen form influence the pattern of calcium oscillations after ICSI fertilization (page 8).

Yours Sincerely,
Dr. Epy Muhammad Luqmqn, M.Sc., DVM
Department of Veterinary Science,
Faculty of Veterinary Medicine,
Kampus C Universitas Airlangga,
Jl. Mulyorejo Surabaya 60115, Indonesia.
Tel.: +62315992785; Fax: +62315993015
Email: epy-m-l@fkh.unair.ac.id

Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@v> Mon, Nov 1, 2021, 1:35 PM
to me

Dear Associated Prof. Epy Muhammad Luqman,

The Editors have now assessed the reviewer response and have concluded that, in its present form, the manuscript is not yet ready for publication in the Journal.

Below you will find the relevant review comments and editorial notes. Acceptance of the paper is contingent upon effectively revising the work by taking these comments into serious consideration, and by responding or rebutting them in detail.

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Kafkas Universitesi Veteriner Fakultesi Dergisi

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Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Mon, Nov 1, 2021, 5:13 PM
to me

Dear Associated Prof. Epy Muhammad Luqman,

This is to acknowledge receipt of your revised manuscript entitled Calcium (Ca2+) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection. You can learn the stage of your manuscript in the review process through the author center. Thank you.
Sincerely,

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Managing Editor
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Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Wed, Nov 17, 2021, 7:31 PM

to me

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The Editors have now assessed the reviewer response and have concluded that, in its present form, the manuscript is not yet ready for publication in the Journal.

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[Message clipped] View entire message

Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Wed, Nov 3, 2021, 11:44 PM

to me

Dear Associated Prof. Epy Muhammad Luqman,

This is to acknowledge receipt of your revised manuscript entitled Calcium (Ca2+) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection. You can learn the stage of your manuscript in the review process through the author center. Thank you.
Sincerely,

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Managing Editor
Kafkas Universitesi Veteriner Fakultesi Dergisi

Kafkas Universitesi Veteriner Fakultesi Dergisi

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Kafkas Üniversitesi Veteriner Fakültesi Dergisi <info@vetdergikafkas.org> Tue, Nov 2, 2021, 2:12 PM

to me

KAFKAS ÜNİVERSİTESİ
VETERİNER FAKÜLTESİ DERGİSİ
Open Access
ISSN : 1309-2251

Dear Associated Prof. Epy Muhammad Luqman,

I am writing to advise that your manuscript entitled "Calcium (Ca2+) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection" has been accepted for publication in an upcoming issue of Kafkas Universitesi Veteriner Fakultesi Dergisi. We will send the proof of your article for your approval prior to publication. You can reach the final information about your manuscript at online manuscript center (http://submit.vetdergikafkas.org). Please find attached Copyright Transfer form. All authors should sign the agreement. Please return a signed hard copy by mail.

Please do not hesitate to contact us at any time if you have questions regarding your manuscript or the publication process by sending an e-mail to info@vetdergikafkas.org
We look forward to publishing your paper.

Sincerely,

Kafkas Universitesi Veteriner Fakultesi Dergisi
Editorial Office
info@vetdergikafkas.org
2 Attachments • Scanned by Gmail

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Wed, Nov 3, 2021, 11:44 AM

to Kafkas

Dear Kafkas University of Veterinary Faculty of Dergisi
Editorial Office
I have submitted a Copyright Transfer form of my manuscript entitled "Calcium (Ca2+) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection" which has been signed by the authors. thank you
--
Dr. Epy Muhammad Luqman
Badan Kerjasama dan Manajemen Pengembangan
Universitas Airlangga
mobile : +628123090594
One attachment • Scanned by Gmail

KVFD-2021-26332

External

Inbox

isa ozaydin <aras_isa@hotmail.com> Wed, Nov 3, 2021, 4:40 AM

to me

Dear Author/s,
You can find the explanation for a sentence in the Abstract section of the English editor of our journal in the attached file. Please make changes according to the suggestion and send the same file back to me.
Best regards
Isa Ozaydin

Isa OZAYDIN, Professor, DVM, MSc, PhD
Editor-in-Chief

Kafkas Universitesi Veteriner Fakultesi Dergisi
(Journal of the Faculty of Veterinary Medicine, Kafkas University)

Kafkas University, Faculty of Veterinary Medicine, Department of Surgery,
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Fax : +90 474 2426853
Mobile : +90 542 4740638

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Wed, Nov 3, 2021, 12:13 PM

to isa

Dear Isa OZAYDIN, Professor, DVM, MSc, PhD
Editor-in-Chief

Kafkas Universitesi Veteriner Fakultesi Dergisi
(Journal of the Faculty of Veterinary Medicine, Kafkas University)

My explanation of your question about a sentence in the Abstract section: The sentence should be deleted because it has been explained in the first sentence as the purpose of the study.

thank you
--
Dr. Epy Muhammad Luqman
Badan Kerjasama dan Manajemen Pengembangan
Universitas Airlangga
mobile : +628123090594

A request to check and fulfill some revisions on your MS of "KVFD-2021-26332"

External

Inbox

fatih büyük <fatihbyk08@hotmail.com> Wed, Nov 3, 2021, 7:43 PM

to me

Dear Author,
I'm writing you on behalf of the Editorial Team as being the Associate Editor.
Here are some revisions on the title and method that need to be addressed before the publication of your manuscript, please see the attached file to find the requested revisions.
You can feedback by this e-mail for your revision.

Sincerely.

Dr. Fatih BÜYÜK
(Associate Editor)

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Thu, Nov 4, 2021, 11:55 AM

to fatih

Dear, Dr. Fatih BÜYÜK
(Associate Editor)
Kafkas Universitesi Veteriner Fakultesi Dergisi

I am submitting a revision of manuscript entitled "Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Vitrified Embryos Produced from Intra-cytoplasmic Sperm Injection (ISCI)" (ID: 26332-KVFD). I had corrected the manuscript according to Dr. Fatih BÜYÜK comments (red color text).

Thank you
--
Dr. Epy Muhammad Luqman
Badan Kerjasama dan Manajemen Pengembangan
Universitas Airlangga
mobile : +628123090594

KVFD-2021-26332

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Inbox

isa ozaydin <aras_isa@hotmail.com> Fri, Nov 5, 2021, 1:06 PM

to me

Dear Authors,
You can find the proof and payment information of your paper attached.

Please check that the current typographed manuscript if it is your last sent revised version of the manuscript.
Please inform any errors in the current typographed manuscript, which is in PDF format, by the below-mentioned
e-mail. Please indicate the errors clearly, if there are any, by indicating which page, column, paragraph, line, header, footer, table, figure, reference.
Due date to claim any corrections: 16 NOVEMBER 2021 (If no request arrives due this date, the manuscript will be printed as it stands).
PLEASE SEND THE PUBLICATION FEE COMPLETELY. ALL THE BANK COMMISSION PAYMENTS SHOULD BE CHARGED BY AUTHORS.
With kind regards.
Isa Ozaydin

Isa OZAYDIN, Professor, DVM, MSc, PhD
Editor-in-Chief

Kafkas Universitesi Veteriner Fakultesi Dergisi
(Journal of the Faculty of Veterinary Medicine, Kafkas University)

Kafkas University, Faculty of Veterinary Medicine, Department of Surgery,
36100 Kars - TURKEY
Phone : +90 474 2426807/5228
Fax : +90 474 2426853
Mobile : +90 542 4740638

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Mon, Nov 8, 2021, 3:26 PM

to isa

Dear Isa OZAYDIN, Professor, DVM, MSc, PhD
Editor-in-Chief
Kafkas Universitesi Veteriner Fakultesi Dergisi
(Journal of the Faculty of Veterinary Medicine, Kafkas University)

I sent a galley proof correction and payment proof for the article entitled “Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Vitrified Embryos Produced from Intra-cytoplasmic Sperm Injection (ISCI)” (ID 26332_KVFD) (attached). Thank you
--
Dr. Epy Muhammad Luqman
Badan Kerjasama dan Manajemen Pengembangan
Universitas Airlangga
mobile : +628123090594

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Sat, Nov 13, 2021, 8:48 PM

to isa

Dear Isa OZAYDIN, Professor, DVM, MSc, PhD
Editor-in-Chief
Kafkas Universitesi Veteriner Fakultesi Dergisi
(Journal of the Faculty of Veterinary Medicine, Kafkas University)

I sent a little correction of a galley proof for the article entitled "Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Vitrified Embryos Produced from Intra-cytoplasmic Sperm Injection (ISCI)" (ID 26332_KVFD). The correct title is "Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Vitrified Embryos Produced from Intra-cytoplasmic Sperm Injection (ICSI)". Correction on the abbreviation of ICSI, not ISCI. Thank you

~~Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra-cytoplasmic Sperm Injection~~

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

Comment [FB1]:
Here is a conflict, your methods and results indicate that the calcium oscillation and intensity have been calculated in both fresh embryo and embryo that was produced by ICSI and vitrified and subsequently thawed, however, the title does not match correctly with that. It currently means that the ICSI embryo tests have been performed prior to vitrification.

Running Title: Ca^{2+} Oscillations, Intensity, Vitrification by ISCI

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

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[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

[(*)] 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)

Article ID: KVFD-2021-26332 Received: 26.07.2021 Accepted: 01.11.2021 Published Online: 01.11.2021

Abstract

This study aims to determine the embryo's intracellular calcium profile and viability using the Intra-Cytoplasmic Sperm Injection (ICSI) method compared to the fresh embryo. In this study, there were 2 groups (T1: fresh embryos, T2: embryos posts 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 indicating a change in embryo quality due to vitrification.
Keywords: *Food production, Calcium, Embryo, Freezing, ICSI, Oscillation*

İntrasitoplazmik Sperm Enjeksiyonu İle Üretilen Vitrifikasyon Öncesi Taze Embriyo ve Embriyoda Kalsiyum (Ca^{2+}) Salınımları ve Yoğunluğu

Öz

Bu çalışmanın amacı, embriyonun hücre içi kalsiyum profilini ve canlılığını, taze embriyoya kıyasla İntra-Sitoplazmik Sperm Enjeksiyonu (ICSI) yöntemiyle belirlemektir. Bu çalışmada 2 grup vardı (T1: taze embriyolar, T2: vitrifikasyon sonrası embriyolar). Bu araştırmanın aşamaları, besiyeri hazırlama, keçi oosit toplama, Kacang keçi oositlerinin *in vitro* olgunlaşması, ICSI yöntemi kullanılarak döllenme ve vitrifikasyon sonrası taze embriyoların ve embriyoların Kalsiyum (Ca^{2+}) yoğunluk profilinin incelenmesini içermektedir. Ca^{2+} yoğunluğunun zaman atlamalı Konfokal Lazer Tarama Mikroskobu (CLSM) kullanılarak ölçülmesi, 3 (üç) noktada, yani nokta 1: kenar, nokta 2: orta ve nokta 3: embriyo örneğinin kenarı. Döllenen embriyolar, T1'in ortalama kalsiyum yoğunluğunun 334.62±8.60 ve T2'nin 408.2±13.67 olduğunu gösterdi. Vitrifikasyon sonrası embriyolarda Ca2+ yoğunluğu taze embriyolardan daha yüksektir. Taze embriyolarda Ca^{2+} salınımları 50 saniyelik ölçüm noktasıyla uyumluyken, vitrifikasyon sonrası embriyolarda 10., 20. erken saniye ve 50. uç aralığından gelen yoğunluk tutarlı değildi. Vitrifikasyon sonrası embriyolarda Kalsiyum (Ca^{2+}) yoğunluğunun taze embriyolara göre daha yüksek olduğu, yoğunlukta değişiklikler yaşayan donmuş embriyolarda kalsiyum (Ca^{2+}) dinamiklerinin vitrifikasyon nedeniyle embriyo kalitesinde bir değişikliğe işaret ettiği sonucuna varılabilir.
Anahtar sözcükler: *Gıda üretimi, Kalsiyum, Embriyo, Dondurma, ICSI, Salınım*

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. ISCI-produced embryos were divided into 2 groups, group T1 which was fresh embryos, and T2 which embryo whith 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.

Comment [FB2]: Please be clear to describe the method performed on individual embryos. It seems like all these methods have been conducted on the ICSI embryo, however, you don't express the fate of the fresh embryo, such as in the oscillation examination step, etc.

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 50 x 9 mm2 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, 15, 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 0.25cc 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 37ºC. 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.

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)*

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 10^{th} initial time interval (296.54) and the lowest at the 40^{th}-sec interval (225.20).

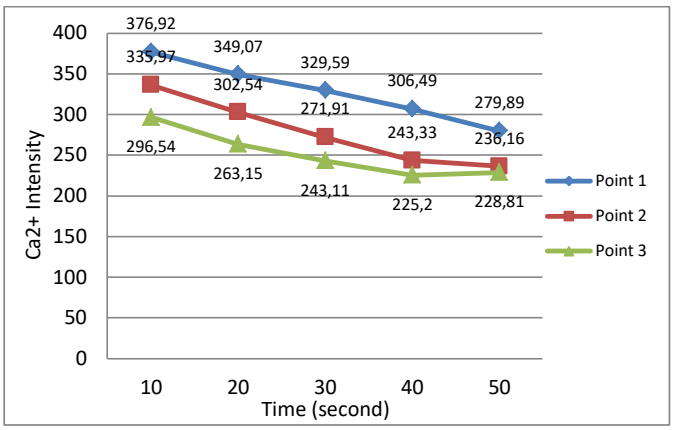

Fig 1. Calcium Oscillation Profile in Fresh Embryo group (T1)

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 40^{th}-sec interval (310.77) and the lowest was at the 10^{th} 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 20^{th} time interval (311.77) and the lowest was at the 50^{th} sec (112.34) *(Fig. 2)*.

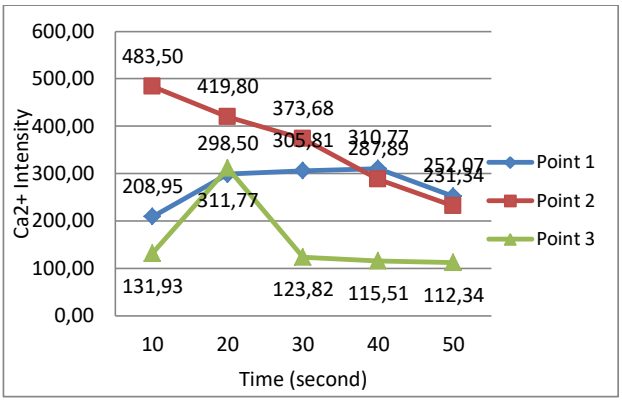

Fig 2. Calcium Oscillation Profile in Embryo post Vitrification (T2)

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) 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 (10^{th} 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 10^{th} 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 50^{th} sec. In frozen embryos, the approximation of the tangent occurs at an interval of 20 sec; the 40^{th} sec; and the 50^{th} 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 50th-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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Calcium (Ca^{2+}) Oscillations and Intensity in Fresh Embryo and Embryo Prior to Vitrification Produced by Intra Cytoplasmic Sperm Injection

Running Title: Ca^{2+} Oscillations, Intensity, Vitrification by ISCI

Abstract

This study aims were to determine the embryo's intracellular calcium profile and viability using the Intra Cytoplasmic Sperm Injection (ICSI) method compared to the fresh embryo. In this study, there were 2 groups (T1: fresh embryos, T2: embryos posts vitrification). Both of them then reviewed the intracellular calcium profile. The stages of this research include 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 timelapse, taken at 3 (three) points, namelypoint 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 in fresh embryos. The oscillations 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 10th and 20th early sec and the 50th end of the 50th-sec interval did not consistent. It can be concluded that the calcium dynamics (Ca^{2+}) in frozen embryos experienced changes in intensity indicating a change in embryo quality due to vitrification.

Keywords: *Calcium, ~~e~~Embryo, ~~f~~Freezing, ICSI, ~~o~~Oscillation*

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 will return to normal when the embryo is thawed again. Drastic

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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 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. The 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 will form in the cell and damage the cell. In the opposite term, if severe dehydration occurs, the cell will experience membrane damage and dies [5].

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 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. While 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].

In embryogenesis of the heart, vasculature, and kidneys, several studies have shown that calcium plays an important role in heart formation 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. Some literature reports that calcium ions as intracellular messengers play a role in kidney formation. This study reported the identification of the temporal and spatial expression of the calcium-binding proteins calbindin-D28k and calbindin-D9k during embryonic kidney formation. In addition, the role of Nuclear Factor Activated in T cells (NFAT) which has implications for signaling pathways for calcium invertebrates was also found [8]. Based on the description above, it is important to know the intracellular calcium profile in embryos produced by the ICSI method that still produces good embryo viability when compared to fresh embryos.

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MATERIAL AND METHODS~~:~~

Ethical ~~a~~Approval

This research was conducted at the Biomedical Laboratory of the Faculty of Medicine, Universitas Airlangga, City, Country. This research has been ethically tested with no.1.KE.061.04.2019 issued by the Animal Care and Use Committee, Universitas Airlangga, Faculty of Veterinary Medicine, City, Country.

Methods

This study used the Completely Randomized Design method and the sample used was a Kacang goat oocyte which was obtained from the Slaughterhouse and has been in the form of an embryo. In this study, there were 2 groups: group T1 which was fresh embryos, and T2 which was embryos post vitrification. Both of them then observed the intracellular calcium profile. The stages of this research include medium preparation, goat oocyte collection, In vitro Maturation of Kacang goat oocytes, fertilization using the ICSI method, and 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 timelapse, taken at 3 (three) 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 are then covered with 250–300 µl mineral oil, and incubated in an incubator with 5% CO_2 at 38^{o}C for 22 h with 98% humidity before being used for in vitro maturation.

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Oocyte Collection

Kacang goat ovaries are obtained from Slaughterhouse then brought to the laboratory in a flask containing 0.95% physiological NaCl with a warm temperature of 37^{o}C. After arriving in the laboratory, the ovaries are 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 are inserted into a sterile petridish then observation of grade oocytes. Only oocytes surrounded with cumulus complexes of more than 3 layers are 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 then incubated in a 5% CO_2 incubator at 38^{o}C for 22 h until expansion of cumulus cells occurs. Then oocyte maturity level is examined.

After 22 h of culture, observations level of oocyte maturity using microscope with magnification of 100x and 400x to observe polar body I. The oocyte is immersed in a medium containing the hyaluronidase enzyme (HYASE-10x ™, Vitrolife®) and left for 30 sec then transferred to the culture medium [9,10].

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Spermatozoa Preparation using Density Gradient Centrifugation

Frozen spermatozoa that have been collected and ready to be used are 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 used for fertilization [11].

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 will 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 [12,13].

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 0.25cc transparent hemistraw, therefore exposed to nitrogen vapor for 10 sec, and placed in a liquid nitrogen container[14].

Embryo Thawing

After being vitrified, the embryos were warmed (warming/thawing) in the air for 10 sec and then in a water bath at 35°C 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 [14].

Embryo Ca^{2+} Oscillation Profile Examination

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 37˚C 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 timelapse. All of the results were analyzed using the TCS-SP2 CLSM software.

Statistical ~~a~~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 data abnormal, the data were verified using the Kruskal~~l~~ Wallis Test then intervariable using Mann–Whitney U Test to find the differentiation of each group and ~~p~~P≤0.05 was considered statistically significant.

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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.

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 (Embryo frozen by vitrification)	408,2 ± 13,67[b]

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

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 shown in *Fig 1*.

Based on the results of *Fig 1*. It is 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).

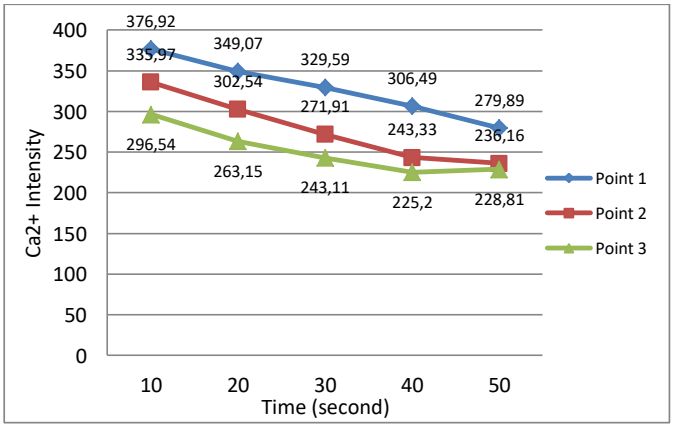

Fig 1. Calcium Oscillation Profile in Fresh Embryo group (T1).

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).*

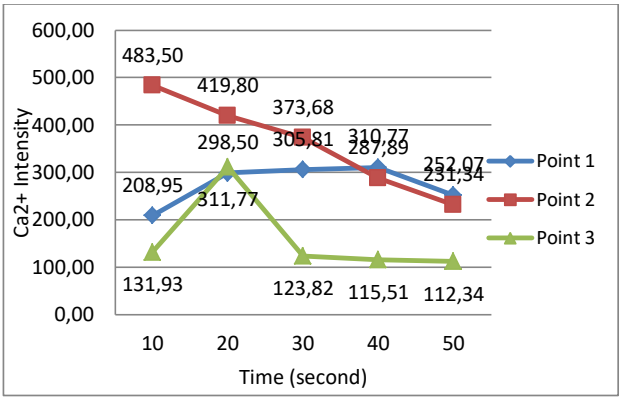

Fig 2. Calcium Oscillation Profile in Embryo post Vitrification (T2).

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) 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 following the report of Bonte et al.[15] which states that the frozen embryo recovery process during the warming process will run quickly and optimally in the right medium. Cellularly in the endoplasmic reticulum, the storage of calcium ions after the warming process will increase 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 will 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 MAP kinase

pathway. ERK stimulation occurs after triggering hypoosmotic cell stress by increasing ERK phosphorylation and inhibiting RVD and keeping cells stable [15,16,17].

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 [18] 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. 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 [18] explained in their research that the transition amplitude of calcium will significantly increase and will 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 [18].

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 [18] explained in his idea that the source of the oocyte and the origin of its shape, both embryonic and frozen form influence the pattern of calcium oscillations after ICSI fertilization. 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[19], 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 [20], who stated that calcium initiation occurred at 18.4±3.8 min after ICSI and was seen every 11 sec [18,19,20].

The difference in the results of each report on calcium analysis in oocytes and embryos does 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 [21] 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 [21].

The pattern of calcium oscillations in vitrified embryos appears to be more likely to occur at contact and pulse oscillations. The same thing is following the research of Nikiforaki [18] 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 showed that there was no significant difference in calcium intensity between fresh and vitrified embryos. This is also following the research of Kim [22], which showed that embryos vitrified using ethylene glycol and DMSO with fresh embryos did 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 frozen embryos. Even though vitrified embryos can survive the freezing process and will be able to increase the survival rate and embryo development, sometimes there will 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 [18, 22,23,24].

In addition, embryos with a vitrification process will experience more calcium shocks or pulsations. This happens because the vitrification process will reduce and damage cortical granules and smooth endoplasmic reticulum. Disturbance in the mitochondria-endoplasmic smooth reticulum will 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. The release of calcium with cryoprotectants induces potentially oocyte activation, fertilization, and embryo formation. Calcium oscillations that occur induce calcium entry into oocytes and embryos and affect meiosis activation and cell division [25,26,27,28,29,30].

The intensity of Calcium (Ca^{2+}) in embryos post vitrification is higher than in fresh embryos. The oscillations of calcium (Ca^{2+}) in fresh embryos was aligned from the measurement point of 50 sec, while in embryos post vitrification the intensity from the initial 10th and 20th-sec intervals and the end of the 50th-sec interval did not match the intensity.

This is due to the dynamics of calcium (Ca^{2+}) in frozen embryos experiencing changes in intensity indicating a change in embryo quality due to vitrification.

Acknowledgement

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed together in completing the research manuscript.

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