

Effects of Addition of Gonadotropin in the Media of Oocyte Maturation on the Embryo Cleavage

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Abstract

This research was performed to find the effects of addition of gonadotropin hormone in oocyte maturation in vitro on embryo cleavage. Gonadotropin hormone such as hMG 0,75 µg/ml and PMSG 15 IU were used in each oocyte maturation in vitro. Maturation media in this research was divided into 4 treatments such as Tc0, Tc+, Tc+hMG, and Tc+PMSG. Each treatment obtained 6 replicates. Oocyte maturation was carried out in each 100 µl TC 199 incubated for 24 hours. Staining of oocyte mature with aceto orcein and in vitro insemination were carried out in the middle of roset. The research result showed that the average percentage of mature oocyte after maturation was 16.67% in the Tc0, 30% in the Tc+, 80% in the Tc+hMG, and 83.33% in the Tc+PMSG. Percentage of cleavage embryo was 5% for Tc0, 11.67% for Tc+, 43.33% for Tc+hMG, and 46.67% for Tc+PMSG. The result of correlation test on mature oocyte and cleavage was 0.932, the result of ANOVA test showed a significant difference, and the result of LSD test showed that there was difference between treatment groups of Tc0 and Tc+ compared to treatment group of Tc+hMG and Tc+PMSG, in both maturation and cleavage. This research concluded that the addition of gonadotropin obtained from hMg and PMSG into oocyte maturation media affected the increase in mature oocyte percentage and cleavage, there was no different result between the addition of hMG and PMSG on the percentage of mature oocyte and cleavage, and there was a correlation of mature oocyte on cleavage.

Experimental article (J Int Dent Med Res 2021; 14(1): 412-418)

Keywords: Gonadotropin, oocyte, in vitro maturation, cleavage.

Received date: 22 July 2020

Accept date: 29 September 2020

Introduction

The development of in vitro fertilization technique particularly relating to optimization of the use of reproductive organ as a source to obtain eggs and sperm cell has important role in improving embryo quality, which until today it is still being studied and developed. One related to in vitro fertilization is the process of maturation of oocytes in vitro which is one of stages in fertilization events to obtain quality embryo¹.

Oocyte maturation in vitro requires a media that is able to be functioned as a place to supply nutrient and at the same time also as a place to

remove nutrient metabolite contained in the media needed for metabolism². The addition of hormone in the maturation media is needed for the development of oocytes to improve oocytes quality so that oocytes potential for fertilization and embryonic development can be improved³. Imperfect completion in the process of maturation in vitro leads to disruption in oocytes growth that certainly affects embryo development. The addition of hormone in the media of oocyte maturation in vitro is meant to improve the oocyte quality so that the fertility rate will increase when fertilization is carried out. Hormonal factors are important for proliferation and differentiation that specifically interact among molecules, therefore the hormonal addition in maturation media allows oocyte to be more able to live in their environment for the purpose of embryonic development⁴.

Hormone that is often added in the oocyte maturation media is gonadotropin hormone such

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as follicle stimulating hormone (FSH) and luteinizing hormone (LH) secreted by pituitary gland⁵. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are the main and primary gonadotropin hormone responsible for oocyte development⁶. The main signal transduction pathway is activated by FSH binding to membrane receptor in granulosa cell⁷, meanwhile LH has important role in follicular maturation and ovulation induction, as well as androgen synthesis stimulation by theca cell⁸. However, other research states that the use of gonadotropin has not been able to increase maturation and potential of embryonic development, leads to disruption on embryo development, and the use of improper gonadotropin concentration leads to negative effects⁹.

Today, gonadotropin has been used to treat infertility. Gonadotropin hormone has important role during oocyte development, so it is necessary to explore the effects of addition of gonadotropin hormone in oocyte maturation in vitro on embryonic development for the development of science in the field of assisted reproductive technology. This research facilitates the readers to understand the effects of addition gonadotropin hormone in oocyte maturation media in vitro on embryonic development.

Materials and methods

hMG Making

As much as 100 ml female urine was collected in the morning and centrifuged within 1.000 x g speed for 15 minutes with 4° C. This centrifugation aims to separate the metabolite cells from the discarded precipitate. The supernatant was filtered and put into an Erlenmeyer flask by utilizing filter paper. The procedures above were repeated until as much as 50 ml supernatant was collected. Next, the urine was used in the glycoprotein isolation examination procedure. It needed 200 µl urine sample, homogenized by adding PBST-PMSF 5 times in the urine sample. The supernatant was put into microtube and was sonicated for 10 minutes, processed in the vortex and centrifuged within 3.000 x g speed for 15 minutes. Cold absolute ethanol in a ratio of 1:1 was added, then it was incubated inside the refrigerator for 1 hour or overnight. The liquid was then centrifuged within 5000 x rpm speed for 15 minutes, the

precipitate was dried to eliminate the smell of ethanol and was added with 20 mM tris-Cl buffer. After that protein isolate was obtained and SDS-PAGE examination was performed.

PMSG Making

The blood of 3.5 months pregnant local race horse was taken through jugular vein. The blood was left in room temperature for 3 hours, the edge of a tube in contact with blood was stabbed and left until 24 hours. It was then centrifuged for 15 minutes within 1000 x rpm speed to separate the serum part from the frozen blood. The supernatant was separated as a ready-to-use serum after went through the 0.2 µm millipore filter or can be stored in -20o C if not used.

Oocyte Collection

Oocyte is originated from local cow ovaries that had entered puberty for more than 18 months. The ovaries were cut in a slaughterhouse and brought hygienically into the laboratory by using thermos in 0,9 (w/v) NaCl containing penicillin G (75µg/ml) and streptomycin sulfate (50µg/ml) maintained at 35-37°C. Cows oocytes was aspirated toward secondary follicle (antral) with 2-5mm section by using a 10 ml syringe of 18 G needles which contained 2 ml of oocyte wash medium.

In Vitro Maturation

Maturation was done in every 100 µl TC199 containing ten oocytes which covered with mineral oil (Sigma) in disposable 36 mm plastic petri dish (Nunc, Denmark). The incubation lasted for 24 hours within 5 % CO₂ incubator with 38,5° C temperature, and 95-99% humidity (Thermoline, USA). The oocyte temperature used 18 G needles associated with 3-5 ml syringes with 500 - 1000 µl oocyte wash medium. Aspiration was done on secondary follicle (2-5 mm diameter). Each treatment was inserted with ten oocytes in treatment medium; Tc0, Tc+, Tc+hMG, and Tc+PMSG with at least six repetitions each. The hMG dosage was 0.75 µg/ml, while the PMSG dosage was 15 IU (10%).

Aceto Orcein Staining

The procedure of collecting egg maturity level data was done through aceto orcein staining solution 1%. The matured oocyte was placed in a glass object with a pipette that had been washed with 0.5% *hyaluronidase enzyme* beforehand to eliminate it from its cumulus cell and rewashed with oocyte wash medium. Next, it was fixed in a 90% acetic acid solution and 70% ethanol in a ratio of 1:3 for 2-3 days, then

covered with a lid supported with Vaseline at the edge of the glass object. The fixed oocyte was stained with aceto orcein solution 1% and left for 2-3 minutes and rinsed with 90% acetic acid, glycerin, and 70% ethanol in a ratio of 1:1:3. Then, the *mature* oocyte development was analyzed under the inverted microscope. Mature oocyte confirmation with chromosome identification reached the metaphase II stage and/or the appearance of polar body I.

In Vitro Fertilization

The sperms used were the local cows' sperms frozen in a container with liquid nitrogen. The sperm were thawed, washed, and centrifuged at 850 rpm for 10 minutes twice. The next step was swams up for 15-20 minutes, and around 30 – 40 µl from the surface of the samples were taken and transferred into the middle part on rosset medium. In the middle rosset medium, 100 µl EBSS associated with 5-6 drops 10 µl of treatment was put on its centripetally on 35 mm plastic petri dish. On the centripetal of rosset medium, there was oocyte that had been matured in the incubator on each treatment medium.

Cleavage Observation

The cleavage observation under the inverted microscope was done after the oocytes were fertilized by spermatozoa for 48 hours in Earle's Balanced Salt Solution (EBSS) medium to see the correlation from the amount of matured oocyte.

Data Analysis

The data analysis used Analysis of Variance (ANOVA). If there was any significant difference, it was then followed with Least Significant Difference (LSD) test to determine the significantly different treatment value.

Results

Oocyte Maturation Level in Maturation Medium

Oocyte maturation level observation in treatment medium was done by using staining preparation with aceto orcein staining solution 1 %. Mature oocyte data was based on polar body I percentage which formed and/or reached the state of chromosome up to the metaphase II stage from nuclear meiosis cleavage as seen in table 1.

The statistical analysis using one-way ANOVA, it showed significant differences

($P < 0.05$) regarding the addition of treatment on maturation medium on mature oocyte formation percentage. To determine the percentage of mature oocyte differences between treatment groups, LSD was conducted, the result was shown in table 3.

Medium	Replicates (n)	Mature oocytes (%)
Tc0	6	16,67 ± 8,16
Tc+	6	30 ± 10,95
Tc+hMG	6	80 ± 12,65
Tc+PMSG	6	83,33 ± 15,06

Table 1. Mean ± SD mature oocyte proportion in maturation medium for 24 hours.

Different value among treatment groups (LSD)	
Tc 0 vs Tc +	13,33
Tc 0 vs (Tc+hMG)	63,33*
Tc 0 vs (Tc+PMSG)	66,67*
Tc + vs (Tc+hMG)	50,00*
Tc + vs (Tc+PMSG)	53,33*
(Tc+hMG) vs (Tc+PMSG)	3,33

Table 2. Least Significant Difference (LSD) among treatment groups on the number of oocyte maturation.

LSD (0,5) = 14,42. Description: * significant difference ($P < 0.05$).

The statistical analysis using one-way ANOVA and followed by LSD showed significant difference between Tc0 and Tc+ treatment group compared to Tc+hMG and Tc+PMSG treatment group on cleavage percentage.

Cleavage

The rate of embryo cleavage of each treatment in oocyte maturation media followed by in vitro fertilization can be seen in Table 3. To determine the effects of each treatment on maturation media on embryo cleavage, the data were transformed into the ANOVA table. The results can be seen in the Table 4. Embryo experiencing and not experiencing cleavage after maturation and fertilization can be seen in figure 3.

Based on statistical analysis using oneway ANOVA, it was showed that there was significant difference result, where ($P < 0.05$) on the addition of hormone in maturation media to the percentage of cleavage. In order to find the difference in the percentage of cleavage among treatment groups, then LSD test was carried and the result was presented in table 5.

Maturation Media	Replicates (n)	Cleavage
Tc 0	6	5 ± 5,48
Tc +	6	11,67 ± 4,08
Tc + hMG	6	43,33 ± 5,16
Tc + PMSG	6	46,67 ± 8,16

Table 3. Proportion of Mean ±SD cleavage after spermatozoa insemination for 48 hours.

The result of statistical analysis using one-way ANOVA that was followed by LSD test showed a significant difference result in the treatment groups of Tc0 and Tc+ compared to the treatment groups of Tc+hMG and Tc+PMSG on the percentage of cleavage.

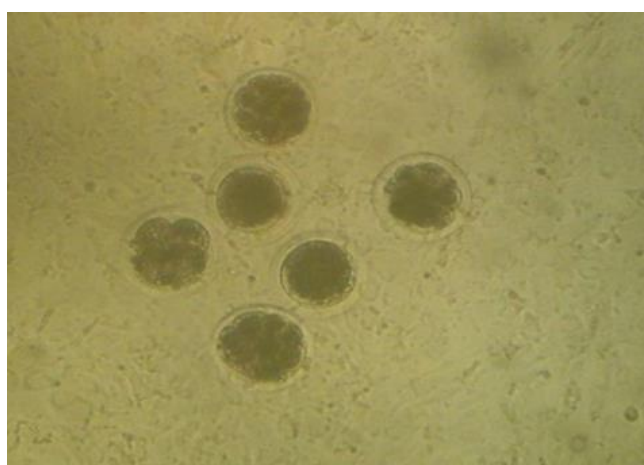


Figure 1. Embryo experiencing and not experiencing cleavage after maturation and fertilization with 100X magnification

	Sum of Squares	df	Mean Squares	Fcount	F-table 0,05
Between Groups	8233.33	3	2744.44	78.41 *	3,10
Within Groups	700.00	20	35.00		
Total	8933.33	23			

Table 4. Oneway ANOVA, the effect of treatment on cleavage.

Note: * there was a significant difference result where (P<0,05).

Different Value among treatment groups (LSD)	
Tc 0 vs Tc +	6,67
Tc 0 vs (Tc+hMG)	38,33*
Tc 0 vs (Tc+PMSG)	41,67*
Tc + vs (Tc+hMG)	31,67*
Tc + vs (Tc+PMSG)	35,00*
(Tc+hMG) vs (Tc+PMSG)	3,33

Table 5. LSD test among treatment groups on the cleavage.

LSD (0,5) = 7,13 Note: * there was significant difference result where (P<0,05).

Mature Oocyte and Cleavage Correlation.

To determine the correlation between mature oocyte and cleavage occurrence, hence, the data was transformed into ANOVA table and followed by statistical analysis using Pearson correlation, in which the result could be seen in table 1. Statistical analysis using Pearson correlation on table 6 showed strong correlation between maturation and cleavage.

	MATURE	CLEAVAGE
MATURE	1	.932(**)
CLEAVAGE	.932(**)	1

Table 6. Mature oocyte and cleavage correlation test.

Description: ** very significant difference (P<0.01).

Discussion

In vitro fertilization is a process of combining eggs and sperm cell to form a new individual outside the body of female mammalian. There are many factors affecting the success of in vitro fertilization, such as optimization media environment condition, oocyte treatment during in vitro fertilization, treatment of spermatozoa during washing and in vitro capacitation, as well as embryo breeding after fertilization. To have optimum media environment, the condition must be resembled in vivo environment and has mechanical, physical, and biochemical function ensuring oocyte and embryo sustainability in vitro.

Mammalian oocyte maturation process is characterized by a change in the morphology of the cell nucleus, a change from immature oocytes (diplotene phase) to the mature stage (metaphase II) so that it is ready to be fertilized. The results of this research showed that oocyte maturation in the maturation media with the addition of FSH and LH led to the increase in the percentage of mature oocytes and provided positive effects on cleavage after in vitro fertilization. This result was in line with the result of previous research that gonadotropin was needed for oocyte competence¹⁰.

The maturation result and cleavage in the treatment group of Tc0 and Tc+ that was significantly difference compared to Tc+hMG dan Tc+PMSG might be caused by bioactivity ratio of FSH: LH contained in hMG and PMSG¹¹. In addition, it also could be caused by the molecule structure of α and β subunit chain that was

different in hMG and PMSG¹² as a comparison in human with FSH and LH composition consisted from the same α subunit, which was 92 amino acid, while FSH β subunit consisted of 110 amino acid and LH β subunit consisted of 121 amino acid¹³. hMG treatment was not significantly different compared to PMSG on the mature oocyte percentage, it might be because bioactivity in FSH and LH was the same, even though their composition and molecule structure of α and β were different, so that biological response produced was still able to stimulate oocyte maturation.

Combination of the role of FSH and LH contained in hMG and PMSG supported classical theory of two cells, the theory of two gonadotropins during folliculogenesis-stereodogenesis stating that LH stimulated androgen production in theca cells, which was subsequently converted into estrogen by stimulating activity of FSH in granulosa cells¹⁴. There were many researchs have showed FSH ability to stimulate oocyte maturation in vitro using various dosage¹⁵. LH had role in facilitating the recruitment of folliculogenesis supported by several research¹⁶. LH has been widely considered as gonadotropin responsible for the onset of the return of meiosis¹⁷. Other research reported that the effect of LH added in the maturation media activated the binding of EGF and EGF receptor, in which it led to the activation of ERK1/2 required to not only facilitate oocyte to achieve metaphase II stage, but also form effective pronuclear¹⁸. However, only FSH was considered as gonadotropin responsible for inducing in vitro maturation on cumulus-wrapped oocyte¹⁹. This is the reason why only cumulus-wrapped oocyte that was able to reach mature stage in vitro, it was because FSH receptor was located in cumulus cell. The uneven uniformity of the cumulus wrapping oocyte at the time of collection from the follicle and continued in vitro maturation was one of the causes why there were oocytes that had not yet reached matured stage although the maturation media was added by gonadotropin²⁰. Besides, it was also caused by condition of oocyte collected was not able to grow individually, so it could not survive in the culture media²¹.

The addition of concentration of gonadotropin combination obtained from the result of extraction and fertilization of menopausal women in the maturation media was

significantly able to increase the percentage of oocyte reaching metaphase II, normal configuration of syndrome, normal chromosome regulation, cortical granule migration, and mitochondrial aggregation²². PMSG is the most glycolysis pituitary glycoprotein group in mammalian placenta with both hormone subunits containing N linked and O linked glycolysis sides²³. The high sialic acid content in PMSG greatly increased half-life compared to other glycoprotein hormones. This also can cause the maturation and cleavage rates produced by PMSG to be slightly higher compared to the media added by hMG, although it was not significant. PMSG also had luteinizing activity that was important for the optimal development of follicles in the pre-ovulatory phase²⁴. Other research reported that the addition of gonadotropin from serum resulted in the high level of maturation²⁵.

The results of this study indicate that the addition of FSH and LH in hMG and PMSG into oocyte maturation influences the embryo division. The addition of FSH and LH combination in maturation media by utilizing TCM-199 could increase in vitro fertilization rate and embryo division²⁶. Supplementation with gonadotropin in maturation media could boost the capacity of oocyte development into embryonic phase, and play an essential role in oocyte competence into metaphase II, and does not prompt the escalation of a chromosome abnormality. Gonadotropin in maturation media acts as an autocrine regulator and paracrine which also involved in protein steroidogenesis and synthesis during embryonic development²⁷. Oocyte without the addition of FSH and LH in maturation media results significantly fewer division rates and tend to experience worse development. The observation on oocyte rate that successfully split after the in vitro fertilization and incubation for 48 hours resulted in different divisions level, i.e., divisions were starting at two cells, four cells, and eight cells. The division rate diversity in this study is allegedly due to the different levels of oocyte maturation during fertilization. The low rate of embryo division in this study is probably due to the oocyte being fertilized in the early stages of maturation. Oocytes that are not fully mature could not split completely, or the process will be inhibited when fertilized. Perfect in vitro fertilization occurs when the division of oocyte core reached the metaphase II during

spermatozoa penetration²⁸. The low cleavage rate hit in this study could be due to the low rate of fertilization that is influenced by spermatozoa mobility which must experience capacitation and acrosome reactions, and the media environment during in vitro fertilization and division²⁹.

The result of correlation test between matured oocyte and cleavage provided positive effects or strong correlation. It showed that the maturation results affected cleavage result, in other words, maturation result was directly proportional with cleavage result. This was in accordance with the result that the more perfect of the level of oocyte maturation, the more oocyte would be fertilized and divided, and the more embryo would grow to reach blastocyst stage. Other research also reported that the addition of FSH and LH hormone in the oocyte maturation media was significantly correlated with the increase in embryonic cleavage after in vitro fertilization³⁰. This research concluded that the addition of PMSG and hMG hormones contained FSH-LH to maturation media can be used as alternative hormones for oocyte maturation in vitro. Oocyte competence reached maturity and the embryonic cleavage stage was stimulated by the coordination of FSH and LH action. This research emphasizes the complexity of gonadotropin transduction signal and its receptor during oocyte maturation in vitro so that it still needs further in-depth studies on gene expression levels related to ligand and receptor interactions as a result of addition of different types and concentrations of gonadotropins to oocyte competence.

Conclusions

The study was that the addition of gonadotropin obtained from hMg and PMSG into oocyte maturation media affected the increase in mature oocyte percentage and cleavage, there was no different result between the addition of hMG and PMSG on the percentage of mature oocyte and cleavage, and there was a correlation of mature oocyte on cleavage.

Acknowledgements

The researcher thanks to Faculty of Medicine of Universitas Airlangga that have been facilitated the implementation of this research.

Declaration of Interest

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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