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Effects of insulin-like growth factor-1 on mitogen-activated protein kinase and maturation promoting factor expressions, and mitochondrial DNA copy number in Kacang goat oocytes

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Abstract

In vitro maturation is a critical step in *in vitro* fertilization, significantly impacting oocyte quality and subsequent embryonic development. This study examines how insulin-like growth factor (IGF)-1 supplementation in the maturation medium affects mitogen-activated protein kinase and maturation promoting factor expressions, as well as the amount of mitochondrial DNA copies in Kacang goat oocytes. Oocytes were collected from Kacang goat ovaries and matured *in vitro* with varying IGF-1 concentrations, including 0.00 (control), 50.00, 100, and 150 ng mL^{-1}. Immunocytochemistry was used to assess mitogen-activated protein kinase and maturation promoting factor expressions, while quantitative polymerase chain reaction quantified mitochondrial DNA copy number. Results showed that the 100 ng mL^{-1} dose of IGF-1 group had substantially increased mitogen-activated protein kinase and maturation promoting factor expressions and also mitochondrial DNA copy numbers compared to the other groups. These findings suggest that IGF-1 supplementation with 100 ng mL^{-1} dose optimally enhances oocyte maturation by activating key signaling pathways and promoting mitochondrial replication. In conclusion, IGF-1 supplementation at 100 ng mL^{-1} is recommended to improve oocyte quality in Kacang goats, potentially enhancing *in vitro* fertilization outcomes.

Introduction

In vitro fertilization (IVF) is a widely researched and developed reproductive technology aimed at improving both the quantity and quality of livestock. *In vitro* maturation (IVM) is a crucial stage in the IVF process. The successful maturation of oocytes through IVM significantly impacts fertilization outcomes, as high-quality mature oocytes lead to higher fertility rates. However, the IVM process does not always yield optimal oocyte quality.[1] Only about 30.00 - 40.00% of oocytes develop into blastocysts through IVF, indicating that embryo development potential is strongly influenced by oocyte quality. The quality of mature oocytes is essential for progression to the blastocyst stage.[2]

Proper supplementation and culture media conditions can enhance oocyte maturation *in vitro*.[3] Optimal IVM may also be influenced by oocyte classification and the addition of growth factors, hormones, and anti-oxidants.[4] Insulin-like growth factor- (IGF)-1 promotes cumulus cell expansion and activates many signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway *via* Ras/Raf. This activation ultimately leads to maturation promoting factor (MPF) reaching the metaphase II stage, a hallmark of oocyte maturation.[5]

Oocyte maturation involves not only the nucleus but also the cytoplasm, with significant changes in distribution and function, particularly in mitochondria. Mitochondria are essential for ATP synthesis, being necessary for oocyte maturation. They also contain their own genome, mitochondrial DNA (mtDNA), which must meet a certain threshold for subsequent processes, such as fertilization, embryogenesis, implantation, and organogenesis, to occur optimally.[6] The IGF-1 can activate pathways related to mtDNA replication, including the MAPK pathway, ultimately activating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and transcription factor A mitochondrial (TFAM), leading to

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mtDNA replication, eventually activating MPF to reach the metaphase II of matured oocyte.[7]

The MAPK pathway plays a crucial role in oocyte maturation, as it is directly associated with the activation of MPF. This activation is a key event in the germinal vesicle breakdown, which initiates the oocyte maturation process. Additionally, mtDNA serves as an indicator of the oocyte's readiness for successful fertilization. To our current understanding, no studies have extensively looked into the influence of IGF-1 supplementation in maturation media on MAPK and MPF expressions, as well as mtDNA quantity in goat oocytes. Therefore, this study investigates the impact of IGF-1 on goat oocyte maturation by assessing MAPK and MPF expressions and mtDNA levels.

Materials and Methods

The materials used in this study included physiological NaCl solution, phosphate-buffered saline, mineral oil (Vitrolife, Göteborg, Sweden), Gamete- 3-(N-morpholino) propanesulfonic acid (G-MOPS; Vitrolife, Göteborg, Sweden), Gamete – In Vitro Fertilization (G-IVF; Vitrolife), recombinant goat IGF-1 (MedikBio, Morrisville, USA), poly-L lysine-coated slides (Sigma-Aldrich, St. Louis, USA), Vaseline (Sigma-Aldrich), hydrogen peroxide (Sigma-Aldrich), antibody extracellular signal-regulated kinase /MAPK (Rockland Immunochemicals, Inc., Limerick, USA), antibody MPF (Bioss, Woburn, USA), methanol, 1.00% aceto-orcein acid (Sigma-Aldrich,), trypsin (Sigma-Aldrich), biotinylated link (yellow) drops (Sigma-Aldrich), streptavidin (red) drops (Sigma-Aldrich), chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich), and methylene green (Sigma-Aldrich). For mtDNA quantification, the materials included proteinase K (Sigma-Aldrich), 2.00 x SensiFAST SYBR Hi-ROX (Bioline, London, UK), 10.00 µM forward primer, and 10.00 µM reverse primer. The equipment used included 35.00 and 65.00 mm Petri dishes (Thermo Fisher Scientific, Waltham, USA), laminar airflow cabinet, beaker, glass slides, cover slips, disposable syringes (1.00, 3.00, and 10.00 mL), thermos, water bath, tweezers, scissors, 18-G needles, Eppendorf micropipettes, refrigerator, CO_2 incubator (Thermo Fisher Scientific), Pasteur pipettes (Merck, Darmstadt, Germany), CX41 microscope (Olympus, Tokyo, Japan), inverted microscope (Meiji Techno, Miyoshi, Japan), and QuantStudio 5 quantitative polymerase chain reaction machine (Applied Biosystems, Waltham, USA).This research was conducted from March to June 2024 and received an ethical clearance certificate (No.1.KEH.025.02.2024) from the Animal Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Kacang goat oocyte collection. Oocytes were collected from the ovaries of Kacang goats obtained from the Pegirian slaughterhouse in Surabaya, Indonesia. The ovaries were cleaned of their attachments and washed with 0.90% physiological NaCl. They were then placed in a beaker filled with NaCl 0.90% and gentamicin (Sigma-Aldrich), and stored in a container at 30.00 - 35.00 ˚C for transport to the laboratory. Oocytes were aspirated from pre-antral ovarian follicles with diameters of 2.00 - 6.00 mm using a syringe (10.00 mL) with an 18-G needle filled with a small amount of maturation medium. Grade 1 and 2 oocytes were selected and washed with maturation medium.

Oocyte maturation. The collected oocytes were divided into four treatment groups for IVM, including control with maturation medium without supplements, treatment group 1 (T1) with 50.00 ng mL^{-1} IGF-1, treatment group 2 (T2) with 100 ng mL^{-1} IGF-1, and treatment group 3 (T3) with 150 ng mL^{-1} IGF-1. Mineral oil was added to stabilize the media droplets. Each maturation droplet (50.00 µL) contained 10 oocytes and was incubated in 5.00% CO_2 at 38.50 ˚C for 22 - 24 hr.

Immunocytochemical analysis of mitogen-activated protein kinase (MAPK) and maturation promoting factor (MPF) expressions. Matured oocytes were placed on poly-L lysine-coated slides, covered with a Vaseline-sealed cover slip, and fixed for 24 hr. They were treated with 3.00% hydrogen peroxide, washed with phosphate-buffered saline, treated with 0.025% trypsin at 37.00 ˚C, and washed again. An ultra-V block was applied, followed by the MAPK primary antibody for 60 min. Biotinylated link and streptavidin were added sequentially, each followed by phosphate-buffered saline washes. Chromogen DAB was added, then the slides were washed and stained with methylene green. Observations were made using an Olympus CX-41 microscope at 400× magnification.[8] Expression was evaluated using the immunoreactive Remmele scale, calculated by multiplying the percentage of positive cells by colour intensity.[8]

Mitochondrial DNA detection by quantitative polymerase chain reaction. The DNA extraction from oocytes was performed using proteinase K, and DNA concentration was measured with a NanoDrop (Thermo Fisher Scientific, Wlmington, USA). A serial standard from control oocytes was created by diluting the DNA extract; 10.00 µL of the original stock DNA was pipetted into the tube 1.00×10^{-1} and mixed, and then 10.00 µL was transferred sequentially through to tube 1.00×10^{-6}. Samples were run on a quantitative polymerase chain reaction machine (QuantStudio 5) with 2.00 x SensiFAST SYBR Hi-ROX, 10.00 µM forward primer, 10.00 µM reverse primer, H_2O, and template DNA. The program included initial denaturation at 95.00 ˚C for 5 min, followed by cycles of denaturation at 94.00 ˚C for 15 sec, annealing at 56.00 ˚C for 30 sec, and extension at 72.00 ˚C for 20 sec.

Data analysis. The MAPK and MPF expressions data were analyzed semi-quantitatively using the Kruskal-

Wallis test, followed by the Mann-Whitney test for significant differences ($p < 0.05$). Copy numbers of mtDNA were analyzed using ANOVA test for significant differences ($p < 0.05$). Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, USA).

Results

Immunocytochemical analysis of MAPK and MPF expressions. Table 1 presents the mean rank scores of MAPK and MPF expressions in Kacang goat oocytes across different experimental groups. The control group showed a MAPK expression mean rank of 17.38, which significantly differed from the treatment groups. Specifically, oocytes supplemented with 50.00 ng mL^{-1} dose of IGF-1 (T1) exhibited a mean rank of 4.50, indicating a marked decrease in MAPK expression compared to the control. Conversely, the IGF-1 supplementation at a dose of 100 ng mL^{-1} (T2) demonstrated a significantly higher mean rank of 23.50, while the 150 ng mL^{-1} IGF-1 group (T3) had a mean rank of 20.63, both suggesting an increase in MAPK expression relative to the control. These data were examined for statistical differences and there were significant distinctions between these groups ($p < 0.05$). For MPF expression, the control group had a mean rank of 11.25. The T1 group showed an increased mean rank of 15.75, while the T2 group exhibited the highest mean rank of 23.25, indicating enhanced MPF expression. The T3 group's mean rank of 15.75 was not significantly different from T1, but it was distinct from the control. These variations underscore the differential impact of IGF-1 concentrations on MPF expression in Kacang goat oocytes The graph clearly delineates the variations in MAPK and MPF expressions, supporting the statistical data presented in Table 1.

Table 1. The mitogen-activated protein kinase (MAPK) and maturation promoting factor (MPF) expressions scores' mean rank in Kacang goat oocytes (n = 25 in each group).

Groups	MAPK expression	MPF expression
Control	17.38^{a}	11.25^{a}
T1	4.50^{b}	15.75^{ab}
T2	23.50^{c}	23.25^{b}
T3	20.63^{ac}	15.75^{ab}

T1, T2 and T3 were supplemented with 50.00, 100, and 150 ng mL^{-1} of insulin-like growth factor, respectively.
^{abc} Different superscripts in each column indicate significant differences ($p < 0.05$).

The scoring in this study was assessed based on immunocytochemical staining expression using the Remmele method, namely the Immunoreactive Scale, as seen in Figures 1 and 2. Positive expression is indicated by a brown colour in the oocytes, while negative expression is indicated by a green colour according to the counterstain used, methylene green. Strong MAPK expression is shown in control, T2, and T3 groups, indicated by the dark brown colour, meanwhile strong MPF expression is shown in T1, T2, and T3 groups. This dark brown colour represents the strong antigen-antibody binding with the DAB chromogen, while considering MAPK in T1 group and MPF in control group, the oocytes show a lighter brown colour compared to the other groups of each variable, respectively. This indicates that the antigen-antibody and DAB chromogen binding regarding MAPK in T1 group is weaker than control, T2, and T3 groups. In addition, considering MPF, control group also shows weaker binding compared to the T1, T2, and T3 groups. Further, this indicates that weaker binding leads to light colour expression, resulting in lower expression scoring values.

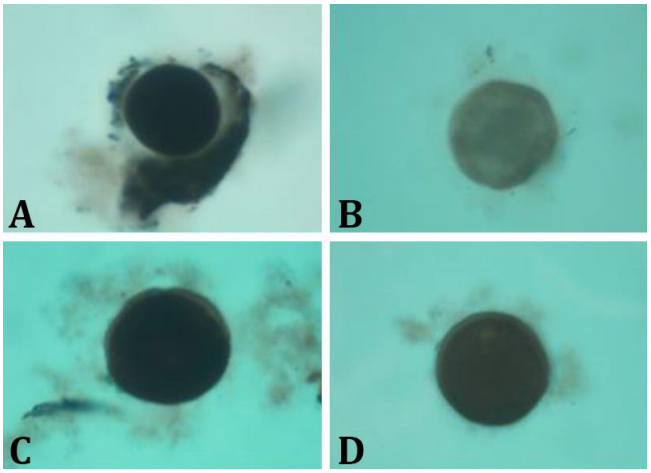

Fig. 1. Mitogen-activated protein kinase (MAPK) expression in oocytes of various groups at 400× magnification. **A)** Control; **B)** Insulin-like growth factor (IGF)-1 supplementation at a dose of 50.00 ng mL^{-1}; **C)** IGF-1 supplementation at a dose of 100 ng mL^{-1}; and **D)** IGF-1 supplementation at a dose of 150 ng mL^{-1}.

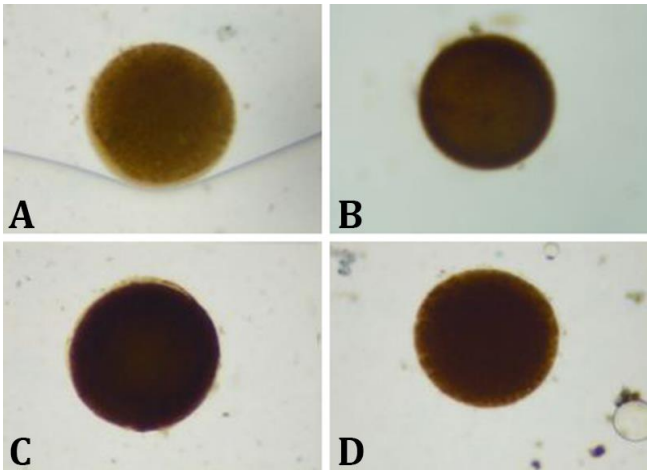

Fig. 2. Maturation promoting factor (MPF) expression in oocytes of various groups at 400 × magnification. **A)** Control; **B)** Insulin-like growth factor (IGF)-1 supplementation at a dose of 50.00 ng mL^{-1}; **C)** IGF-1 supplementation at a dose of 100 ng mL^{-1}; and **D)** IGF-1 supplementation at a dose of 150 ng mL^{-1}.

As Figure 1 showed, the trend is consistent with the idea that IGF-1 increases MAPK expression at a dose of 100 ng mL^{-1}, showing that there is a significant rise. Distinct variations in colour intensity correspond to different degrees of protein expression, which in turn indicate different intensities of antigen-antibody interaction. Oocytes in control group are uniformly dark brown in colour, indicating significant MAPK expression. When comparing the results of IGF-1 supplementation in different groups, this colour intensity can be used as a reference. Lighter brown staining indicates a noticeable decrease in MAPK expression in the T1 group. Reduced IGF-1 concentrations may inhibit MAPK activation, based on these results. The T2 group revealed strong dark brown staining and stronger MAPK expression, indicating that this IGF-1 concentration has the greatest impact on increasing MAPK activity. The T3 group also showed similar result as T2, although with a little less consistent intensity; the MAPK expression is still high and the staining is dark brown. This data point to the idea that enhanced MAPK activity is sustained by high doses of IGF-1.

Figure 2 demonstrated that oocytes in the control group display less brown staining, which means they have reduced MPF expression. This is in keeping with the baseline level of oocyte maturation, which was unaffected by IGF-1 stimulation. Moreover, substantially darker staining relative to the control indicates enhanced MPF expression in the T1 group. It appears that even modest amounts of IGF-1 can activate MPF, speeding up the maturation process of the oocyte. In the T2 group, the dark brown staining is the most pronounced, indicating the highest level of MPF expression compared to the other groups. The ideal concentration of IGF-1 to enhance MPF activity is highlighted by this peak response. In the T3 group, the staining intensity was lower than T2 group, but it was comparable to the T1 group. This indicated that MPF expression was higher in this group than control group. It appears that increasing the concentration of IGF-1 does not lead to a further rise in MPF expression, but rather may reach a plateau or even show a minor decrease in its activity.

Table 2 illustrates mtDNA copy number in each oocyte maturation group. The control group displayed an mtDNA copy number of 3.30 ± 3.89. The T1 group had a significantly higher mtDNA copy number of 100.57 ± 72.83. The T2 group, further increased the mtDNA copy number to 131.53 ± 54.62, indicating a substantial enhancement in mitochondrial replication. However, the T3 group exhibited a significant reduction in mtDNA copy number to 10.97 ± 8.59, which was still higher than the control but lower compared to the T1 and T2 groups. These findings highlight a dose-dependent response of IGF-1 on mtDNA replication, with optimal mitochondrial proliferation observed at 100 ng mL^{-1} IGF-1 supplementation. Table 2 illustrates a dose-dependent influence of IGF-1 on mitochondrial replication, with a maximum response at 100 ng mL^{-1}. The significant reduction in the T3 group highlights the necessity of regulating IGF-1 levels to prevent detrimental impacts on mitochondrial function. This pattern is essential because mitochondria are vital for energy generation, and their quantity directly influences the oocyte's capacity to facilitate fertilization and subsequent embryonic development.

Table 2. Mitochondrial DNA (mtDNA) copy number in each oocyte maturation group (n = 25 in each group).

Groups	Copy number of mtDNA
Control	3.30 ± 3.89 [a]
T1	100.57 ± 72.83 [b]
T2	131.53 ± 54.62 [b]
T3	10.97 ± 8.59 [a]

T1, T2 and T3 were supplemented with 50.00, 100, and 150 ng mL^{-1} of insulin-like growth factor, respectively.

[ab] Different superscripts in each column indicate significant differences ($p < 0.05$).

Discussion

The present research demonstrates that IGF-1 supplementation during IVM may markedly enhance MAPK and MPF expressions and elevate mtDNA copy number in Kacang goat oocytes, with 100 ng mL^{-1} identified as the best dose. At this level, we noted the peak expressions of MAPK and MPF, together with a significant increase in mtDNA replication. These findings underscore the significant function of IGF-1 in facilitating both nuclear and cytoplasmic maturations, hence enhancing oocyte quality and perhaps improving IVF outcomes in animals.[5]

Our findings demonstrated clear variations in MAPK and MPF expressions at the various IGF-1 doses examined. Oocytes subjected to the 100 ng mL^{-1} IGF-1 treatment exhibited markedly elevated MAPK expression compared to the control and other IGF-1 groups. This indicates heightened activation of the MAPK pathway, being crucial for activities, such as germinal vesicle breakdown and advancement to metaphase II.[9,10] The elevated MAPK levels in T2 and T3 groups indicate that IGF-1 may modulate the strength of MAPK activation in a dose-dependent manner. Correspondingly, MPF expression peaked in T2 group, consistent with studies indicating that MAPK activation is intricately linked to cumulus expansion and nuclear maturation, crucial processes for successful fertilization.[11,12] As previously discussed, MAPK/ extracellular signal-regulated kinase activation shuts gap junctions, allowing phosphodiesterase III to convert cyclic adenosine monophosphate to adenosine monophosphate, lowering protein kinase A activity, and activating MPF (cyclin-dependent kinase 1 and cyclin B), causing germinal vesicle breakdown and advancement to metaphase I and II.[11,13]

Upon examining mtDNA replication, oocytes in the T2 group had a significantly increased mtDNA copy number compared to the control and other treatment groups. This indicates that IGF-1 facilitates mitochondrial biogenesis *via* pathways, including MAPK, PGC-1α, and TFAM, so ensuring the oocyte possesses sufficient mtDNA to satisfy the energy requirements of fertilization and early embryonic development.[7,14,15] The mtDNA copy number in metaphase II oocytes is critical for subsequent developmental events. These oocytes have significantly more mtDNA than other cell types, being essential for blastomere survival during embryonic cleavage when mitochondria are not replicated.[9,16] Abnormal mitochondrial numbers are associated with poor oocyte quality in humans and animal models.[17] The reduced mtDNA copy number in the T3 group (150 ng mL^{-1}) relative to T2 group indicates a potential top limit for IGF-1's efficacy in mitochondrial replication; at elevated dosages, IGF-1 may impede this process.

The reason of this reduced efficacy may be related to IGF binding proteins in granulosa cells and oocytes, regulating IGF-1 availability without exceeding normal tissue needs. Changes in IGF binding proteins concentration affect IGF bioavailability in oocytes.[4] In this study, 150 ng mL^{-1} of IGF-1 was less optimal than 100 ng mL^{-1}, possibly due to the tissue saturation at high concentrations, altering the functional mechanisms or inhibiting IGF-1 activity, affecting oocyte maturation and inducing apoptosis.[18]

This dose-dependent response corresponds with data from other species, where optimum IGF-1 dosages differ but generally improve mitochondrial activity and oocyte maturation.[19] These findings reflect those observed in research involving other species, where IGF-1 supplementation has enhanced IVM outcomes in species, such as felines,[20] canines,[10] bovines, porcines,[21] and also ovines.[3] In these animals, IGF-1 facilitates maturation by activating pathways like MAPK and phosphatidylinositol 3-kinase/Akt, which are crucial for cell survival, cumulus growth, and meiotic development.[11,22] Activated Akt has downstream effects that promote granulosa cell proliferation, cumulus expansion, and oocyte maturation.[23] Our findings corroborate this process, since we noted that IGF-1 enhances both MAPK and MPF expressions, comparable with findings from other research in other species.

Furthermore, our research indicates that IGF-1 may activate the phospholipase C pathway, which catalyses the hydrolysis of PIP2 into IP3, resulting in the release of Ca^{2+} and enhanced mitochondrial absorption.[24] The Ca^{2+} release from the endoplasmic reticulum stimulates mitochondrial uptake, changing cell shape and motility, and promoting cell migration and proliferation.[25] This pathway preserves mitochondrial integrity, being essential for oocyte maturation. Additional investigation into IGF-1's influence on calcium homeostasis and mitochondrial dynamics may enhance our comprehension of its function in oocyte development.[26]

Although our findings offer significant insights, some limitations exist. The sample size, while can be adequate for preliminary analysis, might be augmented in subsequent research to validate these tendencies. Moreover, investigating IGF-1 alongside other growth agents or anti-oxidants may uncover possible synergies that enhance oocyte quality.[15] Subsequent research may investigate apoptotic markers or additional indications of mitochondrial integrity to further our comprehension of IGF-1's function in oocyte maturation.[5] Furthermore, it is essential to examine the influence of IGF-1 under diverse IVM circumstances and its effects on post-fertilization embryo development to elucidate its practical uses in livestock breeding.[24]

This work advocates for the utilization of IGF-1, specifically at a concentration of 100 ng mL^{-1}, as an effective means to enhance IVM results in Kacang goat oocytes. Implementing this ideal IVM strategy may boost reproductive efficiency and yield superior results in breeding programs for cattle producers.[27] This research paves the way for advancements in animal IVF techniques, potentially enhancing the efficacy of these technologies in agricultural applications. Future research may concentrate on the practical uses of IGF-1 in diverse livestock environments and assess the economic viability of its incorporation into extensive breeding programs.

This study demonstrates that IGF-1 supplementation in the IVM medium significantly affects the expressions of MAPK and MPF, as well as the mtDNA copy number in Kacang goat oocytes. The optimum IGF-1 concentration for improving these parameters has been found to be 100 ng mL^{-1}. This concentration produced the greatest MAPK and MPF expressions, and mtDNA copy number, highlighting that IGF-1 plays a key part in oocyte maturation and quality. These findings provide useful insights into the improvement of IVM regimens for livestock, with the potential to improve IVF success rates.

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Conflict of interest

The authors declare no conflict of interest.

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