

The Effects of FSH and Glutathione Supplementation to the in Vitro Maturation Media on Mouse Oocyte Maturation

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Abstract. Either Follicle-stimulating hormone (FSH) or glutathione solely has been known to have benefits on oocyte maturation, yet study about combination effects are still limited. This study aimed to observe the effects of FSH and glutathione supplementation to the in vitro maturation (IVM) media on mouse oocyte maturation. Immature oocytes of F1 (C57BL/6xCBA) female mice were matured for 24 hours in Minimum Essential Medium (MEM) Alpha supplemented with 5 µg/mL FSH and 0.15 mM glutathione, in 3 different combinations, i.e. the MEM alpha without any supplementations, supplementation of FSH solely, and combination of FSH with glutathione. A total of 209 immature oocytes were studied to examine the oocyte maturation. Treatment with FSH supplementation alone ($p=0.043$) and in combination with glutathione ($p=0.016$) showed significantly higher oocyte maturation. This study indicated that in vitro oocyte maturation can be augmented by supplementation of IVM medium with FSH, either solely or along with glutathione.

Keywords: FSH, Glutathione, In Vitro Maturation, Medium, Oocyte.

1 Introduction

Suboptimal composition in IVM, is believed as one of the factors related to the lower success rates of IVM. Based on several animal studies, various existing commercial IVM media show the different effects on oocytes maturation and the following preimplantation development [1]. In fact, it still becomes a critical challenge to reveal all the factors contributing to the competence of oocyte maturation and how the control mechanisms work [2]

One of the important components known for oocyte maturation is FSH. FSH may improve the oocyte maturation, fertilization and embryo development. It functions to enhance the maturation of oocytes in isolated cumulus-oocyte complexes (COCs), especially nuclear maturation [3]. The presence of FSH in IVM medium is considered favourable for oocyte quality [3][4][5]. Study by Lee et al (2007) indicated that cumulus cell expansion and nuclear in vitro maturation rate of canine oocyte can be promoted by FSH supplementation to IVM medium. Li et al (2013) signified that the varied concentration of FSH added to IVM medium resulted in different outcomes. According to them, addition 50 or 100 IU FSH to IVM medium exhibited significantly higher on in vitro oocyte maturation when compared with supplementation of 200 IU. Still, the most suitable concentration remains unclear [6].

The importance of reduced form of glutathione for in vitro oocyte maturation has been also reviewed in several studies. It is considered as a strong antioxidant. It may limit free radicals produced in IVM process and prevent the impacts of oxidative stress (OS) on the oocytes, such as prematuration arrest and chromosomal segregation [7]. Naturally, the presence of cumulus cells protects the oocytes from OS through glutathione synthesis [8]. Tatemoto et al (2000) suggested that during IVM, greater DNA damage due to reactive oxygen species (ROS) may occur on denuded-oocytes when compared with COC because of the absence of cumulus cells [9]. In addition, Curnow et al (2011) reported the improving of early and late macaque oocyte maturation after glutathione addition to the IVM medium.[10]

Tabel 1. Compound of IVM medium [11]

Compound (mmol/l)	Medium					
	TCM199	Waymouth MB 752/1	Ham's F-12	MEM	DMEM	HECM
CaCl ₂	1.802	0.82	0.23	1.36	1.36	1.9
MgSO ₄	0.788	3.96	0.58	0.79	0.79	
KCl	5.367	2.01	3	5.37	5.37	3
NaCl	116.359	102.67	130.05	116.36	109.51	113.8
NaHCO ₃		26.66	14	26.19	44.04	25
Na ₂ HPO ₄	1.017	2.5	1.18	1.17	1.04	
DL-alanine	0.561		0.1			
L-arginine	0.332	0.36	1	0.6	0.4	
DL-aspartic acid	0.451	0.45	0.1			0.01
Asparagine						0.01
L-cysteine	6.98×10 ⁻⁴	0.51	0.22			0.01
L-cystine	0.083	0.06		0.1	0.2	
DL-glutamic acid	0.908	1.02	0.1			0.01
L-glutamine	0.684	2.4	1	2	4	0.2
Glycine	0.666	0.67	0.1		0.4	0.01
L-histidine	0.104	0.78	0.17	0.2	0.2	0.01
Hydroxy-L-proline	0.0763					
DL-isoleucine	0.305	0.19	0.03	0.4	0.8	
DL-leucine	0.915	0.38	0.1	0.4	0.8	
L-lysine	0.479	1.64	0.25	0.5	1	0.01
DL-methionine	0.201	0.34	0.03	0.1	0.2	
DL-phenylalanine	0.303	0.3	0.03	0.2	0.4	
L-proline	0.348	0.43	0.3			0.01
DL-serine	0.476		0.1		0.4	0.01
Taurine						0.5
DL-threonine	0.504	0.63	0.1	0.4	0.8	
DL-tryptophan	0.0979	0.20	0.01	0.05	0.08	
L-tyrosine	0.256	0.26		0.23	0.46	
DL-valine	0.427	0.56	0.1	0.4	0.8	
Glucose	5.55	27.75	10	5.55	24.97	
DL-lactate						4.5
Pyruvate					1	
Glutathione	1.62×10 ⁻⁴	0.16				
Hypoxanthine	0.0022	0.18	0.04			

TCM=tissue culture medium; MEM=Minimum Essential Medium; DMEM=Dulbecco's modification of Eagle's medium; mBM-3=Basic salt medium 3; HECM=hamster embryo culture medium.

Since not all commercial IVM media provide those two components in their composition [11], it is still questioned how the effects of that combination on oocyte maturation in vitro. As known advantages of FSH and glutathione for oocyte maturation, we hypothesized that modification of IVM media by FSH and glutathione supplementation may increase the IVM success rate. This study was undertaken to observe the effects of FSH and glutathione supplementation to IVM medium on oocyte maturation and embryo development

2 Materials and methods

2.1 Mice and replicates

F1 (C57BL/6xCBA) female mice were used in this project. Mice were housed in Animal House Monash Medical Center (MMC) and maintained in accordance with associated codes of practice. All animals were approved by the MMC Animal Ethics Committee 'A' under approval no. MMC 2011/84. There were six replicates overall for in vitro oocyte maturation observation. Each replicate involved two mice. Priming and oocyte collecting were done in the afternoon time, almost in the same time for each replicate.

2.2 Media preparation

IVM base medium used was Minimum Essential Medium (MEM) Alpha (Gibco, Invitrogen Corporation) supplemented with 25 mM sodium bicarbonate, 2 mM L-alanyl-L-glutamine (GlutaMAX™, Gibco, Invitrogen Corporation), 1 % penicillin/streptomycin (Gibco, Invitrogen Corporation), and 0.4 % w/v bovine serum albumin (BSA). pH was adjusted to 7.8-7.9 and osmolarity was within 280 ± 5 . It was then supplemented with 5 µg/ml FSH (Folltropin®, Bioniche Animal Health Canada Inc.), and 0.15 mM L-Glutathione reduced (Sigma Chemical Co.) according to 3 different treatments as shown in table 2. For handling medium, MEM (HEPES, Gibco, Invitrogen Corporation) was supplemented with 0.3 % w/v BSA, 2 mM L-alanyl-L-glutamine and 1 % penicillin/streptomycin. pH ranged between 7.2-7.4 and osmolarity was between 260-280. All IVM media and handling media were prepared fresh weekly. Fertilization medium used was Quinn's Advantage® Fertilization (HTF; Sage, In-vitro Fertilization, Inc., Trumbull) supplemented with 2mM L-alanyl-L-glutamine.

Table 2. IVM treatments used in this project

Treatment	Supplementation	Final concentration*
I	-	-
II	FSH	5 µg/ml
III	FSH Glutathione	5 µg/ml 0.15 mM

* osmolarity was assured to be within the range

2.3 Priming and embryo collection

5 IU pregnant mare's serum gonadotropin (PMSG; Folligon, MSD Animal Health, Bendigo) was injected (intra-retroperitoneal) to female mice. 48 hours later, the mice were killed by cervical dislocation. The ovaries were removed and transferred to sterile tubes with warmed handling medium. To retrieve immature oocytes, large antral follicles were punctured with sterile needles no. 29 G. Immature oocytes collected were all in germinal vesicles (GV) stage and categorized as shown in Table 3. The sample of pictures can be seen in figure 1. Immediately, all immature oocytes collected were placed into fresh handling media, then

washed once through IVM base medium and finally divided equally into three different IVM treatment groups.

Table 3. Immature oocytes' categories

Category	Explanation
nude cumulus-oocyte complex (n-COC)	None or very few cumulus cells (< 10) surrounding immature oocyte
partial COC (p-COC)	Several layers of cumulus cells partially surrounding immature oocyte
full COC (f-COC)	Several layers of cumulus cells completely surrounding oocyte

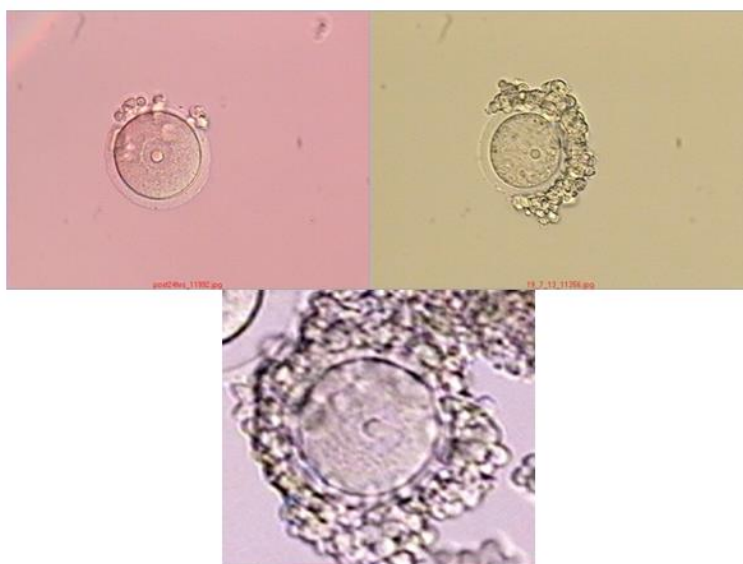


Fig. 1. Immature oocyte collected A. nude COC; B. partial COC; C. full COC

2.4 Maturing oocytes in vitro

Immature oocytes were put into 4-well Nunc dish with IVM media according to three different treatments (after overnight incubation with 37°C, 5% CO₂) for 24-26 hours. Maturation was indicated by expansion of cumulus cells, or the presence of first polar body (PB I).

2.5 Statistical analysis

Statistical analysis was performed using Chi-square test to evaluate the effect of FSH and glutathione supplementation in IVM medium to maturation rate. Statistically significant difference was defined as p value < 0.05.

3 Result

3.1 In vitro oocyte maturation rate

Overall, 209 immature oocytes were retrieved from six replicates. In general, 88 (42.10 %) were nude, 70 (33.49 %) were partial COC and 51 (24.41 %) were full COC. A distribution of oocyte type is given in table 4. Maturation of oocytes correlates significantly with FSH and glutathione supplementation to the IVM medium ($p=0.043$ and $p=0.016$ respectively for treatment 2 and 3).

Table 4. Distribution of immature oocyte number and in vitro maturation rate*

Treatment**	Immature oocyte collection			Total	Mature oocyte Number (rate)
	n-COC	p-COC	f-COC		
I	30 (42.86 %)	24 (34.28 %)	16 (22.86 %)	70	15 (21.43 %)
II	29 (41.42 %)	23 (32.85 %)	18 (25.73 %)	70	26 (37.14 %)
III	29 (42.02 %)	23 (33.33 %)	17 (24.65 %)	69	29 (42.03 %)
Total	88 (42.10 %)	70 (33.49 %)	51 (24.41 %)	209	

* from six replicates** Treatment I = MEM- α without any supplementation; Treatment II = MEM- α with FSH supplementation; Treatment III = MEM- α with FSH and glutathione supplementation.

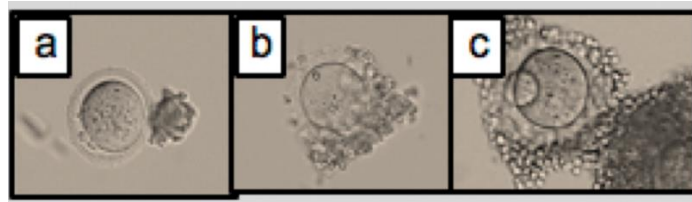


Fig. 2. Mature oocytes A. PB I extruded; B dan C. expansion of COC

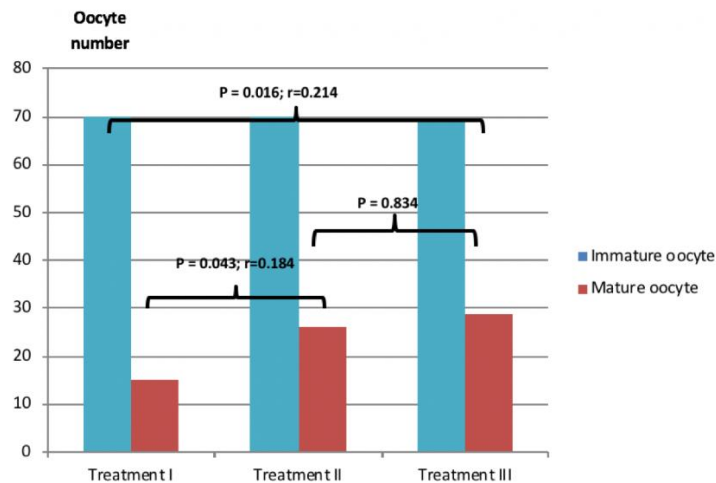


Fig. 3. Proportion of in vitro oocyte maturation between different treatments of IVM media

4 Discussion

The results have indicated that single supplementation of FSH (5 µg/ml) to MEM alpha medium showed a significant difference of in vitro oocyte maturation rate achieved from the treatment without FSH supplementation (P value < 0.05). This finding supports our hypothesis that FSH supplementation to IVM medium may enhance the in vitro oocyte maturation. The result is also in accordance with the previous study involving mouse oocytes done by Eppig et al (2000). Besides, study on bovine oocytes by Ali and Sirard (2005) also highlighted the good effects of FSH addition to maturation culture media. Another study has proven that FSH receptor mRNA exists only in granulosa cells and cumulus cells, not in oocytes isolated from bovine antral follicles (van Tol et al, 1996). It improves GV breakdown in COC, but not in denuded oocytes. It is also considered to produce the substance that may accelerate oocyte meiotic resumption (Byskov et al, 1997). Hence, its supplementation to MEM alpha medium is beneficial. [12],[13],[14],[15]

This project also revealed that addition of 0.15 mM glutathione to IVM medium, along with FSH showed a significant difference of in vitro oocyte maturation rate achieved from the treatment without FSH supplementation (P value < 0.05). Similar to this finding, IVM medium supplemented with 5 mM of glutathione ethyl ester had significantly increased the IVM rates of Macaque oocytes [10]. Supplementation glutathione to the IVM base medium showed its benefit to the oocyte maturation as it acts as the antioxidant agent that prevents the oocytes from OS.

It is known that oocyte maturation, either in vivo or in vitro, should gain both nuclear and cytoplasmic maturation. As cytoplasmic maturation is characterized by some ultra-structural and biochemical changes in oocyte cytoplasm that are necessary for fertilization and further embryo development, it will also influence the oocyte competence in successful fertilization and corresponding embryo development [12],[16]. Miron (2006) highlighted the less competence in cytoplasmic maturation of IVM oocyte compared to in vivo matured oocyte as the cause of low developmental rates of IVM oocytes after cleavage, while the nuclear maturation is alright. [17]

This study had several limitations. Regarding the methods, they were also restricted to only one determined concentration of any supplementations added with only simple combinations of treatments arranged. Indeed, the effect of those supplementations could not be analysed in more details. Moreover, whether different concentrations and combinations may affect the results could not be revealed. No detail evaluations on cytoplasmic maturation should also be improved in the future study for more details. Besides, no separated analysis run on three different groups of immature oocytes collected made it unable to evaluate the influence of cumulus cells to in vitro oocyte maturation in connection with the work mechanism of FSH and/or any other supplements.

5 Conclusion

In conclusion, this study demonstrated that in vitro oocyte maturation can be augmented by supplementing IVM medium with FSH, either solely or with glutathione. Finally, for the future study, improvement for limitations in this study would help to get a better and more reliable result.

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