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Research Article

Effect of Different Types of *In Vitro* Maturation Medium (IVM) on Cumulus Cell Expansion and Nuclear Maturation Rate of Non-vitrified and Post-vitrified Thawed Porcine Follicular Oocytes

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ABSTRACT

Aim: To study the effect of different types of *In Vitro* maturation medium on cumulus cell expansion and nuclear maturation rate of non-vitrified and post vitrified-thawed porcine follicular oocytes.

Methods: Three different *In Vitro* Maturation (IVM) media (IVM-I contained only hormones and growth factors, IVM-II contained porcine follicular fluid along with hormones and growth factors and IVM-III contained porcine follicular fluid, foetal bovine serum and growth factors without any hormone) were prepared using modified tissue culture medium (TCM-199) as a base medium for the study. Porcine oocytes were collected and IVM in above media was done for 48 h.

Results: In case of non-vitrified and vitrified oocytes the rate of cumulus cells expansion did not differ significantly, but nuclear maturation rate differed significantly between IVM media. The nuclear maturation rate was significantly higher in IVM-II and IVM-III than in IVM-I both for non-vitrified and vitrified groups.

Conclusion: Addition of follicular fluid to the IVM media leads to significantly higher nuclear maturation rate for both vitrified and non-vitrified porcine follicular oocytes.

Keywords: Vitrification; *In vitro* maturation; Porcine oocytes; Follicular fluid

INTRODUCTION

The biological and technological advances made during the past few decades gave rise to the development of four generations (1. Artificial insemination (AI) and gamete and embryo freezing; 2. Multiple ovulation and embryo transfer (MOET); 3. *In Vitro* Fertilization (IVF) procedures; 4. Cloning and nuclear transfer) of assisted reproductive technologies. Apart from the historically documented scientific curiosity, the emergence and development of reproductive technologies have been driven by the potential economic gain achievable through increase in the number of offspring from genetically superior animal. Cryopreservation of germinal vesicle (GV) stage oocytes coupled with *In Vitro* maturation (IVM) and *In Vitro* Fertilization (IVF) plays an important role in the on spot fertility preservation and management of genetic resources, low-cost international movement of selected genotypes and rapid cloning procedures. Because of high fecundity of pigs, the need for extra offspring per breeding female is lesser than other domestic animals. However, considering the practical utility, research activity has been intensified in producing large quantities of matured pig oocytes and embryos, through IVM, IVF and *In Vitro* culture (IVC). There is a growing need for transferring genetic material of pig worldwide with minimal health risk and low cost. In fact, it has been argued that any improvement in the swine *In Vitro* embryo production (IVEP) system would revolutionize not only the reproductive management of swine, but also increase the use of pigs for biotechnological and biomedical applications involving the production of pharmaceutical products and as organ donor for

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xenotransplantation [1]. They are also used as a model for studies of human diseases because of their physiological similarities to human [2]. The developmental competence of *In Vitro* matured oocytes is influenced by various factors during IVM, such as maturation environment, type of medium and additives [3-5]. The quality of IVM oocytes influences the subsequent IVEP steps, like *In Vitro* fertilization, embryonic development, foetal development, etc. In comparison with other species, there are limited studies on IVEP in pig especially from vitrified follicular oocytes. This may be due to inefficient vitrification, oocytes maturation and fertilization techniques, poor development capacity of *in vitro* produced embryos and suboptimal embryo culture condition [6]. In view of the above facts, the present study was undertaken to find out the effect of different types of IVM on cumulus cell expansion and nuclear maturation rate of non-vitrified and post vitrified-thawed porcine follicular oocytes.

MATERIALS AND METHODS

Collection of oocytes

Porcine ovaries were collected from local abattoirs, immediately after slaughter of the animal and transported to the laboratory within 1 to 2 h in a flask containing Normal Saline Solution (NSS 0.9%) with antibiotic at environmental temperature (22°C-28°C). In the laboratory, extraneous tissues of the ovaries were removed with the help of a pair of scissors. The ovaries were then washed 3-4 times in NSS containing antibiotic. Follicles measuring 2-8 mm in diameter were selected for collection of oocytes by aspiration technique. A 10 ml disposable syringe attached to an 18 gauge needle was loaded with about 1 ml of Basic Solution (BS) that contained 20% Fetal Bovine Serum (FBS) and 5 % gentamicin prepared in Phosphate Buffered Saline (PBS) at pH 7.2.

Cumulus-Oocyte-Complex (COC) was aspirated along with follicular fluid and oocytes were picked using a sterile Pasteur pipette and transferred in 1 mL wash solution comprising BS and 3.5% sodium pyruvate. The oocytes were washed 3-4 times and classified into four grades (grade A, B, C and D) based on their gross morphology as integrity of cumulus cells [7] and described in Table 1. Only 'A' and 'B' grade oocytes were selected for the study.

Oocyte vitrification and thawing

For vitrification, the oocytes were first equilibrated in equilibration solution (10 minutes) and then exposed to final vitrification solution (30 seconds). Both the equilibration and vitrification procedures were performed at room temperature (24-25°C). The Equilibration Solution (ES) was prepared by adding cryoprotectants (ethylene glycol+ dimethylsulphoxide) 15% (v/v) and sucrose 0.25 M in basic solution. The vitrification solution was prepared by adding cryoprotectants (ethylene glycol+dimethyl sulphoxide) 35% (v/v) and Sucrose (0.5 M)+Polyvenylpyrrolidone (50 mg/mL). Immediately after exposure to vitrification solution, oocytes were loaded in pre labeled Open Pulled straws and plunged directly into liquid nitrogen (LN_2) and finally stored in LN_2 tank for one week [8]. After one week of storage oocytes were subjected to warming at 37°C and removal of cryoprotectant in a step wise manner from higher to lower dilution (oocytes were exposted for 1 min, 2 min and 2 min in 0.5 M, 0.25 M and 0.125 M sucrose in BS respectively) prior to incubation in IVM media.

In Vitro Maturation (IVM)

The IVM media were prepared using tissue culture medium (TCM-199) as a base medium along with additives. Three different IVM media were prepared using the chemical composition as described previously with slight modification [9-11]. The details of the three media are given in Table 2.

The non-vitrified and vitrified-thawed immature oocytes were washed separately 2-3 times in IVM medium. In a 60 mm sterile disposable petridish IVM droplets were prepared by placing 50∝l of IVM medium in each droplet. The droplets were then covered with sterile tissue culture oil (mineral oil). Ten to fifteen oocytes were then transferred into each droplet. The dishes with oocytes were then

Table 1: Grading and selection of oocytes [7].

Grading	Selection of oocytes
Grade A	Oocytes surrounded by 3 or more complete layers of cumulus cells adhered to the zona pellucida and with homogenous nucleus
Grade B	Oocytes surrounded by 2 complete layers of cumulus cells adhered to the zona pellucida and with homogenous nucleus
Grade C	Oocytes surrounded by 1 complete layer of cumulus cells adhered to the zona pellucida
Grade D	Oocytes having less than 1 complete layer of cumulus cells adhered to the zona pellucida and with pyknotic nucleus

Table 2: Preparation of IVM media.

Ingredient	Amount	IVM I		IVM II		IVM III
		Hormone rich	Hormone free	Hormone rich	Hormone free	
TCM-199	Up to 100 ml	+	+	+	+	+
FSH	5 µg/ml	+	-	+	-	-
LH	5 µg/ml	+	-	+	-	-
Oestradiol	1 ng/ml	+	-	-	-	-
Hepes	25 mM	+	+	+	+	+
Sodium Pyruvate	0.22 mg/ml	+	+	+	+	+
Gentamicin	200 ug/ml	+	+	+	+	+
IGF	50 ng/mL	+	+	+	+	+
EGF	10 ng/mL	+	+	+	+	+
PFF [21,23]	10% V/V	-	-	+	+	+
FBS	10% V/V	-	-	-	-	+

placed into a CO_2 incubator maintained at 38.5°C inner chamber temperature, 5% CO_2 and 95% relative humidity for 24 hours for maturation in hormone rich medium followed by incubation for another 24 hours in hormone-free IVM medium at the same condition.

Assessment of cumulus cells expansion

After the completion of 48 h of incubation, maturation of oocytes was assessed based on the cumulus cells expansion by examining COCs under stereo-zoom and phase contrast microscopes. The COCs that had tightly attached cells surrounding the oocyte with a smooth surface over the cumulus were defined as compact while COCs that had cumulus cells detached from the oocyte with matrix visible between cumulus cells were recorded as expanded. To ensure uniformity COCs with the maximum degree of expansion where all layers of cumulus cells expanded even those closest to the oocytes were only considered as expanded [12].

Assessment of nuclear maturation

For the assessment of nuclear maturation expanded COCs were denuded and stained after fixation. The expanded cumulus cells adhering to the zona pellucida were removed by washing the oocytes in 0.1% hyaluronidase droplet for less than 1 minute by gentle pipetting. Denuded oocytes were then fixed in acetic alcohol (acetic acid 1 part and ethyl alcohol 3 parts) and stained with 1% aceto-orcein stain as per the method described by Martin [13]. Oocytes showing extrusion of first polar body and metaphase II were considered as those undergoing complete nuclear maturation. The statistical analysis (Chi-square) of the data was done by using SAS 14.0.

RESULTS

In the present study oocytes with cumulus cells expansion was found to be 87.7%, 93.5% and 95.1% in non-vitrified and 78.8%, 84.2% and 88.6% in vitrified groups for IVM-I, IVM-II and IVM-III media respectively (Table 3). Chi-square test revealed that the rate of cumulus cells expansion of oocytes did not differ significantly between IVM media for both non-vitrified and vitrified oocytes. The rate of nuclear maturation of oocytes in IVM-I, IVM-II and IVM-III was found to be 60.3%, 75.9% and 82.6% in non-vitrified and 42.3%, 57.4% and 66.0% in vitrified groups respectively (Figure 1). Chi-square test showed that the nuclear maturation rate of oocyte differed significantly between IVM media in both non-vitrified (P=0.001) and vitrified (P=0.002) groups and also between non-vitrified and vitrified groups irrespective of medium used (Table 4). The rate of nuclear maturation in both groups was significantly higher in IVM-II and IVM-III media than in IVM-I medium (Table 5).

Table 3: Rate of In vitro maturation (IVM) of non-vitrified and vitrified follicular oocytes in different IVM media based on cumulus expansion.

IVM medium	Non-vitrified				Vitrified			
	No. of oocytes incubated	No. of oocytes with expanded cumulus	Rate of IVM (%)	Chi-square value	No. of oocytes incubated	No. of oocytes with expanded cumulus	Rate of IVM (%)	Chi-square value
IVM-I	106	93	87.74	4.459 (P=0.108)	104	82	78.85	3.78 (P=0.151)
IVM-II	108	101	93.52		108	91	84.26	
IVM-III	104	99	95.19		106	94	88.68	

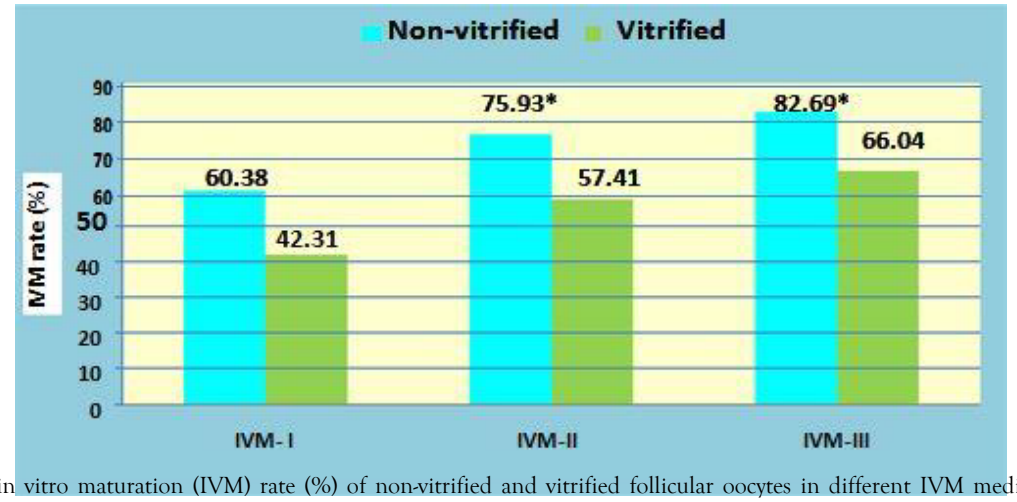

Figure 1: Variation in in vitro maturation (IVM) rate (%) of non-vitrified and vitrified follicular oocytes in different IVM media based on nuclear maturation (*indicates significant P=0.001 difference).

Table 4: Rate of In vitro maturation (IVM) of non-vitrified and vitrified follicular oocytes in different IVM media based on nuclear maturation (NM).

IVM medium	Non-vitrified				Vitrified				Chi-square value between Non-vitrified and vitrified
	No. of oocytes incubated	No. of oocytes with NM	Rate of IVM (%)	Chi-square value	No. of oocytes incubated	No. of oocytes with NM	Rate of IVM (%)	Chi-square value	
IVM-I	106	64	60.38	13.98 (P=0.001)	104	44	42.31	12.242 (P=0.002)	6.862 (P=0.009)
IVM-II	108	82	75.93		108	62	57.41		8.533 (P=0.004)
IVM-III	104	86	82.69		106	70	66.04		7.623 (P=0.006)

Table 5: Rate of In vitro maturation (IVM) of non-vitrified and vitrified follicular oocyte in different IVM media based on nuclear maturation (NM) showing independent chi-square value.

	Non-vitrified			Vitrified		
	IVM-I	IVM-II	IVM-III	IVM-I	IVM-II	IVM-III
No. of oocytes incubated	106	108	104	104	108	106
No. of oocyte with NM	64	82	86	44	62	70
Rate of IVM (%)	60.38	75.93	82.69	42.31	57.41	66.04
IVM-I	_	6.09 (P=0.0135)	13.44 (P=0.0002)	_	4.83 (P=0.0279)	11.897 (P=0.0005)
IVM-II	_	_	1.83 (P=0.1754)	_	_	1.977 (P=0.1596)

DISCUSSION

The results of the present study shows that inclusion of follicular fluid is useful for IVM in porcine oocytes and additional FSH and LH are not essential for the process. Further high IVM rates can be achieved in both vitrified and non-vitrified COCs with the use of follicular fluid but verification reduces IVM rates.

The oocyte maturation process involves the activation and inhibition of enzymes, hormones and growth factors which result in nuclear and cytoplasmic maturation. Nuclear maturation occurs spontaneously and mechanical removal of the oocytes from the follicle is capable of triggering the process, but cytoplasmic maturation occurs more gradually [14]. The identification of substances capable of delaying the nuclear maturation time and thus allowing cytoplasmic and nuclear changes to occur synchronously has been the subject of several studies [15-18]. The significantly higher IVM rate of vitrified oocytes based on nuclear maturation found in IVM II and IVM III as compared to IVM indicated superiority of the former two media in initiation and sustentation of the oocyte maturation process. This could be due to supplementation of IVM II and IVM III media with porcine follicular fluid (FF). Earlier studies documented that maturation medium supplemented with FF provided appropriate environment to bovine oocytes development since it increased the degree of cumulus cells expansion and enhanced embryonic development [19-21]. Follicular fluid consists of electrolytes, hormones, amino acids and growth factors among other components which could aid in cumulus cell expansion and nuclear maturation [19]. It was also reported that pig FF contained a substance that improved the rate of cumulus expansion, nuclear maturation, normal fertilization and normal development which was characterized as an acidic substance having a molecular mass between 10 and 200 KDa that improved the rate of cumulus expansion, nuclear maturation, normal fertilization and development [22,23].

The present findings gain support from the reports of earlier works in porcine, buffalo, bovine and mouse oocytes [24-30]. However, it is important to note that there are significantly lower IVM rate in vitrified oocytes irrespective of the medium used. This might be due to the fact that porcine oocytes are sensitive to cooling at low temperature. Upon cooling, porcine oocytes showed a reduction in membrane potential of the oolemma and various levels of membrane damage which was partly due to the high lipid content in porcine oocytes [31,32]. Cooling induced abnormalities at a chromosomal level, including disorganization of metaphase plates and multipolar spindles in oocytes [33]. Cooling to sub-zero temperature on vitrification might cause abnormal microtubule and micro filament polymerization and thus might prohibit subsequent spindle reorganization and hence disturbed nuclear and cytoplasmic maturation of the germinal vesicle. A reduction in the content of transcripts was demonstrated in vitrified ovine oocytes [34,35]. The block in development of vitrified oocytes might be due to aberrant biochemical processes within oocytes after cryopreservation such as disruption of cytoplasmic protein synthesis critical to the progression from metaphase I to metaphase II [35]. The impaired biochemical process could negatively influence the cytoplasmic maturation of oocytes [36]. Farsani observed that damage to connection between oocytes and cumulus cells after exposure to cryopreservation had adverse effect on IVM after thawing of oocytes [36].

However irrespective of these effects it was found that highest (66.04%) nuclear maturation rate was recorded in vitrified-thawed porcine oocytes in IVM III. This was found to be higher than that reported by earlier works [9,10,26]. Thus we recommend use of IVM III to achieve best maturation rates if vitrified oocytes are to be used. However for non-vitrified oocytes, IVM II or III have best maturation rates.

Gonadotropins, produced by the pituitary are considered to be a major requirement for maintaining follicle integrity and growth and genetic alterations in FSH receptor in humans affect folliculogenesis [37-39]. Indeed, several studies have shown beneficial effects of FSH/LH supplementation on COC maturation and embryo development [40-44]. Recently it has been demonstrated that FSH affects COCs expansion by altering expression of its receptors and connexin and COX-2 [43]. Considering this importance of FSH, we determined if inclusion of FSH and LH would aid in IVM of porcine oocytes. However, to our surprise, we observed that addition of both the gonadotropins had no effect on cumulus expansion or nuclear maturation in the presence of FF. It is possible that FF might itself have sufficient gonadotropins and hence additional amounts would not have any biological effects. Although, we have not studied the effects of IVM media used in the present study on embryo quality, our observations have critical significance on development of cost-effective IVM media. We believe that by simply supplementing self FF in IVM, we can exclude the use of expensive recombinant gonadotropins and reduce the cost without compromising the quality.

CONCLUSION

In summary, addition of porcine follicular fluid with or without supplementation of hormones to the IVM media has higher cumulus cell expansion and nuclear maturation rate for both vitrified and non-vitrified porcine follicular oocytes. However, in general the rates of cumulus expansion and nuclear maturation of oocytes are lower after vitrifications as compared to non-vitrified groups, but better maturation rates can be achieved by use of follicular fluid in absence of FSH and LH. We believe that our study will aid in improving assisted reproductive techniques in the porcine species. The work will also have significance in improving IVM conditions for other species including humans.

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