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Effect of Serum, Follicular Fluid and Hormones in DMEM on *In Vitro* Maturation of Pig Oocytes

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Abstract:
In vitro maturation (IVM) of pig oocytes obtained from abattors was studied based on cumulus-oocyte-complex (COC) and nuclear maturation in DMEM-based media containing 10 per cent foetal calf serum (FCS) or estrous sow serum (ESS), 10 per cent porcine follicular fluid, 15 IU/ml hCG, 10 IU/ml PMSG and 100 ng/ml oestrogen. The oocytes were incubated in the media at 39^{0}C with 5 per cent CO_2 and 95 per cent relative humidity for a period of 44-48 hours. The media contained hormones for the first 24 hours while it was devoid of hormone during the rest 20- 24 hours during oocyte maturation. The highest rate of maturation of oocytes based on COC expansion and nuclear maturation varied from 65.42 per cent and 54.28 per cent respectively in DMEM- based media containing FCS and hormones to 76.50 per cent and 61.53 per cent respectively in that containing ESS and hormones. It was found that ESS was more effective than FCS in increasing pig oocytes maturation in DMEM based media on supplementation with PMSG, hCG and oestrogen. Present findings suggested a synergistic effect of steroid and gonadotropins and other additives on IVM of porcine oocytes.
Key words: *In-vitro* maturation, hormone, follicular fluid, serum.

Introduction:
In vitro production (IVP) of embryos is an important step in realizing the benefits of assisted reproductive technology. In vitro maturation (IVM) of oocytes is of paramount importance towards IVP of embryos. In vitro production of embryos in swine is not as successful as in other livestock species (Abeydeera, 2001; Yoshioka, et.al., 2008). Developmental competence of oocytes into embryos is influenced by several factors that include maturation medium (Gil et.al.,2010) and environment (Park, et.al., 2005). Different supplements including hormones in IVM medium were proved effective for successful IVM of cumulus oocytes complex (COC) and subsequent steps for production of embryos in different species. Work on IVM of porcine oocytes carried out in India is apparently not available in the literature. Hence the present investigation was taken up to study the extent of rate of IVM of pig oocytes in DMEM medium supplemented with sera, follicular fluid and hormones.

Materials and Methods:
Porcine ovaries were collected from the local abattoir soon after the pigs were slaughtered and were transported to the laboratory in a thermos flask containing warm (37°C) Normal saline solution (N.S.S) with 500 mg/ml Gentamicin. The ovaries were washed 3-4 times with warm (37°C) N.S.S. and Phosphate buffered solution containing antibiotics. Oocytes were recovered from the medium size follicles on the surface of the ovary immediately after washing by aspiration technique using 10 ml syringe with 18 G needle and washed three times in washing medium containing 240 mg Hepes and 700 mg Bovine serum albumin fraction V in 100 ml DMEM. IVM droplets were then prepared that contained 100 µl of maturation medium which were placed gently on a 35 mm petri dish for pre-equilibration for 2 hours at 39oC in 5 per cent CO2 with 90-95 per cent humidity in an incubator. The maturation medium contained 1ml foetal calf serum (FCS) or estrous sow serum (ESS), 1 ml porcine follicular fluid (pFF), 100 µl sodium pyruvate, 100 mg gentamicin per 10 ml of DMEM basic solution into which 15 IU/ml hCG, 10 IU/ml PMSG and 100 ng/ml oestrogen (E2) were added in one fraction of it while the other equal fraction had no hormones. The oocytes were then washed three times in warm (39^{0}C) maturation medium and transferred to maturation droplets. The oocytes were subjected to IVM @ 10-12 oocytes per maturation droplet covered with warm (39^{0}C) mineral oil. The petri dish containing oocytes in maturation droplets were kept in an incubator maintained at 39°C with 5 per cent CO_2 and 95 per cent relative humidity for a total period of 44- 48 hours. The maturation droplet contained hormones during first 24 hours of incubation while it was devoid of hormone during the later 20-24 hour of incubation. After the completion of incubation, the expansion of COCs was noted and the proportion of oocytes reaching metaphase II (nuclear maturation) was recorded microscopically by using aceto-orcein stain (Fig 1). Statistical analysis data obtained in the present experiment were analyzed statistically as per Snedecor and Cochran (1994) by SAS Enterprise Guide 4.3.

Results and Discussion

The extents of maturation of oocytes on IVM both on the basis of COC expansion and nuclear maturation are given in Table 1 to 4. The highest rate of maturation of oocytes based on COC expansion varied from 65.42 per cent in DMEM+ FCS + pFF + PMSG + hCG + E2 to 76.50 per cent in DMEM+ ESS + pFF + PMSG + hCG + E2. Somewhat lower rate of oocyte maturation in vitro based on cumulus cell expansion was reported by Gonzales- Figueroa and Gonzales-Molfino (2005) who found that the percentage of oocytes with expansion of cumulus cell layer in San Marcos medium containing sodium pyruvate was 40.9 per cent and it was 42.9 per cent when the medium was combined with pFF. They reported that the rate of oocyte maturation based on COC expansion was increased to 55.6 per cent when SM medium was combined with pFF and gonadotropin. The highest percentage of oocytes found matured in vitro based on nuclear maturation was 54.28 per cent in DMEM + FCS + pFF + PMSG+ hCG + E2 medium and 61.53 per cent in DMEM + ESS + pFF + PMSG + hCG + E2. Wang et. al., (1997) reported 53.84 per cent in vitro matured pig oocytes at M II stage comparable with DMEM containing FCS. Algriany et. al., (2004) recorded that the rate of in vitro nuclear maturation of pig oocytes was 55 per cent in TCM-199 supplemented with cysteamine (100 µm), pFF (10 per cent) and human recombinant FSH (0.05 IU/ml). Kohata et.al., (2013) registered higher (72%) porcine oocytes at metaphase II using NCSU-23 medium supplemented with pFF+ eCG (10 IU/ml) + hCG (10 IU/ml) + E2 (50 ng/m). Lower rate of maturation of pig oocytes in vitro based on M II (51 per cent) was reported by Wu et al. (2001) in NCSU-23 medium supplemented with serum + FSH (0.05 IU/ml). Difference in rate of in vitro maturation of oocytes reported by various workers as compared to present study could be attributed to quality of oocytes, stage of follicles from which oocytes were retrieved, reproductive status of animals, composition of maturation media, temperature and period of incubation, and condition, environment, and methods of in vitro oocytes maturation adopted. DMEM medium the addition of FCS and pFF enhanced rate of IVM of oocytes although non-significantly but supplementation of ESS, and ESS plus pFF did not increase percentage of oocytes maturation significantly on the basis of COC-expansion and nuclear maturation. The percentage of oocyte maturation on the basis of COC expansion increased significantly ($P<0.05$; 0.01) only in medium containing ESS and pFF but not in that comprising FCS and pFF when supplemented with PMSG, hCG and E2. The rate of oocyte maturation based on cumulus cell expansion rose to 61.60, 65.62 and 60.62 per cent when supplemented with PMSG, hCG and E2 respectively from 48.03 per cent in DMEM + ESS + pFF. In vitro studies (Furukawa et al., 1994; Singh and Armstrong, 1997) showed that FSH action could be modulated positively or negatively by follicular fluid components. The expansion of cumulus cells was reported to be induced in vitro by FSH (Hillensjo and Channing, 1980) with the presence of serum required to secrete hyaluronic acid within the cumulus cell complex (Eppig, 1980). The rate of oocyte maturation based on nuclear maturation was not increased significantly in medium that contained either FCS+ pFF or ESS + pFF on supplementation with PMSG, hCG and E2 respectively. The present findings indicated that ESS was more effective than FCS in elevating oocyte maturation in the media on supplementation with PMSG, hCG or E2. The rate of oocytes maturation on the basis of cumulus cell expansion was increased significantly ($P< 0.01$) when the medium comprising FCS, pFF and PMSG/ hCG was supplemented with E2. The rate of nuclear maturation in DMEM + FCS + pFF + PMSG increased significantly with supplementation of E2 but not in DMEM + FCS + pFF + hCG medium. The present findings were indicative of beneficial effect of oestrogen. Kim et al. (2011) also stated that transient E2 supplementation was advantageous for nuclear and cytoplasmic maturation of porcine oocytes. They found that positive effects of E2 on oocyte maturation were achieved by E2 supplementation during only the first half of the IVM period. In the present study also E2 supplementation was done for first 22 hours of in vitro maturation of oocytes. The positive effects of estradiol on cytoplasmic maturation of cattle oocytes were recorded earlier (Younis et.al., 1989) and stated that the function of oestradiol was expressed via oestrogen receptor in cumulus cells and oocytes during IVM. However, the effects of oestradiol on IVM of mammalian oocytes matured in chemically defined medium were variable (Son et. al., 2013). This might explain the non-significant effect of E2 during oocyte maturation in the medium containing FCS, pFF and hCG. The percentage of oocyte maturation on the basis of COC expansion increased significantly ($P<0.01$) from 38.15 to 49.57 when DMEM + FCS + pFF + PMSG was supplemented with hCG. This could be due to sustentation of the action of PMSG with hCG that

had simulation of LH activity. On supplementation of E2 in DMEM + FCS + pFF + PMSC + hCG and DMEM + ESS + pFF + PMSG + hCG media the percentage of oocyte maturation rose significantly (P<0.01; 0.05) from 49.57 to 65.42 per cent and 65.67 to 76.50 per cent respectively. However, nuclear maturation did not register any significant improvement Coy et al. (1999) also suggested that nuclear and cytoplasmic maturation of the porcine oocyte were relatively independent physiological events and that requisites for both were not the same. The present findings might suggest synergistic effect of steroid with gonadotropins and other additives in in vitro maturation condition of porcine oocytes that could improve the rate of oocyte maturation based on cumulus cells expansion.

Conclusion:

The present study was carried out to study the development competence of porcine oocytes matured in vitro using DMEM medium. The highest rate of maturation of oocytes based on COC expansion and nuclear maturation varied from 65.42 per cent and 54.28 per cent respectively in DMEM- based media containing foetal calf serum and hormones to 76.50 per cent and 61.53 per cent respectively in that containing estrous sow serum and hormones.

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TABLE I.Effect of foetal calf serum, porcine follicular fluid and hormones in DMEM on oocytes maturation based on COC expansion during IVM showing independent chi-square values

COC EXPANSION		DMEM	DMEM+FCS	DMEM+FCS+ pFF	DMEM+FCS +pFF+ PMSG	DMEM+FCS+pFF+ hCG	DMEM+FCS+ pFF+E_2	DMEM+ FCS+ pFF+	DMEM+FCS+pFF+	DMEM+ FCS+ pFF+	DMEM+FCS+ pFF+ hCG+E_2
No. of oocytes incubated		98	67	112	249	258	219	238	269	243	254
No. of oocytes matured		25	22	41	95	109	99	118	176	145	158
Maturation percentage		25.51	32.83	36.60	38.15	42.24	45.20	49.57	65.42	59.67	62.20
Independent Chi-square values	DMEM	-	1.05 NS	2.99 NS	4.97*	8.48**	11.20**	16.45**	46.20**	32.60**	38.15**
	DMEM+FCS	-	-	0.26 NS	0.64 NS	1.96 NS	3.22 NS	5.90**	23.54**	15.22**	18.57**
	DMEM+ FCS+ pFF	-	-	-	0.08 NS	1.03 NS	2.24 NS	5.17*	26.79**	16.35**	20.53**
	DMEM+FCS+pFF+PMSG	-	-	-	-	0.88 NS	2.39 NS	6.46**	38.56**	22.79**	29.10**
	DMEM+FCS+pFF+hCG	-	-	-	-	-	0.42 NS	2.68 NS	28.49**	25.20**	20.43**
	DMEM+FCS+ pFF+E_2	-	-	-	-	-	-	0.88 NS	20.67**	9.67**	13.70**
	DMEM+ FCS+ pFF+ PMSG+ hCG	-	-	-	-	-	-	-	13.02**	4.94*	7.95**
	DMEM+FCS+pFF+PMSG+hCG+E_2	-	-	-	-	-	-	-	-	1.81 NS	0.58 NS
	DMEM+ FCS+ pFF+ PMSG+E_2	-	-	-	-	-	-	-	-	-	0.34 NS
	DMEM +FCS +pFF+hCG+E_2	-	-	-	-	-	-	-	-	-	-

TABLE II. Effect of foetal calf serum, porcine follicular fluid and hormones in DMEM on nuclear maturation of oocytes during IVM showing independent chi-square values

nuclear maturation		DMEM	DMEM +FCS	DMEM +FCS+ pFF	+FCS +pFF+ PMSG	+FCS+p FF+ hCG	DMEM +FCS+ pFF+E_2	+ FCS+ pFF+ PMSG+	+FCS+p FF+ PMSG+	+ FCS+ pFF+ PMSG+	CS+ pFF+
No. of oocytes incubated		27	38	29	23	27	37	26	35	36	23
No. of oocytes matured		4	8	7	6	8	12	10	19	17	12
Maturation percentage		14.81	21.05	24.13	26.08	29.62	32.43	38.46	54.28	47.22	50.00
Independent Chi-square values	DMEM	-	0.41 NS	0.77 NS	0.99 NS	1.71 NS	2.58 NS	3.81 NS	10.81**	7.20**	7.97**
	DMEM+FCS	-	-	0.09 NS	0.20 NS	0.62 NS	1.24 NS	3.70 NS	7.20**	4.46**	5.17**
	DMEM+ FCS+ pFF	-	-	-	0.03 NS	0.21 NS	0.55 NS	1.31 NS	5.98**	3.67**	4.35**
	DMEM+FCS+pFF+PMSG	-	-	-	-	0.07 NS	0.27 NS	0.84 NS	4.50**	2.63**	3.28**
	DMEM+FCS+pFF+hCG	-	-	-	-	-	0.05 NS	0.46 NS	3.77NS	1.20 NS	2.63 NS
	DMEM+FCS+ pFF+E_2	-	-	-	-	-	-	0.24 NS	3.50NS	1.67 NS	2.30 NS
	DMEM+ FCS+ pFF+ PMSG+hCG	-	-	-	-	-	-	-	1.50NS	0.47 NS	0.93 NS

TABLE III. Effect of estrous sow serum, porcine follicular fluid and hormones in DMEM on oocytes maturation based on COC expansion during IVM showing independent chi-square values

nuclear maturation		DMEM	DMEM+ FCS	DMEM+ FCS+ pFF	DMEM+ FCS +pFF+	DMEM+ FCS+pF F+ hCG	DMEM+ FCS+ pFF+E_2	DMEM+ FCS+ pFF+	DMEM+ FCS+pF	DMEM+ FCS+ pFF+	DMEM+ FCS+ pFF+
No. of oocytes incubated		27	38	29	23	27	37	26	35	36	23
No. of oocytes matured		4	8	7	6	8	12	10	19	17	12
Maturation percentage		14.81	21.05	24.13	26.08	29.62	32.43	38.46	54.28	47.22	50.00
Independent Chi-square values	DMEM	-	0.41 NS	0.77 NS	0.99 NS	1.71 NS	2.58 NS	3.81 NS	10.81**	7.20**	7.97**
	DMEM+FCS	-	-	0.09 NS	0.20 NS	0.62 NS	1.24 NS	3.70 NS	7.20**	4.46**	5.17**
	DMEM+ FCS+ pFF	-	-	-	0.03 NS	0.21 NS	0.55 NS	1.31 NS	5.98**	3.67**	4.35**
	DMEM+FCS+pFF+PMSG	-	-	-	-	0.07 NS	0.27 NS	0.84 NS	4.50**	2.63**	3.28**
	DMEM+FCS+pFF+hCG	-	-	-	-	-	0.05 NS	0.46 NS	3.77NS	1.20 NS	2.63NS
	DMEM+FCS+ pFF+E_2	-	-	-	-	-	-	0.24 NS	3.50NS	1.67 NS	2.30NS

	DMEM+ FCS+ pFF+ PMSG+hCG	-	-	-	-	-	-	-	1.50^{NS}	0.47^{NS}	0.93^{NS}
	DMEM+FCS+pFF+PMSG+hCG+E_2	-	-	-	-	-	-	-	-	0.35^{NS}	0.03^{NS}
	DMEM+ FCS+ pFF+ PMSG+E_2	-	-	-	-	-	-	-	-	-	0.14^{NS}
	DMEM +FCS +pFF+hCG+E_2	-	-	-	-	-	-	-	-	-	-

TABLE IV : Effect of estrous sow serum, porcine follicular fluid and hormones in DMEM on nuclear maturation of oocytes during IVM

Sr. No	**Medium**	**Serum**	**pFF**	**Hormone**	**Oocytes**			**Chi-square value**
					Number Incubated	**Number matured**	**Matura-Tion rate (%)**	
1.	DMEM	ESS	---	---	27	8	29.62	
2.	DMEM	ESS	pFF	---	39	14	35.89	
3.	DMEM	ESS	pFF	PMSG	34	16	47.05	
4.	DMEM	ESS	pFF	hCG	36	18	50.00	
5.	DMEM	ESS	pFF	E_2	38	17	44.73	10.18 ^{NS}
6.	DMEM	ESS	pFF	PMSG+hCG	27	14	51.85	
7.	DMEM	ESS	pFF	PMSG+hCG+ E_2	26	16	61.53	
8.	DMEM	ESS	pFF	PMSG+ E_2	25	14	56.00	
9.	DMEM	ESS	pFF	hCG+ E_2	29	17	58.62	

NS Non Significant

TABLE IV : Effect of estrous sow serum, porcine follicular fluid and hormones in DMEM on nuclear maturation of oocytes during IVM

Sr. No	**Medium**	**Serum**	**pFF**	**Hormone**	**Oocytes**			**Chi-square value**
					Number Incubated	**Number matured**	**Matura-Tion rate (%)**	
1.	DMEM	ESS	---	---	27	8	29.62	
2.	DMEM	ESS	pFF	---	39	14	35.89	
3.	DMEM	ESS	pFF	PMSG	34	16	47.05	
4.	DMEM	ESS	pFF	hCG	36	18	50.00	
5.	DMEM	ESS	pFF	E_2	38	17	44.73	10.18 ^{NS}
6.	DMEM	ESS	pFF	PMSG+hCG	27	14	51.85	
7.	DMEM	ESS	pFF	PMSG+hCG+ E_2	26	16	61.53	
8.	DMEM	ESS	pFF	PMSG+ E_2	25	14	56.00	
9.	DMEM	ESS	pFF	hCG+ E_2	29	17	58.62	

NS Non Significant

COC expansion		DME M	DME M+ FSS	DME M+ FSS	DME M+ES S	DME M+ES S+	DME M+ES S+	DMEM+ ESS	DMEM+ ESS	DME M+ FSS +	DMEM+ ESS
No. of oocytes incubated		98	69	102	125	128	127	134	315	219	207
No. of oocytes matured		25	29	49	77	84	77	88	241	149	150
Maturation percentage		25.51	42.02	48.03	61.60	65.62	60.62	65.67	76.50	68.03	72.46
Independent Chi-square values	DMEM	-	0.41 NS	0.77 NS	0.99 NS	1.71 NS	2.58 NS	3.81 NS	10.81**	7.20 **	7.97 **
	DMEM+FCS	-	-	0.09 NS	0.20 NS	0.62 NS	1.24 NS	3.70 NS	7.20**	4.46 **	5.17 **
	DMEM+ FCS+ pFF	-	-	-	0.03 NS	0.21 NS	0.55 NS	1.31 NS	5.98**	3.67 **	4.35 **
	DMEM+FCS+pFF+PMSG	-	-	-	-	0.07 NS	0.27 NS	0.84 NS	4.50**	2.63 **	3.28 **
	DMEM+FCS+pFF+hCG	-	-	-	-	-	0.05 NS	0.46 NS	3.77 NS	1.2NS	2.63 NS
	DMEM+FCS+ pFF+E_2	-	-	-	-	-	-	0.24 NS	3.5NS	1.67 NS	2.30 NS
	DMEM+ FCS+ pFF+ PMSG+ hCG	-	-	-	-	-	-	-	1.50 NS	0.47 NS	0.93 NS
	DMEM+FCS+pFF+PMSG +hCG+E_2	-	-	-	-	-	-	-	-	0.35 NS	0.03 NS
	DMEM+ FCS+ pFF+ PMSG+E_2	-	-	-	-	-	-	-	-	-	0.14 NS
	DMEM +FCS +pFF+hCG+E_2	-	-	-	-	-	-	-	-	-	-

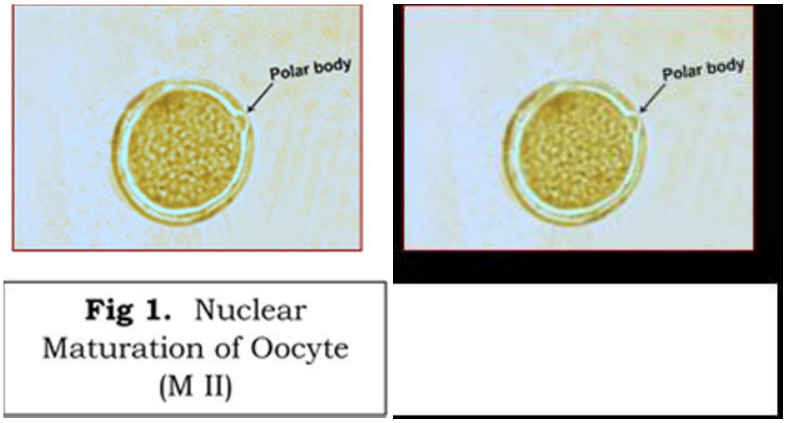

Fig 1. Nuclear Maturation of Oocyte (M II)

References:

Abeydeera, L.R. (2001). In vitro fertilization and embryo development in pigs. Reprod. (Cambridge, England), 58: 159-173.

Algriany, O.; Bevers, M.; Schoevers, E.; Colenbrander, B. and Dieleman, S. (2004). Follicle size-dependent effects of sow follicular fluid on in vitro cumulus expansion, nuclear maturation and blastocyst formation of sow cumulus oocytes complexes. Theriogenology, 62(8): 1483-1497.

Coy, P.; Ruiz, S.; Romar, R.; Campos, I.; Gadea, J. (1999). Maturation, fertilization and complete development of porcine oocytes matured under different systems. Theriogenology, 51: 799-812.

Eppig, J.J. and O'Brien, M.J. (1996). Development in vitro of mouse oocytes from primordial follicles. Biol. Reprod., 54(1): 197-207.

Furukawa, K.; Yamato, M.; Kokawa, N. and Nakano, R. (1994). Purification of high-molecular- weight follicles-stimulating hormone binding inhibitor in porcine follicular fluid. Eur J.Endocrinol., 130: 625-33.

Gil, M.; Cuello, C.; Parrilla, I.; Vazquez, J.; Roca, J. and Martinez, E. (2010). Advances in swine in vitro embryo production technologies. Reprod. Domestic. Anim., 45(2): 40-48.

Gonzales-Figueroa, H. and Gonzales-Molfino, H.M. (2005). Maturation of pig oocytes in vitro in a medium with pyruvate. Brazilian J. Med. Bio. Reach., 38: 869-872.

Hillensjo, T. and Channing, C.P. (1980). Gonadotropin stimulation of steroidogenesis and cellular dispersion in the cultured porcine cumuli oophori. Gamete Research, New York, 3: 233-240.

Kim, J.S.; Song, B.S.; Lee, S.R.; Yoon, S.B.; Huh, J.W.; Kim, S.U.; Kim, E.; Kim, S.H.; Choo, Y.K.; Koo, D.B. and Chang, K.T. (2011). Supplementation with estradiol-17-β improves porcine oocyte maturation and subsequent embryo development. Fertil. Steril., 95: 2582-2584.

Kohata, C.; Izquierdo-Rico, M.J.; Romer, R. and Funahashi, H. (2013). Development competence and relative transcript abundance of oocytes derived from small and medium follicles of prepuberatal gilts. Theriogenology, 10: 1-9.

Park, J.I.; Hong, J.Y.; Yong, H.Y.; Hwang, W.S.; Lim, J.M. and Lee, E.S. (2005). High oxygen tension during in vitro oocyte maturation improves in vitro development of porcine oocytes after fertilization. Anim. Reprod. Sci., 87: 133-141.

Singh, B. and Armstrong, D.T. (1997). Insulin like growth factor-I, a component of serum that enables porcine cumulus cells to expand in response to follicles- stimulating hormone in vitro. Biol. Reprod., 56: 1370-1375.

Snedecor, G.W. and Cochran, W.G. (1994). Statistical Methods. 8th Edn., Oxford and IBH Publishing Co., New Delhi.

Son, J.; Malaweera, D.B.O.; Lee, E.; Shin, S. and Cho, J. (2013). Development of in vitro produced porcine embryos according to serum types as macromolecule. J. Vet. Sci., 14(3): 315-321.

Wang, W.H.; Abeydeera, L.R.; Cantley, T.C. and Day, B.N. (1997). Effects of oocyte maturation media on development of pig embryos produced by in vitro fertilization. J. Reprod. Fertil., 111: 101-108.

Wu, J.; Emery, B. R. and Carrell, D. T. (2001). In vitro growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. Biol. Reprod., 64: 375–381.

Yoshioka, K.; Suzuki, C. and Onishi, A. (2008). Defined system for in vitro production of porcine embryos using a single basic medium. J. Reprod. Dev., 54: 208-213.

Younis, A.I.; Brackett, B.G. and Fayrer-Hosken, R.A. (1989). Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. Gamete Res., 23: 189-201.

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