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Effects of monopotassium phosphate and oviduct cells on the in vitro fertilized mice embryos development*)

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Summary

The study investigated the effects of three different KH_2PO_4 concentrations (0.59 mM; 1.19 mM; 2.38 mM) in Whitten's medium and co-culture on in vitro two-cell blocks and their development to the blastocyst stage of inbred BALB/C mice embryos following in vitro fertilization.

Standard IVF and IVC procedures were used and mouse oviduct cells were used as a co-culture. The results demonstrated that various concentrations of potassium were not effective on the cleavage rates. 1.19 mM KH_2PO_4 with and without co-culture groups (P<0.05)had the highest rate of reaching the 4-to 6- and 8-cells. At the 72nd hour, the 1.19 mM KH_2PO_4 groups without (55.26%) and with co-culture (44.77%) (P<0.05) displayed the most satisfactory development to the morula stage. Although 0.59 mM and 2.38 mM KH_2PO_4 were found to be insufficient to develop up to morula, 0.59 mM KH_2PO_4 with co-culture may have beneficial effects (31.57%; P<0.05). On the other hand, despite the high concentration of KH_2PO_4 (16.90%) supporting a more effective development rate to the morula than the lower level (P<0.05), it was noted that co-cultures having a high KH2PO4 were not beneficial. KH_2PO_4 with and without (30.00%) co-culture (26.01%) (P<0.05) demonstrated the most beneficial development of the blastocyst stage.

Keywords: mouse, IVF, IVC, embryos, co-culture, 2-cell block

Necessary conditions for healthy development of embryos have not been achieved completely so far for *in vitro* studies. Researchers have not completely elucidated the effects of chemical substances in the female genital tract such as Ca^{++}, Mg^{++}, NaCl and K^+ on fertilization and embryo development (2, 5, 6, 9, 11). However, it was reported that oviduct cells and high concentration of potassium in the oviduct play a significant role in embryo development (1, 2, 9, 11, 20). The oviduct possesses a high proportion of potassium and when *in vitro* fertilization and culture is carried out in media containing a high proportion of potassium, embryos may reach the blastosyst stage at a better rate (9). Contrarily, when embryos cultured in media containing a low concentration of potassium, overcome the 2-cellblock seen in inbreed mice may be overcome and the embryos can reach the blastocyst stage more easily (9, 17, 25).

In many mammal species, a block occurs at various stages of *in vitro* development of embryos (2, 4, 7, 14, 15). In mice, this block occurs in the 2-cell stage. It has been reported that, this cell block occurring in species is due to the beginning of the genomic activation of the embryo, decrease of DNA synthesis, sensitivity to nutritional matter needed for the development of the embryo and placement of embryos under *in vitro* conditions (4, 16, 24). Although there is a high ratio of K^+ in the oviduct fluid (4-6, 9, 18), it has been reported that increasing K^+ and high KH_2PO_4 concentration has a negative effect on the *in vitro* development of mouse embryos (9, 17).

The aim of this study has been to determine different (0.59 mM, 1.19mM, 2.38 mM) KH_2PO_4 concentrations' and the co-culture of mice oviduct cells' effects to overcome the block formation at the 2-blastomer stage and to develop up to the blastocyst stage in *in vitro* culture of *in vitro* fertilized inbred BALB/C mice oocytes.

Material and methods

In the study, 5 to 8-week old female and 8 to 10-week old male BALB/C in breed mice were used. The mice were exposed to periods of 10 hours of darkness and 14 hours of light and fed *ad libitum*.

Mediums for handling, *in vitro* fertilization and culture of oocytes/embryos. M2 medium was preferred for handling and Whitten's media were prepared for *in*

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vitro culture in laboratory (15). In this medium, three concentration of KH_2PO_4 were used (0.59 mM in low concentration groups, 2.38 mM in high concentration groups, 1.19 mM control group). The TYH medium (7, 8, 23) was used as the fertilization medium.

Collection and preparation of sperm. Sperm was collected from the cauda epidydimis of the cervical dislocated male mice. The epidydimises were washed 3 times in M2 medium, one time in the capacitation petri dish containing 600 µl TYH medium covered with mineral oil. Following this, they were squeezed into 600 µl TYH medium by using forceps. The collected sperm was incubated for 1.5-2 hours at 37°C in 5% O_2, 5% CO_2 and 90% N_2 gas mixture for capacitation.

Collection of oocytes. The female mice were superovulated by i.p injections of PMSG (5 I.U) and hCG (5 I.U) 48 hours apart oocytes were collected by puncturing the ampullas within the M2 medium 12 hours after hCG injection. Selected oocytes were washed twice in 50 µl TYH medium and placed in fertilization drops containing 25 µl TYH covered with mineral oil by adjusting 10-30 to each drop. Fertilization dishes were placed in the incubator at 37°C in 5% O_2, 5% CO_2 and 90% N_2 gas mixture.

Collection of oviduct cells and preparation of co-culture. Healthy-looking oviduct cells with moving cilia, were obtained by washing the inside of the oviduct with M2 medium. The cells were washed twice in a 50 µl washing petri dish covered with mineral oil and containing 3 different concentrations of KH_2PO_4. The oviduct cells were placed into three co-cultere groups by delivering 7-10 cell layers.

***In vitro* fertilization and embryo culture.** Capacitated sperm was added up to 2.5-3 × 10^6 spermatozoa/mL a final concentration in drops. The sperm and oocytes were incubated to fertilize the ova for 4-6 hours in a gas mixture (5% O_2, 5% CO_2 and 90% N_2) at 37°C. Than, presumptive embryos were distributed into co-culture and non-co-culture drops (50 µL) for each groups. The oocyte/embryos were then cultured for 96 hours. Development of the embryos were checked every 24 h until 96th hours of *in vitro* culture.

The χ^2 test and t-test were used in the statistical analyses of the study.

Tab. 1. Development by the end of 48 hours of the embryos cultured following in vitro fertilization and their distribution according to the stages of development

Groups	Total oocytes	Total cleaved oosit (%)		Degenerated oocytes	Developmental stages of embryos (%)*			
		24th h.	48th h.		2-4-cell	4-6-cell	8-cell	16-cell
0.59 mM KH_2PO_4	175	84 (48.00)^a	105 (60.00)^a	21 (12.00)^a	72 (68.57)^a	33 (31.43)^b	0 (0.0)^b	0 (0.0)^a
0.59 mM KH_2PO_4, Co-culture	179	103 (57.44)^a	123 (68.71)^a	26 (14.52)^a	75 (60.97)^a	37 (30.08)^b	11 (8.94)^a	0 (0.0)^a
1.19 mM KH_2PO_4	182	101 (55.4)^a	120 (65.93)^a	10 (5.49)^a	61 (50.83)^a	55 (45.83)^a	4 (3.33)^a	0 (0.0)^a
1.19 mM KH_2PO_4, Co-culture	181	103 (56.90)^a	123 (67.95)^a	18 (9.94)^a	84 (68.29)^a	27 (21.95)^b	11 (8.94)^a	1 (0.81)^a
2.38 mM KH_2PO_4	177	90 (50.84)^a	109 (61.58)^a	25 (14.12)^a	75 (68.80)^a	31 (28.44)^b	3 (2.75)^a	0 (0.0)^a
2.38 mM KH_2PO_4, Co-culture	178	95 (53.37)^a	111 (62.35)^a	22 (12.35)^a	71 (63.96)^a	35 (31.53)^b	5 (4.50)^a	0 (0.0)^a

Explanations: a, b – the difference between the rates with different letters in the same column is significant ($P < 0.05$), * percentages were calculated according to the number of embryos showing cleavage

Results and discussion

At 24th hours, different concentrations of potassium and co-culture in the first 24 hours did not statistically affect the first cleavage rates of the embryos (tab. 1). At the 48th hours, the cleavage rates increased in all groups. The development speed of embryos up to 4-6-cell stage in the 1.19 mM KH_2PO_4 concentration group was higher (45.83%) than other groups ($P < 0.05$) (tab. 1).

At the end of 72 hours of the *in vitro* culture, significant differencess were determined in the morula stage ($P < 0.05$; tab. 2). Among the groups in the morula stage, there was difference only in the control group; the most prominent change was observed in the morula stage (tab. 2).

Tab. 2. Development by the end of 72 hours of the embryos cultured following *in vitro* fertilization and their distribution according to the stages of development

Groups	Total embryo*	Developed embryo	Degenerated embryo (%)***	Developing embryos (%)**			
				8 Blast.	8-16 Blast.	Morula	Compact morula
0.59 mM KH_2PO_4	105	52 (49.53)^a	53 (50.47)^a	18 (34.61)^a	17 (32.69)^b	6 (11.53)^e	11 (21.15)^a
0.59 mM KH_2PO_4, Co-culture	123	76 (61.79)^a	47 (38.21)^a	20 (26.31)^a	20 (26.31)^b	24 (31.57)^c	12 (15.78)^a
1.19 mM KH_2PO_4	120	76 (63.34)^a	44 (36.66)^a	14 (18.42)^a	10 (13.15)^a	42 (55.26)^a	10 (13.15)^a
1.19 mM KH_2PO_4, Co-culture	123	67 (54.48)^a	56 (45.52)^a	11 (16.41)^a	21 (31.34)^b	30 (44.77)^b	5 (7.46)^a
2.38 mM KH_2PO_4	109	71 (65.14)^a	38 (34.86)^a	22 (30.98)^a	27 (38.02)^b	12 (16.90)^d	10 (14.08)^a
2.38 mM KH_2PO_4, Co-culture	111	56 (50.46)^a	55 (49.54)^a	16 (28.57)^a	12 (21.42)^b	16 (28.57)^d	12 (21.42)^a

Explanations: a, b, c, d, e – the difference between the rates with different letters in the same column is significant ($P < 0.05$); * based on the number of embryos showing cleavage at 48 hours; ** rates were calculated using the number of developing embryos;*** embryos smaller than 8 blastomers were classified as degenerated

At the end of 96 hours of *in vitro* culture, developmental stages in all groups were evaluated as morula and early blastocyst-blastocyst. The best developments to the early blastocyst and blastocyst stages were examined in with and without co-culture control groups ($P < 0.05$, tab. 3).

There was no difference between the cleavage rates of the groups within the first 24 and 48 hours after fertilization. This shows that different KH_2PO_4 rates are not effective on cleavage in the first 24 hours of the developmental stage. The maximum cleavage rate was reached at 48th h after IVF. Although not statistically significant, co culture has increased number of cleaved embryos. This in turn suggests the idea that, oviduct epithelium cells may play a supportive role in media containing different potassium rates. Co culture with oviduct cells (12, 13, 19, 21) may have beneficial effect to overcoming the block *in vitro* by using glucose in the media (1). On the other hand, the fact that cleavage rates were similar in all groups supported the idea of Roblero and Riffo (22) and Chatot et al. (7) stating that high potassium rates are not essential during the cleavage of embryos. However, although embryo cleavages were slightly higher when co-culture was used. It reminded the view of Borland et al. (6), in which the first division stage occurred in the oviduct region containing high potassium under *in vivo* conditions and that potassium originating from oviduct cells.

Tab. 3. Development by the end of 96 hours of the embryos cultured following *in vitro* fertilization and their distribution according to the stages of development

Groups	Total embryos*	Developed (%)	Degenerated*** (%)	Developing embryos (%)**	
				Morula – compact Morula	Early blastocyst – blastocyst
0.59 mM KH_2PO_4	105	43 (40.96)^{b}	62 (59.04)^{b}	30 (28.57)^{a}	13 (12.38)^{c}
0.59 mM KH_2PO_4, Co-culture	123	51 (41.47)^{b}	72 (58.53)^{b}	31 (25.20)^{ab}	20 (16.26)^{bc}
1.19 mM KH_2PO_4	120	67 (55.84)^{a}	53 (44.16)^{a}	31 (25.83)^{ab}	36 (30.00)^{a}
1.19 mM KH_2PO_4, Co-culture	123	63 (51.22)^{b}	60 (48.78)^{b}	31 (25.20)^{ab}	32 (26.01)^{ab}
2.38 mM KH_2PO_4	109	30 (27.53)^{c}	79 (72.47)^{c}	18 (16.51)^{b}	12 (11.00)^{cd}
2.38 mM KH_2PO_4, Co-culture	111	30 (27.03)^{c}	81 (72.97)^{c}	22 (19.81)^{ab}	8 (7.20)^{d}

Explanations: a, b, c, d, e – the difference between the rates with separate letters in the same column is significant ($P < 0.05$); * based on the number of embryos showing cleavage at 48 hours; ** percentage rates were calculated using the number of dividing embryos; *** embryos in the stage before the morula stage were classified as degenerated

Significant differences occurred in the development of embryos at the 72nd hour of the *in vitro* culture up to 8-16 blastomer and morula stages ($P < 0.05$; tab. 2). The highest rate of reaching the morula stage was seen in the control group 55.26% ($P < 0.05$). The lowermost development up to morula occurred in the low KH_2PO_4 group (tab. 2; $P < 0.05$). These results suggested that addition of oviduct cells to the 1.19 mM KH_2PO_4 group negatively affected embryo development. It has also brought about the idea that these cells supported the negative effects created by the low and high concentration of KH_2PO_4. While the reasons are not known for the drop occurring in the rate of development up to morula and compact morula stages when oviduct cells are added to the 1.19 mM KH_2PO_4 group, this is thought to be due to oviduct cells rapidly consuming the nutritional matter necessary for embryo development. This idea is supported by the opinions of Bavister (3) and Menezo et al. (19), stating that the nutritional matter contained in the medium with co-culture should be sufficient enough to cover the requirements of embryos and cells. The best development to the early blastocyst and blastocyst stage of cleaved embryos occurred in the media containing 1.19 mM KH_2PO_4 without and with co-culture ($P < 0.05$; tab. 3). In the light of the findings obtained at the 72nd and 96th hours of the culture, it was seen that high potassium supported development of embryos up to the morula; however, that this positive effect turned negative when the embryos were developed up to the early blastocyst and blastocyst stages (tab. 2, 3). This observation is supported by the opinion of Roblero et al. (22), stating that high potassium has a positive effect until morula, and that following this stage the value of potassium in the medium should be lowered for blastocyst development.

Although these results are parallel to the idea of Willey et al. (25) that embryos in the medium containing a low concentration of potassium reach to the blastocyst stage earlier than high concentration of potassium. They contradict the opinion of Erbach et al. (10) stating that embryos developing in the medium containing a high concentration of potassium overcome the 2-cell block and reach the blastocyst stage at higher rate. Dumoulin et al. (9) stated that although having a high concentration of potassium, in culture media would have a negative effect on embryo development and that embryos were very sensitive to high potassium. Contrarily Roblero and Riffo (22) indicated that high potassium has a positive effect on *in vitro* development of mice embryo before blastocyst until the compact stage. In this study at the 96th hour of the culture high potassium groups with and without co-culture were found to be significantly lower than other groups ($P < 0.05$) (tab. 3). These results are correlated with Dumoulin et al. (9).

In this study, in view of development rates, the difference in potassium concentration were found to be ineffective on cleavage; however, that with respect

developing rate up to blastocyst stage, the low concentrations of KH_2PO_4 had less detrimental effect compared to high concentrations without ($P > 0.05$) and especially with co-culture ($P < 0.05$), (tab. 3). Also, while it was determined that embryos in the co-culture groups in the first 48 hour period (tab. 3) showed slightly better development than embryos in the groups without co-culture ($P > 0.05$). By the end of 96 hours, this beneficial effect had been reversed except in the low potassium group. This result is parallel to the views of many researchers (3, 19) stating that the medium used in co-culture studies should cover the nutritional requirements of the embryo and cells. The decrease in the development rates, occurring in the co-culture groups by the end of 96 hours of *in vitro* culture, is thought may be due to the medium drops not being refreshed throughout the culture and especially due to the insufficiency of nutritional matter necessary for embryo development in co-culture.

While it has been reported that mice embryos developing under *in vitro* conditions reach hatching and hatched blastocyst at the end of 96 hours (14-16). In this study, it has been determined that mice embryos cultured under *in vitro* conditions developed in the compact morula, early blastocyst, blastocyst and partially in the hatching blastocyst stage. This result was found to be parallel to the opinion of Knobil and Neil (16), when compare to the *in vivo* development of embryos, in *in vitro* culture embryos start to cleavage after a delay of at least an hour and this delay reaches one whole day until the blastocyst stage.

As a result, in this study it was concluded that; the co-culture with oviduct epithelium cells may be partially beneficial for embryo development and the potassium applied at different rates has no effect on overcoming of the 2-cell block occurring in the *in vitro* development. In the *in vitro* development of mice embryos, low and normal potassium with and without co-culture would be more beneficial compared to high KH_2PO_4, to reach an optimal result, normal amounts of KH_2PO_4 would be appropriate. Different concentrations of KH_2PO_4 in embryo culture media may be examined for every developmental stage of embryos such as cleavage, morula and blastocyst stages.

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