The Influence of Microinjection of Foreign Gene into the Pronucleus of Fertilized Egg on the Preimplantation Development, Cell Number and Diameter of Rabbit Embryos

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ABSTRACT : The aim of this *in vitro* study was to test the effect of microinjection (Mi) of foreign gene into the rabbit egg pronucleus and epidermal growth factor (EGF) addition on the blastocyst rate, the cell number and the diameter of embryos, and to determine possible relationships between embryo cell number and embryo diameter. Blastocyst rate was significantly decreased in gene-Mi (G-Mi/E0) group (63.1%) comparing to intact ones (83.5%, $p_1<0.05$). The addition of EGF at 20ng/ml (G-Mi/E20) or 200 ng/ml (G-Mi/E200) to gene-Mi embryos did not affect blastocyst rate (65.6 and 55.2% resp.). As a control for Mi, the eggs were microinjected with the same volume of phosphate-buffered solution (PBS-Mi) instead of the gene construct solution. Cell numbers and embryo diameters were measured from embryo images obtained on confocal laser scanning microscope. Bonferroni-modified LSD test showed that the embryo cell number in PBS-Mi group was significantly lower ($p_1<0.05$) and in gene-Mi group was tended to decrease compared with intact embryos. Embryo diameter was not different among experimental groups. No effect of EGF given at any doses both on the cell number and embryo diameter was found. A positive correlation between cell number and embryo diameter was observed in all groups of embryos. Since embryo diameter was not changed under the influence of Mi or EGF addition in this study, this seems to be more conservative characteristics of the embryo morphology. These results suggest that the pronuclear microinjection compromises developmental potential of embryos, decreasing blastocyst rate and embryo cell number, whilst embryo diameter is not affected. No effects of EGF on studied parameters were confirmed. Declined quality of Mi-derived embryos is caused by the microinjection procedure itself, rather than by the gene construct used. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 2 : 171-175)***

Key Words : Rabbit, Egg, Blastocyst, Microinjection, Cell Number, Embryo Diameter

INTRODUCTION

Microinjection of gene construct into the pronucleus or the perivitelline space of fertilized egg is one of techniques for transgenic animal production. The main problem using this *in vitro* approach is a decrease in viability of obtained embryos, which is resulted in declined rate of morula and blastocyst stage embryos, as well as in higher percentage of degenerated and developmentally arrested ova (Chrenek et al., 1998). The most useful criterium of the quality of early preimplantation embryos is the evaluation of developmental stage of the embryo basing on visual inspection of their morphology under the light microscope. However such evaluation is often confused and subjectively influenced. Moreover, developmental stage of preimplanted embryo does not reflect its developmental potential after the transfer to the recipient.

In the earlier report the embryo cell number was proposed as a valid indicator of embryo quality (Papaioannou a Ebert, 1988). Cell number of bovine *in vitro* produced blastocysts varied depending on the morphological grade, and later developing blastocysts were of poor quality as proved by the cell number (Jiang et al., 1992). It was observed that embryos developing quickly to the blastocyst stage had a higher total cell number than embryos developed more slowly (Iwasaki et al., 1990). Another non-invasive parameter for the testing of embryo viability seems to be an embryo diameter. Mori et al. (2002) have reported a high positive correlations between the cell number and the diameter in bovine embryos at each stage collected on days 7-9 after *in vitro* fertilization. Embryo diameter is positively dependent on size of blastomeres, which may contribute to the efficiency of embryo cloning in rabbit, using blastomeres as donors of nuclei (Ju et al., 2003). Therefore, the diameter of the embryo may be potentially used for the viability testing of the expanded blastocysts. However, there is no useful information regarding the cell number, embryo diameter and correlation between its in microinjection-derived embryos. The measurement of embryo diameter and cell number using nuclear staining with vital permeant dyes (Hoechst 33342, DAPI, SYTO a.o.) will enable the non-invasive selection of the embryos with best developmental potential without its destruction. Although positive effects of such growth factors as insulin-like growth factor I (IGF-I) or epidermal growth factor (EGF) on preimplantation embryos *in vitro* were documented in rabbit (Herrler et al., 1998), pig (Wei et al., 2001), cattle (Makarevich and Markkula, 2002; Sirisathien et al., 2003) and mouse (Demeestere et al., 2004), effects of EGF neither on the embryo cell number nor on the embryo diameter of microinjection-derived

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embryos has been reported yet for any species.

The aim of this study was to test (1) the effect of pronuclear microinjection and EGF addition on the development of rabbit eggs to blastocyst stage, the total cell number and the diameter of rabbit embryos, and (2) to determine possible relationships between embryo cell number and embryo diameter.

MATERIALS AND METHODS

Egg collection

Female New Zealand White rabbits, kept on the local farm, were treated with PMSG (Werfaser, Alvetra und WERFFT, Wien, Austria) i.m. at 20 IU/kg live weight, 72 h before mating. Immediately prior to mating, the females were injected i.m. with hCG (Werfachor, Alvetra und WERFFT) at 40 IU/kg live weight, afterwards the females were mated with a male of proven fertility from the same breed. The pronuclear stage eggs were flushed from the oviduct of the slaughtered animals with phosphate-buffered saline (PBS, Gibco-Life Technologies, Lofer, Austria). As rabbit eggs in *vivo* are normally surrounded by mucin coat produced in oviduct, which can hinder fixation of the egg during the microinjection procedure, we flushed rabbit oviducts at 19 to 20 h post coitum (hpc), the time when the mucin coat has not been formed yet (Joung et al., 2004). The flushed eggs were evaluated morphologically and the eggs with both pronuclei were selected and divided into two groups: 1) intended for gene microinjection (G-Mi), and 2) non-microinjected (intact) eggs.

Gene microinjection and embryo culture

Gene microinjection (Mi) was carried out using an Olympus microscope equipped with an Alcatel micromanipulation unit (France) and Eppendorf microinjector (Germany) in CIM- (CO_2-independent) medium supplemented with 10% fetal calf serum (FCS; both from Gibco-Life Technologies). The eggs were immobilized with a holding pipette by air suction. Approximately 1-2 picoliters of the human factor VIII gene construct (about 300 copies) were microinjected into male pronuclei using a micropipette with a filament inside (Chrenek et al., 2004). Swelling of the pronucleus indicated successful microinjection. Both microinjection-intended (G-Mi) and non-Mi (intact) embryos were kept in separate wells of the same culture plate, so that both groups were almost equally exposed to microscope light during the procedure. As a control for Mi, the eggs were microinjected with the same volume of PBS, pH 7.2 (PBS-Mi) instead of gene construct solution. After the microinjection, the eggs were cultured in 4-well dishes (Nunc, Roskilde, Denmark), containing 600 µl of k-DMEM medium (Gibco-Life Technologies, Lofer Austria) supplemented with 10% FCS and epidermal growth factor (Sigma-Aldrich, Taufkirchen, Germany; at 0, 20, 200 ng/ml) in 5% CO_2 and 39°C till 92-96 hpc (blastocyst stage).

Determination of embryo cell number and embryo diameter

Total cell number and embryo diameter were determined in blastocysts stained with fluorochrome propidium iodide (PI). Embryos were removed from culture medium, washed (3×5 min) in PBS supplemented with polyvinylpyrrolidone (PBS-PVP, 4 mg/ml), and then fixed for 5 min in neutral buffered 3.7% formalin solution (Sigma-Aldrich). For membrane permeabilization, embryos were incubated for 15 min in 0.5% Triton X100 in PBS. Fixed and permeabilized embryos were stained with propidium iodide (5 µg/ml in PBS) for 20 min to visualize all blastomeres. After the washing, embryos were placed on a coverslip and covered immediately with 6 µl of Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). The coverslip was mounted on a slide with small drops of nail polish. The slides were stored at -20°C until analysis, which was usually performed within 2 weeks. Embryos were analyzed using a confocal laser scanning microscope Olympus IX 70 (Japan) equipped with the UplanApo 40× objective and Fluoview 300 scanning unit. Total cell numbers of blastocysts were counted from overlay stereoimages, which were acquired and processed using Fluoview version 1.2 software.

Embryo diameters, excepting zona pellucida, were measured from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a 40× objective and 10× eyepiece. The diameter of the embryo without zona pellucida was the mean of two measurements made perpendicularly to each other.

Statistical methods

All experiments on the pronuclear microinjection, embryo culture and determination of embryo cell number and diameter were performed in five replicates. In each experiment only blastocysts or expanded blastocysts were selected for the cell staining and confocal analysis.

Experimental data were statistically evaluated as follows. The occurrence of embryos in various treatment groups according to the stage of development was evaluated by x^2-(Chi -square) test using contingency table method. Individual multiple comparisons of the occurrence probability within stages, i.e. between treatment groups were evaluated by *u*-test. Basic variational-statistical characteristics for the embryo cell number and embryo diameter were computed both for each treatment group and for whole experimental material. Differences between groups were estimated using one-way analysis of variance

Table 1. Influence of Mi (G-Mi) and EGF (E0, E20, E200) addition on the preimplantation development of rabbit embryos *in vitro*

Treatment groups	No. embryos N	Stages of embryo development, n (%)				Embryo cleavage n (%)
		1-cell	2-16-cell	Morulas	Blastocysts	
Intact	109	13 (11.9)	-	5 (4.6)	91 (83.5) [a]	96 (88.1)
G-Mi/E0	141	28 (19.9)	9 (6.4)	15 (10.6)	89 (63.1) [b]**	113 (80.1)
G-Mi/E20	125	18 (14.4)	10 (8.0)	15 (12.0)	82 (65.6) [b]**	107 (85.6)
G-Mi/E200	143	35 (24.5)	16 (11.2)	13 (9.1)	79 (55.2) [b]**	108 (75.5)

[a] vs. [b] differences within the column are significant. * -$\alpha \leq 0.05$, **- $\alpha \leq 0.01$, $\alpha = p_1$ (Chi-square-test and *u*-test).

Table 2. Basic variational-statistical characteristics of embryo cell number and embryo diameter (μm) in relation to effects of Mi (G-Mi, PBS-Mi) or EGF addition (E0, E20, E200)

Group, no.		Traits	N	$\bar{x}$	SD	SE	CV %	Skewness	Kurtosis	r
Intact, i	1	Cell no.	25	120.80[a]	21.22	4.24	17.57	-0.205	-0.319	0.560**
		Diameter		167.52	32.22	6.44	19.23	0.479	-0.839	
G-Mi/E0	2	Cell no.	24	95.67[a,b]	39.56	8.08	41.35	-0.522	-0.710	0.591**
		Diameter		152.75	41.22	8.41	26.98	1.309	2.094*	
G-Mi/E20	3	Cell no.	19	116.0[a,b]	33.18	7.61	28.60	0.857	0.629	0.570*
		Diameter		147.53	28.61	6.56	19.39	0.153	-0.747	
G-Mi/E200	4	Cell no.	20	119.0[a,b]	30.12	6.73	25.31	-0.486	0.712	0.707**
		Diameter		159.50	29.85	6.68	18.72	1.270	1.077	
PBS-Mi	5	Cell no.	15	90.67[b]	29.41	7.59	32.43	-0.209	-0.360	0.626*
		Diameter		141.40	20.76	5.36	14.68	-0.495	0.736	
Total	T	Cell no.	103	109.32	33.09	3.26	30.27	-0.414	0.573	0.601**
		Diameter		155.03	32.83	3.23	21.18	0.943**	1.424**	

[a] vs. [b] differences within the column concerning cell number are significant. * $\alpha \leq 0.05$, ** $\alpha \leq 0.01$, $\alpha = p_1$ (Bonferroni multiple comparison test).

with fixed effects. Individual comparisons between groups were performed using modified LSD Bonferroni test. Parameters of linear regression function of dependency of the embryo diameter (y) on the embryo cell number (x) were estimated for each treatment group. Individual regression functions between groups were estimated by comparing regression coefficients b_1 (slope of regression function) for parallelism and by comparing b_0 coefficients (intercept of absolute value) for equality of regression functions. Pearson correlation coefficients were used to identify associations between two variables. All statistical analyses were performed according to Grofik and Fľak (1990) using statistical package SPSS for Windows, Release 6.0, 1989-1993.

RESULTS

Blastocyst rate was significantly decreased in all gene-microinjected groups (63.1; 65.6 and 55.2%) comparing to intact ones (83.5%, $p_1<0.05$). The addition of EGF at any concentration did not affect blastocyst rate of gene-microinjected embryos. Embryo cleavage beyond 2-cell stage was in range of 75.5 to 88.1% with no significant effect of Mi or EGF additions (Table 1). Developmental rate in PBS-Mi group (n = 23) was comparable to those in all Mi-groups, (blastocyst rate -61%; embryo cleavage -79%), which is enough to confirm decreased developmental potential of embryos microinjected with PBS.

In several our experiments totally 341 blastocyst stage embryos were obtained. Of them 103 embryos were fluorescent-stained for the determination of total cell number and embryo diameter. The stages of the embryos were presented by blastocysts or expanded blastocysts, whilst hatching or hatched blastocysts were excluded from the analysis.

Basic variation-statistical characteristics of embryos according to groups of treatment are presented in Table 2. The least blastocyst cell number and embryo diameter were observed in the group PBS-Mi, whilst both these traits were maximal in intact group of embryos. In average for all groups, blastocyst cell number was 109.32±3.26 and embryo diameter was 155.03±3.23 μm. Cell number in whole embryo material showed a higher variability (CV = 30.27%), than embryo diameter (CV = 21.18%). A higher variability among cell number was found in microinjected groups G-Mi/E0 (CV = 41.35%) and PBS-Mi (CV = 32.44%), whilst a lower variability was determined in the intact group (CV = 17.57%). Embryo diameter was maximally varied in the gene-Mi group (CV = 26.96%) and minimal variability was noted in the PBS-Mi group (CV = 14.68%). The intact embryos were, in the range of variability, found between the G-Mi and PBS-Mi embryos (CV = 19.23%).

A positive correlation was found between blastocyst cell number and embryo diameter in all tested groups. Minimal correlation was observed in the intact group (r = 560**) and maximal correlation was in the G-Mi/E200 group (r = 0.707**). Correlation coefficients were homogenous, i.e. no

Table 3. One-way ANOVA of embryo cell number and embryo diameter

Traits		Groups (G) $f_G = 4$	Error (e) $f_e = 98$	Modified LSD bonferroni test
Embryo cell number	MS	3927.4401	979.2109	1:5**
	F	4.011**		
Embryo diameter	MS	2070.2089	1037.6130	No difference
	F	1.995		

$F_{0.05}$ (4.98)-2.463. $F_{0.01}$ (4.98)-3.513.

differences between groups were found. For whole experimental material, blastocyst cell number was positively correlated with embryo diameter (r = 0.601**).

Using one-way ANOVA with Bonferroni-modified LSD test (Table 3) a significant difference in blastocyst cell number was found between the intact (group 1) and PBS-Mi (group 5; $p_1<0.01$) embryos. Microinjection with the gene (G-Mi/E0) tended to decrease embryo cell number, but this influence was not significant. No significant effect of the EGF addition on the cell number in the gene-Mi embryos was found. There were no significant differences between groups in embryo diameter.

DISCUSSION

The microinjection of gene construct in our study did not affect the cleavage rate of embryos, but significantly lowered blastocyst rate compared to intact embryos. Microinjection of foreign gene into the pronucleus of eggs can result in DNA rearrangement during integration and cause decreased yield of blastocyst stage embryos (Voss et al., 1996). Present results, obtained on the embryos microinjected with blank solution (PBS-Mi), demonstrated that embryo cell number and embryo diameter were comparable to those in the gene-microinjected embryos (G-Mi). These data together with earlier observation of Canseco et al. (1994) exclude the influence of the gene preparation. It is possible that other factors, associated with the microinjection itself, for example, mechanical damage by microinjection pipette, exposure of the zygote to a microscope light of a higher intensity, or their combination may result in decreased quality of Mi-derived embryos. The embryo diameter and cell number are non-invasive markers of embryo quality, as their determination does not require destructing the embryo, when vital dye staining with is used. From this viewpoint we choose these two parameters for the evaluation of rabbit embryos developed from the gene-microinjected eggs.

Usually in our *in vivo* experiments rabbit embryos after microinjection are cultured for further 22 h and then are transferred to recipients at 6-8-cell stage. In the present study the embryos were cultured until 92-96 hpc, and the embryo viability *in vitro* was evaluated on the basis of the progression of cleaving embryos to the blastocyst stage. The embryo cell number was significantly lower after microinjection with PBS-Mi, in embryos injected with the gene the cell number had a tendency to decrease. Since the embryo diameter was not changed under the influence of Mi or the EGF addition in this study (Table 3), this parameter seems to be more conservative characteristics of the embryo morphology. The zona pellucida is an extracellular coat, which surrounds the mammalian embryo between the zygote and the blastocyst stage. The thickness of the mammalian zona pellucida has been measured in numerous studies, in most mammals it is approximately 10 μm. There is no consistent opinion yet on using the zona thickness as a marker of embryo quality (rev. by Van Soom, 2003). Basing on our observations, the zona pellucida can mask differences in embryo diameters between more advanced and less advanced embryos, because early blastocysts, containing less cells, showed larger thickness of zona than expanded blastocysts, which contained more cells. In our study we measured diameter of embryos excepting zona pellucida. Correlation coefficients determined between the cell number and the diameter of rabbit blastocysts in our study (r -range from 0.560 to 0.707) were higher than those reported (Mori et al., 2002) in Day 7 and Day 8 bovine *in vitro* produced blastocysts (r = 0.372-0.496), but were comparable with Day 9 blastocysts (r = 0.532, 0.711). Since these authors measured embryo diameter including zona pellucida, we may assume that this difference in correlation coefficients was due to the difference in zona thickness between early and expanded blastocysts. Therefore, measurement of blastocyst diameter omitting zona pellucida seems to be more exact in the point of interrelation between embryo diameter and embryo cell number.

The microinjection procedure in our study decreased blastocyst rate and cell number, but did not affect embryo diameter. Although embryo diameter is assumed to be potential marker for the viability testing of bovine expanded blastocysts (Mori et al., 2002) this statement has not been confirmed in rabbit microinjected embryos. It is also possible that a functional state of embryos is more susceptible to consequences of micromanipulations than a morphological state. The decline in the blastocyst cell number after the pronuclear microinjection is, more likely, realized either via repression of cell proliferation, or via induction of cell apoptosis, or via both mechanisms. Therefore, the percentage of proliferating (Markkula et al., 2001) or apoptotic cells (Makarevich et al., 2005 in press) could better reflect functional state of *in vitro* manipulated embryos.

The comparison of coefficients of variation between groups indicated that the cell number in the intact embryos is relatively homogenous value, whilst in microinjected groups (G-Mi/E0; PBS-Mi) the cell number varies, indicating that the embryo response to Mi procedure might be individual. Our observations show that EGF did not improve blastocyst rate and embryo cleavage of microinjected embryos. Moreover, this growth factor did not influence the cell number and the diameter of embryos derived from both gene- or PBS-microinjected rabbit eggs.

In conclusion, these results suggest that pronuclear microinjection affects developmental potential of embryos, decreasing the blastocyst rate and the embryo cell number, but it does not affect the embryo diameter. Decreased quality of Mi-derived embryos is more likely due to the microinjection procedure itself, but not due to the gene construct. No effects of EGF on studied parameters were confirmed. Despite positive correlation between the cell number and embryo diameter, the latter parameter does not seem to be a flexible marker of the viability of Mi-derived embryos.

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