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Establishment of Efficient Microinjection System in the Porcine Embryos

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ABSTRACT

Transcription activator like effector nucleases (TALENs) are artificial restriction enzymes generated by fusing a TALE DNA binding domain to a DNA cleavage domain which remove and introduce specific genes to produce transgenic animals. To investigate the efficient laboratory techniques for the injection of TALEN mRNA, pEGFP-N1 commercial plasmid were microinjected into porcine parthenogenetic and *in vitro* fertilization (IVF). In Experiment 1, to investigate injection time, compared 4 different time durations (2 hr, 4 hrs, 6 hrs & 8 hrs) after post activation of parthenogenetic embryos and after 6 hrs of co-incubation with sperms in IVF embryos. There were significant difference (P<0.05) in development to the blastocysts (4.4, 8.9, 3.9, 0.6%), GFP expression in blastocysts (1.3, 5.7, 2.3, 0.0%) which injected after post activation of 4 hrs compared with other 3 groups. IVF embryos after 2 hrs and 4 hrs injected were expressed GFP significantly higher than rest of two groups (P<0.05). In Experiment 2, compared development of 2 different concentrations (20 ng/μl and 50 ng/μl) of EGFP injection. There were significant difference (P<0.05) between two treatments which has higher cleavage (58.8 vs 41.9%), blastocysts development rate (13.0 vs 11.1%) and GFP expressed blastocysts (5.7 vs 0.0%) in 20 ng/μl than the 50 ng/μl in parthenogenetic embryos. In IVF embryos, only 20 ng/μl injected embryos were expressed GFP (4.2%) after 7 days of incubation and 77.3 vs 64.7% of cleavage, 26.4 vs 23.5% development to blastocysts. In Experiment 3, three different volumes (5, 10 and 20 pl) were microinjected into porcine embryos to determine the most appropriate volume. Out of 3 groups, significantly higher development rates of cleavage (68.3, 58.0, 29.3%), blastocysts (11.7, 12.7, 0.5%) and GFP expressed blastocysts (2.9, 7.8, 0.0%) were shown in the 10 pl group (P<0.05). In conclusion, these results imply that 20 ng/μl concentration, 10 pl of volume and injection at 4 hrs after post activation for parthenogenetic and 2～4 hrs after IVF, 20 ng/μl concentration and 10 pl volume for IVF embryos were more effective microinjection conditions.

(Key words : TALEN, embryos, porcine, parthenogenesis, IVF)

INTRODUCTION

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome (Baker, 2012). The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it (Laible, 2009). There are several techniques used to produce transgenic animals. Reproductive cloning and embryonic microinjection are two major methods which enable generation of profoundly genetically modified animals (Galli *et al.*, 2010). Embryonic microinjection is a technique that was developed in the mouse, involves the direct introduction of a DNA construct into the fertilized egg. Cloning by somatic cell nuclear transfer has been successfully achieved by both fusing of a donor cell with and injecting an isolated donor cell nucleus into an enucleated oocyte (Li *et al.,* 2009). Cytoplasmic microinjection in the embryos refers to the process of using a glass micropipette to inject a liquid substance at a microscopic or borderline macroscopic level. For processes such as cellular or pronuclear injection, the target embryos is posi- tioned under the microscope and two micromanipulators of one holding the pipette and one holding a microcapillary needle usually between 0.5 to 5 μm in diameter are used to penetrate the cell membrane. In this way, the process can be used to introduce a vector into a single embryo. Pronuclear microinjec- tion of DNA is a technique

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used to create transgenic organisms by injecting genetic material into the nucleus of a fertilized oocyte (Gordon & Ruddle, 1983). This technique is commonly used to study the role of genes using mouse animal models (Burke *et al.,* 2004). In order for pronuclear injection to be successful, the genetic material must be injected while the ge- netic material from the oocyte and sperm are separate (Gordon & Ruddle, 1983).

Transcription activator like effect or nucleases (TALENs) have recently emerged as a revolutionary genome editing tool in many different organisms and cell types (Sun & Zhao, 2013). The site specific chromosomal double strand breaks introduced by TALENs significantly increase the efficiency of genomic modifiation (Chen & Gao, 2013; Moscou & Bogdanove, 2009). Applications include studying gene functions in model organisms, improving traits in crop plants and livestock, generating disease models, and treating genetic disorders in humans (Carlson *et al.,* 2012). Recent studies have tested the modularity of the TALE DNA binding code and have demonstrated that custom TALEs can be designed to recognize specific DNA sequences in a number of different cell types including plant and mammalian cells (Sun & Zhao, 2013). As TALENs have enabled the efficient introduction of targeted alterations in a number of model organisms that were previously difficult or impossible to genetically manipulate such as fruit fly,roundworm, zebra fish, frog, rat and pig (Schmid & Haass, 2013).

Current study was based on the determination of most appropriate microinjection time, concentration and volume for the injection of TALEN mRNA into porcine parthenogenetic and IVF embryos and within the research period studied about the best applicable standards for the highly efficient blastocyst production in parthenogenetic and IVF embryos. Used pEGFP-N1 as marker gene for the microinjection helps to identify blastocysts by the expression of GFP. Little researches and inadequate information about porcine TALEN experiments leads to this study which explores the microinjection time after oocytes post activation, IVF embryo incubation, volume of microinjection and concentration of microinjection for the efficient blastocyst production has been identified within the experimental period.

MATERIALS AND METHODS

1. *In Vitro* Maturation of Porcine Oocytes

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated. Pig ovaries were collected from the slaughterhouse and transported to the laboratory washed with prewarmed saline water, and follicles were aspirated using 18 gauges needle syringe. Selected cumulus-oocytes complexes (COCs) were washed for 3 times in the TLH-PVA and cultured in maturation medium con- sist culture media (TCM-199, Life Technologies, CA, USA) supplemented with 20 μg/ml of eCG (Intervet, Netherlands) / hCG (Intervet) and, 0.2 mM sodium pyruvate, 15 μg/ml kana- mycin, 15 μg/ml epidermal growth factor, and 10 μg/ml insulin for 22 h (39℃, 5% CO_2). After 22 hours of incubation, oocytes were transfer to the culture media contains above formulation without hormone for another 22 h of incubation 39℃, 5% CO_2 conditions. After 44 hours of total incubation, oocytes stripped of cumulus cells by denuding of 0.1% (w/v) hyaluronidase and oocytes with a dark, uniform cytoplasm and a visible first polar body were used for embryo production of IVF and parthenogenesis.

2. Production of Parthenogenetic Embryos

Parthenogenetic activation of oocytes accomplished by submerging oocytes in activation medium contains 0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 mM HEPES. In between two platinum wire electrodes, follow by two successive 60 msec pulses (1.2 kV/cm) of direct current (DC) channeled by a BTX Electro-Cell Manipulator (Genetronics. Inc, CA, USA). Parthenogenetic embryos put into post activation media consist 7.5 μg/ml cytochalasinB in PZM-5 media for 4 hrs in the incubator at 39℃, 5% CO_2 in air at saturating humidity immediately after exposure to the electrical activation stimulus transfer to post activation media and keep in the incubator. After 4 hours of incubation in post activation media, parthenogenetic embryos were transferred to the 25μl of PZM5 drops covered by mineral oil. Parthenogenetic embryos were cultured at 39℃, 5% CO_2, 5% O_2 at saturating humidity. Parthenogenetic embryos were taken for the injection of pEGFP after the post activation of 2, 4, 6 and 8 hours intervals. After 7 days of incubation, embryos were examined the quality, blastocyst rate (%) and checked for the GFP expression from the blastocysts.

3. Production of IVF Embryos

The basic medium used for IVF was referred to as modified Tris-buffered medium (mTBM). Oocytes cultured for 44 h in maturation medium were mechanically stripped of cumulus by gentle aspiration with a pipette. Denuded oocytes were washed

three times in mTBM medium and groups of 30～35 oocytes transferred to each well of four-well Nunc multidishes (Nunc, Denmark) containing TALP medium, and equilibrated overnight at 38.5℃ under 5% CO_2. Sperm suspensions (250μl) from each treatment group were added to each fertilization well to obtain final concentration of 5×10^4 cells/ml. After incubation of 6 hours IVF embryos were taken for the microinjection of TALEN mRNA and cultured in the PZM5 medium 25 μl drops covered by mineral oil and incubated at 39℃, 5% CO_2, 5% O_2 at saturating humidity. After 7 days of incubation, embryos were examined the quality, blastocyst rate (%) and checked for the GFP expression from the blastocysts.

4. Cytoplasmic Microinjection of pEGFP-N1

pEGFP-N1 (Clontech, USA) encodes a red shifted variant of wild type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (Excitation maximum = 488 nm; emission maximum = 507 nm). pEGFP-N1 were prepared at a final concentration of 20 ng/μl and 50 ng/μl and backfilled in glass injection capillaries. Individual zygotes were fixed by suction to a holding pipet, while the injection capillary was pushed though the zona pellucida and cell membrane. Approximately 5 pl, 10 pl and 20 pl plasmid solution was injected into the cytoplasm using an Fem- tojet express (Eppendorf, Germany).

5. Experimental Design

In Experiment 1, it was compared development and expression rates of embryos in four different time intervals microinjection of pEGFP-N1 for the porcine parthenogenetic and IVF embryos after post activation and IVF respectively. Different time of 2 hr, 4 hrs, 6 hrs and 8 hrs after post activated embryos in parthenogenetic activated and IVF embryos after 6hrs of co-incubation with sperms were taken for injection and after injection embryos were cultured in PZM-5 media for further development. Embryos without any treatment, controls were cultured in PZM-5 media without giving microinjection. After 2 days of incubation, cleavage of embryos were counted and after 7 days of incubation, examined and counted GFP expressed blastocysts under the UV light.

In Experiment 2, it was compared development and expression rates of embryos in two different concentrations of microinjection 20 ng/μl and 50 ng/μl into parthenogenetic and IVF embryos. Two different concentrations (20 ng/μl and 50 ng/μl) of pEGFP-N1 were microinjected with respective time interval which given highest GFP expressed blastocysts in the experiment 1. After microinjection, embryos were cultured in 25 μl PZM5 media drops covered by mineral oil. After 2days of incubation in PZM5 media drops in the incubator, cleaved embryos were counted and after 7 days of incubation, examined and counted GFP expressed blastocysts under the fluorescence microscope under UV light.

In Experiment 3, compared development and expression rates of three different volumes of injection in to parthenogenetic and IVF embryos. 5 pl, 10 pl and 20 pl volumes were compared by injecting to parthenogenetic and IVF embryos after selecting the best time and concentration of pEGFP-N1 microinjection from Experiment 1 and 2. Controls were cultured in PZM5 media without microinjection. After 2 days of incubation, cleavage of embryos were counted and after 7 days of incubation, examined and counted GFP expressed blastocysts under the fluorescence microscope under UV light.

6. Statistical Analysis

Data were analyzed by one-way ANOVA with a general linear model procedure using the SPSS System (version 21.0 IBM, USA), followed by the least significant difference mean separation procedure when treatments differed at $P<0.05$.

RESUTLS

1. Development of Embryos after pEGFP-N1 Injection at Different Time

Developmental potentials of parthenogenetic and IVF embryos after injection of pEGFP-N1 in different time intervals were shown in Table 1 and 2, respectively. The embryo development to blastocyst rate and GFP expressed blastocysts of 4 hrs after post activation group were significantly higher than other three groups ($P<0.05$), however there was no significantly difference between cleavage rates in four groups. The development competence of embryos upto blastocyst rate and GFP expressed blastocysts of 2 hrs and 4 hrs after IVF groups were significantly higher than rest of two groups ($P<0.05$). There was no significantly difference between the cleavages of the treated groups in the early stages of development of embryos.

2. Development of Embryos after pEGFP-N1 Injection at Different Concentrations

Developmental potential of parthenogenetic and IVF embryos

Table 1. Development of porcine parthenogenetic embryos after cytoplasmic microinjection of PEGF-N1 at different time after postactivation

Time after post-activation (hrs)	Number of embryos (%)			
	Injected	Cleaved	Develop to BL*	GFP expressed in BL*
Control	158	128(81.1)[a]	38(24.1)[a]	
2	158	75(47.5)[b]	7(4.4)[b]	2(1.3)[b]
4	158	77(48.7)[b]	14(8.9)[c]	9(5.7)[a]
6	154	72(46.8)[b]	6(3.9)[b]	4(2.6)[b]
8	158	72(45.6)[b]	1(0.6)[b]	0(0.0)[b]

[a,b] Values in the same column with different superscripts are different(p<0.05).
* Blastocyst.

Table 2. Development of porcine IVF embryos after cytoplasmic microinjection of PEGF-N1 at different time after end of co-incubation with sperms

Time after IVF (hrs)	Number of embryos (%)			
	Injected	Cleaved	Develop to BL*	GFP expressed in BL*
Control	126	82(65.1)[a]	21(16.7)[a]	
2	126	55(43.7)[b]	8(6.4)[b]	3(2.4)[a]
4	126	46(36.6)[b]	6(4.8)[b]	3(2.4)[a]
6	126	51(40.5)[b]	3(2.4)[b]	2(1.6)[a]
8	126	45(35.8)[b]	1(1.6)[b]	1(0.8)[a]

[a,b] Values in the same column with different superscripts are different(p<0.05).
* Blastocysts.

after injection of pEGFP-N1 in two different concentrations were shown in Table 3 and 4. The cleavage rate, blastocyst rate and GFP expressed blastocysts were significantly higher (P< 0.05) in 20 ng/μl groups than that of 50 ng/μL. Developmental potential of IVF embryos were significantly greater in 20 ng/μl than 50 ng/μl which clearly implies cleavage rate (P<0.05), blastocysts rate and GFP expressed blastocysts were at significantly higher.

3. Development of Embryos after pEGFP-N1 Injection at Different Volume

Table 3. Development of porcine parthenogenetic embryos after cytoplasmic microinjection of pEGFP-N1 at different concentrations

Concentration (ng/μl)	Number of embryos (%)			
	Injected	Cleaved	Develop to BL*	GFP expressed in BL*
20	640	370(57.8)[a]	83(13.0)[a]	36(5.7)[a]
50	117	49(41.9)[b]	13(11.1)[b]	0(0.0)[b]

[a,b] Values in the same column with different superscripts are different(p<0.05).
* Blastocysts.

Table 4. Development of porcine IVF embryos after cytoplasmic microinjection of pEGFP-N1 at different concentrations

Concentration (ng/μl)	Number of embryos (%)			
	Injected	Cleaved	Develop to BL*	GFP expressed in BL*
20	334	258(77.3)[a]	88(26.4)[a]	14(4.2)[a]
50	51	33(64.7)[b]	12(23.5)[b]	0(0.0)[b]

[a,b] Values in the same column with different superscripts are different(p<0.05).
* Blastocysts.

Table 5 and 6 shows the developmental potential of parthenogenetic and IVF embryos after injection of pEGFP-N1 in three different volumes (5 pl, 10 pl and 20 pl). The cleavage rate, development to blastocyst rate and GFP expressed blastocysts were significantly different 10 pl than other 2 groups (P<0.05). There was no significant difference between 5 pl and 10 pl blastocysts rates, however the GFP expressed blastocysts rates were significantly (P<0.05) higher than the 5 pl volume microinjected group of blastocysts. The cleavage rate and blastocysts rates in the 5 pl and 10 pl volume microinjected groups were have no significant difference, however the 10 pl volume microinjected group was significantly different when comparing the other groups (P<0.05).

4. Development of IVF Embryos after Injection of mRNA apoE TALEN at Different Concentrations

Developmental potential of IVF embryos after injection of mRNA apoE TALEN at different concentrations was shown in Table 7. In the table, it was shown that the embryos cleavage

Table 5. Development of porcine parthenogenetic embryos after cytoplasmic microinjection of pEGFP-N1 at different volumes

Volume (pl)	Number of embryos (%)			
	Injected	Cleaved	Develop to BL*	GFP expressed in BL*
Control	205	161(78.5)[a]	46(22.4)[a]	
5	205	140(68.3)[b]	24(11.7)[b]	6(2.93)[b]
10	205	119(58.0)[c]	26(12.7)[b]	16(7.80)[a]
20	205	60(29.3)[d]	1(0.5)[c]	0(0.00)[b]

[a~d] Values in the same column with different superscripts are different(p<0.05).
* Blastocysts.

Table 6. Development of porcine IVF embryos after cytoplasmic microinjection of pEGFP-N1 at different volumes

Volume (pl)	Number of embryos (%)			
	Injected	Cleaved	Develop to BL*	GFP expressed in BL*
Control	120	72(60.0)[a]	22(18.3)[a]	
5	120	62(51.7)[b]	16(13.3)[b]	3(2.5)[b]
10	120	57(47.5)[b]	12(10.0)[b]	8(6.7)[a]
20	120	25(20.8)[c]	1(0.8)[c]	0(0.0)[b]

[a~d] Values in the same column with different superscripts are different(p<0.05).
* Blastocysts.

rate, development to blastocyst rate and GFP expressed blastocysts were significantly higher in 20 ng/μl than 50 ng/μl (P< 0.05).

DISCUSSION

TALEN has been proven to be a valuable methodology for species in which it was difficult to generate targeted mutations for desired genes. In this study, production of blastocysts in laboratory by using TALEN mRNA injection and determine the best conditions of injections changing injection time interval, volume, and concentration. Although there are many of researches and experiments conducting with using TALENs, there are still few publications mentioning about the exact conditions for the microinjection.

Table 7. Development of porcine IVF embryos after cytoplasmic microinjection of mRNA apoE TALEN at different concentrations

	Number of embryos (%)		
	Injected	Cleaved	Develop to BL*
PBS	115	68(59.1)	17(14.8)
TALENs mRNA (20ng/μl)	117	72(61.5)[a]	14(12.0)[a]
TALENs mRNA (50ng/μl)	118	67(56.8)[b]	12(10.2)[b]

[a,b] Values in the same column with different superscripts are different(p<0.05).
* Blastocysts.

Considering microinjection of TALENs into porcine parthenogenetic embryos and IVF embryos, the three factors (time, volume and concentration) which was conduct in this experiment should be considered. According to results the best time for injection is early after fertilization when the ova have two pronuclei. When the two fuse to form a single nucleus, the injected DNA may or may not be taken up. If the DNA is incorporated into the genome, it is done so randomly (Christian *et al.*, 2010). Because of this, there is always a chance the gene insert will not be expressed by the GMO (Genetic Modified Organism), or may even interfere with expression of gene on the chromosome. Moreover in parthenogenetic embryos shown higher development competence when the microinjection done in early stage of after post activation. Microinjection at 2 hrs after post activation not given higher blastocysts as expected in IVF in the parthenogenetic embryos. Although microinjection at 4 hrs after post activation did not shows clear difference between the cleavage rates, its articulate higher development capability of blastocysts and GFP expressed blastocysts in later stage of embryo development. In previous studies, it shows that time of microinjection is important regarding different types embryos using for the injection (Isoe *et al.*, 2007; Khoo *et al.*, 1993; Luna *et al.*, 2007). However these results imply cytoplasmic microinjection in early embryo development period will increases the embryo development and increases competence of development of embryos up to blastocysts stage. GFP expression of the blastocysts had some variation regarding expression intensity and amount of cells GFP expressed in the

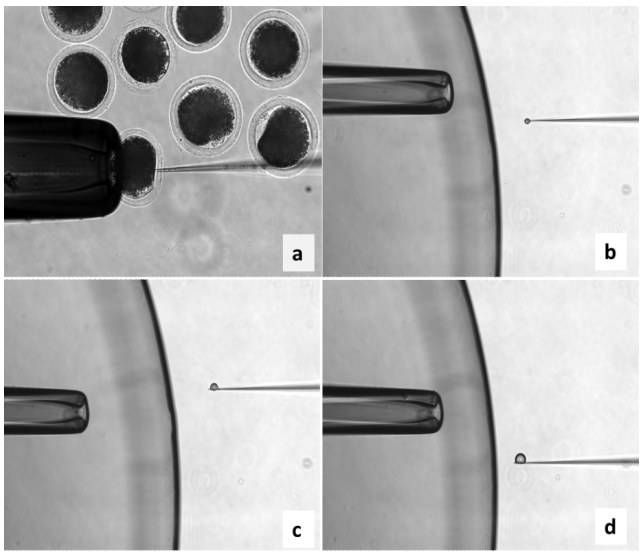

Fig. 1. Cytoplasmic microinjection of EGFP in to parthenogenetic and IVF embryos with different volume. (a) Injection of EGFP in to embryos (×200), (b) Injection volume adjusted to 5 pl, the diameter near to 20 μm of the injection sphere (×100), (c) Injection volume adjusted to 10 pl, the diameter near to 28 μm of the injection sphere (×100), (d) Injection volume adjusted to 20 pl, the diameter near to 33 μm of the injection sphere (×100).

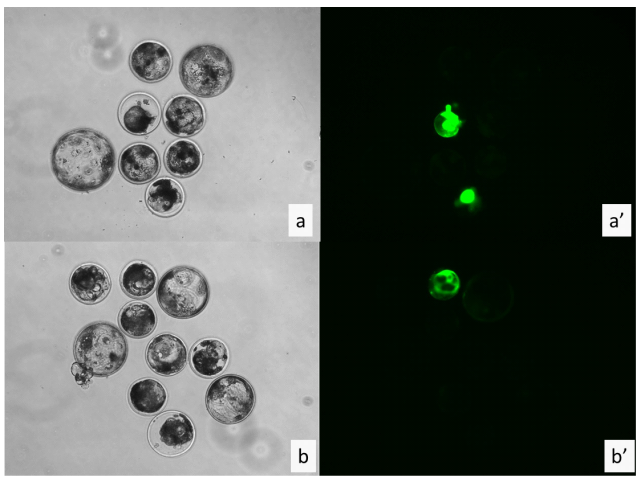

Fig. 2. Development of porcine parthenogenetic embryos contrast to injection time after activation (a and b images of normal light at ×100, a` and b` images of UV light at ×100).

blastocysts (Fully or partial expression). With the time taken to injection, development competence of embryos will be reduced and the gene expression ability will be decreases in the blastocysts.

Concentration for microinjection will be critical factor for

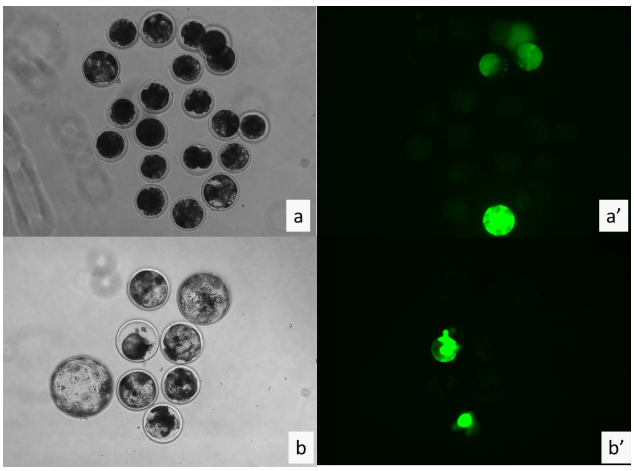

Fig. 3. Development of porcine IVF embryos contrast to injection time after IVF (a and b images of normal light at ×100, a` and b` images of UV light at ×100).

the embryo development and higher concentration will have detrimental effects to the embryonic development and decreases competence of development of embryos up to blastocysts stage (Gagne *et al.*, 1995). In parthenogenetic and IVF embryos which used to inject 50 ng/μl of concentration groups could not has ability to develop any blastocysts or GFP expressed blastocysts. Besides cleavage rate were significantly lower and the cytoplasmic appearance were in pale color which implies lower capability of development. Concerning 20 ng/μl concentration injected embryos in both parthenogenetic and IVF embryos shows higher cleavage rates, blastocyst rate and GFP expressed blastocysts. In previous studies (Davidson *et al.*, 2010; Kinoshita *et al.*, 2000). They described around 20 ng/μl concentration of injection more effective and reduce the stress to the embryos and leads to higher development competence up to blastocysts. The 20 ng/μl concentration was given the best condition of microinjection and higher capability of development in porcine embryos.

Injection volume is another crucial factor to be taken to account in the microinjection. Higher volume may destruct embryos cytoplasmic materials and cause to higher damages to the embryos. From the three volumes used within the experiment, 10 pl microinjected embryos shows the higher blastocysts rates and cleavage rates. Within the cytoplasmic injection, observed cytoplasmic destruction when injecting higher volumes such as 20 pl in to the embryo. Appropriate volume and concentration of microinjection reduce the stress of embryos and competence of embryonic development will be higher and previous studies

shows that volume of 10 pL appropriate for the microinjection (Kato *et al.*, 2013).

Other than preplanned experiments, IVF embryos were used to observe the development competence of cytoplasmic microinjection of mRNA apoE TALEN at two different concentrations. Similar results shown in the mRNA apoE TALEN injection in the IVF and 20 ng/μl concentration injected embryos has significant different cleavage and blastocysts development compared with the 50 ng/μl concentration injected group.

A conventional method to investigate the functions of these factors is to modify the genetically modified organism after direct injection of the molecules of interest into the embryos (Kinoshita *et al.,* 2000). The transgenic method may be applied as another tool to modify both the quality and quantity of maternal factors that accumulate as transgene products in oocytes (Scholze & Boch, 2011). This study leads to maximize the rate of development in microinjected embryos with using correct conditions and time. Specially in using TALEN for the gene targeting and gene editing, embryonic developments of microinjected embryos were critical factor towards the successful research.

At last the present study demonstrates importance of the time, concentration and the volume of cytoplasmic microinjection for the development of embryos. Those three factors are critical for the development competent of embryos and early stage of microinjection, 20 ng/μl concentration and 10 pl of volume gives higher outcomes form the embryos.

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