J Heart Valve Dis
Vol. 13. No. 5
September 2004

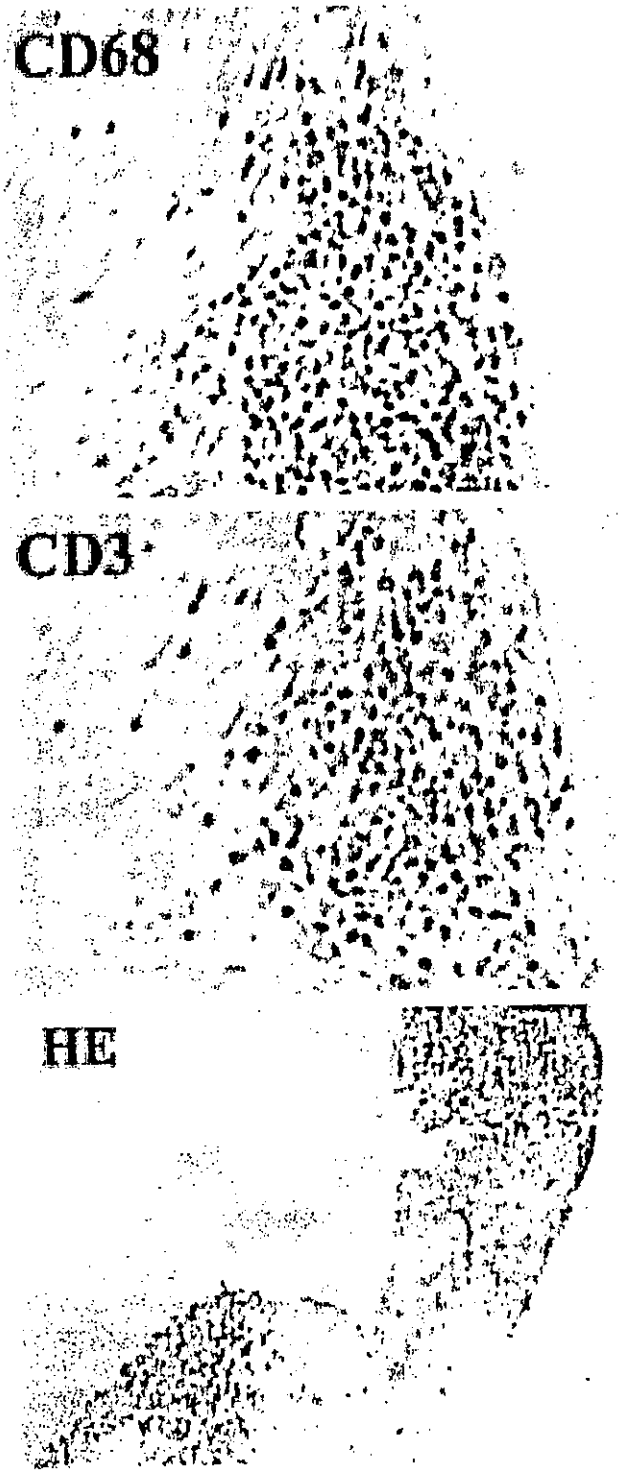

Figure 2: Histological and immunohistochemical findings of pig # 6 (cryopreserved graft; not decellularized, not cell-seeded). There were two to three regions of focal spindle or round cell proliferation in the cusp. CD68-positive cells were observed focally at the interstitium of the cusps, together with CD3-positive cells. HE: Hematoxylin and eosin staining.

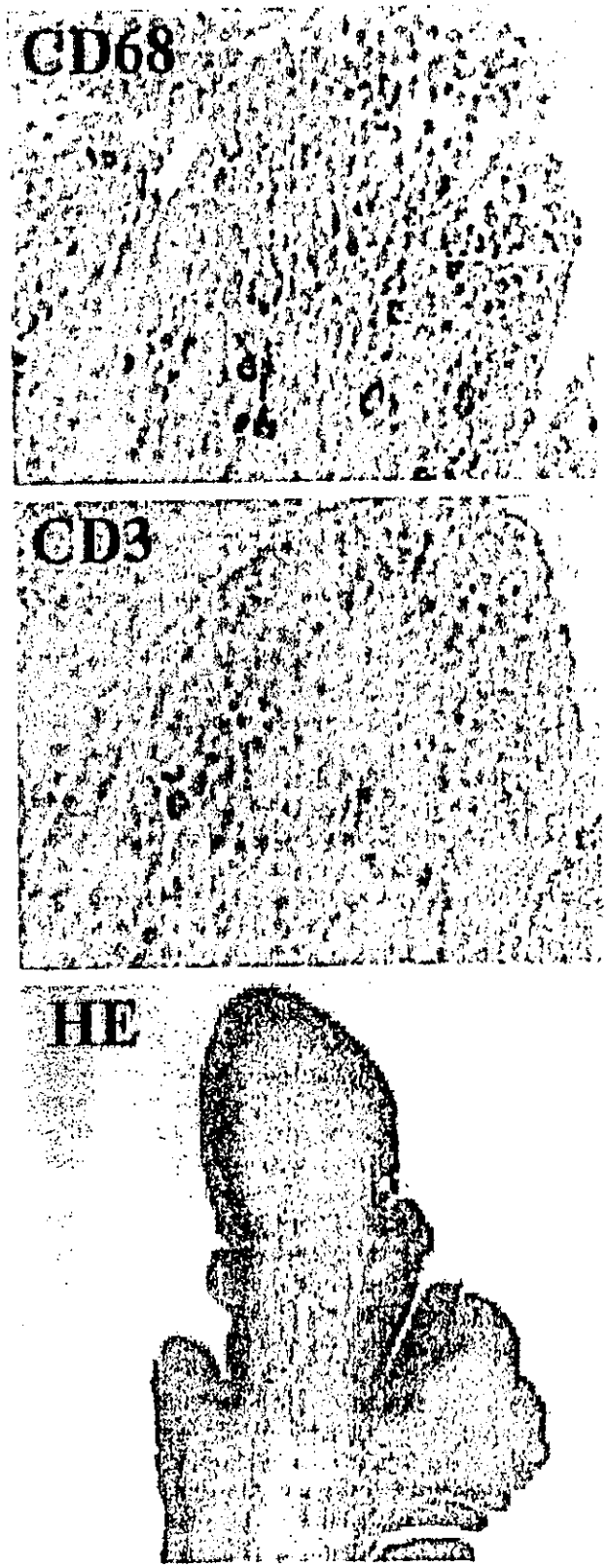

Figure 3: Histological and immunohistochemical findings of pig # 5 (cryopreserved graft but not decellularized or cell-seeded). There were multi-layer cell linings predominantly localized at the cusp surface. CD68-positive cells were observed at the multi-layer cells, and CD3-positive cells were also present. HE: Hematoxylin and

cells were also present around these cells (Table II; Fig. 3). No polymorphonuclear neutrophil leukocytes were observed in the valve tissue of either of these pigs.

Discussion

The preservation, clinical application, and long-term outcome of cryopreserved homografts have been evaluated previously (9-11). It has also been shown that fibroblast viability persists in cryopreserved allografts after implantation, and that these cells express the genes for fibroblast growth factor and procollagen (12), which can maintain the structure of the tissue. It has also been demonstrated that collagen synthesis of the cryopreserved allograft was relatively well maintained (13). However, fibroblasts in the donor allograft were unable to survive for long, because of apoptosis (14). The durability of the homograft valve is related to the viability of the fibroblasts that maintain the valve matrix (15), and therefore the loss of fibroblasts may result in a loss in homograft durability.

Endothelial cells exhibit strong antigenicity, but cannot survive under ischemic conditions. Moreover, during the cryopreservation process endothelial cells lose their ability to proliferate (16,17). This raises the possibility that homograft durability is related to the extent of endothelial cell denudation. Thus, the homograft will lose its endothelial cells and fibroblasts, and eventually become a non-viable tissue.

The fate of implanted cryopreserved homografts has been demonstrated in previous clinical and pathological studies (18,19). After implantation, the homograft loses its endothelium, cellularity, and the layered architecture, after which calcified deposits and hematoma/mural thrombi are seen to form. The homograft cusps become flattened and thinned, with obliteration of the usual corrugated pattern. These investigators concluded that the cryopreserved allograft is morphologically non-viable, and also suggested that the early influx of macrophages and T lymphocytes was limited after implantation. Over the longer term, inflammatory cells were not found, and thus the degeneration could not be attributed to immunological responses.

The results of previous studies have indicated that, after allograft implantation, donor cells disappear rapidly and are then replaced by recipient endothelial and interstitial cells (20-22). It would be expected that influx of the recipient cells would progress into the allograft and maintain valvular function. Thus, the present authors speculated that if valvular tissue were to be decellularized prior to implantation, then the recipient cells would readily repopulate and proliferate upon this substrate.

In the present study in a mini-pig model, right ventricular outflow tract replacement was performed with a cryopreserved and decellularized allograft and an 'only-cryopreserved' allograft, and the short-term results evaluated. In the decellularized group, H&E staining revealed that the surfaces of the graft were repopulated by endothelial cells for up to one month. By contrast, in the only-cryopreserved group, endothelial cells were almost completely lost, the trilaminar tissue architecture had disappeared, and macrophages and T-lymphocytes were found in the valve. Endothelialization of the decellularized cusps occurred more densely than in the only-cryopreserved cusps. Furthermore, in the decellularized group, inflammatory cells infiltrated less than in the only-cryopreserved cusps. Decellularization may precede repopulation of the recipient cells.

In the present study, no significant differences were observed between the cell-seeded and non-cell-seeded groups. The endothelial cells play an important role in preservation of the subendothelial cellular and matrix components (23), and this influence may become apparent after implantation. With the static seeding approach used in the present investigation, the endothelial cells were seeded heterogeneously.

Steinhoff et al. (7) reported that with only static reseeding, complete recellularization with endothelial cells and myofibroblasts occurred, at four and 12 weeks after implantation, although the unseeded group recellularized only with endothelial cells. The duration of reseeding used by these authors was longer (8 days) than was used in the present study (2 days). Furthermore, these other investigators performed the reseeding with myofibroblasts for six days and with endothelial cells for two days, whereas only endothelial cells were used for seeding in the present study. For further evaluation of these procedures, the duration of static reseeding and the type of reseeding cells used are important. It is possible that confluent endothelial cell coverage before implantation might be preferable, and if the grafts were to be seeded using a bioreactor (24,25), then confluent cell coverage might be established.

Several points regarding this graft procedure remain unclear. The first is thrombogenicity - the evaluation of which is difficult because the operative procedure itself is associated with thrombus formation. In addition, in the present study neither anticoagulation nor anti-platelet therapy were initiated, and a limited period of anticoagulation therapy may indeed be necessary with this graft procedure. Furthermore, at the microscopic level the surface of the graft was found to be rough, and this may provide a suitable substrate for the thrombus formation observed in the present study. If confluent coverage of the graft surface with endothelial cells were to be established before implantation, then thrombus formation may be reduced.

In conclusion, a decellularized allograft was recellu-

larized with endothelial cells for up to four weeks. In comparison with the cryopreserved allograft, the inflammatory response was reduced by decellularization.

References

1. Shinoka T, Breuer CK, Tanel RE, et al. Tissue engineering heart valves: Valve leaflet replacement study in a lamb model. Ann Thorac Surg 1995;60(6 Suppl.):S513-S516
2. Shinoka T, Ma PX, Shum-Tim D, et al. Tissue-engineered heart valves. Autologous valve leaflet replacement study in a lamb model. Circulation 1996;94(9 Suppl.):II164-II168
3. Sodian R, Hoerstrup SP, Sperling JS, et al. Early in vivo experience with tissue-engineered trileaflet heart valves. Circulation 2000;102(19 Suppl.3):III22-III29
4. Elkins RC, Dawson PE, Goldstein S, Walsh SP, Black KS. Decellularized human valve allografts. Ann Thorac Surg 2001;7(5 Suppl.):S428-S432
5. Goldstein S, Clarke DR, Walsh SP, Black KS, O'Brien MF. Transpecies heart valve transplant: Advanced studies of a bioengineered xeno-autograft. Ann Thorac Surg 2000;70:1962-1969
6. Bader A, Schilling T, Teebken OE, et al. Tissue engineering of heart valves: Human endothelial cell seeding of detergent acellularized porcine valves. Eur J Cardiothorac Surg 1998;14:279-284
7. Steinhoff G, Stock U, Karim N, et al. Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits: In vivo restoration of valve tissue. Circulation 2000;102(19 Suppl.3):III50-III55
8. Shinoka T, Imai Y, Ikeda Y. Transplantation of a tissue engineered pulmonary artery. N Engl J Med 2001;344:532-533
9. Niwaya K, Knott-Craig CJ, Santangelo K, Lane MM, Chandrasekara K, Elkins RC. Advantage of autograft and homograft valve replacement for complex aortic valve endocarditis. Ann Thorac Surg 1999;67:1603-1608
10. Niwaya K, Knott-Craig CJ, Lane MM, Chandrasekara K, Overholt ED, Elkins RC. Cryopreserved homograft valves in the pulmonary position: Risk analysis for intermediate-term failure. J Thorac Cardiovasc Surg 1999;117:141-147
11. Niwaya K, Sakaguchi H, Kawachi K, Kitamura S. Effect of warm ischemia and cryopreservation on cell viability of human allograft valves. Ann Thorac Surg 1995;60:S114-S117
12. Song YC, Yao LY, Kneebone JM, Lupinetti FM. Effect of cryopreservation and histocompatibility on type I procollagen gene in aortic valve graft. J Thorac Cardiovasc Surg 1997;114:421-427
13. Kano M, Masuda Y, Tominaga T, et al. Collagen synthesis and collagenase activity of cryopreserved heart valves. J Thorac Cardiovasc Surg 2001;122:706-711
14. Hibert SL, Luna RE, Zhang J, et al. Allograft heart valves: The role of apoptosis-mediated cell loss. J Thorac Cardiovasc Surg 1999;117:454-462
15. O'Brien MF, Stafford EG, Gardner MAH, Pohler PG, McGriffin DC. A comparison of aortic valve replacement with viable cryopreserved and fresh allograft valves, with a note on chromosomal studies. J Thorac Cardiovasc Surg 1987;94:812-823
16. Yankah AC, Wottge HU, Muller-Hermelink HK, et al. Transplantation of aortic and pulmonary allografts, enhanced viability of endothelial cells by cryopreservation, importance of histocompatibility. J Card Surg 1987;1(Suppl.):209-220
17. Lang SJ, Giordano MS, Cardon-Cardo C, Summers BD, Staiano-Coico L, Hajjar DP. Biochemical and cellular characterization of cardiac valve tissue after cryopreservation or antibiotic preservation. J Thorac Cardiovasc Surg 1994;108:63-67
18. Mitchell RN, Jonas RA, Schoen FJ. Pathology of explanted cryopreserved allograft heart valve: Comparison with aortic valves from orthotopic heart transplants. J Thorac Cardiovasc Surg 1998;115:118-128
19. Mitchell RN, Jonas RA, Schoen FJ. Structure-function correlations in cryopreserved allograft valves. Ann Thorac Surg 1995;60:S108-S113
20. Braun J, Hazekamp MG, Koolbergen DR, Sugihara H, Goffin YA, Huysmans HA. Identification of host and donor cells in porcine homograft heart valve explants by fluorescence in situ hybridization. J Pathol 1997;183:99-104
21. Koolbergen DR, Hazekamp MG, Kurver M, et al. Tissue chimerism in human cryopreserved homograft valve explants demonstrated by in situ hybridization. Ann Thorac Surg 1998;66(6 Suppl.):S225-S232
22. Koolbergen DR, Hazekamp MG, de Heer E, et al. The pathology of fresh and cryopreserved homograft heart valves: An analysis of forty explanted homograft valves. J Thorac Cardiovasc Surg 2002;124:689-697
23. Walluscheck KP, Steinhoff G, Haverich A. Endothelial cell seeding of native vascular surfaces. Eur J Vasc Endovasc Surg 1996;11:290-303
24. Zeltinger J, Landeen LK, Alexande HG, Kidd ID, Sibanda B. Development and characterization of tissue-engineered aortic valve. Tissue Eng 2001;7:9-22
25. Hoerstrup SP, Sodian R, Sperling JS, Vacanti JP, Mayer JE, Jr. New pulsatile bioreactor for in vitro formation of tissue engineered heart valves. Tissue Eng 2001;6:75-79

Why does Developmental Potential Differ between Oocytes from Prepubertal and Adult Pig?

Mitsutoshi Yoshida, Nao Miyazaki, Jyunnko Matsuba and Tadayuki Yamanouchi

Laboratory of Animal Reproduction, Faculty of Agriculture,
Kagoshima University, Kagoshima 890-0065, Japan

ABSTRACT

Experiments were carried out to identify potential factors of differences in developmental ability between oocytes from prepubertal and adult pig. Follicular oocytes collected from prepubertal or adult pig at a local slaughterhouse were cultured *in vitro* and assessed for maturation, pronuclear formation and development using *in vitro* fertilization, electrical activation, somatic cell-nuclear transfer, and the microinjection of ooplasmic components. The rates of blastocyst formation of embryos generated from *in vitro* fertilization, electrical activation or somatic cell-nuclear transfer were two folds higher in oocytes resulting from adult pig than those in oocytes resulting from prepubertal gilts. However, the microinjection of ooplasmic components or RNA obtained from adult oocytes matured *in vitro* into prepubertal oocytes matured *in vitro* significantly increased the rates of blastocyst formation of parthenotes compared with the control ($P<0.01$). These results indicated that *in vitro* matured oocytes derived from adult pig had an abundant store of ooplasmic factor RNA effective for enhancing the ability of oocytes to develop blastocyst.

Key words: sexual maturity, developmental ability, *in vitro*, RNA, pig oocytes

INTRODUCTION

Successful methods for *in vitro* maturation of oocytes in farm animals could provide a sufficient supply of oocytes and embryos for their possible application in basic research and biotechnology.

The viability to term of pig embryos resulting from *in vitro* fertilization of oocytes matured *in vitro* has been reported by several investigators (1-3). However the developmental ability of oocytes from prepubertal pig is decreased compared to those from their adult counterparts (3-12), and the precise reason for this largely remain unknown. The overcome of the developmental deficiency of oocytes derived from prepubertal animals could be a step forward to the use of ovaries of prepubertal gilts routinely collected in large quantities at a local slaughterhouse.

This paper summaries our recent experiments to identify potential factors of differences in developmental ability between oocytes from prepubertal and adult pig.

Effect of the sexual maturity of the oocyte donor on *in vitro* maturation and fertilization of pig oocytes

The first experiment was carried out to examine the effects of sexual maturity of oocyte donor animals on *in vitro* maturation of pig oocytes and on their subsequent fertilizing and developmental ability *in vitro*. Follicular oocytes collected from prepubertal or adult pig at a local slaughterhouse were cultured, fertilized, developed *in vitro* and assessed nuclear maturation, fertilization and development. The use of oocytes resulting from adult pig significantly increased the rates of blastocyst formation of oocytes after *in vitro* maturation and fertilization: the rate of blastocyst formation was two folds higher in adult oocytes than those in prepubertal oocytes ($P<0.01$). However, there were no differences in the rates of nuclear maturation, fertilization and cleavage between prepubertal and adult oocytes. The results of this experiment indicated that sexual maturity of donor animals affected on the rate of *in vitro* production of blastocyst. This is consistent with the reports that zygotes generated *in vivo* or *in vitro* resulting from prepubertal gilts did not possess the same capacity for *in vitro* development as do zygotes resulting from adult pig (3-8).

Effect of the sexual maturity of the oocyte donor on parthenogenic activation and development of pig oocytes

The second experiment was carried out to examine the effects of sexual maturity of oocyte donor animals on parthenogenic activation of pig oocytes and on their subsequent developmental ability *in vitro*. Follicular oocytes collected from prepubertal or adult pig at a local slaughterhouse were matured, electrically activated (DC pulse of 100V/mm for 50μsec), developed *in vitro* and assessed pronuclear formation and development. The use of oocytes resulting from adult pig significantly increased the rate of blastocyst formation of parthenotes after activation ($P<0.01$): the rate of blastocyst formation was two folds higher in adult oocytes than those in prepubertal oocytes. However, there were no differences in the rates of pronuclear formation and cleavage between prepubertal and adult oocytes. The result indicated that sexual matuarity of donor animals affected on the rate of in vitro production of parthenogenic blastocyst and was consistent with the recent reports (11, 12).

Effect of the sexual maturity of the oocyte donor on *in vitro* development of somatic cell nuclear transfer embryos

The third experiment was carried out to examine the effect of sexual maturity of oocyte donor animals on the development of nuclear transfer embryos obtained using enucleated oocytes recovered from prepubertal or adult pigs. Follicular oocytes collected from prepubertal or adult pig at a local slaughterhouse were matured, enucleated, electrically fused with fibroblasts of a Crown miniature pig (using a needle-type electrode), developed *in vitro* and assessed the fusion and development. The use of enucleated oocytes resulting from adult pig significantly increased the rate of blastocyst formation of reconstructed embryos after nuclear transfer ($P<0.01$): the rate of blastocyst formation was two folds higher in adult oocytes than those in prepubertal oocytes. However, there were no differences in the fusion rate and the cleavage rate between prepubertal and adult oocytes. The results indicated that sexual maturity of donor animals affect on the rate of *in vitro* production of nuclear transfer embryos and the cytoplasm of adult oocytes had a beneficial effect for enhancing the ability of reconstructed embryos to develop blastocyst compared with the counterparts. This is consistent with the findings that the use of adult oocytes as a cytoplast source improves the efficiency of *in vitro* production of nuclear transfer embryos (9-12).

Effect of microinjection of cytoplasmic components of adult oocytes on parthenogenic development of prepubertal oocytes

The fourth experiment was carried out to examine the effect of microinjection of cytoplasmic components of adult oocytes on parthenogenic development of prepubertal oocytes. Follicular oocytes collected from prepubertal or adult pig at a local slaughterhouse were matured *in vitro*. Cytoplasmic components were isolated from pools of zona-free oocytes at metaphase II stage and suspended in saline (10 oocytes/6μl). Matured oocytes resulting from prepubertal donor were microinjected with cytoplasmic components (5pl/oocytes) either from adult or prepubertal oocytes, electrically activated, and assessed *in vitro* development. The microinjection of cytoplasmic components of adult oocytes significantly increased the rate of blastocyst formation of parthenotes ($P<0.01$). However, there was no difference in the cleavage rate between prepubertal and adult oocytes. The result indicated that the cytoplasm of adult oocytes had an abundant store of components effective for enhancing the ability of oocytes to develop blastocyst compared with the counterparts.

Effect of microinjection of RNA of adult oocytes on parthenogenic development of prepubertal oocytes

The fifth experiment was carried out to examine the effect of microinjection of cytoplasmic RNA of adult oocytes on parthenogenic development of prepubertal oocytes. Follicular oocytes collected from prepubertal or adult pig at a local slaughterhouse were matured *in vitro*. Total RNA was isolated from pools of zona-free oocytes at metaphase II stage using RNeasy Total RNA Kit and then eluted in the DEPC-treated MiliQ water. Matured oocytes resulting from prepubertal donor were microinjected with cytoplasmic RNA (5pl/oocytes) from adult oocytes, electrically activated, and assessed *in vitro* development. The microinjection of cytoplasmic RNA of adult oocytes significantly increased the rate of blastocyst formation of parthenotes compared with the control ($P<0.01$), whereas there was no difference in the cleavage rate. The result indicated that the cytoplasm of adult oocytes had an abundant store of components RNA effective for enhancing the ability of oocytes to develop blastocyst.

CONCLUSION

The results of our recent experiments showed that the factor(s) which promoted embryonic developmental ability existed in RNA of oocytes, as well as in the cytoplasmic components of oocytes derived from adult pig.

The cytoplasm of oocytes and early embryos is literally the "stuff of life" and ooplasm harbors the factors responsible for the events of fertilization/activation, maternal and paternal genome remodeling, epigenetic programming, embryonic genome activation and early development (13). It was clear that the difference of the developmental ability of pig oocytes depending on sexual maturity of donor originated from the difference in cytoplasmic maturation but not nuclear maturation. However, the results of this study provide the way to overcome of the developmental deficiency of oocytes derived from prepubertal animals by the application of the cytoplasmic components or RNA of adult oocytes.

Further investigations are in progress to identify the substance(s) in pig ooplasm and to clarify the its kinetic mechanism during maturation and development.

REFERENCES

[1] Mattioli M, Bacci ML, Galeuti G and Seren E. Developmental competence of pig oocytes matured and fertilized *in vitro*.Theriogenology 1989;31:1201-7.

[2] Yoshida M, Mizoguchi Y, Ishigaki K, Kojima T, Nagai T. Birth of piglets derived from in vitro fertilization of pig oocytes matured in vitro. Theriogenology 1993;39:1303-1311.

[3] Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 2001;56:17-29.

[4] Pinkert CA, Kooyman DL, Baumgartner A, Keisler DH. In-vitro development of zygotes from superovulated prepubertal and mature gilts. J Reprod Fertil 1989;87:63-66.

[5] Hajdu MA, Knight JW, Canseco RS, Krisher RL, Velander WH, Pearson RE, Gwazdauskas FC. Effect of culture conditions, donor age, and injection site on in vitro development of DNA microinjected porcine zygotes. J Anim Sci 1994;72:1299-1305.

[6] Grupen CG, McIlfatrick SM, Ashman RJ, Boquest AC, Armstrong DT, Nottle MB. Relationship between donor animal age, follicular fluid steroid content and oocyte developmental competence in the pig. Reprod Fertil Dev 2003;15:81-87

[7] Peters JK, Milliken G, Davis DL. Development of porcine embryos in vitro: effects of culture medium and donor age. J Anim Sci 2001;79:1578-1583.

[8] Sherrer ES, Rathbun TJ, Davis DL. Fertilization and blastocyst development in oocytes obtained from prepubertal and adult pigs. J Anim Sci 2004;82:102-8.

[9] Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M. Production of cloned pigs from in vitro systems. Nat Biotechnol 2000;18:1055-9.

[10] Kuhholzer B, Hawley RJ, Lai L, Kolber-Simonds D, Prather RS. Clonal lines of transgenic fibroblast cells derived from the same fetus result in different development when used for nuclear transfer in pigs. Biol Reprod 2001;64:1695-8.

[11] Hyun SH, Lee GS, Kim DY, Kim HS, Lee SH, Kim S, Lee ES, Lim JM, Kang SK, Lee BC, Hwang WS. Effect of maturation media and oocytes derived from sows or gilts on the development of cloned pig embryos. Theriogenology 2003;59:1641-9.

[12] Ikeda K, Takahashi Y. Comparison of maturational and developmental parameters of oocytes recovered from prepubertal and adult pigs. Reprod Fertil Dev 2003;15:215-21.

[13] Malter HE. Cytoplasmic transfer in Mammalian eggs and embryos. Methods Mol Biol 2004;254:313-24.

For Journal of Reproduction and Development

Optimization of Calcium Concentrations in Fusion and Activation Media for Production of Cloned Embryos from Miniature Pig Somatic Cells

Kazuchika MIYOSHI[1)], Yasuto YUKI[1)] and Mitsutoshi YOSHIDA[1)]

[1)]*Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*

Running head: CALCIUM IN FUSION AND ACTIVATION MEDIA

Correspondence: K. Miyoshi (e-mail: kmiyoshi@agri.kagoshima-u.ac.jp)

Mailing address: Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan.

Abstract

The optimal concentration of calcium for activation of porcine oocytes and the effect of the presence or absence of calcium in a fusion medium on the *in vitro* development of cloned embryos derived from miniature pig somatic cells after activation with the optimal calcium concentration were examined. Treatment with cytochalasin B after applying electric pulses in the presence of 0.1 mM calcium decreased the second polar body extrusion rate of activated oocytes. When oocytes were treated with cytochalasin B, more oocytes developed into blastocysts after applying electric pulses in the presence of calcium at 0.1 mM compared with 0-0.05 or 1.0 mM. Fusing in the absence of calcium decreased the blastocyst formation rate of cloned embryos without activation but improved the development into blastocysts of the embryos when they were activated after fusion. These results show that the optimal concentration of calcium in an activation medium for the development of porcine parthenogenetic oocytes is 0.1 mM when they are treated with cytochalasin B after activation. Removing calcium from a fusion medium improves the development of cloned embryos derived from miniature pig somatic cells when they are activated in the presence of 0.1 mM calcium after fusion.

Keywords: Calcium, Miniature pig, Oocyte activation, Nuclear transfer, Embryo development

Abstract in Japanese

ミニブタ体細胞クローン胚の作出に用いる融合および活性化用培地中におけるカルシウム濃度の最適化

三好和睦・遊木靖人・吉田光敏

鹿児島大学農学部家畜繁殖学研究室

本研究では、ブタ卵子の電気的活性化に用いる培地中のカルシウム濃度を最適化した。さらに、最適化されたカルシウム濃度をミニブタ体細胞クローン胚の活性化に応用して、融合用培地中におけるカルシウムの有無がクローン胚の体外発生に及ぼす影響について調べた。ブタ卵子を 0.1 mM のカルシウム存在下で活性化した場合には、電気パルス印加後のサイトカラシン B 処理により活性化卵子の第 2 極体放出率が低下した。電気パルス印加後の卵子をサイトカラシン B で処理した場合には、0.1 mM のカルシウム存在下で活性化した区において 0-0.05 あるいは 1.0 mM の区と比較して高い胚盤胞形成率が得られた。融合用培地からカルシウムを除去することにより、融合後に活性化処理をしなかったクローン胚の胚盤胞形成率は低下した。しかし、活性化処理をした場合には、カルシウム不在下で融合することによりクローン胚の胚盤胞形成率が改善された。これらの結果から、電気パルス印加後にサイトカラシンBで処理する場合には、ブタ卵子の活性化に最適なカルシウム濃度は 0.1 mM であることが示された。また、融合用培地からのカルシウムの除去は、0.1 mM のカルシウム存在下で活性化したミニブタ体細胞クローン胚の発生を改善することが明らかとなった。

Introduction

Some research groups have succeeded in cloning piglets from differentiated cells [1-12]. However, the efficiency of cloning, when measured as development to offspring as a proportion of embryos transferred into recipient females, was less than 1% in most of the previous reports and widespread use of this technology in a number of animal agriculture and biomedical applications has been limited. This suggests that the cloning protocols containing fusion of donor cells and recipient oocytes and activation of the fused embryos are not yet optimized.

Fusion and/or activation in pig somatic cell nuclear transfer studies has been mainly induced by electric stimulation [1-12]. Oocyte activation induced by electric pulses is initiated by an elevation of intracellular calcium and the intracellular calcium transient increase is triggered by an influx of extracellular calcium immediately after electric stimulation [13]. Recently, it was reported that increasing the calcium concentration from 0.1 mM to 1.0 mM in an activation medium can enhance the blastocyst formation rates of porcine parthenogenetic oocytes and fibroblast nuclear transfer embryos [14]. However, the optimal concentration of calcium for activation of porcine oocytes and cloned embryos is unclear.

It is considered that the level of maturation/meiosis/mitosis promoting factor (MPF) in recipient oocytes is an important factor for the development of cloned embryos and cloning efficiency is enhanced when nuclei of differentiated cells are exposed to enucleated oocytes with high levels of MPF for a few hours before activation [15,16]. This can be achieved by using metaphase II-arrested oocytes with high MPF activity as recipients and fusing donor cells without concurrent activation of recipient oocytes. The concurrent activation could be avoided by fusing donor cells and recipient oocytes under calcium-free conditions [7] and the comparatively high efficiency of pig cloning

(5.5%) was obtained by this method [12]. However, the developmental potential of pig cloned embryos produced by this method has not been directly compared with that of embryos fused in the presence of calcium.

On the basis of the results from previous reports described above, it is suggested that concentrations of calcium in fusion and activation media affect the developmental potential of cloned embryos. The present study determined the optimal concentration of calcium for activation of pig oocytes. An additional objective was to examine the effect of the presence or absence of calcium in a fusion medium on the *in vitro* developmental competence of cloned embryos derived from miniature pig somatic cells after activation with the optimal calcium concentration.

Materials and Methods

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline at 36-38 C. Oocytes were aspirated from antral follicles (2-5 mm in diameter) with an 18-gauge needle fixed to a 5-ml syringe. The follicular contents were pooled in test tubes and maintained at 38.5 C. Oocytes that had an evenly granulated cytoplasm and were surrounded by at least three uniform layers of compact cumulus cells were recovered from the collected fluid. About 50 oocytes were transferred to 200 μl of TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 3.05 mM D-glucose, 0.91 mM Na pyruvate, 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor (Sigma Chemical Co., St. Louis, MO), 10 IU/ml eCG (Teikoku-Zoki Co., Tokyo, Japan), 10 IU/ml hCG (Teikoku-Zoki), 0.1 mg/ml amikacin sulfate (Meiji Seika Co., Tokyo, Japan), and 0.1% (w:v) polyvinylalcohol [17] that had been previously covered with paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) and equilibrated in an atmosphere of 5% CO_2 in air at 38.5 C. After culture of oocytes for 38-42 h under 5% CO_2 in air at 38.5 C, cumulus cells were removed by vortexing with 0.1% (w:v) hyaluronidase (Sigma). Oocytes with a polar body, indicating successful nuclear maturation, were selected for the present study.

Activation and culture of oocytes

Denuded oocytes were washed once in a medium composed of 250.3 mM sorbitol, 0.5 mM $Mg(CH_3COO)_2$, 0.3 mM HEPES, and 0.2% (w:v) BSA (sorbitol medium) with $Ca(CH_3COO)_2$ at various concentrations and then placed between two wire electrodes (1 mm apart) of the chamber slide with 15 ml of the sorbitol medium. Direct-current pulses of 100 V/mm were applied twice to the oocytes for a duration of 50 μsec at

intervals of 30 min. The oocytes were cultured in 100 μl of porcine zygote medium-3 [18] with a modification in which 0.05 mg/ml gentamicin was replaced with 0.1 mg/ml amikacin sulfate (mPZM-3) under 5% CO_2, 5% O_2, and 90% N_2 at 38.5 C. Some oocytes were incubated in 100 μl of mPZM-3 supplemented with 2.2 μg/ml cytochalasin B for 2 h after activation and then transferred into the same medium without cytochalasin B. The oocytes were examined for cleavage and blastocyst formation at 2 and 7 days of culture, respectively. At the end of the culture period, all blastocysts were counted for nuclei after Hoechst staining. The blastocysts were placed on slides with a drop of mounting medium consisting of glycerol and PBS (9:1) containing 0.1 mg/ml Hoechst 33342 (Sigma). A cover slip was placed on top of the blastocysts and the edge was sealed with nail polish. The numbers of nuclei were counted under ultraviolet light.

Experiment 1

The activation status in oocytes applied the electric pulses in the sorbitol medium with 0.1 or 1.0 mM $Ca(CH_3COO)_2$ with or without cytochalasin B treatment was examined. At 6 h after activation, the oocytes were mounted, fixed for 48-72 h in 25% (v:v) acetic acid in ethanol at room temperature, stained with 1% (w:v) orcein in 45% (v:v) acetic acid, and examined for polar body extrusion and pronuclear formation under a Nomarski differential interference microscope (Olympus Co., Tokyo, Japan). The oocytes with a pronucleus were regarded as activated oocytes.

Experiment 2

The developmental potential of oocytes activated under the same conditions as those in experiment 1 was examined.

Experiment 3

The developmental potential of oocytes applied the electric pulses in the sorbitol medium supplemented with 0, 0.01, 0.05, 0.1, 0.5, or 1.0 mM $Ca(CH_3COO)_2$ followed by treatment with cytochalasin B was examined.

Experiment 4

The effect of the presence or absence of calcium in a fusion medium on the developmental competence of somatic cell nuclear transfer embryos with or without activation using the optimal calcium concentration decided in the previous experiments.

A kidney was obtained from a 2-yr-old female Clawn miniature pig (Japan Farm Co., Kagoshima, Japan) and transported to the laboratory in PBS. After washing with PBS, the renal cortex tissue was chopped into small pieces. The tissue was enzymatically digested with 0.2% (w:v) trypsin (Sigma) in PBS for 12-15 h at 4 C with stirring. The digested tissue was allowed to settle for 5 min and the supernatant containing disaggregated cells was mixed with the same volume of HEPES-buffered TCM-199 supplemented with 10% (v:v) FCS and centrifuged at 500g for 5 min. The cell pellet was resuspended and cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS under 5% CO_2 in air at 37 C. After reaching confluence, cells were passaged. The cells were used as donors for nuclear transfer between passages 5 and 10 of culture. The cells were allowed to grow to confluency and continued to culture for additional 5-6 days. A single cell suspension was prepared by standard trypsinization immediately prior to nuclear transfer.

In vitro-matured and denuded oocytes were cultured in 100 μl of mPZM-3 supplemented with 0.5 μg/ml demecolcine (Sigma) and 0.05 M sucrose for 1 h. The

oocytes with a protruding membrane were transferred into Hepes-buffered TCM-199 with the osmolarity adjusted to 300 mOsm by adding sucrose supplemented with 0.5 μg/ml demecolcine, 7.5 μg/ml cytochalasin B, and 10% FCS and then the protrusion was removed by aspiration with a 15-μm inner diameter glass pipette [19]. A single donor cell was inserted into the perivitelline space of each enucleated oocyte using the same glass pipette. Cell-oocyte complexes were transferred to mPZM-3 and kept in a CO_2 incubator adjusted to 5% CO_2 in air at 38.5 C until fusion.

The chamber for fusion was a 60-mm dish filled with 7 ml of the sorbitol medium with or without 0.1 mM $Ca(CH_3COO)_2$. Two stainless-steel wires (100-μm diameter) were used as electrodes and they were attached to micromanipulators. The single cell-oocyte complex was sandwiched between the electrodes and oriented with the contact surface between the cytoplast and the donor cell perpendicular to the electrodes. Membrane fusion was induced by applying a single direct-current pulse of 25 V for a duration of 20 μsec with a prepulse of alternating-current field of 5 V, 1 MHz for 2 sec using an LF 101 Fusion Machine (Nepa Gene Co., Chiba, Japan). Following the fusion pulse, the complexes were cultured for a period of 2 h in 100 μl of mPZM-3 supplemented with 2.2 μg/ml cytochalasin B. Fusion was determined by microscopic examination at 1 h after applying the pulse. At 2 h after culture, some fused embryos were activated using the same methods as those for oocytes in the sorbitol medium supplemented with 0.1 mM $Ca(CH_3COO)_2$ and treated with cytochalasin B for 2 h. The culture and evaluation methods of embryos were also the same as those described above.

Statistical analysis

All percentage data were subjected to an arcsin transformation in each replicate.

The transformed values and the numbers of cells in blastocysts were analyzed using one-way (experiment 3) or two-way (experiments 1, 2, and 4) ANOVA followed by Fisher's protected least significant difference test. A probability of $P<0.05$ was considered statistically significant.

Results

Experiment 1

The percentages of activated oocytes (85.5-92.9%) were not affected by the concentrations of calcium in the activation medium and the presence or absence of treatment with cytochalasin B (Table 1). However, the second polar body extrusion rate (13.7%) of activated oocytes after applying electric pulses in the presence of 0.1 mM calcium and treatment with cytochalasin B was significantly ($P<0.05$) lower than those (51.9-65.3%) of activated oocytes that were not treated with cytochalasin B regardless of calcium concentrations in the activation medium.

Experiment 2

There were no significant differences in the percentages of cleaved oocytes (63.5-85.3%) among different treatment groups (Table 2). However, the blastocyst formation rate (38.8%) of oocytes activated in the presence of 0.1 mM calcium and treated with cytochalasin B was significantly ($P<0.05$) higher than those (14.3-16.7%) of oocytes in other treatment groups. No significant differences in the mean numbers of cells (52.2-63.8 cells) in the blastocysts were observed among different treatment groups.

Experiment 3

The percentage of cleaved oocytes (76.8%) at 0.1 mM was significantly ($P<0.05$) higher than that (52.0%) at 0 mM, although the value was not different from those (56.0-69.6%) at 0.01-0.05 and 0.5-1.0 mM (Table 3). More oocytes (28.6%) developed to the blastocyst stage at 0.1 mM ($P<0.05$), although the value was not different from that (23.0%) at 0.5 mM. There were no significant differences in the mean numbers of