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# *In vitro* production of canine blastocysts

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ABSTRACT

Though blastocyst production *in vitro* has been successful in several animal species, a culture system to produce viable and normal canine blastocysts *in vitro* remains to be established. In this study, we report the development of an *in vitro* culture system for canine preimplantation embryos produced via parthenogenetic activation (PA) and somatic cell nucleus transfer (SCNT). Our results show that the medium developed by us, named "Qingdao Agricultural University's (QAU)-4 medium", successfully breaks the developmental arrest observed at the eight-cell stage in canine embryos grown in other culture systems. The blastocysts produced in QAU-4 displayed normal blastocyst structures, including a clear inner cell mass and blastocyst cavity. We also found that blastocyst formation in PA embryos cultured in QAU-4 medium was quite high, though this was not so in the case of SCNT embryos. However, supplementation of QAU-4 medium with 100 nM of scriptaid caused a sharp increase in blastocyst formation in SCNT embryos. After culture, hatched blastocysts were also observed to successfully adhere to collagen-coated dishes, where further growth and differentiation occurred. To our knowledge, this is the first *in vitro* canine preimplantation embryo culture system that can successfully produce canine blastocysts.



## 1. Introduction

Dogs are one of the most important animals domesticated by man, and currently serve as companions, workers in farms and protective service sectors, as well as test animals in medical research. Although research in the field of advanced reproductive technologies (ARTs) and embryonic stem cells has seen rapid progress in mouse, porcine, bovine, and ovine animal systems, ARTs in canine systems have been lagging.

IVF and somatic cell nucleus transfer (SCNT) techniques in canines have been established only recently [1,2]. However, researchers have not been able to evaluate the quality of embryos produced via IVF and SCNT as effective embryo culture systems for canines have not yet been established. The lack of a canine-specific IVC system has severely limited research on canine embryo development and embryonic stem cells and suggests that blastocysts must be isolated from oviducts ~8–12 days after mating [3–6]. Further experiments can be carried out on these blastocysts; however, due to the unique oocyte maturation and fertilization events in dogs, the age of the blastocysts obtained by oviduct flushing is hard to discern.

The primary stumbling block in the establishment of an effective canine IVC system is the lack of an optimized culture medium for canine embryos. Although several media, such as modified synthetic oviduct fluid (mSOF) [7], porcine zygote medium (PZM)-5, and G1/G2 [8], have been utilized as embryo culture media in attempts to set up a canine IVC system, none have been effective. However, Kim et al., report that the G1/G2 medium can support the growth of canine SCNT embryos *in vitro* till the blastocyst stage; conversely, no cavities were observed in the 'blastocysts' obtained in this study, indicating flawed developmental morphologies [8].

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In this study, we report the development of a medium named "Qingdao Agricultural University's (QAU)-4 medium", for the *in vitro* culture of canine embryos produced by parthenogenetic activation (PA) and SCNT till the blastocyst stage. Blastocysts obtained by embryonic culture in QAU-4 medium displayed normal morphologies (blastocyst cavity and an obvious inner cell mass (ICM)). Furthermore, these blastocysts also successfully hatched and grew on collagen-coated dishes. The medium we have developed can serve as an essential platform for ART and embryonic stem cell research in canines.

## 2. Material and methods

### 2.1. Reagents

Unless specified, all reagents were obtained from Sigma-Aldrich. QAU-4 medium was produced and ordered from Osight biological technology co., LTD (Osight@yeah.net; Qingdao, China). Component of QAU-4 medium were shown in Table 1.

### 2.2. Animals

All experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and specifically approved by the institutional Animal Care and Use committee of Qingdao Agricultural University, China. Female dogs (Beagles, n = 11) between 2 and 6 years of age bred in the laboratory animal unit were used in this study. All dogs were housed in temperature-controlled rooms, fed regular diets, and routinely attended by a veterinarian in Bolong experimental animal co., LTD (Qingdao, China).

### 2.3. Isolation of in vivo matured oocytes from canine oviducts

Matured oocytes were collected according to a previously described procedure [9]. In brief, after serum progesterone levels ranging from 16 ng/mL to 20 ng/mL (which occur during the ovulation period) were detected in female dogs, a surgical procedure was used to obtain oocytes 2 days after ovulation. This was done by flushing the oviduct with flushing medium, to obtain cumulus cell-oocyte complexes once the ovary bursa split open. The cumulus cells surrounding the oocytes were removed by a fine-flamed glass needle. Only oocytes with the first polar body and perivitelline space having diameters <25 μm were used for further experiments.

**Table 1**
Components of QAU-4 medium.

| Component | Concentration (mM) |
|---|---|
| NaCl | 81–106 |
| KCl | 0.1–7.2 |
| $NaHCO_3$ | 25 |
| $KH_2PO_4$ | 1.2 |
| Na Lactate | 6.6 |
| $CaCl_2$ | 1.75 |
| $MgSO_4$ | 0.5–1.0 |
| Na Pyruvate | 0.3 |
| Glucose | 0.5–1.5 |
| Glutamine | 1–2 |
| Taurine | 3.5 |
| NEAA | 2% |
| EAA | 1% |
| EDTA | 0.01 |
| BSA | 4 mg/ml |
| MEM Vitamin | 0.1–0.5% |
| pH | 7.2–7.4 |

### 2.4. Somatic cell nucleus transfer (SCNT) and parthenogenetic activation (PA)

Ear skin tissues surgically collected from a 9-year-old dog and maintained at room temperature (20–25 °C) were transported to the laboratory within 2 h of collection. After washing, the tissue was minced, and the cells obtained were cultured in DMEM supplemented with 10% fetal bovine serum at 37.5 °C in a humidified atmosphere containing 5% $CO_2$. The cultured cells were then collected and stored in liquid nitrogen.

For nuclear transfer, canine oocytes were enucleated in the medium supplemented with 5 μg/mL cytochalasin B. One donor somatic cell was injected into the perivitelline space of each enucleated oocyte and fused using two pulses of DC at 3 kV/cm for 15 μs The fused couplets were activated by calcium ionophore treatment for 5 min followed by treatment with 1.9 mM of 6-(Dimethylamino) purine (6-DMAP) in mSOF medium for 4 h.

For PA, matured oocytes were activated using two DC pulses at 1.5 kV/cm for 25 μs in 270 mM mannitol solution supplemented with 0.1 mM $Ca^{2+}$ and 0.15 mM $Mg^{2+}$. The second polar body extrusion was inhibited by treatment with 6-DMAP in mSOF medium for 4 h.

### 2.5. Embryo culture

After treatment, embryos were cultured either in mSOF or QAU-4 medium for 8 days at 38 °C in a humidified atmosphere containing 5% $CO_2$, 5% $O_2$, and 90% $N_2$. To detect the effect of scriptaid (SCR) on canine SCNT embryo culture, only QAU-4 medium was employed.

The addition of SCR, which is a histone deacetylation inhibitor, has been found to significantly improve blastocyst formation of SCNT embryos in many species [10–14]. To detect the effect of SCR on canine SCNT embryo development, the embryos were cultured in QAU-4 medium with/without 100 nM SCR for 24 h after 6-DMAP treatment, and then cultured in QAU-4 medium for another 7 days. In all experimental setups, the medium was changed on days two, four, and six after activation; the ratios of embryos that had developed to the two-cell, eight-cell, and blastocyst stages were observed at 24 h, 72 h, and Day 8, respectively. After culture, hatched blastocysts were cultured in collagen-coated dishes in the same media they were exposed to during somatic cell culture.

### 2.6. Measurement of total cell numbers in blastocysts

All blastocysts were washed three times in polyvinyl alcohol (PVA)-PBS and fixed in 4% formaldehyde for 40 min at room temperature. Following this, the blastocysts were washed again in PVA-PBS, before being mounted in mounting solution with 2 μg/mL DAPI. Cell count was obtained by counting the number of nuclei exhibiting blue fluorescence using a fluorescence microscope (Nikon, Tokyo, Japan).

### 2.7. Real-time quantitative PCR (RT-qPCR)

Messenger RNA (mRNA) was extracted from two blastocysts using the dynabeads mRNA DIRECT Kit (Ambion, Thermo, USA) followed by routine cDNA synthesis by reverse transcription of RNA using an oligo$(dT)_{12-18}$ primer in accordance with the manufacturer's protocol.

RT-qPCR with the three pairs of primers [15] listed in Table 2 was performed using a Applied Biosystems®(7500, Waltham, MA) real-time PCR machine. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, with the GAPDH mRNA as an internal control. Three independent experiments were performed with each sample in

**Table 2**
Primers and amplification conditions used for real-time PCR.

| Genes | Primer sequences | Size(bp) | Temperature |
|---|---|---|---|
| *OCT4* | F: GAGGCTCTGCAGCTCAGTTT<br>R: AGCCCAGAGTGGTGACAGAC | 502 | 60 °C |
| *SOX2* | F: AGTCTCCAAGCGACGAAAAA<br>R: CCACGTTTGCAACTGTCCTA | 189 | 55 °C |
| *GAPDH* | F: GGAGAAAGCTGCCAAATATG<br>R: ACCAGGAAATGAGCTTGACA | 194 | 57 °C |

triplicate in each case.

### 2.8. Statistical analysis

All statistical analyses were performed using the SPSS software (v19.0, IBM, USA). The percentages of embryos that developed to the blastocyst stage under each experimental setup were analyzed using the Chi-square test. Results with *P* values of less than 0.05 were considered significant. All experiments were repeated three times.

## 3. Results

### 3.1. Effect of medium on the development of canine embryos obtained via PA and SCNT

As shown in Fig. 1, the percentages of parthenogenetic canine embryos developing till the two-cell and eight-cell stages were similar in mSOF and QAU-4 media. However, the percentages of embryos developing up to the blastocyst stage in QAU-4 medium were significantly higher ($P < 0.01$) than those of embryos grown in mSOF medium ($58.89\% \pm 8.39$, $n = 7$ and $6.67\% \pm 11.55$, $n = 1$, respectively). This indicates that the QAU-4 medium can effectively support canine blastocyst formation *in vitro*.

Similar results were observed in SCNT-derived embryos at the two-cell stage, namely, that there was no difference in the percentages of embryos developing till the 2-cell stage between mSOF medium and QAU-4 medium. However, significantly more embryos grown in QAU-4 medium ($P < 0.05$) developed up to the eight-cell stage compared to those grown in mSOF medium ($78.33\% \pm 20.21$, $n = 9$ and $11.11\% \pm 19.24$, $n = 2$, respectively). Stable blastocyst formation was also observed to be significantly higher ($P < 0.01$) in QAU-4 medium than in mSOF medium ($26.11\% \pm 6.74$, $n = 3$ and 0, respectively), though the percentages of blastocysts formed were very low (Fig. 2).

### 3.2. Effect of scriptaid (SCR) on the development of canine embryos obtained through SCNT

Our results indicate that the percentages of blastocysts formed in SCR-supplemented QAU-4 medium were significantly higher than those formed in QAU-4 medium alone ($58.33\% \pm 14.43$ and $26.11\% \pm 6.74$, respectively, $P < 0.01$, $n = 24$). As shown in Fig. 2B, these blastocysts were well-formed with distinct ICMs and cavities. Average total cell number of blastocysts in control group was $83 \pm 30.51$ ($n = 3$) vs. $207.43 \pm 88.76$ ($n = 7$) of SCR treated group (Fig. 3); however, we were unable to carry out statistical analyses for this data due to low sample sizes.

### 3.3. Effect of SCR on the expression of canine SCNT blastocyst stem cell markers

To further test the quality of canine embryos produced by QAU-4, the expression levels of the reprogramming-related genes, *OCT-4* and *SOX-2* were measured via RT-qPCR in canine SCNT blastocysts. As expected, our results indicate that the relative expression levels of *OCT-4* were significantly higher in blastocysts grown in QAU-4 medium supplemented with SCR ($P < 0.05$) as compared to those grown in QAU-4 medium alone. However, the expression levels of *SOX-2* were not found to be affected with SCR treatment (Fig. 4).

### 3.4. Blastocyst outgrowth

To test the viability of the blastocysts obtained in the QAU-4 + SCR group, these blastocysts ($n = 2$) were further cultured on a collagen-coated dish with DMEM and 10% fetal bovine serum (FBS). The blastocysts were found to attach to the dish, following which ICM-like cells formed clones that further differentiated into fibroblast-like cells in morphology; finally, we also observed that trophectoderm (TE)-like cells were present, and reached confluence after 5 days of culture (Fig. 5).

## 4. Discussion

Dogs play important roles in human lives as companions, workers, and model organisms for medical research, especially since they are known to share similar emotional [16] and genetic

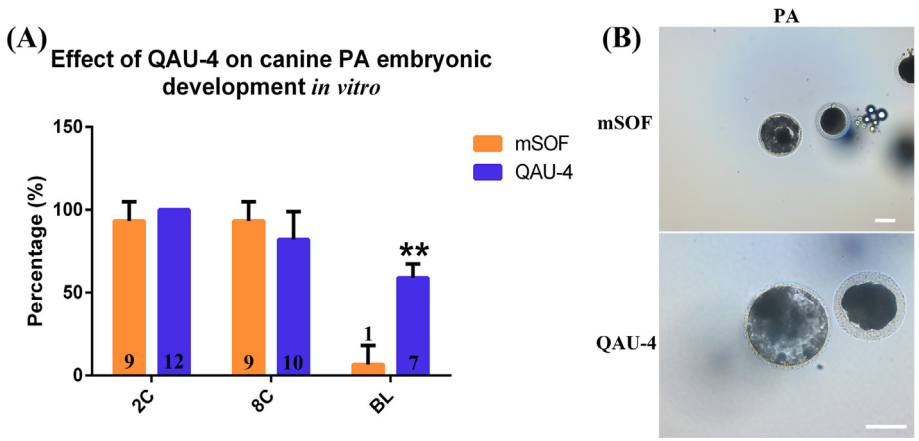


**Fig. 1.** Effect of QAU-4 on the *in vitro* development of canine embryos obtained via parthenogenetic activation (PA). (A) Percentages of canine PA embryos developing to the two-cell (2C), eight-cell (8C), and blastocyst (BL) stages. (B) Representative images of the embryos for each group are shown here. Values represent the mean ± SD of the mean of three independent experiments. $^{**}P < 0.01$. Scale bar = 100 μm.

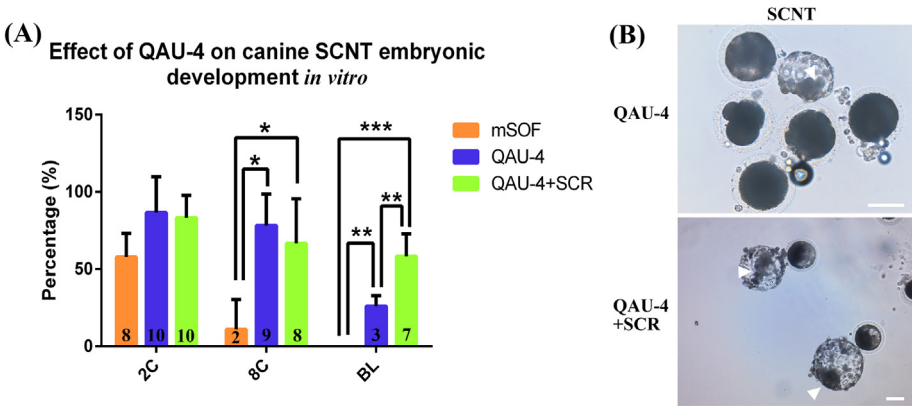


**Fig. 2.** *In vitro* development of canine SCNT embryos in different media. (A) Percentages of canine SCNT embryos developing to the two-cell (2C), eight-cell (8C), and blastocyst (BL) stages in mSOF, QAU-4, or QAU-4+SCR media. (B) Blastocysts cultured with and without SCR. Values represent the mean ± SD of the mean of three independent experiments. The arrows indicate the inner cell mass (ICM). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scale Bar = 100 μm.

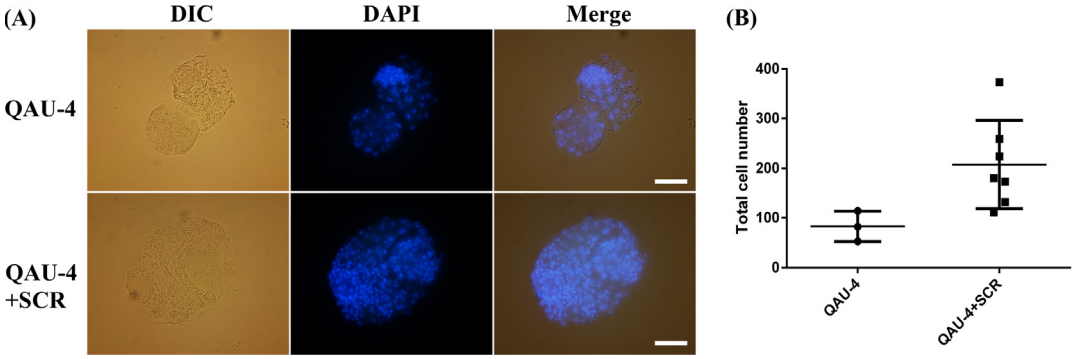


**Fig. 3.** Measurement of total cell numbers in canine blastocysts cultured *in vitro*. Canine SCNT embryos were cultured in the presence or absence of SCR for 24 h, following which, total cell numbers in each blastocyst was counted using DAPI staining. (A) DAPI staining (blue) of cell nuclei. (B) Total cell numbers in blastocysts. Scale Bar = 100 μm.

disease [17] characteristics with human beings. Given their importance to human beings, ARTs specifically designed for canines are likely to be useful tools for increasing the numbers of dogs exhibiting desirable sets of characteristics such as those that make good pets or elite service dogs, as well as in treating or correcting genetic diseases in these animals. Unfortunately, advancements in embryo development, embryonic stem cell research, and therefore, ARTs specific to canines have been limited due to the lack of a system by which pre-implantation embryos can be developed and grown.

In this study, we have developed a medium, QAU-4, which can support *in vitro* canine embryo development up to the blastocyst stage. Although relatively high blastocyst development rates were observed in PA embryos grown in this medium, it is possible that these rates could be due to the use of good quality oocytes (which were matured *in vivo*) in this study. We further evaluated the effectiveness of QAU-4 medium in sustaining growth and development of SCNT embryos. Compared to mSOF medium, we were able to achieve a blastocyst development rate of >20%, which was similar to that seen in other animal cloning systems that have used *in vitro*-matured oocytes as recipients for somatic nuclei in SCNT [18–20]; the reason for such low blastocyst development rates with mSOF medium could be likely due to either poor medium formulation of mSOF or insufficient reprogramming of somatic nuclei. However, since a higher rate of blastocyst development was achieved when the QAU-4 medium was supplemented with SCR, it is likely that the low rate of blastocyst development in SCNT embryos grown in just QAU-4 medium is caused by reprogramming problems. Because of the difficulty in oocyte collection from dogs, the effect of SCR on IVF and PA embryos could not be evaluated in the present study. However, based on the previous reports on other species, we speculated that SCR shows no effect on IVF or PA embryonic development of canine. However, this should be confirmed by conducting further experiments.

Almost all the blastocysts produced via embryonic culture in the QAU-4 medium displayed normal blastocyst structure, including ICM-like structures. Due to difficulties in oocytes collection, markers of epiblast, primitive endoderm (hypoblast) and trophectoderm didn't be detected in the present study. Further, *in vivo*

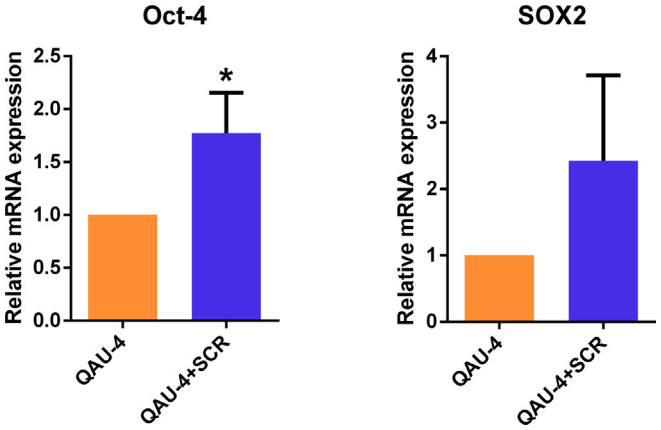


**Fig. 4.** Expression levels of the reprogramming genes *OCT4* and *SOX2* in canine SCNT blastocysts were determined by real-time quantitative PCR (RT-qPCR). Values represent the mean ± SD of the mean of three independent experiments. $^{*}P < 0.05$.

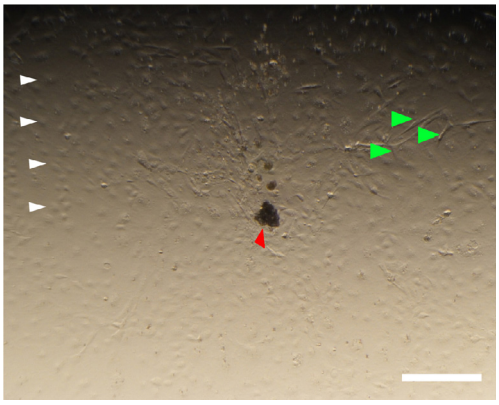

**Fig. 5.** Outgrowth of canine SCNT blastocysts. Canine SCNT blastocysts obtained after culture in QAU-4+SCR were cultured in collagen-coated dishes with DMEM and 10% fetal bovine serum (FBS). The presumptive inner cell mass (ICM red arrow) formed a colony, and some of these cells differentiated into fibroblast-like cells in morphology (Green arrow). Trophectoderm (TE) cells are indicated with white arrow. Scale bar = 100 μm.

development of the blastocysts should be check after establishment of blastocyst transfer system in canine.

We also found that after hatching, these blastocysts could adhere to and grow on collagen-coated dishes in cell culture medium, and that the ICM-like cells were able to form clone-like structures that gradually differentiated into shuttle-like cells even in the absence of growth factors. On closer examination, the TE-like cells and ICM-like cells obtained from these blastocysts displayed different morphologies, indicating that SCNT blastocysts have at least two kinds of cells; however, these observations need to be further tested and confirmed.

Composition of the QAU-4 medium is based on the mSOF medium. One of our previous studies has established that supplementing growth media with EDTA can help in overcoming the eight-cell-stage arrest and improve genomic activation in canine embryos cultured *in vitro* [21], EDTA supplementation itself is not sufficient for further development. We have introduced additional nutrients to the QAU-4 medium composition (Table 1). Since both media show almost equal capacity in supporting the growth of canine embryos till the eight-cell stage, we believe that the additional nutrients in the QAU-4 medium are necessary for canine embryo development after genomic activation, which happens at the eight-cell stage (See review [22]).

Since canine embryos are extremely sensitive to the build-up of breakdown -products of metabolism, we were careful to change the growth medium every 2 days. Water is another important factor for success. Generally, endotoxin level in water should less than 25EU in clinic injection but <5EU is suitable for canine embryo culture. A commercial water underwent human embryo culture tested should be used in medium. If lab-made de-ion water was employed, make sure the UV lamp of the de-ion water machine still work well. Furthermore, a low oxygen environment to reduce the incidence of ROS toxicity is essential.

Patent application for the QAU-4 media formulation has been submitted to the company. To our knowledge, this is the first report of a successful canine IVC system capable of producing normal blastocysts *in vitro*. In conclusion, we believe that the development of our new medium, QAU-4, which is capable of supporting *in vitro* canine preimplantation embryo culture, can facilitate research on canine embryonic stem cells and embryo growth.

## Conflicts of interest

The authors declare that there are no conflicts of interest that would prejudice the impartiality of this scientific work.

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