Royana: Successful Experience in Cloning the Sheep

Saeed Kazemi Ashtiani, Ph.D.1, 2, 3, Mohammad Hossein Nasr-Esfahani, Ph.D.2, 3, 4, *, Sayyed Mortaza Hosseini, D.V.M.2, Fariba Moulavi, B.Sc.2, Mahdi Hajian, M.Sc.2, Mohsen Frouzanfar, Ph.D.5, Parvaneh Abedi, B.Sc.2, Maryam Meamar, B.Sc.2, Mojtaba Rezazadeh Valojerdi, Ph.D.5, 6, Hamid Gourabi, Ph.D.1, 2, 3, 9, Abdolhossein Shahverdi, Ph.D.1, 2, 3, Hossein Baharvand, Ph.D.1, 6, Ahmad Vosough Dizaj, M.D.1, 2, 3, Hossein Imani, Ph.D.3, Poopak Eftekhar-Yazdi, Ph.D.3, Mahdi Vojgani Ph.D.3, Mahdi Safahani D.V.M.4, Ramin Radpour, M.Sc.9, Iman Salahshouri, M.Sc.9

1. Stem Cells Department, Cell Sciences Research Center, Royan Institute, ACECR, Tehran, Iran
2. Clinical and Experimental Embryology Department, Cell Sciences Research Centre, Royan Institute, ACECR, Isfahan, Iran
3. Department of Clinical and Experimental Embryology, Reproductive Medicine Research Centre, Royan Institute, ACECR, Tehran, Iran
4. Isfahan Fertility and Infertility Center, Isfahan, Iran
5. Faculty of Basic Science, Islamic Azad University, Marvdasht Branch, Iran
6. Anatomy Department, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
7. Clinical Sciences Department, Faculty of Veterinary, Tehran University, Tehran, Iran
8. Obstetric and Gynecology Unit, Foola Dairy Complex, Social Security Association, Isfahan, Iran
9. Genetic Department, Reproductive Medicine Research Centre, Royan Institute, ACECR, Tehran, Iran

* Corresponding Address: P. O. Box: 8185968443, Clinical and Experimental Embryology Department, Cell Sciences Research Centre, Royan Institute, ACECR, Isfahan, Iran

Email: mh.nasr-esfahani@royaninstitute.org

Abstract

Received: 22/Oct/2007, Accepted: 3/May/2008
Objective: This study describes our experiences in reproductive cloning using two different procedures resulting in birth of the first successfully cloned sheep in Iran and the Middle-East, nick-named "Royana".

Materials and Methods: Abattoir-derived sheep oocytes were enucleated after in vitro maturation for 18-20hrs and then reconstructed by ear-derived sheep somatic cells using two different procedures of renucleation (subzonal, intracytoplasmic), embryo culture (coculture, sequential medium) and embryo transfer (intra fallopian, intra uterine). Pregnancy status and fetal development were followed regularly and elective cesarean was inducted on day 145 of pregnancy. Histopathological and genetical examinations were performed on either aborted and delivered clones for confirmation different aspects of cloning.

Results: The two procedures were both efficient in producing early and/or advanced cloned embryos, establishing early and/or advanced stages of pregnancy till delivery. Four pregnancies were detected; one was failed at early pregnancy, one aborted on day 90, one was still born and the fourth delivered to a healthy male lamb nick named "Royana".

Conclusion: Many different approaches have been developed for mammalian cloning which all are judged by their ultimate potency for establishment of successful pregnancies terminated to healthy/viable clones. As a preliminary study toward establishment of the technology, this study also successfully examined the competency of two procedures of somatic cell nuclear transfer (SCNT). However, the overall low efficiency of SCNT indicates that many different aspects of the technology remain to be dissolved.

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Driesch and others separated the blastomeres at the two-cell stage (6). They produced identical, but dwarfed embryos. Eleven years later, the same experiment with similar results was performed by Hans Spemann in a vertebrate (salamander), using human baby hair, to separate the cells (4, 6). By considering the results of these experiments, Spemann (7) proposed the transfer of nuclei from more advanced developmental stages to zygotes from which the genetic material had been removed as a valuable method for producing cloned mammals. However the unavailability of an efficient handling system hampered the application of the mammalian procedure for almost 80 years (4, 5), Willadsen (8) could produce the first live lambs after transferring nuclei from 8 to 16-cell sheep embryos into enucleated MII oocytes. However, this work was rested by the ever fame work of Wilmut and his co-workers (9) in cloning «Dolly» which named appropriately «the second creation»(10). Since then, somatic cell nuclear transfer (SCNT) efforts has spanned to about 35 different kinds of mammals including live stocks, laboratory animals and even endangered mammals in over 160 laboratories across at least 37 nations by attracted scientists (11). Even though, the numbers of live birth clone are still limited to certain countries.

Application possibilities of cloning in research, industry and agriculture are theoretically almost limitless (4, 6). In fact, the enormous potential applications of somatic cell nuclear transfer can be divided into two general areas: biomedical and agricultural (12, 13). In addition to its practical applications, cloning has become an essential tool for understanding basic laws of biology including the gene expression, reprogramming, differentiation, X-inactivation, as well as many other topics (12, 14-17).

Considering the great potential of farm animal cloning, pharmaceutical protein production or cell/xenotransplantation (18, 19), there is an obvious need for the countries to make them skill forced in the field of nuclear transfer. In this regard, the paper describes successful experience of mammalian cloning for the first time in Iran and the Middle East.

**Materials and Methods**

**Chemicals**

All chemicals used in the present study were purchased from the Sigma Chemical Company (St. Louis, MO, USA) and media from Gibco (Life Technologies, Rockville, MD, USA) unless otherwise specified. All media solutions were adjusted at PH 7.4 and osmolarity of 270-280 mOsmol/kg H2O.

**Oocyte preparation and in vitro maturation**

Ovaries were obtained from a local abattoir and maintained in normal saline (0.9%) at 28-32°C during transport to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2-6mm in diameter via a hypodermic needle (1.2 mm internal diameter) attached to 2ml syringe containing 0.5ml aspiration medium (H-TCM 199 with Earle’s salts [Gibco, 22340]) supplemented with 10% fetal calf serum (FCS) and 50 IU/ml heparin and then transferred into sterile 10 ml conical tubes (TPP, Switzerland). The tubes were placed into warm bath (38°C) and the follicular material was allowed to settle for 10-15 min. The resulting pellet was transferred into a petri dish (Falcon, 1005) containing 3.4ml diluting/washing medium (H-TCM 199 with Earle’s salts [Gibco, 22340]) supplemented with 10% FCS and examined for COCs under a stereo microscope. COCs with at least 2-3 compact layers of cumulus cells were selected and washed three times in 200μl of washing droplets and two times in 200μl droplets of maturation medium. Washed COCs were then co-cultured in groups of 5 into 100μl of maturation medium over a previously established monolayer of vero cells (approximately 1×10⁶ cells/ml) (20). Maturation medium was comprised of TCM199 (Gibco, 31150) containing 2.5 mM Na pyruvate, 1mM l-glutamine, 100 IU penicillin- 100 μg/ml, 10% (v/v) FCS, 10 μg/ml FSH, 10 μg/ml LH and 1μg/ml estradiol-17β at 38.5°C, 5% CO₂, and maximum humidity (Labotect C200, Germany).

**Collection and culture of ear fibroblasts**

Skin biopsies were obtained from the ear of two 7-9 months old Afshari lambs (male and female) with high race purity. The ear tissues were cut into small pieces (2mm²) and then cultured as tissue explants in 25mm² culture flasks (TPP, Switzerland) containing DMEM (Gibco, 11965-092) plus 10% FCS (Gibco, 10270-106) and 1% penicillin/streptomycin (Gibco, 15140-122) at 38.5°C, 5% CO₂, and humidified air. The fibroblast cells had formed monolayers surrounding the tissue explants after 5-7 days in culture, the explants were removed and the fibroblast cells were cultured to become confluent. Cultured cells were routinely maintained in 30 mm culture dishes (Falcon, 3001) until passage 15 and then stored frozen. For each passage, confluent cells were disaggregated by incubation in a 0.25% trypsin-EDTA (Gibco, 25200-072) solution for 3 min. Two third of the suspension was allocated into two new dishes for further passageing and the remaining part was diluted in 10% dimethyl sulfoxide (Sigma D-5879), 50% FCS and 40% DMEM solution; followed by being aliquoted equally into three cryotubes (Nunc, Denmark), then stored overnight in 80°C before being plunged into liquid nitrogen.

**Preparation of donor cells**

Frozen-thawed adult ear fibroblast cells between passages 10-15 were used as donor cells, which were plated into a 30-mm culture dishes and cultured in DMEM supplemented with 10% fetal bovine serum (FCS) until nearly 70 to 80% confluency. These cells were then subjected to serum starvation (0.5% FCS in DMEM) for 4-6 days. Immediately prior to SCNT, donor cells
were collected after trypsinization and then resuspended in either Hepes-TCM199 (H-TCM199) or Hepes synthetic oviduct fluid (H-SOF1) containing 0.5% FCS (9) as indicated in the procedure I and II.

**Procedures**

During this study, in vitro production of sheep SCNT embryos and their transfer into the synchronized recipients were performed according to the two different procedures commonly used for mammalian SCNT but with some modifications. Procedure I comprised sub-zonal insertion of the donor cell for enucleation (9), culture of embryos in medium conditioned by vero cells (20) and transfer of the early stages embryos in to the fallopian tubes of the recipients (21). Procedure II, in contrast, comprised intracytoplasmic insertion of the donor cells for reconstruction (22), prolonged culture of the SCNT embryos in sequential medium till day 8 (23) and transfer of advanced embryos in to the uterine horn (9). Figure 1 represents the schematic pattern of experiments carried out during this study known as procedure I and II.

**Procedure I**

**Media**

The base media used during this procedure were TCM 199 and H-TCM 199 + 10% FCS named in this section as base medium: TCM and HTCM respectively. Embryo culture was carried out in TCM with vero cells co-culture.

**Enucleation**

At 20-22hrs after the onset of maturation (hpm), oocytes were stripped free of the surrounding cumulus cells by gentle pipetting with a narrow-bored micropipette in 300 IU/ml hyaluronidase (Sigma, H-3506) prepared in HTCM. Only oocytes with homogenous cytoplasm and first polar body extruded were selected and incubated in HTCM containing cytochalasin B (CB 7.5 μg/ml; Sigma, C-6762) for 15min before being enucleated in the same medium at 38.5°C. Enucleation was performed by aspirating a small amount of cytoplasm from directly beneath the first polar body using a beveled 25μm outside diameter glass pipette and an inverted microscope (Olympus equipped with Narishige micro manipulators). The removed cytoplasm was observed in 10 μg/ml Hoechst 33342 (Sigma) under a fluorescent microscope (x100) to determine successful enucleation. Oocytes retaining the karyoplast were discarded. Enucleated oocytes were then returned to the maturation medium at 38.5°C until 26-27hpm.

**Nuclear transfer, fusion and activation**

For nuclear transfer, successfully enucleated oocytes were removed from the maturation medium and were transferred back into HTCM micro drops with central droplets of serum-starved fibroblast cells in H-TCM199+0.5% FCS. Well-rounded small donor cells (karyoplast) were deposited adjacent to the oocytes through the same slit made during enucleation. For fusion, reconstructed oocytes were first washed in fusion medium (0.3M mannitol, 0.1mM MgSO₄), then transferred into a fusion chamber between two platinum wires filled with fusion medium. Cell fusion was induced approximately 27–28hpm by two direct current (DC) electric pulses (1.75 kV/cm for 80μs and 1 second inter delay; Cryologic®, Australia) perpendicular to the two fusion partners. An alignment current of 800KHZ was applied just before and after the DC pulses to assure better alignment and fusion of the couplet. Fusion or successful reconstruction was examined 1hr after the pulse delivery. Reconstructed oocytes were then washed twice in TCM and cultured under light paraffin oil in TCM microdrops for 1.5 to 2hrs before activation was inducted. The reconstructed embryos were activated by 5-min exposure to 5μM ionomycin (Sigma I-0634), washed thoroughly thereafter in TCM. Further activation was carried out using 6-DMAP (Sigma, D-2629: 2 mM in TCM) for 4hrs before being washed 3 times in TCM and co-cultured in vero-TCM for the next 1or 2 days. Fragmented embryos were discarded and good quality embryos (at stages of 4 to 8 cells) were transferred in micro liters of HTCM to the fallopian tube of synchronized, freshly ovulated ewes using thin, TDT-catheters (CCD-France). Based on availability of good quality embryos, 2, 3 to 8 embryos were transferred for each recipient.

**Procedure II**

During the second course of studies, completely different approaches were tested based on the following items: 1) improved embryo culture procedure (sequential SOF medium) which could consistently results in 40% blastocyst production in ovine fertilized oocytes, 2) injection of donor cell into the cytoplast i.e.: enucleated oocytes (22) and 3) lazar assisted hatching to overcome zona hardening (24).

**Media**

The base media used during this course of study was SOF medium in a sequential manner of SOF1&2 for days 1-3 and 3-8 of the in vitro embryo development respectively. SOF1 and HEPES supplemented SOF1 (H-SOF1) were also used during embryo reconstitution.

**Enucleation and nuclear transfer**

Oocyte preparation, enucleation was similar to procedure I except for media (SOF1 or H-SOF1) and time of enucleation (24-26hpm). Nuclear transfer was carried out immediately by direct insertion of somatic donor cell into the cytoplast through the same slit, made during enucleation. Care was taken to transfer the donor cell with the minimum amount of media. Apparently enucleated-reconstructed oocytes were then cultured for at least 3hrs before fusion in numbered microdrops. Meanwhile, removed karyoplasts were observed in...
carded. On day 3rd, good quality embryos were selected were scored daily and fragmented embryos were dis-
muted by 5-min exposure to 5 μM ionomycin, washed thoroughly thereafter in SOF1. Further activation was
carried out using 6-dimethyl aminopurine (6-DMAP: 2 mM in SOF1) for 4 hrs before being cultured in SOF1
for the first 3 days of embryo development. Embryos were scored daily and fragmented embryos were dis-
carded. On day 3rd, good quality embryos were selected and a slit was made in zona by assisted laser hatching
(ZILOS, Hamilton-Thorne Research, Beverly, MA). These embryos were further cultured in SOF2 for the
next 3 to 4 days before embryo transfer. At this time, 1, 2 or 3 good quality blastocysts (expanding or hatched/
hatched blastocysts) were transferred in micro liters of SOF2 to uterine horn of synchronized ewes using thin,
TDT-catheters (CCD-France).

Recipient preparation, embryo transfer and monitoring
Thirty two adult Afshari sheep were kept in a field in June (December-
March; 15 ewes for procedure I) or non-breed season (March-June; 17 ewes for procedure II), were selected
based on good body condition scoring and previous fec-
cundy. The animals were maintained at the free stall
experimental farm of the Royan Institute in Isfahan,
Iran. The animals were fed at an alfalfa based (at li-
birum) supplemented with concentrate (twice in 24hrs)
and natural light day program. Estrus was synchronized
with the insertion of an intra-vaginal progestagen im-
pregnated sponge (40 mg fluoroestrogen acetate, FGA, Chronogest®, Intervet International, The Netherlands).
For either 7 or 14 days in procedure I and II, respec-
tively. 48hrs before sponge removal, ewes received 500IU
single injection of pregnant mare serum gonadotrophin
(Folligon®, Intervet International, Holland). Single injec-
tions of 45 mg prostaglandin (Estrumate®, Shering-
Plough, Levallois, France) and an ovulation stimulator
(hCG, Chrolon®, Holland) were administered on the
morning of day 14th (day of sponge removal) and the
day after sponge removal (Day 0 of estrous). Recip-
ients were deprived from food and water 24 and 12hrs
before embryo transfer. Embryo(s) were transferred in
20 μL H-TCM199+10% fetal calf serum (FCS) (proc. I)
or H-SOF (proc. II) via a TDT-catheter (CCD-France)
surgically (25) into the fallopian tube or the top of the
uterine horn (through opening previously made by 18
gauge needle) ipsilateral to the ovary bearing at least
one functional corpus luteum.
Pregnancy status and fetal development was determined
using regular noninvasive monitoring of recipients on
days 30-35 of embryo transfer using a high-resolution
7.5 MHz probe. Status of pregnancy was assessed by
visualization of pregnancy sac through ultrasound car-
ried out on days 35 and 60 post transfer. From day 140,
the ewes were controlled for any sign of changes in
body temperature, restlessness, or grazing. From 142-
145, daily sonographical exams were carried out to
evaluate the precise conditions of placenta, fetus and
mother. C-section was inducted by the early signs of
placental detachment under local analgesia (lidocaine,
Pasture, Iran).

Genetic analysis
Genomic DNA was extracted from liver tissues of dead
fetuses or white blood cells from the live born lamb and
their corresponding surrogate mothers. Twelve micro-
satellite markers (as depicted in figure 2) were examined
using methods described elsewhere (26). Briefly, micro-
satellite band patterns were generated following micro-
satellite variant repeat PCR (MVR-PCR) and size frac-
tionation of reaction products were determined by 12%
polyacrylamide gel electrophoresis. Data was confirmed
using the ABI373A DNA sequencer (Applied Biosys-
tems/Perkin–Elmer) and analyzed by GENOTyper
software (Applied Biosystems/Perkin–Elmer). The
resulting microsatellite alleles for the nuclear transfer
derived fetuses and the lamb were compared with those donors the skin biopsies were obtained, and con-
trast with those from the recipient ewes that carried
the respective pregnancies. To determining the exact
number and chromosomal structure of the resulting
lamb and fetus in respect to their corresponding donor
cells, karyotyping was also done from cultured blood
lymphocytes by Gimsa Trypsin Gimsa Banding (GTG
banding) method.

Results
In vitro studies
Of 624 ovaries collected during procedure I and II,
totally about 4820, 3373 and1414COCs were retrieved,
matured in vitro and selected for nuclear transfer,
respectively. Table 1 summarizes the results obtained
during this study and Table 2 represents the results of
preimplantation embryo development in successfully
reconstituted, activated oocytes of procedure I and II.

In vivo studies
Table 3 also indicates the details of embryo transfer and
the pregnancy outcome come in procedure I and II.

Procedure I
During the first course of experiments, two pregnancies
were observed under ultrasound on day 35 post trans-
fer, resulting in 2.5% pregnancy per embryo transfer. Of these two pregnancies, one was further confirmed on day 60 (Royan-SHE-C1), while the other was lost (Royan-SHE-C3). Upon the removal of the uterine horn for the latter pregnancy, no fetal tissue was observed, suggesting complete loss of pregnancy. The former was continued until day 145 when the emergency cesarean carried out due to severe placental detachment and a very weak fetal heart beat under ultrasound. The delivered lamb was an immature (crump to tail: 32cm, 3.35kg) which was lost immediately after delivery due to acute apnea. A complicated peritonitis was observed as such the peritoneum was tightly adhered to the uterus at greater curvature- the possible cause of

Table 1: The efficiency of embryo reconstitution (EER) associated with the number of ovine oocytes retrieved, cultured and used during different stages of somatic cell nuclear transfer using adult ovine ear fibroblast cells

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Retrieved oocytes</th>
<th>Ovaries</th>
<th>Successfully manipulated</th>
<th>Successful enucleation (%)</th>
<th>In vitro matured</th>
<th>In vitro enucleated</th>
<th>Successfully reconstituted</th>
<th>EER (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>286</td>
<td>1578</td>
<td>576</td>
<td>626</td>
<td>493</td>
<td>498</td>
<td>(78)</td>
<td>342</td>
</tr>
<tr>
<td>II</td>
<td>338</td>
<td>1795</td>
<td>681</td>
<td>553</td>
<td>553</td>
<td>389</td>
<td>(16.8)</td>
<td>247</td>
</tr>
<tr>
<td>Total</td>
<td>624</td>
<td>3373</td>
<td>1441</td>
<td>1179</td>
<td>1046</td>
<td>882</td>
<td>(74.8)</td>
<td>589</td>
</tr>
</tbody>
</table>

EER is expressed as a % of enucleated oocyte.

Table 2: The results of preimplantation embryo development associated with the number of successfully reconstituted ovine oocytes produced during procedure I and II of this study with adult ovine ear fibroblast cells

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Fragments-D2 (%)</th>
<th>Cleavages-D2 (%)</th>
<th>5-8 cell-D2 (%)</th>
<th>Degenerated-D2 (%)</th>
<th>Fragmentation-D2 (%)</th>
<th>Uncleaved (%)</th>
<th>Recipient 5.5,8 cell-D2 (%)</th>
<th>Recipient 5-8 cell-D2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>324</td>
<td>139</td>
<td>57</td>
<td>112</td>
<td>80</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>II</td>
<td>247</td>
<td>75</td>
<td>46</td>
<td>99</td>
<td>73</td>
<td>31</td>
<td>18</td>
<td>71</td>
</tr>
<tr>
<td>Total</td>
<td>589</td>
<td>175</td>
<td>103</td>
<td>192</td>
<td>103</td>
<td>34</td>
<td>49</td>
<td>52</td>
</tr>
</tbody>
</table>

* Percentage were expressed in relation to number of cleaved day 2 embryos.

Table 3: Pregnancy outcome following transfer of ovine nuclear transferred embryos produced during experimental procedure I and II with ovine adult ear fibroblast cells

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No. reconstituted embryos</th>
<th>No. embryos transferred</th>
<th>No. recipients</th>
<th>Establishment pregnancy (%)</th>
<th>% Establishment pregnancy embryos transferred</th>
<th>% Isolated blastocysts</th>
<th>% Established pregnancy</th>
<th>% Established pregnancy embryos transferred</th>
<th>Live offspring (%)</th>
<th>% Established pregnancy embryos transferred</th>
<th>% Live offspring</th>
<th>No. offspring</th>
<th>Cloning efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>342</td>
<td>80</td>
<td>2 (15)</td>
<td>13.3</td>
<td>2.5</td>
<td>1</td>
<td>1 (6.7)</td>
<td>1 (1.3)</td>
<td>0</td>
<td>0.29</td>
<td>1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>247</td>
<td>28*</td>
<td>2 (17)</td>
<td>11.8</td>
<td>7.1</td>
<td>1</td>
<td>1 (5.9)</td>
<td>1 (3.6)</td>
<td>1</td>
<td>0.4</td>
<td>1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>580</td>
<td>108*</td>
<td>4</td>
<td>12.5</td>
<td>3.7</td>
<td>2</td>
<td>2 (6.3)</td>
<td>2 (1.9)</td>
<td>1</td>
<td>0.34</td>
<td>1</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Consisted of 18 blastocyst and 10 compact embryos.
undetectable running of parturition. Necropsy finding unraveled a retarded pulmonary system with non-structural absolute atelectasia.

Fig 1: Schematic representation of experimental designs:
Procedure I: enucleated oocytes were reconstituted at 26-28hrs post maturation (hpm) by subzonal insertion of donor cells, followed by immediate fusion and activation on 2 hours post fusion using ionomycin + 6-DMAP. Reconstructed embryos (2 to 8 cells with no fragmentation), obtained as a result of co-culture over Vero cells, were transferred surgically to the fallopian tube of the synchronous ewes.
Procedure II: apparently enucleated oocytes were immediately renucleated by intracytoplasmic donor cell insertion and rested in incubator for 3 to 4 h while enucleation was confirmed. Reconstituted oocytes were then imposed to electrical pulses followed by further activation using ionomycin and 6-DMAP. Activated embryos were cultured in SOF1 for 3 days. At this time, embryos were subjected to laser hatching before being cultured in SOF2. On day 7 to 8, blastocyst(s) were transferred surgically to the uterine horn of the synchronous ewes.

Two confirmed pregnancies were detected at day 35, resulting in 7.1% pregnancy per embryo transfer. Of two pregnancies, one continued till day 95 (Royan-SHE-C4) and then aborted to a 13 cm (crump to tail) morphologically normal female fetus having well formed heart, lung, visceral apparatuses and urogenital system. However, a mild purulative vaginal muca was observed just before abortion. The second pregnancy (Royan-SHE-C2 nick named Royana) was continued till day 145 under precise monitoring when ultrasound revealed a fully matured healthy fetus within a slightly detaching placenta. Elective cesarean was preceded by two IM injections of dexametazin (Daropakhsh, Iran). After delivery, the lamb immediately started breathing. The lamb was dried off, oxygen therapy was provided via a face mask, and the lamb was positioned in sternal recumbency. Blood gas and electrode values were monitored as if it was in normal range. Despite of some major arrhythmia in heart beat and respiratory distress, the general status was improved at the next hours when Royana received his first colostrums via stomach tube. One hundred and fifty days after, when drafting the manuscript, Royana was a ealthy lamb fostering by his mother in a pasture.

Microsatellite analysis
The results of microsatellite analysis clearly revealed that all three lambs obtained during this study were genetically identical to their donors. Figure 2 shows the molecular profiling and DNA fingerprinting of Royana and his respective somatic donor, which are identical and different from his foster mother. Karyotyping analysis also revealed a normal diploid chromosome number (2n=54) in cloned lambs, their foster mother and relative donors.

Histopathological analysis
The tissues were prepared from both the viscera of each aborted lambs, as well as the placenta of the 3 lambs. On histopathological exam, marginal mild necrosis at the apical lobe of lung, liver and kidney were detected in still birth lamb (Royan-SHE-C1) whereas the aborted fetus (Royan-SHE-C4) showed no obvious sign of
abnormality. The placenta of both the aborted fetus and the still birth lamb had clear cellular apoptosis and premature atrophy, while the signs was modest for the live lamb.

Discussion

The cloning technique is an extension of research that has been ongoing for over 50 years using nuclei derived from embryonic and fetal cells as well as completely differentiated adult’s cells (6). The ability to obtain a fully term mammal via somatic cell reprogramming was our basic start point toward establishment genetically modified animals and more importantly therapeutic cloning. Accordingly, during 2004-2006 a series of interrelated experiments were carried out to defining the most suitable conditions for in vitro culture of live stocks embryos (mainly cattle and sheep) as an essential prerequisite for embryo reconstitution studies. A series of preliminary studies were then undertaken to establish the best settings for oocyte enucleation, renucleation and parthenogenetic activation, donor cells preparation and cell cycle adjustment, recipient endometrial preparation and embryo transfer. Since the cattle and sheep are the two major live stocks, the cloning efforts were concentrated mainly to cattle and then the sheep. Despite of initial major successful achievements in the field of bovine cloning, the project shifted to the sheep, concerning financial problems in cattle.

In the experimental procedure I, the reconstructed oocytes were co-cultured in TCM199 with Vero cells (20). Despite of using co-culture system to overcome developmental block, the developmental rate was retarded. Therefore, it was decided to transfer the reconstructed embryos into fallopian tube as early as possible and to transfer higher numbers of embryos to increase the chance of achieving an established pregnancy. Although this procedure was undertaken to overcome in vitro deficiencies, two pregnancies (2.5% per embryos transferred and 13.3% per recipients) were achieved. One of the pregnancies successfully continued to term, even though, it was reported that embedding embryos in agar chips were necessary to protect the embryos from immunocompetent cells present in the oviductal lumen (21). Experimental procedure II was mainly designed to overcome retarded embryo development in procedure I. Thus, sequential semi-defined embryo culture medium, SOF 1 & 2 (23), in which cloned embryos could develop as a reasonably acceptable rate (18.2% blastocyst development for SCNT) were established (Table 2). Although it was suggested that in vitro culture environment exerted significant effects on embryo development (23, 27), embryo culture conditions had not been optimized for cloned embryos and the effects of culture conditions on the successful initiation of clonal development had not been examined (27). Therefore it is proposed that the block to development observed in procedure I may be caused by adverse culture conditions, such as imbalances in the concentrations of certain constituents (27, 28).

During the procedure II of this study, the technique for embryo reconstitution was also changed from that conventionally insertion of donor cell into the perivitelline space (9) to more recently applied method known as intracytoplasmic donor cell injection (22). This method may have the advantage of overcoming the low fusion efficiency reported in the former procedure. On the other hand, the possibility of proper expulsion of donor cell into the recipient cytoplasm and possible damages to nucleus should be considered (29). The technique of intracytoplasmic cell injection was first accredited to Wakayama et al (22) which led to successfully cloned, two generation of mice. This technique was then applied for SCNT production of the other animals such as pig and cow with results comparable to the routine technique of sub zonal donor cell injection (30, 31). It is of note that Wakayama was the first who applied piezo-actuated microinjection system due to very high sensitivity of mouse oocytes to the enucleation-renucleation processes (22). Other researchers also proposed piezo-actuated microinjection for improving the survival rate after intracytoplasmic injection (32). Therefore, it is likely that the excessive lysis observed during reconstruction of oocytes in procedure II (16.8%) may be contributed to the method of intracytoplasmic renucleation which causes excess pressure into the cytoplasm. However, it has been reported that this modified procedure requires prolonged manipulation of donor cells for nucleus isolation which is not only labor intensive but also can reduce the overall cloning efficiency due to the damages to the isolated nucleus (29, 33). The later technique was further improved by conducting whole-cell injection bypassing both the fusion and nucleus isolation process, thus simplifying the cloning procedure. This technique was used successfully for producing panda-rabbit interspecies cloned embryos (33). Our experience also confirms that this technique is a simple, less labor-intensive method of cloning.

Despite of acceptable rate of embryo production in the procedure II (table 2), unlike naturally fertilized embryos, the reconstructed embryos were collapsed and degenerated during blastocyst expansion and prior to hatching. It was believed that this phenomenon might be related to spontaneous progressive hardening of the zona pellucida (34). Therefore, laser assisted hatching was performed on 8-16 cell embryos which drastically improved hatchability of blastocysts as compared to non assisted embryos (24). In our experience, the second procedure as modified in our hand may provide a simple cloning procedure for labs starting cloning.

Limited overall success rates in terms of live born offspring (0.34% per reconstructed embryos), achieved during this study, is not a new surprising phenomenon. Published data reveal that on average only about 0-3% of the reconstructed embryos (number of live offspring as a percentage of the number of nuclear transfer embryos) resulted in live offspring, irrespective of the species, the donor cell type or technique (35). Considering that unsuccessful cloning experiments is unlikely to be pub-
lished, the actual cloning success rate will be substantially lower. Even though we were on the way of mastering this technology, overall efficiency of cloning is still the main milestone despite broad application of the technology.

Conclusion

Although our study was not a comparative study, but our experience may reveal that procedure II may provide a simple method for future cloning. We are hopping to use this procedure for production of transgenic live stock.

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This study is dedicated to Dr. Saeed Kazemi Ashtiani; the beloved passed Director of Royan Institute. Although it is difficult to accept that he is not with us any longer, as we promised him, we try our best to keep what he brought to our research center and follow his way. May he rest in peace.

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