Effects of DNA methyltransferase inhibitor RG108 on methylation in buffalo adult fibroblasts and subsequent embryonic development following somatic cell nuclear transfer

H.L. Sun12*, L.N. Meng†*, X. Zhao1, J.R. Jiang1, Q.Y. Liu1, D.S. Shi1 and F.H. Lu1

1Animal Reproduction Institute, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning, China
2Reproductive Medicine Department, Binzhou Medical University Hospital, Binzhou, China

*These authors contributed equally to this study.
Corresponding authors: D.S. Shi / F.H. Lu
E-mail: ardsshi@gxu.edu.cn / lfhgggg@163.com

Received January 19, 2016
Accepted March 18, 2016
Published September 2, 2016
DOI http://dx.doi.org/10.4238/gmr.15038455

Copyright © 2016 The Authors. This is an open-access article distributed under the terms of the Creative Commons Attribution ShareAlike (CC BY-SA) 4.0 License

ABSTRACT. Buffalo are characteristic livestock of the Guangxi Zhuang Autonomous Region of China, but their low reproductive capacity necessitates the use of somatic cell nuclear transfer (SCNT). We investigated the effects of RG108 on DNA methylation in buffalo adult fibroblasts, and on subsequent SCNT embryo development. RG108 treatment (0, 5, 10, 20, and 100 µM) had no effect on cell morphology, viability, or karyotype (2n = 48), and cell growth followed
a typical “S” curve. Immunohistochemistry showed that relative DNA methylation gradually decreased as RG108 concentration increased, and was significantly lower in the 20 and 100 μM groups compared to the 0, 5, and 10 μM treatments (0.94 ± 0.03 and 0.92 ± 0.05 vs 1.0 ± 0.02, 0.98 ± 0.05, and 0.98 ± 0.09, respectively; P < 0.05). Quantitative polymerase chain reaction revealed that DNMT1 gene expression of fibroblasts administered 10, 20, and 100 μM RG108 was significantly lower than those in the 0 and 5 μM groups (0.2 ± 0.05, 0.18 ± 0.07, and 0.3 ± 0.09 vs 1.0 ± 0.12 and 1.4 ± 0.12, respectively; P < 0.05). Treatment with 20 μM RG108 resulted in the lowest expression levels. Fibroblasts incubated with 20 μM RG108 for 72 h were used as donor cells to generate SCNT embryos. A greater number of such embryos developed into blastocysts compared to the non-treated group (28.9 ± 3.9 vs 15.3 ± 3.4%; P < 0.05). RG108 treatment can modify DNA methylation in buffalo adult fibroblasts and promote development of subsequent SCNT embryos.

Key words: Buffalo adult fibroblast; Methylation; RG108; Somatic cell nuclear transfer

INTRODUCTION

The birth of “Dolly” the sheep, the first mammal cloned using somatic cell nuclear transfer (SCNT), proved that differentiated somatic cells can be returned to a totipotent state in oocyte cytoplasm (Schnieke et al., 1997). However, the efficiency of SCNT remains low (<5%). Furthermore, the occurrence of abnormalities such as increased abortion rates, high birth weight, and perinatal death has been reported in such cloned animals (Sawai et al., 2011). Of the various processes associated with SCNT, aberrant epigenetic modification in somatic cell nuclei is considered to be the most important factor determining cloning efficiency (Holliday and Pugh, 1975; Ohgane et al., 2001; Kishigami et al., 2006; Sawai et al., 2011). This phenomenon is principally attributed to incomplete reprogramming of the differentiated somatic nuclei (Oswald et al., 2000). Epigenetic modifications, such as DNA methylation and histone acetylation and phosphorylation, do not involve changes in gene sequences.

DNA methylation is a covalent chemical modification of DNA, in which the methyl group of the coenzyme S-adenosyl-l-methionine is transferred to cytosine residues in CpG dinucleotides (Holliday and Pugh, 1975). In natural reproduction, male and female gametes have relatively low levels of DNA methylation, while embryos are further demethylated during early development (Oswald et al., 2000). However, somatic cells have been shown to exhibit higher degrees of DNA methylation than early embryos. Highly methylated somatic cells are used to generate cloned embryos, which in turn have been revealed to be abnormally hypermethylated (Bourc’his et al., 2001; Ohgane et al., 2001). The present study was designed to assess whether treating donor cells with pharmacological agents to remove epigenetic marks before SCNT enhances their ability to be reprogrammed. In bovine SCNT embryos, DNA methylation levels are high and successful development rate is low (Enright et al., 2005). Jafarpour et al. (2011) reported that cloned bovine embryos treated with 5-aza-2’-deoxycytidine
(AzC) cannot develop beyond the 8-16-cell stage (Jafarpour et al., 2011). AzC is a synthetic antineoplastic drug that inhibits DNA methyltransferases (DNMTs). Given the cytotoxicity of nucleoside analogues, including AzC, a novel type of non-nucleoside small-molecule DNMT inhibitor, RG108, was developed by drug design (Brueckner et al., 2005; Siedlecki et al., 2006). RG108 causes only low toxicity during demethylation, and demonstrates substantial potential as an antitumor drug (Siedlecki et al., 2006).

Buffalo are characteristic livestock of the Guangxi Zhuang Autonomous Region of China, but their poor reproductive capacity entails the use of advanced reproductive technology in the form of SCNT. Therefore, we chose this animal as the focus of the current research, in which the effects of RG108 on DNA methylation in buffalo adult fibroblasts and on subsequent embryonic development after SCNT were investigated. This was carried out to improve the efficiency of SCNT in buffalo, and ultimately ameliorate their reproductive capacity using this technique.

MATERIAL AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA). All culture medium was sterilized by 0.22-μm filtration (Millipore, Billerica, MA, USA). Animal welfare and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals, and were approved by the animal Ethics Committee of Guangxi University.

Buffalo adult fibroblast culture and RG108 treatment

Ear skin tissue samples (2 mm³) from adult buffalo were enzymatically digested with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) for 30 min, then 0.2% collagenase for 45 min. Disaggregated cells were washed three times in Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), before being centrifuged at 1500 rpm to obtain cell pellets. These cells were resuspended and cultured on a 60-mm culture dish in a humidified atmosphere of 5% CO₂ in air at 38.5°C. After 7-10 days of culture, confluent cells were detached for passage by 5-min incubation with an enzymatic solution (0.25% trypsin and 0.05% EDTA). Confluent seventh-passage fibroblasts were treated with different concentrations of RG108 (0, 5, 10, 20, and 100 μM) for 72 h, and subjected to subsequent experiments.

Cell growth curve and viability analysis

Various concentrations of RG108 (0, 5, 10, 20, and 100 μM) were added to suspensions of buffalo adult fibroblasts. These cells were adjusted to a density of 2-3 x 10⁴ cells/mL, and then 500 μL was placed in each well of a 24-well plate for culturing. Every 24 h, cells in each well were trypsinized, and harvested to assess growth using a hemocytometer (three wells per concentration). Trypan blue was used to detect the number of viable cells (Mohana Kumar et al., 2007).

Karyotype analysis

Buffalo adult fibroblasts treated with the above concentrations of RG108 for 72 h
and grown to 60-70% confluence were arrested in metaphase using 0.2 μg/mL demecolcine for 2.5 h in a humidified atmosphere of 5% CO₂ in air at 38.5°C. Cells were detached with enzymatic solution before hypotonic treatment with 0.8% sodium citrate for 30 min at 37°C. After centrifugation at 1200 rpm for 3 min, cells were resuspended and fixed with an acetic acid:methanol (1:3) fixative solution for 20 min at 37°C, then dropped onto a slide to obtain chromosome spreads. Finally, samples were stained with 10% Giemsa solution for 10 min, washed three times with distilled water, and air dried. Metaphase chromosomes were analyzed under an BX41 Olympus (Tokyo, Japan) microscope.

Immunohistochemistry

Buffalo adult fibroblasts treated with different concentrations of RG108 for 72 h and grown to 60-70% confluence on coverslips were subjected to immunohistochemistry. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature. For permeabilization, cells were treated with 1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature. Next, they were incubated with 1% bovine serum albumin (BSA) in PBS for 1 h, and after three washes, were incubated with mouse anti-5-methylcytidine primary antibody (1:300; Abcam) overnight at 4°C. Following a further three PBS washes, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-mouse immunoglobulin G, 1:1500; Cell Signaling Technology) for 1 h at room temperature in the dark. After three PBS washes, fluorescent immunostaining of global DNA methylation in fibroblasts was then detected using an LSM 510 META Laser Scanning Microscope (Zeiss, Oberkochen, Germany). Nuclear fluorescence intensity was measured with the Zeiss imaging software.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (TaKaRa, Kusatsu, Japan). Quantification of all gene transcripts was carried out using an ABI 7500 Fast Real-Time System (Applied Biosystems, Foster City, CA, USA). Primers for all targets were designed with the Oligo 6.0 software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA). Details of qPCR primers are given in Table 1. The qPCR mixture (20 μL per reaction) contained 10 μL SYBR Premix Ex Taq (TaKaRa), 0.3 μL each primer (10 nM), 0.4 μL 50X ROX Reference Dye II, 1 μL complementary DNA, and 8.3 μL double-distilled H₂O. Cycling conditions consisted of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 55°C for 1 min. Relative expression profiles of target genes were calculated using the 2^(-DDCt) method, with normalization to β-actin transcript levels.

In vitro fertilization (IVF)

Fertilization and culture of oocytes were performed as described previously (Kubota et al., 1998). Briefly, a frozen semen straw (0.25 mL per straw) was thawed in a 37°C water bath, and spermatozoa were washed in 1.5 mL modified Tyrode’s medium. After in vitro maturation culture for 22-24 h, oocytes were co-incubated with sperm in a 30-μL drop of improved Tyrode’s medium under mineral oil. Fertilization was achieved by a 24-h co-incubation in a
humidified atmosphere of 5% CO₂ in air at 38.5°C. Adhering cumulus cells were completely removed by repeated aspiration in embryo culture medium (CM; TCM-199 supplemented with 3% FBS), with which denuded presumptive zygotes were subsequently washed twice.

<table>
<thead>
<tr>
<th>Table 1. Details of primers used for quantitative polymerase chain reaction analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>DNMT1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>DNMT3A</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>β-actin</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

SCNT

Production of SCNT embryos from buffalo adult fibroblasts was performed as reported by Shi et al. (2007). In brief, trypsinized adult fibroblasts were transferred to the perivitelline space of enucleated oocytes with a 25-μm micropipette. The reconstructed couplets were transferred to a 25-μL drop of fusion medium (0.28 M mannitol, 0.1 mM CaCl₂, 0.1 mM MgSO₄, 5 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid, and 0.1% BSA) covered with mineral oil, and placed on a micromanipulator equipped with two platinum needle electrodes. Cell fusion was accomplished by applying an alternating-current pulse of 2 V for 1 s, then three direct-current pulses of 1 kV/cm for 15 μs using a BTX ECM 2001 Electro Cell Manipulator. Couplets were then incubated in embryo CM for 30 min at 38.5°C, and their fusion was inspected under an inverted microscope (Nikon, Tokyo, Japan). Activation of reconstructed embryos was induced by exposure to 5 μM ionomycin in CM for 5 min, and subsequent incubation with 2 mM 6-dimethylaminopurine in CM for 3 h.

Embryo culture

Embryos derived from IVF or SCNT were co-cultured with granulosa cell monolayers in 30 μL CM covered with mineral oil, in a humidified atmosphere of 5% CO₂ in air at 38.5°C. After in vitro culture for 48 and 144 h, cleavage and development to the blastocyst stage were examined, respectively.

Statistical analysis

Experiments were repeated at least six times. Differences between treatment methods were analyzed by one-way repeated-measures analysis of variance using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). In all tests, P < 0.05 was considered statistically significant.

RESULTS

Effect of RG108 on growth of buffalo adult fibroblasts

The morphology of buffalo adult fibroblasts was not affected by administration of various concentrations of RG108 (0, 5, 10, 20, and 100 μM; Figure 1). Moreover, the growth
of cells under each RG108 treatment was similar to that of the control group, demonstrating
an “S”-shaped curve (Figure 2). After cells had been stained with trypan blue, no significant
difference was observed in viability among the various RG108 concentration groups (Figure
3; $P > 0.05$). In addition, approximately 90% of adult fibroblasts treated with various RG108
levels exhibited a normal karyotype ($2n = 48$; Figure 4).

Figure 1. Buffalo adult fibroblasts treated with different concentrations of RG108 for 72 h (100X magnification).
A, B, C, D, and E. represent treatment with 0, 5, 10, 20, and 100 µM RG108, respectively.

Figure 2. Growth curves of buffalo adult fibroblasts after treatment with different concentrations of RG108.

---

Genetics and Molecular Research 15 (3): gmr.15038455
Effect of RG108 on DNA methylation in buffalo adult fibroblasts

After treatment of buffalo adult fibroblasts with different concentrations of RG108 (0, 5, 10, 20, and 100 μM) for 72 h, fluorescent immunostaining of global DNA methylation was carried out, as shown in Figure 5A. The relative intensity of DNA methylation in the fibroblasts gradually decreased with increasing RG108 concentration. The 20 and 100 μM groups showed significantly lower relative DNA methylation than the 0, 5, and 10 μM groups (Figure 5B; P < 0.05). However, there was no difference in relative intensity between the 20 and 100 μM treatments (P > 0.05).

Figure 3. Effect of different RG108 concentrations on the viability of buffalo adult fibroblasts.

Figure 4. Effect of different RG108 concentrations on buffalo adult fibroblast karyotype. A. Normal diploid buffalo adult fibroblasts. B. Chromosome pairing. C. Diploidy rate among the groups of buffalo adult fibroblasts treated with different concentrations of RG108.

Effect of RG108 on DNA methylation in buffalo adult fibroblasts

After treatment of buffalo adult fibroblasts with different concentrations of RG108 (0, 5, 10, 20, and 100 μM) for 72 h, fluorescent immunostaining of global DNA methylation was carried out, as shown in Figure 5A. The relative intensity of DNA methylation in the fibroblasts gradually decreased with increasing RG108 concentration. The 20 and 100 μM groups showed significantly lower relative DNA methylation than the 0, 5, and 10 μM groups (Figure 5B; P < 0.05). However, there was no difference in relative intensity between the 20 and 100 μM treatments (P > 0.05).
Effect of RG108 on expression of DNA methylation-related genes (DNMT1 and DNMT3A) in buffalo adult fibroblasts

Our qPCR analysis of DNA methylation-related gene (DNMT1 and DNMT3A) expression in fibroblasts treated with various concentrations of RG108 (0, 5, 10, 20, and 100 µM) for 72 h is shown in Figure 6. The values obtained were normalized to β-actin transcript levels. Relative expression of DNMT1 in the 10, 20, and 100 µM treatment groups was significantly lower than that of the other two groups (P < 0.05). In particular, fibroblasts administered 20 µM RG108 exhibited the lowest DNMT1 transcript levels. However, no difference was detected in the expression of the DNMT3A gene among the various RG108 concentration groups (P > 0.05). Thus, fibroblasts treated with 20 µM RG108 were chosen for subsequent experiments.

Figure 5. Effect of different RG108 concentrations on DNA methylation in buffalo adult fibroblasts. A. Fluorescence intensity of buffalo adult fibroblasts treated with different RG108 concentrations (100X magnification). A 1, 2, 3, 4, and 5 represent treatment with 0, 5, 10, 20, and 100 µM RG108, respectively. B. Relative DNA methylation level in buffalo adult fibroblasts treated with different RG108 concentrations. Different superscript letters depict significant differences between concentrations (P < 0.05).
Somatic cell nuclear transfer in buffalo

Figure 6. Relative expression of DNA methylation-related genes (DNMT1 and DNMT3A) in buffalo adult fibroblasts treated with different RG108 concentrations. Different superscript letters depict significant differences between concentrations (P < 0.05).

Effect of RG108 treatment of buffalo adult fibroblasts on in vitro development of subsequent SCNT embryos

To investigate its effect on in vitro development of subsequent SCNT embryos, buffalo adult fibroblasts were administered 20 μM RG108 for 0, 24, 48, 72, and 96 h before being used as donor cells. There was no difference in the cleavage rate of SCNT embryos among the various groups (Table 2; P > 0.05). However, the blastocyst formation rate of such embryos derived from buffalo adult fibroblasts treated with 20 μM RG108 for 72 h was significantly higher compared to that of the 0, 24, and 96 h groups (28.9 vs 15.3, 17.9, and 15.7%, respectively; P < 0.05). No difference was detected between the groups treated for 48 and 72 h (26.2 vs 28.9%, respectively; P > 0.05).

Table 2. Effect of RG108 treatment time on the development of somatic cell nuclear transfer embryos derived from buffalo adult fibroblasts.

<table>
<thead>
<tr>
<th>RG108 concentration (μM)</th>
<th>Treatment time (h)</th>
<th>No. of reconstructed embryos</th>
<th>No. of two-cell embryos (%)</th>
<th>No. of blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0</td>
<td>282</td>
<td>200 (70.9 ± 3.7)</td>
<td>43 (15.3 ± 3.4)</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>95</td>
<td>75 (78.9 ± 3.2)</td>
<td>17 (17.9 ± 3.9)</td>
</tr>
<tr>
<td>20</td>
<td>48</td>
<td>84</td>
<td>64 (76.2 ± 3.3)</td>
<td>22 (26.2 ± 3.6)**</td>
</tr>
<tr>
<td>20</td>
<td>72</td>
<td>211</td>
<td>169 (80.1 ± 4.1)</td>
<td>61 (28.9 ± 3.9)</td>
</tr>
<tr>
<td>20</td>
<td>96</td>
<td>83</td>
<td>64 (77.1 ± 3.0)</td>
<td>13 (15.7 ± 4.0)*</td>
</tr>
</tbody>
</table>

Values within parentheses represent means ± standard errors of means. **Values with different superscript letters in the same column are significantly different (P < 0.05).
SCNT constitutes a valuable tool in a number of life science applications, including the production of valuable transgenic animals (Schnieke et al., 1997) and regenerative medicine (Gurdon and Melton, 2008). Although some animals have been obtained by SCNT, the associated cloning efficiency remains low. Previous studies have indicated that SCNT efficiency is affected by many factors, but abnormal epigenetic reprogramming is thought to be foremost among these. Epigenetic changes include DNA methylation, histone modification and phosphorylation, and ubiquitination. Any donor cell faults will be propagated during subsequent SCNT embryonic growth, and affect further development and differentiation (Kang et al., 2001; Zhang et al., 2015b).

Many papers have suggested that highly methylated somatic donor cells are used to produce cloned embryos, which in turn, have been shown to be abnormally hypermethylated (Dean et al., 2001). In fact, aberrant reprogramming of DNA methylation has been reported in early cloned embryos (Bourc’his et al., 2001; Kang et al., 2001). To improve nuclear transfer efficiency, several epigenetic remodeling drugs, such as the DNA methylation inhibitors AzC and RG108 have been used in efforts to ameliorate the development of cloned embryos (Ding et al., 2008). The latter, a new type of non-cytidine DNA methylation inhibitor, can markedly reduce DNA methylation in tumor cells and parthenogenetic embryos in vitro and in vivo (Graça et al., 2014; Zhang et al., 2015a). It has been demonstrated that treatment of lung adenocarcinoma cells with RG108 decreases methylation of the RASSF1A gene, inducing apoptosis (Rui and Wei, 2009). In the present study, we first demonstrated that appropriate concentrations of RG108 (≤100 μM) had no effect on buffalo adult fibroblast morphology, and treated cells exhibited typical “S”-shaped growth curves. These results indicated that the biological characteristics of such cells are not negatively affected by RG108 treatment. Furthermore, their karyotype appeared normal under the various RG108 concentrations tested.

Modification of DNA methylation is catalyzed by DNMTs, including DNMT1, DNMT3A, and DNMT3B. DNMT1 maintains methylation patterns during DNA replication, while DNMT3A and DNMT3B principally establish de novo methylation states of specific genomic regions. This process plays a key role in maintaining genomic stability, activating or suppressing gene expression, and regulating genomic imprinting. In bovine SCNT embryos, methylation of the satellite I region is significantly lower than that observed using IVF (Wang et al., 2011). In addition, Stresemann et al. (2006) found that RG108 treatment can inhibit methyltransferase activity in various tumor cells. In our study, we established that methylation in buffalo adult fibroblasts gradually decreases as RG108 concentration increases, while qPCR revealed that expression of the DNMT1 gene is reduced after treatment of these cells with this drug. Furthermore, use of RG108-administered buffalo adult fibroblasts as donor cells to investigate subsequent in vitro development of SCNT embryos showed that incubation with a low dose of this DNMT inhibitor (20 μM) for 48 or 72 h can improve blastocyst formation rate. These results are consistent with a study by Jin et al. (2008), in which the treatment of fetal fibroblasts with 50 μM RG108 significantly increased the developmental competence of cloned pig embryos. Moreover, RG108 improves the reprogramming efficiency of murine-induced pluripotent stem cells (Shi et al., 2008). Results reported by Li et al. (2009) also indicate that genome-wide methylation in mouse embryos is decreased by RG108 treatment. Our results suggest that RG108 can decrease DNA methylation levels in buffalo SCNT donor cells, and facilitate development of the resulting embryos.
Somatic cell nuclear transfer in buffalo

In conclusion, treatment of buffalo adult fibroblasts with an optimal concentration of RG108 not only has no negative effect on the biological characteristics of donor cells, but can also decrease DNA methylation and improve the in vitro developmental competence of buffalo SCNT embryos. By inference, the development of such embryos is related to the DNA methylation status of donor cells. However, further study is required in regard to the full-term development of buffalo SCNT embryos from donor cells treated with RG108.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank the buffalo ovary collectors. Research supported by the China High Technology Development program (#2011AA100607), the China Natural Science Foundation (#31560633), the Guangxi Science Foundation (#2012GXNSFFA060004), and Research Fund for the Doctoral Program of Higher Education of China (#20114501110001).

REFERENCES


References:

Genetics and Molecular Research 15 (3): gmr.15038455


---

Genetics and Molecular Research 15 (3): gmr.15038455