INCREASE HISTONE ACETYLATION DURING THE FIRST MITOTIC CELL CYCLE IMPROVES PREIMPLANTATION DEVELOPMENT AND THE QUALITY OF MOUSE EMBRYOS GENERATED BY ROUND SPERMATID INJECTION (ROSI)

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Abstract. This study examined the effects of Scriptaid, a histone deacetylase inhibitor (HDAC) during the first mitotic cell cycle on preimplantation development of mouse embryos generated by injection of round spermatid into mature oocytes. In the first experiment, we treated ROSI embryo with 0, 50, 250, 500, 1,000 nM Scriptaid for 16 hours after injection and activation. We found the intensity of histone acetylation and diameter of ROSI male pronucleus are proportional to the increase of concentration of Scriptaid; however, the rate of ROSI embryo development to blastocyst was highest at 250 nM Scriptaid (51.6 %) compared with the other concentrations (19.4 %, 34.4 %, 35.3 %, and 8.6 %, respectively). In the second experiment, 250 nM Scriptaid is used to test optimal timing (6, 10, 16 hours) for Scriptaid treatment on preimplantation development of ROSI embryos. The results showed that the 10-hour to 16-hour treatment groups resulted in the highest development of ROSI embryos to the blastocyst stage (56 % and 57 %, P < 0.05) compared with 6-hour group (23 %). In conclusion, increasing histone acetylation at concentration of 250 nM Scriptaid with 10-hour treatment increased the intensity of histone acetylation and decondensation of the male DNA, finally increase the rate of ROSI embryos developing to blastocyst and blastocyst quality.

Keywords: Histone acetylation, histone deacetylase inhibitor, mouse, preimplantation development, pronuclear formation, ROSI, round spermatid, Scriptaid.

1. INTRODUCTION

Previous studies have demonstrated that using round spermatids after completion of second meiosis process injecting into mature oocyte can produce a fertilized embryo; some cases can produce a full-term development (Ogura et al., 1994; Yanagimachi, 2005; Hirabayashi et al., 2009). Until recently, development rate of ROSI embryos to full-term development is still significantly lower than that of fertilized embryo generated by mature spermatozoa for many reasons (Kimura et al., 1994; Kishigami et al., 2004; Kishigami et al., 2004b; Yanagimachi, 2005), typically due to abnormal DNA hypermethylation of...
spermatid-derived male genomes in mouse zygotes (Kishigami et al., 2006), and hypoacetylation in male pronuclear formation (Van Thuan N et al., unpublished data). Histone deacetylase inhibitors (HDAC inhibitors, HDACi) are chemical compounds that inhibit histone deacetylases resulted in increased histone proteins acetylation (Miller et al., 2003; Thiagalingam et al., 2003), and widely used both in basic research and medications such as anticonvulsants and anticaner (Marks et al., 2000; Dokmanovic et al., 2007). However, HDACi such as Trichostatin A (TSA) and valproic acid are well known potent teratogens using zebrafish, xenopus and mice (Gurvich et al., 2005; Svensson et al., 1998). Van Thuan N (Thuan et al., 2009) reported that Scriptaid, an HDACi that increases translation during protein synthesis, was able to increase histone H3 acetylation in pronuclei of cloned mouse zygote, enhance zygotic gene activation, finally improve the development of cloned inbred and hybrid mouse to the full-term. Some reports have suggested that treatment of ROSI embryos with TSA for 10 hours after injection resulted in improved the development of ROSI embryos to full-term (Kishigami et al., 2006). However, TSA is more toxic than Scriptaid for embryo treatment, and there have been no reports on the effects of Scriptaid on ROSI embryo development. Therefore, in this study, we investigated the effects of Scriptaid with different concentrations and treatment timing on ROSI male pronuclei formation and preimplantation development in the mouse.

2. MATERIALS AND METHODS

2.1. Animals

ICR females were used as oocyte donors, and female mice were injected with 5 IU of pregnant mare serum gonadotropin (PMSG) and 5 IU of human chorionic gonadotropin (hCG) 48 hours later to induce super-ovulation. ICR male was used to collect mature spermatozoa and round spermatid for the production of embryos via ICSI and ROSI.

2.2. Intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI)

Mature spermatozoa were collected from cauda epididymis, and round spermatid were collected from testis of ICR male mice. After collection, the cumulus cells were dispersed with 0.1 % hyaluronidase in drops of Hepes-buffered CZB (Hepes-CZB) medium. The oocytes were transferred to new drops of Hepes-CZB and were denuded of almost all cumulus cells by gentle pipetting. Collected oocytes were selected and cultured in new drops of mCZB at 37 °C in an atmosphere of 5 % CO2 in air until use. For ICSI embryos, mature oocytes were injected with mature spermatozooa under microscopy (ICSI) and transferred to culture drop of mCZB medium for culture. For ROSI embryos, mature oocytes were injected with round spermatid after it had been ruptured membrane and removed cytoplasm by 8 µm microinjection needle.

2.3. Scriptaid treatment for ROSI oocytes

In experiment 1, the reconstructed ROSI oocytes were activated by 10 mM SrCl2 in Ca2+-free CZB medium for 6 hours in the present of 50, 250, 500, and 1.000 nM Scriptaid then subsequently cultured in mCZB with Scriptaid at the different concentrations as
above for the next 18 hours and 96 hours after activation. After three washes in mCZB, ROSI embryos were cultured in the same medium for examining histone acetylation of male pronuclear formation and for preimplantation development. In experiment 2, the reconstructed ROSI oocytes were activated by 10 mM SrCl₂ in Ca²⁺-free CZB medium for 6 hours in the present of 250 nM Scriptaid then subsequently cultured in mCZB with Scriptaid for the next 0 hour for group of 6-hour treatment, 4 hours for group of 10-hour treatment, and 10 hours for group of 16-hour treatment. Groups of control were ICSI embryos and ROSI embryos without Scriptaid treatment. The preimplantation development rates of ROSI embryos and ICSI embryo were examined from two-cell to the expanded blastocyst stages at 96 hours after activation.

2.4. Immunofluorescence procedures

The methods for immunofluorescence and measure the diameter of male pronucleus were as described by Van Thuan et al. (Thuan et al., 2009). Primary antibodies were mouse monoclonal anti-bromodeoxyuridine (Roche Diagnostics, Mannheim, Germany) and rabbit polyclonal anti-acetyl-histone H3-K9 (Upstate Cell Signaling Solutions, Charlottesville, VA, USA). The secondary antibodies were Alexa-Fluor-568-labeled goat anti-mouse IgG and Alexa-Fluro-488-labeled chicken anti-rabbit IgG antibodies. The DNA was stained for 30 minutes with 2 mg/ml 4,6-diamidino-2-phenylindole (Molecular Probes Inc., Eugene, OR, USA). To diminish errors in measuring nuclear volumes, the embryos in each repeat experiment were mounted on glass slides with same volume (9 µL) of glycerol, and then samples were observed by using a Nikon Inverted Microscope Eclipse Ti-U.

2.5. Statistical analyses

Student’s t-test was used to calculate the significance of any differences between experimental groups in the immunofluorescence studies. Each experiment was repeated at least four per treatment. The data were subjected to arcsine transformation for each replication. The transformed values were analyzed using one-way ANOVA, and \( P < 0.05 \) was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effects of different concentration of Scriptaid on the intensity of histone acetylation, the diameter of ROSI male pronucleus, and preimplantation development

Figure 1 showed that when converting the intensity of histone acetylation of the male pronucleus of ICSI embryo group to 1, the ROSI male pronucleus without Scriptaid treatment was very low (0.3). Meanwhile, the intensities of histone acetylation were increased to 0.7, 1.3, 3.2, and 4.7 at Scriptaid concentrations of 50, 250, 500, and 1000 nM, respectively. This result indicated that the intensity of histone acetylation was very low in male pronucleus compared with that of the male pronucleus of ICSI zygote at 16 hours after activation. The result also showed the intensity of histone acetylation was proportional to the concentration of Scriptaid treatment. At concentration of 250 nM, there
was a higher increase of histone acetylation than in ICSI zygotes; however, the difference was not significant. According to this result, when treating ROSI oocytes with a concentration of 250 nM Scriptaid, the intensity of histone acetylation was close to that of the male pronucleus of the ICSI zygote.

**Figure 1.** The intensity of histone H3 lysine 9 acetylation (aH3-K9) in male pronucleus of ROSI zygotes at 16 hours post-activation after being treated with 0, 50, 250, 500, and 1000 nM Scriptaid. The mean value of aH3-K9 intensity of ICSI zygote (Possible control group) at 16 hours post-ICSI was set at 1, and the average value of fluorescence intensity observed in each experiment group was expressed relatively to this value. The experiment was repeated 4 times. The number of zygotes in each experimental group was 8, and was used to measure the intensity of histone acetylation. Bars show the S.E.M. Values with different superscripts at each column are significant difference.

**Figure 2.** The mean diameter of male pronucleus of ROSI zygotes at 16 hours post-activation after being treated with 0, 50, 250, 500, and 1000 nM Scriptaid. Control group is ICSI zygote at 16 hours post-ICSI. The experiment was repeated 4 times. The number of zygotes in each experimental group was 8, and was used to measure the intensity of histone acetylation. *P<0.05 for comparison of male pronuclear diameter of ICSI zygote and of ROSI zygote with different concentration of Scriptaid.

The results of diameter of male pronucleus of ROSI zygotes that were treated with 0, 50, 250, 500, and 1000 nM Scriptaid at 16 hours after activation are showed in Figure 2. The results showed that the diameter of male pronucleus of ROSI zygote was significantly
smaller than that of ICSI zygote. Previous studies have reported that the size of male pronucleus is dependent on decondensation of chromatin, which is the result of acetylation of histone proteins (Bui et al., 2010). The results in this study showed that the diameter of the male pronucleus was increased proportionally with the increase of Scriptaid processing concentration. The results indicated that at concentrations of 50, 250, and 500 nM Scriptaid, the diameter of the male pronucleus of ROSI zygote increased close to that of the male ICSI pronucleus (P > 0.05). While increasing the concentration up to 1,000 nM, the male pronucleus size of the ROSI zygote was significantly larger than that of the ICSI zygote group (P < 0.05) (Figure 2).

The effect of different concentrations of Scriptaid on the development of ROSI embryos is shown in Table 1. The results showed that there was no difference among groups of Scriptaid-treated ROSI embryos, non-Scriptaid-treated ROSI embryos, and ICSI embryos that developed up to the 2-cell stage embryos. However, at the blastocyst stage, the percentage of embryos that developed to blastocyst in the group of non-Scriptaid-treated and the Scriptaid-treated groups with concentrations of 50, 500, and 1000 nM were significant difference for the ICSI group. In group of 250 nM Scriptaid treatment, although lower than the ICSI group, the difference was not significant (76.6% vs 64.5%, respectively, Figure 3).

**Table 1.** The effect of different concentration of Scriptaid on the development of ROSI embryos

<table>
<thead>
<tr>
<th>Experiment groups</th>
<th>No. of oocyte injection</th>
<th>No. (%) of 2-cell embryos</th>
<th>No. (%) of blastocyst</th>
<th>Average cell number of Blastocyst (±S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICSI</td>
<td>32</td>
<td>30 (93.7)a</td>
<td>23 (76.6)a</td>
<td>76.2 ±2.9a</td>
</tr>
<tr>
<td>ROSI (-)</td>
<td>37</td>
<td>31 (83.7)a</td>
<td>6 (19.4)b</td>
<td>34.1±4.1b</td>
</tr>
<tr>
<td>ROSI (50 nM)</td>
<td>34</td>
<td>32 (94.1)a</td>
<td>11 (34.4)c</td>
<td>48.8±5.2c</td>
</tr>
<tr>
<td>ROSI (250 nM)</td>
<td>35</td>
<td>31 (88.6)a</td>
<td>16 (51.6)d</td>
<td>64.4±6.8d</td>
</tr>
<tr>
<td>ROSI (500 nM)</td>
<td>37</td>
<td>34 (91.8)a</td>
<td>12 (35.3)c</td>
<td>60.3±5.2d</td>
</tr>
<tr>
<td>ROSI (1,000 nM)</td>
<td>39</td>
<td>35 (90.7)a</td>
<td>3 (8.6)b</td>
<td>49.6±2.2c</td>
</tr>
</tbody>
</table>

This experiment was repeated 4 times. Values with different superscripts at each column are significant difference (P < 0.05).

**Figure 3.** Development of ROSI embryos and ICSI embryos to blastocysts. A. ROSI blastocyst derived from Scriptaid-untreated group, B. ROSI blastocysts derived from 250 nM Scriptaid-treated group, C. ICSI blastocysts derived from ICSI embryos. Scale bar: 100 µm
The results also showed that the average cell number of blastocysts derived from ROSI groups with or without treatment was lower than that of the ICSI group \((P < 0.05, \text{Table 1 and Figure 4})\). However, with the concentration of 250 nM Scriptaid, the average cell number of ROSI was highest compared with the other Scriptaid-treated and untreated groups (Table 1 and Figure 4).

*Figure 4.* The cell number of ROSI and ICSI blastocyst at 96 hours after sperm injection for ICSI or after activation for ROSI. A. The number of cells of ROSI blastocyst without Scriptaid treatment, B. the number of cells of ROSI blastocyst with 250nM Scriptaid treatment, C. The number of cells of ICSI blastocyst. Blue is the nuclei of the embryo cell. Scale bar: 100 µm

### 3.2. Effects of timing treatment of 250 nm Scriptaid on the development of ROSI embryos to 2-cell embryos and the blastocyst stage

*Table 2.* Effects of timing treatment of 250 nM Scriptaid on the development of ROSI embryos

<table>
<thead>
<tr>
<th>Experiment groups</th>
<th>No. of oocyte injection</th>
<th>No. (%) of 2-cell embryos</th>
<th>No. (%) of blastocyst</th>
<th>Average cell number of Blastocyst (±S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICSI</td>
<td>18</td>
<td>14 (77.8)(^{a})</td>
<td>9 (64.3)(^{a})</td>
<td>71.2 ±3.9</td>
</tr>
<tr>
<td>ROSI 6 hrs</td>
<td>17</td>
<td>13 (76.4)(^{a})</td>
<td>3 (23.1)(^{b})</td>
<td>64.1±4.2</td>
</tr>
<tr>
<td>ROSI 10 hrs</td>
<td>20</td>
<td>16 (80.0)(^{a})</td>
<td>9 (56.3)(^{a})</td>
<td>65.4±4.7</td>
</tr>
<tr>
<td>ROSI 16 hrs</td>
<td>16</td>
<td>14 (87.5)(^{a})</td>
<td>8 (57.1)(^{a})</td>
<td>66.2±5.4</td>
</tr>
</tbody>
</table>

This experiment was repeated 4 times. Values with different superscripts at each column are significant difference \((P < 0.05)\).

Based on the results of experiment 1, when treating ROSI zygote with 250 nM Scriptaid, the intensity of histone acetylation and the size of the male pronucleus of ROSI embryos were equivalent to ICSI and improved the development of ROSI embryos to the blastocyst stage. Therefore, in the second experiment, we chose 250 nM Scriptaid to study the optimal duration for Scriptaid treatment (6 hours, 10 hours, and 16 hours) on the preimplantation development of ROSI embryos. The results showed that there was no significant difference of ROSI embryos developing up to 2-cell embryos in the experimental groups as well as in the ICSI embryos group \((P > 0.05)\). At 96 hours after activation, the results showed that the percentage of ROSI embryos that developed to blastocysts when treated with 250 nM Scriptaid for 6, 10, and 16 hours was 22 %, 56 % and 57 %, respectively. There was no statistical difference between the group of 10-hour and 16-hour treatment, however, the group of 6-hour scriptaid treatment gave the lowest and statistically significant difference compared with the group of 10-hour and 16-hour. However, the number of cells in ICSI and in ROSI blastocysts showed no statistical difference.
This result suggests that the optimal timing for Scriptaid treatment enhanced the development of ROSI embryos is 10-16 hours after activation. With a concentration of Scriptaid of 250 nM, the results improved the quality of embryos through the total number of blastomere in ROSI blastocysts that were not different compared with fertilized embryos by ICSI technology.

4. CONCLUSION

The results of this study indicated that treatment of ROSI embryos with 250 nM Scriptaid for 10 or 16 hours after activation resulted in increased the histone acetylation and the diameter of the male pronucleus, eventually leading to the increase in ROSI embryo developed to the blastocyst stage as well as the quality of ROSI embryos. This study has very important implications regarding to application of ROSI technique in treatment of infertility caused by not having spermatozoa in human.

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REFERENCES


GIA TĂNG HISTONE PROTEINS ACETY HÓA TRONG KỲ PHÂN CHIA ĐẦU TIÊN ĐÃ CẢI THIỆN QUÁ TRÌNH PHÁT TRIỂN SÓM VÀ CHẤT LƯỢNG CỦA PHỒI CHUỘT ĐƯỢC TẠO RA BẰNG KỸ THUẬT ROSI

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Tóm tắt. Nghiên cứu này đã khảo sát sự ảnh hưởng của Scriptad, một chất ức chế histone proteins acety hóa trong chu kỳ phân bào đầu tiên đối với sự phát triển của phôi chuột được tạo ra bằng cách tiêm tiền tinh trùng (round spermatid) vào tế bào trứng (ROSI). Trong thí nghiệm 1, phôi ROSI được xử lý với các nồng độ Scriptad khác nhau 0, 50, 250, 500, và 1.000 nM Scriptad trong 16 giờ kể từ khi kích hoat trứng sau khi ROSI. Kết quả cho thấy rằng, cương độ acety hóa và degree của tiền nhân được ROSI tỷ lệ thuận với sự gia tăng nồng độ scriptad. Tuy nhiên tỷ lệ phôi ROSI phát triển thành phôi nang cao nhất ở nhóm xử lý với nồng độ 250 nM Scriptad (51.6 %) so với các nồng độ khác (19.4 %, 34.4 %, 35.3 % và 8.6 %). Trong thí nghiệm thứ 2, với nồng độ tối ưu của Scriptad là 250 nM, chúng tôi đã khảo sát khoán thời gian tối ưu nhắm đến xử lý phôi ROSI là 6 giờ, 10 giờ và 16 giờ sau khi kích hoat. Kết quả cho thấy rằng phôi ROSI phát triển đến phôi nang là cao nhất trong nhóm xử lý 10 giờ (56 %) hoặc 16 giờ (57 %), trong khi nhóm xử lý 6 giờ chỉ đạt được khoảng 23 %. Kết luận, gia tăng acety hóa của histone proteins trong khoảng 10 hoặc 16 giờ sau khi kích hoat bằng 250 nM Scriptad đã làm gia tăng cương độ acety hóa histone proteins, gia tăng kích thước của tiền nhân đức và cuối cùng gia tăng tỷ lệ phôi ROSI phát triển đến phôi nang cũng như phạm chất của phôi nang.

Từ khóa: Chuột, histone acety hóa, quá trình phát triển som, ROSI, Scriptad, tiền nhân, tiền tinh tử, ức chế histone acety hóa.

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