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The Effect of C-Jun on Leydig Cells Proliferation of Elemental Status in Rongchang Piglets

[1]Xiaobo Zheng, [2]Lijuan Qing, [1]Zhiwan Zheng, [2]Jiao Li, [1]Rong Xiao, [1]Libin Wang,
[2]Yinzhu Yao, [1]Boqing Wu, [1]Huihao Xu, [2]Maosen Xu, [2]Jing Li, [2]Qun Wu and [2]Liang Ge
[1]Department of Veterinary Medicine, Rongchang Campus,
[2]College of Animal Science and Technology, Southwest University, Chongqing, China

Abstract: Leydig cells were isolated from the testis of 3 week old Rongchang piglets. Its activity, purity and function were qualified before being cultured *in vitro*. Then, they were respectively treated with 0.125, 0.25, 0.5, 1 and 2 μmol L^{-1} c-jun ASODNs and the expression of c-jun mRNA were detected by FQ-PCR. To study the effect of c-jun on proliferation of elemental status leydig cells in piglets, the leydig cells had been treatedwith c-jun ASODNs and the growth inhibition ratio of leydig cells were detected by MTT method and cell cycle phase distribution were examined by flow cytometer analysis. The FQ-PCR assays results indicated that c-jun mRNA expression decreased with the rise of c-jun ASODNs concentration. Only 0.125 μmol L^{-1} c-jun ASODNs could reduce the expression of *c-jun* gene significantly ($p<0.01$). And the expression no longer went down when the concentration was up to 1 μmol L^{-1}. Through MTT analysis, it is found that c-junASODNs could inhibit the growth of leydig cells significantly ($p<0.01$). There is a positive correlation between c-jun ASODNs concentration and growth inhibition ratio of leydig cells ($r = 0.8966$).

Key words: C-jun, piglet, leydig cells, proliferate, China

INTRODUCTION

About 95% of testosterone in animal were synthesized and secreted by leydig cells whose secretion function is influenced by a wide variety of factors, for instance growth factors (such as IGF-1, TGFβ1) endothelin-1, inhibin and activin, glucocorticoid, leptin and so on (Xiao and Wang, 2002). As a matter of fact, a large number of researches indicated that these factors are realized by regulating the *proto-onco* gene related to them (such as c-jun, c-fos, c-myc, etc.). By means of *in situ* hybridization and immunohistochemistry, researchers found that c-jun was expressed in leydig cells and that the expression was related to the synthesis and secretion of testosterone in leydig cells (Xu *et al*., 2005; Yuan *et al*., 2006; Hall *et al*., 1991). As a *proto-onco* gene existing widely in prokaryotes and eukaryotes genome, c-jun belongs to normal component of cells. The expression product of c-jun is very important to the growth and differentiation of cells with its coding product existing in nuclei. It participates in the signal transduction path of cells meanwhile and can stimulate the proliferation related gene expression and DNA synthesis. The number of leydig cells is one of the key factors to testosterone secretion and also is directly related to cell proliferation and apoptosis. Cell proliferation is the result of cell circulation and operation in cell cycle in the order of (G0) G1→S→G2→M. Under the influence of all sorts of cell cycle control mechanism, different genes in cell are activated and expressed in strict accordance with the time sequence which results in the completion of three important transitions in the cell cycle. The transitions of G1/S, G2/M and mid-term/late-term in M phase. When influenced by mitogen stimulated factors, such as growth factor, the proliferation related gene and its expression product will change and lead to different regulation function in cell cycle. But, as the effective factor of MPAK signal pathway, c-jun still remains a puzzle as to its effect on proliferation of piglets leydig cell. Therefore, in order to further explore the role of c-jun in testosterone secretion process in leydig cell in piglets, it is necessary to research the influence that c-jun acts on proliferation in piglets leydig cell. This research plans to cultivate piglets leydig cell *in vitro*. In the elemental status, artificial synthesized c-jun ASODNs were used to specifically block the transcription of c-jun, then real time fluorescent quantitation technique was employed to detect the expression quantity of c-jun mRNA. Meanwhile, the growth activity and cell cycle of leydig cell were observed. It is expected that the relationship between the

Corresponding Author: Xiaobo Zheng, Department of Veterinary Medicine, Rongchang Campus, Southwest University, Chongqing, China

expression of c-jun mRNA and the proliferation in leydig cell will be clarified and the action mechanism of c-jun in the testosterone secretion process will be further explored.

MATERIALS AND METHODS

Animals: Rongchang pig was provided by national Rongchang pig breed conservation field (Numbers: C5001027) in Rongchang District in Chongqing.

Synthesis of c-jun ASODNs: C-jun ASODNs was design and synthesized according to the sub-sequence 5'-ATGACTGC AAAGATGGAA-3' of pig c-jun sequence (LOCUS: S83515) in NCBI GenBank with the help of Primer Premier Version 5.0 software. Namely, the sequence of c-jun ASODNs was 5'-TTCC ATCTTTGCAGTCAT-3'. Antisense tat-ODNs, as the comparison of c-jun ASODNs was the complementary sequence of 53995413 basic group in *HIV-tat* gene and its sequence was 5'-CATTTCTTGCTCTCC-3' (Habert *et al*., 2001). The above antisense-nucleic acids were synthesized by Invitrogen Biotechnology Limited Company and its beginning and ending 3 basic groups were modified by thiophosphoric acid.

The separation and cultivation of leydig cells: The collected pig leydig cells were processed according to the methods of Mauduit *et al*. (1992) and Zhang *et al*. (1999) and were cultivated in the condition of 37°C, 5% CO_2 and saturated humidity.

The identification of leydig cells

Trypan blue exclusion experiment: Prepare apinoid blood cell counting plate and special slide glass, put the slide glass on the blood cell counting plate and expose a bit of the top of the counting plate. Blend cell suspension with 0.4 g L^{-1} trypan blue fluid at the ratio of 9-1. Then, take a small amount of trypan blue cell mixture liquid and drop the liquid to the edge of slide glass so that the cell mixture liquid can the interspaces between counting plate and slide glass. Use microscope to randomly pick 3 visual fields to count the numbers of dead cells and living cells within 3 min. The cell-vigor calculation:

$$\text{Ratio of living cells} = \left[\frac{\text{Living cell number}}{\text{Living cell number} + \text{dead cell number}}\right] \times 100\%$$

The detection of 3 β-HSD activity operate according to the method of Wang *et al*. (2003) leydig cell is a blue corpuscular positive cell in cytoplasm observed by microscope. About 10 random visual fields are observed for each sample and the number of masculine cells in 100 cells in each visual field is counted. The purity quotient of leydig cells in cell suspension is expressed by the positive cell ratio:

$$\text{Positive cell ratio} = \frac{\text{Total number of 3B} - \text{HSD positive cells}}{\text{Total cell number}} \times 100\%$$

Identification of leydig cell function cells are cultured according to before. After culture of 48 h, the cells are treated in hCG of different concentrations: 0, 5, 10, 25 and 50 IU mL^{-1} with each treatment repeated for 3 times. The cells will continue to be cultivated for 24 h and then the culture fluid will be collected and centrifuged under 1500 r min^{-1} for 5 min. Detect testosterone concentration by getting the clear supernatant liquid.

Detect the expression of c-jun mRNA in leydig cells: Add c-jun ASODNs or tat ODNs into leydig cells (concentration: 0.125, 0.25, 0.5, 1, 2 μmol L^{-1}) and cultivate for 24 h in the condition of 37°C, 5% CO_2 saturated humidity. Then detect the expression of c-jun mRNA in leydig cells with fluorescent quantitative method. The control group was used at the same time.

Inhibition ratio of cell proliferation with MTT: Inoculate leydig cells into 96 pore plates (about 10^5 cells for each pore) and cultivate the cells in the condition of 37°C, 5% CO^2, saturated humidity. After disposing the cells with c-jun ASODNs or tat ODNs (concentration: 0.125, 0.25, 0.5, 1, 2 μmol L^{-1}) for 20 h, add 5 mg mL^{-1}, 20 μL of MTT into each pore and cultivate at 37°C for 4 h, absorb away MTT and add 150 μL DMSO into each pore and allow reaction for 15 min in 37°C. On ELISA reader, choose the wavelength of 490 nm, zero set with blank group, then detects extinction value (A) for each hole:

$$\text{Inhibition ratio}(\%) = \left(\frac{1 - \text{A experiment group}}{\text{A comparison group}}\right) \times 100\%$$

Detect the number of cells in cell cycle with flow cytometry: Cultivate for 24 h in the condition of 37°C, 5% CO_2 and saturated humidity, remove former culture fluid, digest for 5 min with 0.1% trypsin then add equal amount of complete medium to end the digestion and repeatedly blow to guarantee uniformity. Remove unicell suspension into centrifuge tube, centrifuge for 3 min in 4°C then discard supernatant and misce bene the remaining cell suspension (0.5 mL or so) and eject the cell suspension by syringe into 4°C ,1.5 mL cold dehydrated alcohol (99%) misce bene thoroughly, then store it at 4°C for the night (at least 18 h of fixation). Adjust the concentration of fixed cell suspension to 106 cells mL^{-1}, use DNA dyeing liquid

(among which the concentration of propidium iodide is 0.05 mg mL^{-1}, the concentration of RNA enzyme a is 0.1 mg mL^{-1} and the content of TritonX-100 is 1%) to dye for 30 min at 4°C without exposure to light. Detect 5000 cells with BD FACSCalibur, calculate the number of cells in G0/G1 phase, S phase, G0/M phase, observe the distribution features of cell cycle.

Statistic analysis: The experimental results are shown by $\bar{x}\pm SD$ with SPSS 11.5 for Windows statistical software being used to analyze data. Single factor material was verified by t-test, the materials between different groups were analyzed by one way ANOVA. The data has statistical significance in the condition of $p<0.05$.

RESULTS

The vitality test: After the unicell suspension went through dyeing with trypan blue, colorless transparent living cells and light blue dead cells can be seen through microscope. According to the formula of living cells rate, the rate of living cells is calculated at between 94.33 and 97.67%. In general requirement, the rate of living cells cultured *in vitro* should be not <95%. Therefore, the cell suspension with a rate of living cells >95% can be chosen to culture.

The identification of purity: After cell suspension with a living cells rate >95% goes through dyeing by 3β-HSD, blue corpuscular positive cell can be observed through microscope. The result of calculation is that the rate of 3β-HSD positive cells is above 90% which meets the test requirement that the purity of leydig cells should not be <90%.

The reactivity of leydig cells cultured *in vitro*: From Table 1, it can be observed that when exposed to stimulations of different concentrations of hCG, the testosterone secretion of leydig cells cultured *in vitro* will increase with the increases of hCG concentration. Compared with the control group, 5 IU mL^{-1} hCG can increase the testosterone secretion of $p<0.05$ which reaches the peak at 50 IU mL^{-1} when the concentration increases further, the testosterone secretion does not change very much. Thus, the functions of leydig cells in this experiment are fine.

The expression of c-jun mRNA in leydig cells after treatment with different concentrations of c-jun ASODNs: Ct value of each gene was obtained through analysis of Bio-Rad CFX Manager software and gene expression analysis (Table 2) was conducted according to $2^{-\Delta\Delta Ct}$ method from which it has been concluded that compared with the control group, any concentration of antisense tat ODNs has no effect on the expression of c-jun mRNA but c-jun ASODNs can significantly reduce the expression of c-jun mRNA ($p<0.01$). At the same time, compared with each antisense tat ODNs group of different concentrations, the c-jun ASODNs of corresponding concentration has extremely blocking function to the expression of c-jun mRNA ($p<0.01$). When the concentration of c-jun ASODNs reaches 1 μmol L^{-1}, the expression of c-jun mRNA no longer decreases.

Table 1: Effect of different doses of hCG on testosterone synthesis in leydig cells

hCG concentration (IU mL^{-1})	Testosterone content (ng mL^{-1})
0	2.99±0.15
5	4.64±0.65[a]
10	7.01±0.82[a]
25	9.38±1.01[a]
50	22.85±1.77[b]
100	20.63±0.94[b]

Compared to the control group; [a]$p<0.05$ significant difference; [b]$p<0.01$ extremely significant difference

Table 2: Expression of c-jun mRNA of leydig cells after being treated by c-jun ASODNs of different concentrations

Groups	Δct (c-jun-GAPDH)	$-\Delta\Delta Ct^{\diamond}$	$2^{-\Delta\Delta Ct}$
Control group	0.95±0.10	0	1.00±0.06
0.125 μmol L^{-1} tat ODNs	0.81±0.23	0.14±0.26	1.10±0.20
0.25 μmol L^{-1} tat ODNs	0.70±0.13	0.25±0.17	1.19±0.14
0. 5 μmol L^{-1} tat ODNs	0.79±0.03	0.15±0.13	1.11±0.10
1 μmol L^{-1} tat ODNs	0.76±0.02	0.18±0.11	1.14±0.09
2 μmol L^{-1} tat ODNs	0.87±0.20	0.07±0.29	1.05±0.21[a]
0.125 μmol L^{-1} c-jun ASODNs	2.89±0.18	-1.94±0.16	0.26±0.03[a]
0.25 μmol L^{-1} c-jun ASODNs	3.75±0.46	-2.80±0.56	0.14±0.06[a]
0.5 μmol L^{-1} c-jun ASODNs	5.12±0.69	-4.17±0.61	0.06±0.02[a]
1 μmol L^{-1} c-jun ASODNs	6.31±0.35	-5.36±0.45	0.02±0.01[a]
2 μmol L^{-1} c-jun ASODNs	6.66±0.71	-5.72±0.79	0.02±0.01[a]

[a]$p<0.01$; [a]Indicates that the expression of c-jun mRNA is significantly lower than that in the control group and that in the tat ODNs group with corresponding concentration; $^{\diamond}$Target sample - control sample

The effect of c-jun ASODNs of different concentrations on leydig cell proliferation: From Fig. 1, the inhibition rate of leydig cell proliferation increases with the rise of c-jun ASODNs concentration but this trend was not seen in the antisense tat ODNs group of corresponding concentration. c-jun ASODNs can significantly suppress the proliferation of leydig cells ($p<0.01$) and compared with tat ASODNs group, the difference was remarkable ($p<0.01$). It is also found that the concentrations of c-jun ASODNs are obviously positive correlated with growth inhibition rate ($r = 0.8966$). When the concentration of c-jun ASODNs was 2 μmol L^{-1}, the inhibition of leydig cell proliferation has decreased but compared with 1 μmol L^{-1} c-jun ASODNs, the difference was not significant ($p>0.05$).

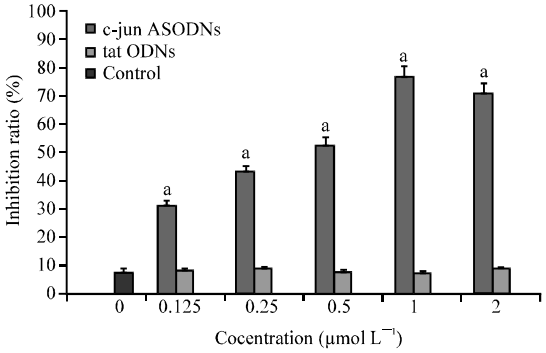

Fig. 1: Effect of different concentrations of c-jun ASODNs on proliferation in leydig cells. a indicates that c-jun ASODNs significantly inhibits the growth rate of leydig cells' proliferation, compared with control group and corresponding concentration tat ODNs group ($p<0.01$)

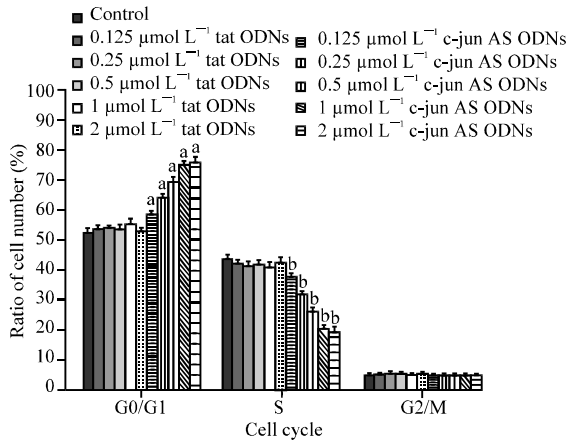

Fig. 2: Effect of different c-jun ASODNs contrations on the cell cycle of leydig cells. a and b represent that the cell number has significant difference between control group and corresponding concentration tat ODNs group ($p<0.01$)

The effect of different concentrations of c-jun ASODNs on leydig cell cycle: As is shown in Fig. 2, compared with the control group, 0.125 μmoL L^{-1} of c-jun ASODNs can significantly reduce the number of leydig cells that enter into S phase ($p<0.01$) and the cell number blocked in G0/G1 phase increased, respectively by 5.78, 11.36, 16.73, 22.25, 23.36% for each. The blockages of c-jun ASODNs to leydig cell cycle mainly occur in G0/G1 phase.

DISCUSSION

The evaluation for leydig cell *in vitro* can be confirmed through its vigor, purity and testosterone secretion. Typan blue rejection experiment was chosen to test the vigor of leydig cell. Generally speaking, the rate of living cells cultured *in vitro* should not be <95%. In addition, the synthesis of testosterone from leydig cell is a series of enzymatic reaction which mainly includes cytochrome P450 family (such as P450 scc, P450 c17, etc.) and hydroxyl steroids dehydrogenation enzymes (such as 3-HSD, 17-HSD, etc.). As the key enzyme that induces the catalysis of isomerization reactions between D^5 and D^4, 3β-HSD has organization specificity in its expression (Conley and Bird, 1997). Therefore in the test, the purity of leydig cell *in vitro* was determined by 3β-HSD test. The main functions of leydig cell are synthesis and secretion of Testosterone (T) which is controlled by LH or hCG. So, the reaction of leydig cell to hCG stimulation was selected as the function identification index. In the test, the leydig cells were stimulated with concentrations of 0, 5, 10, 25, 50 and 100 IU mL^{-1}. At the beginning, the secretion of testosterone increased with the increase of concentrations but stabilized when the concentration reaches 50 IU mL^{-1}. HCG receptors reside in leydig cell membranes when hCG combines with its receptors, a series of reactions will start, triggering the regulation of testosterone secretion. The numbers of hCG were less than the number of receptor at the beginning and hCG promoted the secretion of testosterone. When the concentration of hCG is very high, the receptors are saturated and testosterone secretion no longer increases. In addition, the fact that hCG can stimulate the number of testosterone secretion progressively with the increase of concentration can also illustrate that the function of enzymes related to leydig cells' testosterone synthesis is in good condition. To sum up the vigor, purity and function state of leydig cells meet the requirements for the test, therefore they are suitable to study the related mechanism of secretion of testosterone in the following research.

Effective control of the expression of c-jun is the key to studying the effects of c-jun on leydig cell proliferation. ASODN is antisense DNA sequences which can be complementary with specific target mRNA or target DNA. It can specifically inhibit the translation of target mRNA without affecting the normal expression of other near genes, thus stopping expression of the corresponding genes which makes it possible to separately study the influence of target genes on cell development, the cell cycle and the cell function (Deng and Bao, 2003). Artificially synthesized antisense c-jun ASODNs was used to block the expression of c-jun mRNA in this experiment and its blocking effect was detected by fluorescence quantitative RT-PCR (Zhu *et al*., 2009). The results showed that 0.125 μmol L^{-1} of c-jun ASODNs can significantly block the expression of c-jun mRNA and the

blocking effect was enhanced with the increase of concentration but when the concentration reaches 1 μ mol L^{-1}, the blocking effect levels off. This suggests that c-jun ASODNs can effectively control the expression volume of c-jun mRNA. Shuanghu Yuan uses c-jun ASODNs to study the effect of proto-oncogene c-jun on Leydig cell's testosterone secretion in basic state in rats. In his study, 0.03125, 0.125, 0.5 and 1 μmol L^{-1} doses of c-jun ASODNs were chosen which can significantly block the expression of c-jun (Yuan *et al*., 2003).

As one of the transcription factors in AP-1 family, c-jun has a wide control range, for example the central nervous system, bone tissue, cardiovascular system, reproductive system and so on. But, the regulating effect mainly takes place in the transcription. At first, after c-jun is itself transcribed and coded, it expresses the corresponding protein jun which can form by means of leucine zipper, homologous or heterogenous dimers to compose the active AP-1. Next AP-1 can be combined with the DNA specificity sequence to regulate the expression of some genes and accordingly regulate a series of physiological processes. For instance, it can identify and combine the transcription of related genes, such as DNA cis-regulatory elements TRE activing collagen enzyme and cycle protein. And it participates in the regulation of cell proliferation (Fang and Zhang, 2002). In order to validate the effect of c-jun on leydig cell proliferation in this study, researchers observed the effect on cell proliferation activity and cell cycle of the blocked c-jun expression in leydig cell. The results found that along with the increase of c-jun ASODNs concentration, the growth inhibition rate of leydig cell increases gradually and the number of cells that were blocked between G/G1 phases and entered into S phase went down significantly. This shows that c-jun can promote the growth of leydig cell and in the cell cycle it promotes the transition from G0/G1 phase to S phase. A mass of researches indicate that c-jun can promote the secretion of testosterone in leydig cell. There are 2 major ways to enhance the secretion of testosterone in leydig cell. Enhance the ability of individual cell to systhesize testosterone and increase the total number of leydig cells that secrete testosterone (Xiao *et al*., 2004). Shuanghu Yuan reported that the c-junASODNs could dose-dependently inhibit leydig cell's testosterone in rats in basic state (Yuan *et al*., 2006). So, c-jun possibly regulates the secretion of testosterone in leydig cell through controlling the number of leydig cell (such as cell proliferation). And such regulation is reflected in the process of transition from G0/G1 phase to S phase in cell cycle. About the control of cell cycle, the core is the time-phase activation of Cdks which mainly depends on the expression of cyclins. In different cell cycles, cyclins differs in the amount and kinds of expression but the expressions of Cdks in the whole cell cycle are almost always the same (Gao *et al*., 2010). So, the change of cell cycle depends on the expression of cyclins. In the G1 phase, cyclin D (including D1-D3) and cyclin E are mainly expressed, they can respectively combine and activate Cdk 4/6 and Cdk 2 to promote the change from G1-S. The peak expression level of cyclin D appears only in G1 phase, its expression can increase after the stimulation from growth factors (Morgan, 1997). As effect factor of the signal pathways of Mitogen Activated Protein Kinase (MAPK) stimulated by growth factors, it has the likelihood to promote the expression of cyclin D. So, it is well-founded that the experiment has proved that c-jun can promote the process of the G1/S phase in leydig cell cycle. In addition, the ell cycle inhibitory protein (CKI) can inhibit the activity of cdks, the downregulated expression of CKI can increase the activity of Cdks. CKI not only can combine with cdk alone but also can combine with Cdk-cyclin compound. For example, INK4 family (a-d) can be peculiarly combined in Cdk 4/6 and stop its combination with cyclinD; Cip/Kip family (Wafl/Cip1, Cip2, Kip2) can particularly be combined with Cdk4/6-CyclinD compound in G1 phase. So, further research is needed for the function mechanism governing c-juns promotion of G/S transition in cell cycle.

CONCLUSION

As is detected by flow cytometer, researchers found that the number of leydig cells in S phase decreased significantly and that in G0/G1 phase increased obviously ($p<0.01$) after being treated by 0.125 μmol L^{-1} c-jun ASODNs. This shows that c-jun can promote the growthof leydig cell and in the cell cycle it promotes the transition from G0/G1 phase to S phase.

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