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·Original Article·

Erythropoietin gene transfer into rat testes by *in vivo* electropo-ration may reduce the risk of germ cell loss caused by cryptorchidism

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Abstract

Aim: To investigate the effects of rat Erythropoietin (Epo) on spermatogenesis by transferring rat *Epo* gene into cryptorchid testes by means of *in vivo* electroporation. **Methods:** Sprague-Dawley rats with surgically-induced unilateral cryptorchidism were divided into three groups: the first group was given intratesticular injections of pCAGGS-*Epo* (pCAGGS-*Epo* group), the second group was given intratesticular injections of pCAGGS (pCAGGS group), and the third group were given intratesticular injections of phosphate-buffered saline (PBS group). At the same time, square electric pulses of 30 V were applied six times with a time constant of 100 ms. One or two weeks after injection, each testis was weighed and the ratio of the total number of germ cells to that of Sertoli cells (G/S ratio) was calculated to evaluate the impairment of spermatogenesis. Ten testes taken from each of the three groups were examined at each time point. **Results:** The testicular weight after the injection of pCAGGS-*Epo* or pCAGGS control plasmid was (0.85 ± 0.08) g and (0.83 ± 0.03) g, respectively, at week 1 (P = 0.788) and (0.62 ± 0.06) g and (0.52 ± 0.02) g, respectively, at week 2 (P = 0.047). At week 1, spermatids and sperm were more abundant in testes with pCAGGS-*Epo* than those in the control testes. At week 2, spermatids and sperm were hardly detected in either group. The G/S ratio was 23.27 ± 6.80 *vs*. 18.63 ± 5.30 at week 1 (P = 0.0078) and 7.16 ± 3.06 *vs*. 6.05 ± 1.58 at week 2 (P = 0.1471), respectively. **Conclusion:** The transfer of *Epo* to rat testes by *in vivo* electroporation may reduce the risk of the germ cell loss caused by cryptorchidism. *(Asian J Androl 2005 Dec; 7: 369–373)*

Keywords: electroporation; gene transfer techniques; erythropoietin; spermatogenesis; *Epo*

1 Introduction

Impaired spermatogenesis is an adverse effect of cryptorchidism in many mammals; however, the impairment can be reversed by orchidopexy soon after birth [1]. One of the models widely used to investigate testicular function was the experimental cryptorchid model. In rats, testicular weight has been found to decrease in 4 days to 2 weeks after an experimental induction of cryptorchidism [2]. Histopathologically, seminiferous tubules were reduced in diameter, and only a few tubules showed active spermatogenesis [3].

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Erythropoietin (Epo), a hematopoietic cytokine, regulates erythrocyte production by acting on the proliferation, differentiation, and apoptosis of erythroid progenitor cells [4]. It has been demonstrated that Epo stimulated steroidogenesis in the testis. It influenced Leydig cell steroidogenesis of rat *in vitro* by stimulating testosterone production [5], and intravenous Epo administration increases testosterone production [6], although the mechanism by which this occured has not been completely clarified.

In vivo gene transfer techniques have become popular tools in gene therapy and biological analysis to the various organs of rats, mice and others and several different methods have been developed thus far [7]. Virus-mediated gene transfer was the most widely used as it has a high gene transfer rate; however, it was also a high-risk biohazard. Although non-viral vectors such as lipid-mediated systems were safer and easier, the transfection efficiency rate was relatively low [8]. *In vivo* electroporation has been shown to be an efficient method of transferring genes to the tissues of living animals [9]. This system indiscriminately delivered DNA segments to any type of tissue cell and had markedly higher transfer efficiency rates than other non-viral transfer systems.

We investigated the effects of rat Epo on spermatogenesis by transferring rat *Epo* genes into cryptorchid testes by means of *in vivo* electroporation.

2 Materials and methods

2.1 Plasmid vectors

Plasmid pCAGGS-*Epo* [10] was constructed by inserting rat *Epo* cDNA into a unique XhoI site between the CAG promoter and the 3'-flanking sequence of rabbit *β-globin* genes in the pCAGGS expression vector [11]. The empty pCAGGS was used as a control. DNA was diluted in phosphate-buffered saline (PBS) to 1.0 μg/μL immediately before injection.

2.2 Experimental procedures

All experimental procedures were conducted in strict accordance with the Kobe University Guide for the Care and Use of Laboratory Animals. Seven-week-old male Sprague-Dawley rats had cryptorchidism surgically induced [12]. Under general anesthesia (sodium pentobarbital), the right testis of each rat was briefly surgically exposed when a mid-line incision was made, gubernaculum was cut and then the testis was replaced in the abdominal cavity. At the same time, each plasmid DNA (50 μg) diluted in 1.0 μg/μL of PBS was injected into the right testis with a 27-gauge needle connected to a 1-mL capacity syringe. Immediately after injection of the plasmid DNA, we applied square electric pulses of 30 V six times per 100 ms with a disk-type electrodes of an electro-square porator T820 (BTX, San Diego, CA, USA). Later, to evaluate the likelihood of testicular damage, we analyzed the testis sections that had been injected with the 50 μL of PBS solution containing no DNA. The testes were removed so that we could evaluate spermatogenesis and testicular weight at weeks 1 and 2. Ten testes taken from each of the three groups were examined at each time point.

2.3 X-gal staining

Plasmid pCAGGS-*lacZ* expresses *Escherichia coli* β-galactosidase in the cytoplasm [11]. Rats were killed 2 days after the injection of 50 μg of pCAGGS-*lacZ*, and then the testes were rapidly dissected. The testes were fixed in 0.25 % glutaraldehyde and then stained with a 5-brome-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) solution mixture prepared as follows: 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6 \cdot 3H_2O$, 0.2 mmol/L $MgCl_2$, 1 mg/mL X-gal. Sections 4 μm in thickness were mounted on silanized slides, and were then counterstained with eosin.

2.4 Total tissue RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

At weeks 1 and 2 after gene transfer, the rats received either pCAGGS-*Epo* or pCAGGS and then were killed. The total RNA was extracted using a TRIzol reagent (Life Technologies, New York, NY, USA) and treated with RNase-free DNase. Complementary (c)DNA was prepared from 1 μg of total RNA by random priming, using a RNA PCR kit (TaKaRa, Kyoto, Japan) and PCR reactions were performed with a thermocycler (Program Temp Control System PC-800, ASTEC, Fukuoka, Japan). The lengths of the products were 323 bp for *Epo* mRNA and 207 bp for *GAPDH* mRNA. The primer sequences were as follows: *Epo* (sense: 5'-TCTGACTGACCGCGTTACTC-3', antisense: 5'-GACTTTGGTATCTGGGACGG-3'), *GAPDH* (sense: 5'-CTGGCGTCTTCACCACCA-3', antisense: 5'-AGTTGTCATGGATGACCT-3').

2.5 Evaluation of spermatogenesis

The testes were fixed in 10 % buffered formaldehyde, embedded in paraffin for sectioning and stained with hematoxylin and eosin. The numbers of germ cells and Sertoli cells were counted and the ratio of the total number of germ cells to that of Sertoli cells (G/S ratio) was calculated. Although the impairment of maturation or the decrease of numbers of germ cells in the testes with hypospermatogenesis was observed, the number of Sertoli cells was stable. The impairment of spermatogenesis was reflected in the G/S ratio.

2.6 Statistical analysis

The statistical significance of difference was evaluated by unpaired *t*-test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Localization of pCAGGS-lacZ gene expression

X-gal staining was positive in the interstitial cells and the spermatogonium-like cells located close to the basement membrane of the seminiferous tubule in the pCAGGS-lacZ-injected testes (Figure 1). No instances of pathological damage were noted in this treated area.

3.2 RT-PCR detection of Epo mRNA in testes injected with pCAGGS-Epo

Epo mRNA was detected only in the testes injected with pCAGGS-*Epo* and not in those injected with pCAGGS; while control *GAPDH* mRNA was detected in both of the groups (Figure 2). We were also able to confirm the gene transfer into the testes by *in vivo* electroporation.

3.3 Testicular weight

One week after the injection, there was no difference found in the testicular weight between pCAGGS-*Epo*-injected rats ($n = 10$) and pCAGGS-injected rats ($n = 10$): (0.85 ± 0.08) g *vs*. (0.83 ± 0.03) g, respectively ($P = 0.788$). The testicular weight 2 weeks after the injection was significantly heavier in pCAGGS-*Epo*-injected rats ($n = 10$) than that in pCAGGS-injected rats ($n = 10$): (0.62 ± 0.06) g *vs*. (0.52 ± 0.02) g, respectively ($P = 0.047$) (Table 1). The testicular weight where there had been no gene transfer (PBS) was (0.89 ± 0.05) g at week 1 ($n = 10$) and (0.55 ± 0.07) g at week 2 ($n = 10$) after the operation. No difference between the PBS group and pCAGGS group was noted.

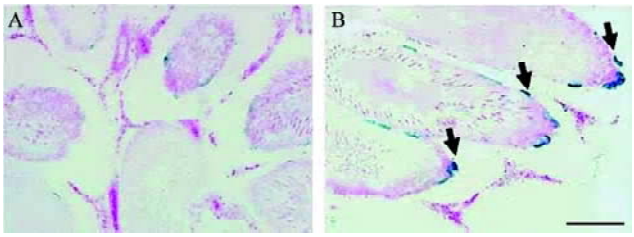

Figure 1. Expression of the *lacZ* gene observed 48 h after transfection of 100 mg of pCAGGS-*lacZ* plasmid to the rat testis by *in vivo* electroporation (A, B × 200). The gene expression was detected by X-gal staining (arrow). Bar = 50 μm

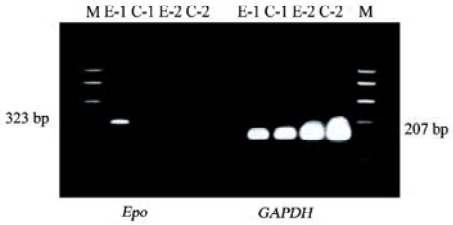

Figure 2. Reverse transcription-polymerase chain reaction (RT-PCR) detection of *Epo* mRNA in testes injected with pCAGGS-*Epo*. Testes injected with pCAGGS-*Epo* (lane E-1) or pCAGGS (lane C-1) were resected 1 week after the gene transfer took place. Testes injected with pCAGGS-*Epo* (lane E-2) or pCAGGS (lane C-2) were resected 2 weeks after the gene transfer took place. The lengths of the products were 323 bp for *Epo* mRNA and 207 bp for *GAPDH* mRNA. Lane M shown DNA size markers.

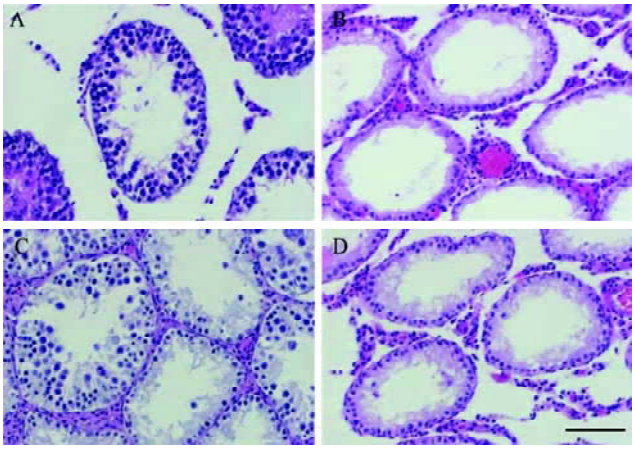

Figure 3. Hematoxylin and eosin staining of testes that were transfected with pCAGGS by *in vivo* electroporation and were resected 1 week after the injection (A) and 2 weeks after the injection (B). Hematoxylin and eosin staining of testes that were transfected with pCAGGS-*Epo* by *in vivo* electroporation and were resected 1 week after the injection (C) and 2 weeks after the injection (D). The magnification is × 200 in A, B, C and D. Bar = 50 μm.

3.4 Histochemical findings

One week after the pCAGGS injection, spermatogonia, spermatocytes, spermatids and a few sperm were detected in the testes (Figure 3A). At week 1, spermatids and sperm were more abundant in the testes with pCAGGS-*Epo* than that in the testes with pCAGGS (Figure 3C). The G/S ratios 1 week after the injections with pCAGGS-*Epo* and pCAGGS were 23.27 ± 6.80 and 18.63 ± 5.30, respectively (P = 0.0078) (Table 1). At week 2, spermatids and sperm were hardly detected in either group (Figure 3B, D). The G/S ratios 2 weeks after the injections with pCAGGS-*Epo* and pCAGGS were 7.16 ± 3.06 and 6.05 ± 1.58, respectively (P = 0.1471) (Table 1). These results demonstrated that the G/S ratio in the pCAGGS-*Epo* group was larger than that in the pCAGGS group at week 1 after the induction of cryptorchidism, but not after 2 weeks. The G/S ratios in the group where there were no gene transfer (PBS) was 19.98 ± 5.37 at week 1 and 6.36 ± 3.14 at week 2, repectively. No difference was seen between the PBS group and pCAGGS group.

4 Discussion

The present study was the first report to demonstrate that the *Epo* gene can be transferred into rat testes by *in vivo* electroporation and that germ cell loss due to cryptorchidism might be reduced to some extent as a result of the transfer. In a previously published report [13], the testicular weight of rats decreased 6 days after the induction of cryptorchidism. Similarly, in this study, the weight of the right testes that had been injected with pCAGGS had decreased by the 7th day after the induction of cryptorchidism. Some reports demonstrated that *Epo* stimulates spermatogenesis or steroidogenesis, for example, Epo might reduce the risk of germ cell loss in boys with cryptorchidism [14]; Epo influenced rat Leydig cell steroidogenesis *in vitro* by stimulating testosterone production [5], and in humans, intravenous Epo administration increased testosterone production [6]. Moreover, a recent report described how Epo receptors were detected in rat Leydig cells [15]. We suggested that in the present study, the reversal of cryptorchidism-induced germ cell loss was the result of the transfection of *Epo* genes into Leydig cells. As described previously, the *lacZ* gene was transfected by *in vivo* electroporation and was expressed in the interstitial cells or spermatogonium-like cells that were located close to the basement membrane of the seminiferous tubule. In the testis, interactions among Sertoli cells, peritubular myoid cells, Leydig cells and germ cells were thought to be essential for spermatogenesis to occur [16]. We suggested that the reversal of cryptorchidism-induced germ cell loss was a result of the interactions of the cells into which the *Epo* gene was transfected.

Table 1. Right testicular weight and G/S ratio in each group.

Group (n)	Testicular weight (g)	G/S ratio
E-1 (10)	0.85 ± 0.08	23.27 ± 6.80[c]
C-1 (10)	0.83 ± 0.03	18.63 ± 5.30
N-1 (10)	0.89 ± 0.05	19.98 ± 5.37
E-2 (10)	0.62 ± 0.06[b]	7.16 ± 3.06
C-2 (10)	0.52 ± 0.02	6.05 ± 1.58
N-2 (10)	0.55 ± 0.07	6.36 ± 3.14

Results are mean ± SD, [b]$P < 0.05$, compared with group C-2; [c]$P < 0.01$, compared with group C-1; E-1: testis injected with pCAGGS-Epo and resected at 1 week after gene transfer; C-1: testis injected with pCAGGS and resected at 1 week after gene transfer; N-1: testis with no gene transfer and resected at 1 week after the operation of cryptorchidism; E-2: testis injected with pCAGGS-Epo and resected at 2 weeks after gene transfer; C-2: testis injected with pCAGGS and resected at 2 weeks after gene transfer; N-2: testis with no gene transfer and resected at 2 week after the operation of cryptorchidism.

Quantitative analysis of germ cell numbers has been used to objectively assess spermatogenesis. Prior studies have demonstrated a significant correlation between sperm concentration in the ejaculate and the total spermatogenesis (sum of all germ cells), total spermatid count (sum of early and late spermatids) and the ratio of late spermatids per Sertoli cell [17]. Such studies have also proposed a simplified and rapid technique, which quantified spermatogenesis by determining the mean number of late spermatids per seminiferous tubule in the human testis [18]. The quantification of germ cell numbers and its relation to Sertoli cell numbers has also been developed and used extensively in rat studies to evaluate spermatogenesis or estimate daily sperm production [19, 20]. The present study demonstrated how *Epo* gene transduction significantly increased the total number of germ cells per Sertoli cell (G/S ratio).

In the present study, we showed that the G/S ratio in rats injected with pCAGGS-*Epo* was larger than that in rats injected with pCAGGS at week 1 after the induction of cryptorchidism, but not at week 2. The testes of the rats in the pCAGGS group weighed less than those of the rats in the pCAGGS-*Epo* group at week 2 after in-

duction of cryptorchidism, but not at week 1. We had thought that Epo could reduce the deleterious effect of surgical cryptorchidism on spermatogenesis, but the impaired spermatogenesis did not show any signs of recovery. At week 1 there was no significant difference in the destruction of seminiferous tubules between the two groups, but the reduction of the number of germ cell in the pCAGGS group was observed more than in the pCAGGS-*Epo* group. It was considered that the difference in the testicular weight would be primarily caused by the loss of seminiferous tubules rather than the pathological changes in the tubules such as the G/S ratio. Therefore, no significant difference was observed in the testicular weight at week 1. At week 2 after the induction of cryptorchidism, the more progressive destruction and loss of seminiferous tubules were observed in the control testes more than that in the *Epo*-transfected testes. Therefore, testicular weight decreased significantly in the control, compared with the *Epo*-transfected testes. In contrast, germ cells themselves in the remaining tubules were more severely impaired by cryptorchidism at week 2 than they were at week 1, resulting in the two groups displaying the same G/S ratio.

In conclusion, we demonstrated that *Epo* gene transfer in rat testes by *in vivo* electroporation was efficient and it might to some extent reduce the loss of germ cells caused by cryptorchidism.

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