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# Evaluation of Apoptotic Genes Expression and Its Protein after Treatment of Cryptorchid Mice

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## ABSTRACT

**Introduction:** Cryptorchidism has been proved to cause apoptosis in germ cells in respond to changes in the stimulation levels of specific physiological events. The purpose of this study was to determine whether treatment of bilateral cryptorchidism was associated with alterations in testicular gene expression. **Methods:** To induce bilateral cryptorchid model, immature mice were anesthetized and a small incision was made in the abdominal skin and peritoneum, then fat pad at the upper end of testis was sutured to the peritoneum. Transcript level of Bax, Bcl-2 proper, p53 and survivin mRNA and protein were determined after performing the two treatment methods: surgical return of testis into scrotum (Exp1) and transplantation of spermatogonial stem cells with later orchidopexy (Exp2), performed 2 and 3 months after heat exposure, respectively. **Results:** RT-PCR data showed decreased levels of p53 and Bax expression as well as decreased levels of Bcl-2 mRNA in treatment groups especially after transplantation compared with control group. The expression of survivin 140 was increased significantly after treatment, whereas that of survivin 40 was lower especially in the orchidopexy group. Immunohistochemistry staining showed that the intensity of Bax expression mainly was decreased in treated cryptorchid testis and rates of Bcl-2 were increased significantly, but expression of p53 and survivin proteins did not changed significantly after treatment. **Discussion:** These observations suggest that cell-type-specific and many apoptotic systems control germ cell apoptosis after treatment of cryptorchidism. *Iran. Biomed. J. 16 (2): 77-83, 2012*

*Keywords:* Gene expression, Transplantation, Apoptosis

## INTRODUCTION

Complex network of signals are associated with male germ cells when prepared to survive or die [1]. Like other cell types, male germ cells respond to external signals and to their internal milieu by activating intracellular signaling pathways that revealed their fate [2].

A greater understanding of these signaling pathways is emerging from studies of the spermatogenic defects [3]. Testicular hyperthermia provides an *in vivo* model system to study the regulation of a form of stress-induced germ cell apoptosis and can be used to examine the possible apoptotic gene interactions [4]. It was shown that competitive interactions of the pro- and anti-survival Bcl-2 family proteins regulate the activation of the proteases which dismantle the cell [5]. Our previous work demonstrated that many signal pathways play an important role during germ cell apoptosis in induced cryptorchid testis [6].

Bax, from the Bcl-2 family, is a promoter apoptotic member required for normal spermatogenesis [7]. Bax-knockout mice are infertile as a result of the accumulation of premeiotic germ cells and the absence of mature haploid sperm [8].

Bcl-2 is the first member to be identified of a growing family of genes that regulates cell death in either a positive or negative fashion [9]. It is an important anti-apoptotic mitochondrial protein over-expressed in the germ cells of the heat-stressed testis [10].

The tumor suppressor p53 is a point inducer of apoptosis. This protein is a positive regulator of Bax gene expression [11]. It has been shown that p53 is an inhibitor of cell cycle progression or inducing cell apoptosis in response to stress or DNA damage found in high concentrations in the testis [12].

Among the mammalian apoptosis regulators, the inhibitor of apoptosis protein BIRC-5/survivin plays roles in both apoptosis and with the regulation of chromosome segregation/cytokinesis during mitosis [13]. This gene is a potential molecular marker of

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spermatogenesis whose expression is altered in specific spermatogenic disorders.

It was demonstrated that the level of survivin mRNA expression is correlated with spermatogenic failure in a cryptorchid mouse model [14].

To determine whether members of *Bcl-2* gene changed patterns of expression after spermato-genesis improvement, transcript level of Bax, Bcl-2 proper, p53 and survivin mRNA and its protein were determined after performing the two treatment methods: surgical return of testis into scrotum (Exp1) and transplantation of spermatogonial stem cells with later orchidopexy (Exp2).

Following our previous work [15], we demonstrated that the spermatogonia isolated from a bilateral cryptorchid mouse have the ability to differentiate for regenerating spermatogenesis. On the other hand, while the orchidopexy is a routine practice for cryptorchidism treatment, trans-plantation may thus prove to be a promising technique for preservation of fertility for severely damaged cryptorchid testes that have scarce spermatogonia [15].

## MATERIALS AND METHODS

***Animals.*** Immature NMRI mice (under 2 months of age) were purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran). The mice were anesthetized with an injection of 1.6 ml/kg mixture of ketamine and xylazine. A cut was made along the skin in the right and left upper abdominal region and the adipose tissue of the caput epididymis was sutured to the inner peritoneal wall. In orchidopexy group (Exp1) two and three months after surgery, the right abdominal testis and epididymis of bilateral cryptorchid mice were returned to the scrotum through the inguinal canal; sutures were used to connect the organ to the scrotum. In Exp2 group, donor cells obtained from testis of bilateral cryptorchid mice 8 weeks after surgery were transplanted to transplantation group (Exp2), and then testis returned to the scrotum. The mice that were grown with other groups with the same conditions were used as a control group. Experimental and control animals were sacrificed by cervical dislocation at 2 and 8 weeks after treatment. In each group, we examined at least 5 animals. All the animal experimentation protocols were approved by the Institutional Animal Care and with the help of the Committee of Tarbiat Modarres University (Tehran, Iran).

***Reverse transcription polymerase chain reaction analysis (RT-PCR).*** Total RNA was extracted from the testis tissue with RNX plus™ kit (CinnaGen, Tehran, Iran) according to the manufacturer's recommendations. Reverse transcription was performed with a Revers transcription kit (Fermentas, Tehran, Iran). One microgram of total RNA was used as a template and RT-generated cDNA encoding Bax, Bcl-2 proper, survivin and tumor suppressor protein p53 were amplified with PCR (Fermentas, Tehran, Iran). The sequences of the primers for the amplification of cDNA were as follows:
The primers were designed as follows (5′→3′):

β2 microglobulin (NM-009735)
Forward: TGACCGGCCTGTATGCTATC
Reverse: CACATGTCTCGATCCCAGTAG

Bax (NM-007527)
Forward: GCTGCAGACATGCTGTGGATC
Reverse: TCACAGCCAGGAGAATCGCAC

Bcl-2 (NM-00177410)
Forward: ACCGTCCTGACTTCSCACAS
Reverse: CGTGTGCAGATGCCGGTTCCA

P53 (NM-011640)
Forward: AGAGACCGCCGTACAGAAGA
Reverse: GCATGGGCATCCTTTAACTC

Survivin (AF-115517)
Forward:
TCGCCACCTTCAAGAACTGGCCCTTCCTGGA

Reverse1:
GTTTCAAGAATTCACTGAVGGTTAGTTCTT
Reverse 2: GGCTTCTGACAATGCTTG

In this study, the expressions of three survivin mRNA variants were examined. Primer srv86 (5′-TCGCCACCTTCAAGAACTGGCCCTTCCTGGA), when paired with primer srvas311 (anti-sense 5′-GTTTCAAGAATTCACTGAVGGTTAGTTCTT) was expected to generate PCR amplicons of 225 bp (survivin 140) or 144 bp (survivin 40), depending on the mRNA splice form. The primer srvas86, when paired with srvas6380 (anti-sense 5′ GGCTTCTGA CAATGCTTG), was expected to generate a PCR amplicon of 332 bp (survivin 121).

***Immunohistochemistry analysis.*** For immune-histochemical staining, the sections were first deparaffinized and rehydrated, then washed in PBS for 5 minutes. The sections were placed into 2N HCl at 95°C for 30 min. Subsequently, they were washed in PBS for 5 minutes. After being blocked in 3% Triton in PBS with normal goat serum (Sigma-Aldrich, Germany) at 37°C for 30 min, the sections were treated with monoclonal antibody against Bax (1:100, Sigma-Aldrich, Germany), Bcl-2 proper (1:200, Chemichon,



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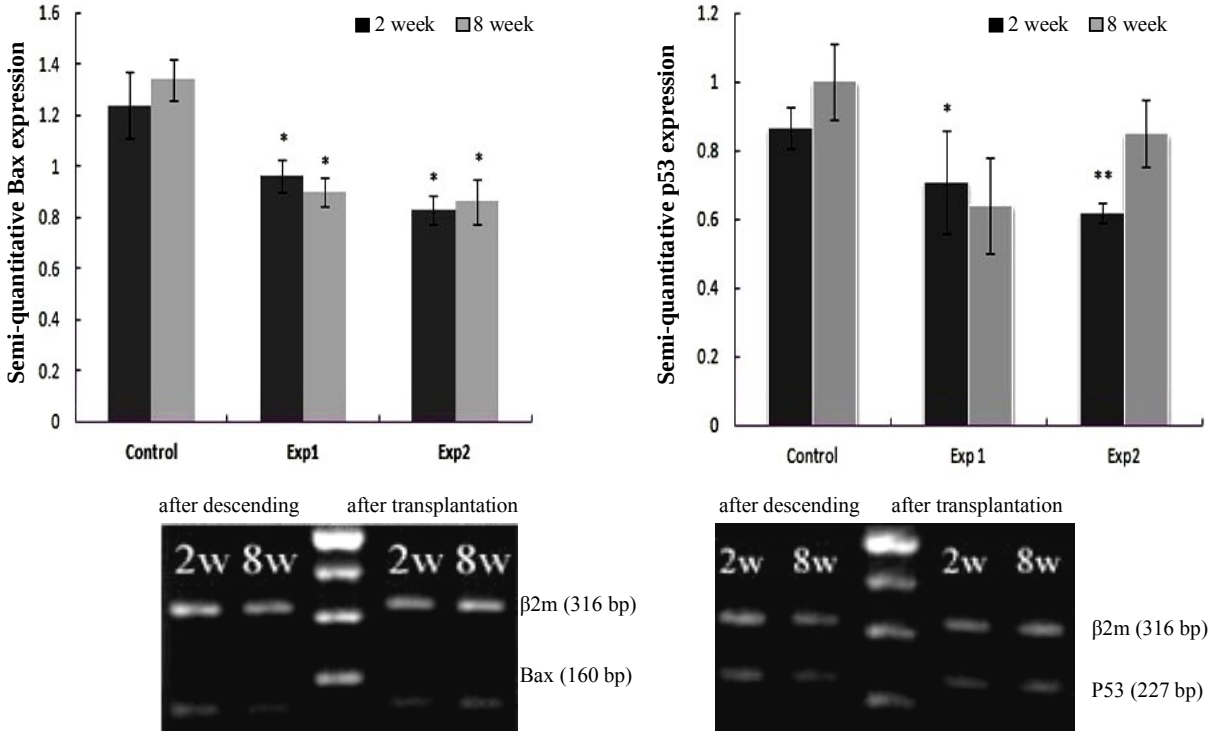



**Fig. 1.** Expression of Bax and p53 mRNA after treatment in cryptorchid mouse testis. mRNA levels were determined by semi-quantitative analysis to $\beta_2$m in the control, Exp1 (after descending) and Exp2 (after transplantation) groups. *, significant difference with control group; **, significant difference with the after descending group.

UK), p53 (1:20, Chemichon, UK) and survivin (1:500, Sigma-Aldrich) and left at 4°C for 48 h. After being extensively washed in PBS, the second antibody FITC (1:100, Sigma-Aldrich, Germany) was applied at room temperature for 2 h. The sections were extensively washed in PBS and mounted with glycerol phosphate. Control staining comprised the same process excluding the reaction against the Bax, Bcl-2 proper, p53 and survivin antibodies.

***Statistical Analysis.*** The quantified gel results were measured with UVIdoc software, then analyzed by performing ANOVA and Tukey's tests with $P<0.05$, considered as statistically significant. The mean and standard deviation was also calculated for each value.

## RESULTS

***Expression of Bax and p53 mRNA after treatment in cryptorchid mouse testis.*** To detect the semi-quantitative expression and variation of Bax and p53 mRNA in the testis of the treated cryptorchid mice, their mRNA levels were analyzed 2 and 8 weeks after treatment by semi-quantitative RT-PCR method. Statistical analysis showed that Bax expression in treated groups in comparison to control group was decreased significantly in 2 and 8 weeks after treatment. Expression of p53 in Exp1 group in comparison to control and Exp2 groups was significantly decreased after 2 weeks (Fig. 1).

***Expression of Bcl-2 proper and survivin mRNA after treatment in cryptorchid mouse testis.*** In comparing with control group, the Bcl-2 proper mRNA level in the Exp2 group was decreased significantly after 2 weeks. The mRNA expression level of survivin 140 was increased significantly especially after transplantation when compared with the control group. However, survivin 40 mRNA showed a decrease after orchidopexy and after transplantation and expression of survivin 40 was increased gradually (Fig. 2).

***Expression of Bax and p53 proteins after treatment in cryptorchid mouse testis.*** Analysis of gene product, determined by immunohistochemistry, indicated a decrease in Bax expression after treatment as compared with the control group. Cytoplasmic expression of p53 protein was observed in different germ cells of seminiferous tubules. Decrease of p53 expression in two treatment groups compared with the control group has been shown in Figure 3.



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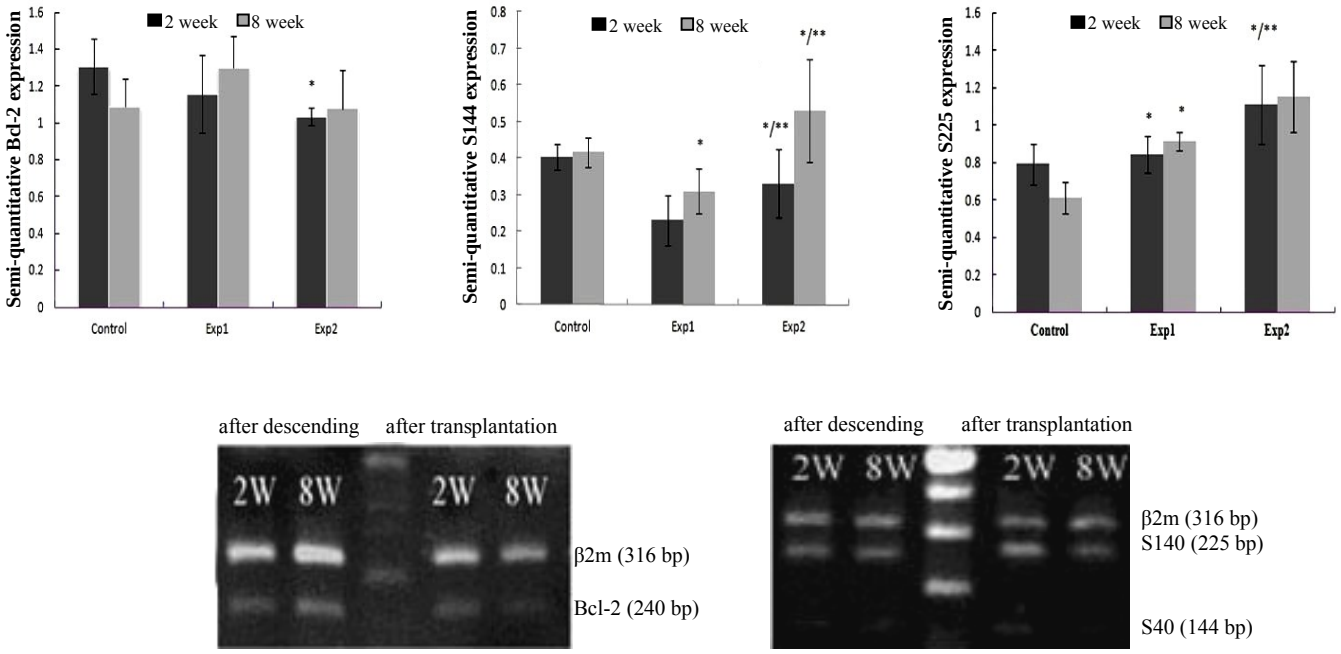



**Fig. 2.** Expression of Bcl-2 proper and survivin mRNA after treatment in cryptorchid mouse testis. mRNA levels were determined by semi-quantitative analysis to $\beta_2$m in the control, Exp1 (after descending) and Exp2 (after transplantation) groups. *, significant difference with control group. **, significant difference with the after descending group.

***Expression of Bcl-2 and survivin proteins after treatment in cryptorchid mouse testis.*** In Exp1 group, Bcl-2 expression was decreased significantly compared with the control group, but in Exp2 group, Bcl-2 expression was increased in comparison with control and Exp1 groups. Different expressions of survivin protein were shown in nucleus and cytoplasm of seminiferous tubular germ cells. In two treatment groups, survivin expression was diminished when compared to the control group (Fig. 4).

## DISCUSSION

Testicular hyperthermia provides an *in vitro* model system to study the regulation of a form of stress-induced germ cell apoptosis and can be used to examine the possible protein-protein interactions within the Bcl-2 family [6].

To determine whether members of *Bcl-2* gene changed patterns of expression during germ cell proliferation and during differentiation, quantitative analysis was conducted by RT-PCR and immune-histochemistry after treatment of this model.

Zini *et al.* [16] showed in their experimental study that cryptorchidism results in apoptosis, and orchidopexy reduces the level of apoptosis in undescended testis. The most common treatment for this condition over the last 50 years has been surgical correction by orchidopexy [17]. We attempt, for the first time, to define the role of apoptotic genes in a cryptorchid mouse model using a germ cell transplantation technique, established by injecting donor germ cells into seminiferous tubules of recipient mice [18]. This technique was used to evaluate the apoptotic pathways through reconstitution of spermatogenesis. According to the previous investigation [19], p53 was suggested to take part in a number of pathways that were shown to be associated essentially with the maintenance of genome stability. P53 seemed to control cell growth arrest, induction of apoptosis: cell differentiation and DNA repair [19]. Based on this data, we investigated the expression of the p53 mRNA and p53 protein. Our findings in the present study showed down-regulation of p53 mRNA after treatment. The reduction of p53 level in different phases would reflect the regeneration of germ cells and recovered spermatogenesis [20]. It was shown that the expression of Bax was carried out in association with p53, since the p53 protein acts as a Bax promoter [11].

Immunohistochemically, we showed a weak cytoplasmic Bax staining of tubular cells in general, but a remarkable finding in the present study is the preferential Bax staining in spermatogonia and spermatocyts. This finding is in accordance with that of Rodriguez *et al.* [21] that showed in the testes of



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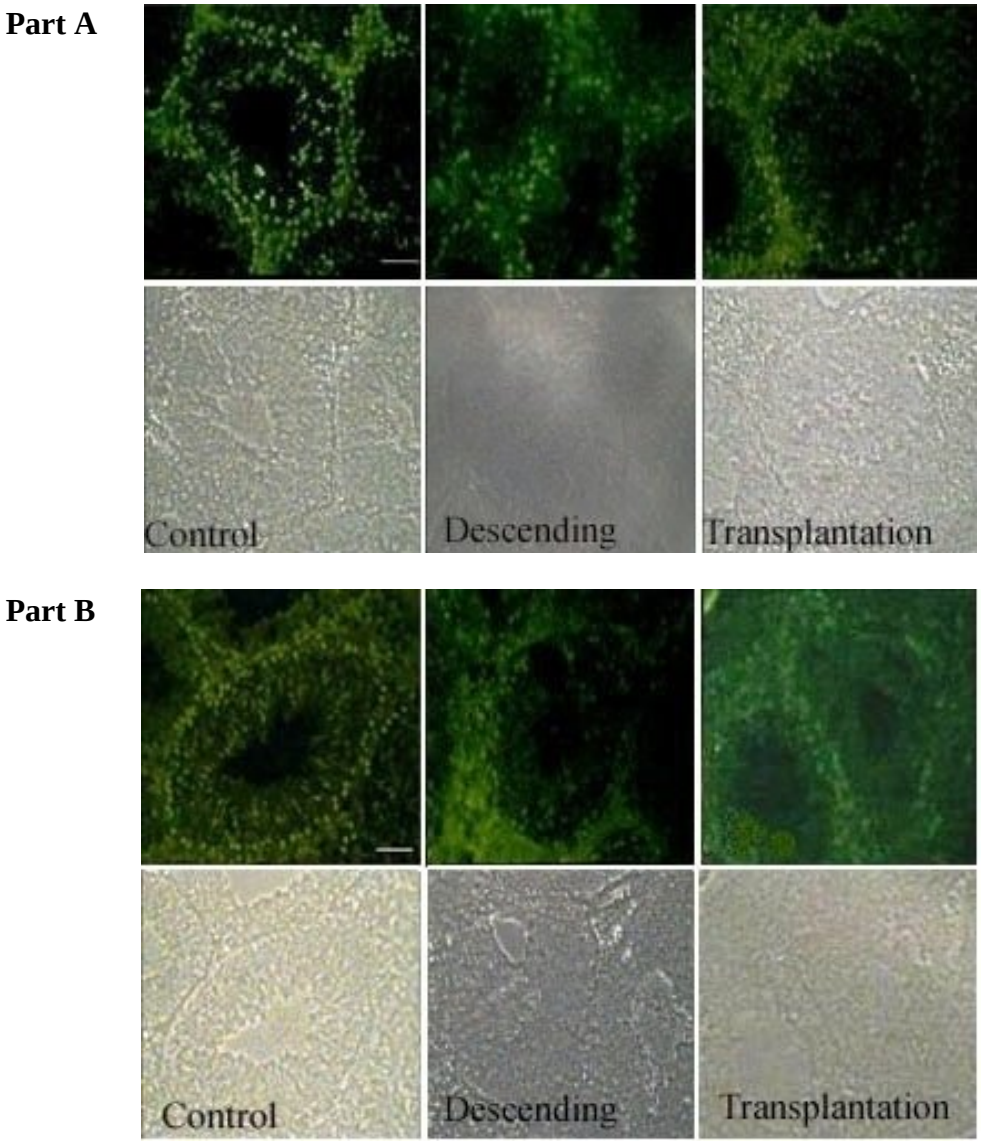



**Fig. 3.** Immunohistochemical analysis of Bax (partA) and p53 (part B) protein expression in the testis of mouse in the control, Exp1 (after descending treatment) and Exp2 (after transplantation treatment) groups.

normal adult mice, Bax is expressed at low levels and is restricted to spermatogonia [22]. The distribution of Bax expression in this study suggests that Bax is important for germ cell differentiation and maturation in mice. Gradually, decrease of Bax expression after treatment can establish the anti-apoptotic role of this gene during reconstruction of spermatogenesis.

Among most of the Bcl-2 family members expressed in the testis, Bcl-2 was not detected in the testis, but was found in mature spermatozoa in the epididymis [23]. Upregulation of Bcl-2 protein after transplantation may indicate that Bcl-2 promotes cell survival by inhibiting apoptosis [4].

A recent *in vitro* study has demonstrated that Bcl-2 proper is a substrate for the key executioner caspase such as caspase-3 that cleaves the C-terminal domain of the Bcl-2 proper during apoptosis and abolishes its anti-apoptotic activity [24].

The presence of distinct isoform of survivin, that are expressed differentially, suggests that survivin plays a complex role in regulating apoptosis. There is no direct evidence whether survivin acts as an inhibitor of apoptosis in testis.

BIRC-5/survivin RNA and protein exhibit rhythms of expression throughout the seminiferous epithelial cycle [26]. Therefore, it is reasonable to speculate that the differential expression of survivin might affect the balance between cell proliferations and programmed cell death.

We suggest that decreased levels of p53 and Bax and the increase survivin levels might represent the mechanisms by which germ cell apoptosis declines after treatment of cryptorchidism.



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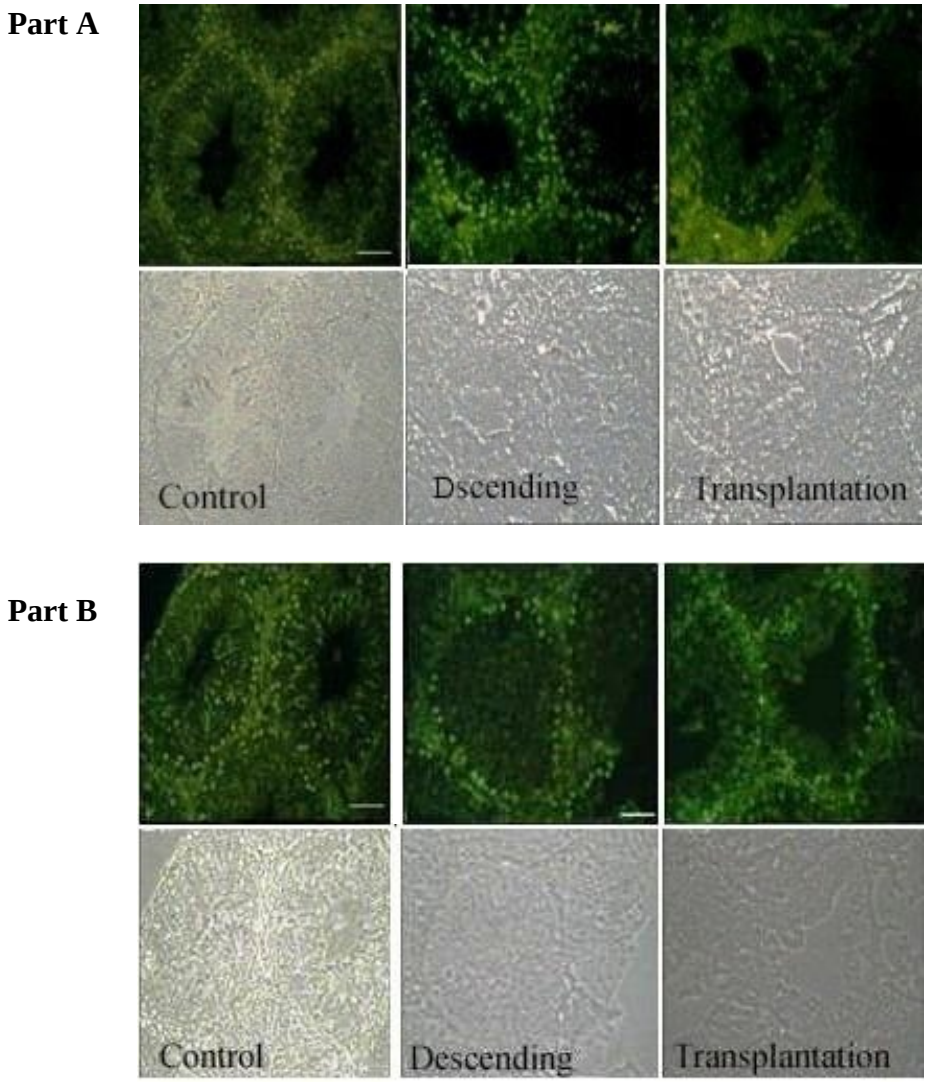



**Fig. 4.** Immunohistochemical analysis of Bcl-2 proper (part A) and survivin protein (part B) in the testis of mouse in the control, Exp1 (after descending treatment) and Exp2 (after transplantation treatment) groups.

According to Yan findings [25], our immune-histochemistry staining showed that the germ cell types, that are the most prone to die during spermato-genesis, are spermatogonia and spermatocytes.

We provided evidence for the involvement of pro- and anti-apoptotic genes in reconstruction of spermatogenesis. Furthermore, understanding of the regulation of germ cell apoptosis may allow new targeted approaches to male contraceptive development and treatment of some forms of male sub-fertility.

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