

Preparing Rat Spermatogonial Transplantation Recipient by Treating the Pregnant Female Rats with Busulfan

¹Boyang Yu, ¹Fenhua Luo, ^{1,2}Taodi Liu, ¹Daiyan Liu, ¹Linhong Liu
¹Yan Zhang and ¹Yingji Wu

¹Key Laboratory of China Education Ministry for Research of Mammal Reproductive Biology and Biotechnology, Inner Mongolia University, 010021 Hohhot, China

²Inner Mongolia Medical College, 010050 Hohhot, China

Abstract: Successful spermatogonial stem cells transplantation is largely dependent on the preparation of the recipient. In the present study, to acquire more efficient rat recipients, the response of pregnant female rats to treatment with 7.5-12.5 mg busulfan per kg of body weight was determined in terms of the birth rate, survival rate, body weight, testicular mass, histology, expression of certain germ cell genes as measured by real time PCR and fertility rate of male offspring. The analysis suggested that a dosage of 10 mg busulfan per kg administered to pregnant female rats at 13.5 day post pregnancy effectively prepares male pups to be recipients because it leads to the maximal deletion of endogenous spermatogonial stem cells with minimal effects on the recipients' health.

Key words: Busulfan, germ cells, infertility, rat, spermatogonial transplantation

INTRODUCTION

Spermatogenesis is a complex process by which stem cells in the seminiferous tubules of the testes develop into male gametes (spermatozoa) (Wang *et al.*, 2010). Studies of spermatogenesis have become more common since a new technique, spermatogonial transplantation was established. Donor germ cells are transplanted into the testes of infertile recipient animals where they generate donor derived colonies of spermatogenic cells after which donor derived sperm cells develop (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Spermatogonial transplantation which has been widely used to study the process of spermatogenesis and cell-cell interactions in the testes has high potential for preserving the genetic stock of endangered species for restoring fertility in infertile animals and for producing transgenic animals with genetically modified germline cells and it is also a potentially powerful tool in assisted reproductive technology (Brinster, 2002).

Successful SSC transplantation is largely dependent on the preparation of the recipient which involves the destruction and blockade of endogenous germ cells and spermatogenesis to allow donor SSCs to translocate from the lumen to the basal compartment of the seminiferous tubules and begin donor derived spermatogenesis (Johnston *et al.*, 2000). The efficacy of

donor SSC engraftment is increased by treatments that remove endogenous stem cells and increase niche accessibility. Ideally, the cells most affected by treatment should be the type A spermatogonia and in particular, the As spermatogonia which is the stem cells of the testes. Several methods have been used in different species to prepare effective recipients. The best mouse recipient model is the dominant White spotting (W) homozygous mutant male which is congenitally infertile and lacks endogenous germ cells because of a mutation in the c-kit receptor tyrosine kinase (Clouthier *et al.*, 1996). Several other techniques have been developed to reduce the number of germ cells in the testes including irradiation (Shinohara *et al.*, 2000), experimental cryptorchidism (Jegou *et al.*, 1984) heat treatment (Young *et al.*, 1988; Ma *et al.*, 2011), cold ischemia (Schlatt *et al.*, 1999) Gonadotropin Releasing Hormone (GnRH) antagonist treatment use of genetically sterile animals (Brinster and Zimmermann, 1994) and sterilizing drug administration (Brinster and Avarbock, 1994).

One common method for removing endogenous germ cells from the testes of wild type animals and creating space for donor stem cell engraftment is treatment with a sublethal dose of the chemotherapeutic agent Busulfan, a DNA-alkylating agent (Hemsworth and Jackson, 1963; Brinster and Zimmermann, 1994; Ogawa *et al.*, 1997). Busulfan targets the slowly proliferating As

spermatogonial (Dunn, 1974; Ma *et al.*, 2011). Brinster and Zimmermann suggested that busulfan may disrupt junctions between Sertoli cells and thus aid in the movement of transplanted spermatogonia into the basal compartment (O'Shaughnessy *et al.*, 2008). Busulfan can eradicate almost all of the endogenous germ cells in the testes of a recipient, thereby creating an empty space in the adluminal and basal compartments that is surrounded by Sertoli cells and the Sertoli cell basement membrane (Ogawa *et al.*, 1999a, b). This empty space facilitates the migration and localization of donor SSCs into the testes of the recipient. Injections of busulfan have been carried out in several series of animals such as mice (Brinster, 2002), rats (Jiang and Short, 1995; Ogawa *et al.*, 1999a; Hamra *et al.*, 2002), hamsters (Ogawa *et al.*, 1999b), rabbits, cats, dogs (Dobrinski *et al.*, 1999), cow (Izadyar *et al.*, 2002; Oatley *et al.*, 2002), pigs, horse (Dobrinski *et al.*, 2000), goats (Honaramooz *et al.*, 2003) and monkeys (Schlatt *et al.*, 2002; Nagano *et al.*, 2001). Sensitivity to busulfan is species specific and the sterilizing dose of busulfan is also species specific. In mice, 40-44 mg busulfan per kg of busulfan is the most common range of doses used and it has been proven that pup recipients appear to support higher levels of donor derived spermatogenesis than do adults. Recipient rats are usually prepared by administering two injections of 10 or 15 mg busulfan per kg of busulfan. However, the chemotherapeutic preparation of adult and pup rat recipients has been plagued by problems of high sensitivity to the toxic effects of busulfan and incomplete removal of the endogenous spermatogonia (Brinster *et al.*, 2003). Mouse recipients are prepared by treating the pregnant mouse with 40 mg busulfan per kg busulfan and with busulfan treatment at 12.5 days postcoitum (dpc); treatment has resulted in long term infertility in male offspring. The day of vaginal plug detection was considered as 0.5 days dpc. Bollag first reported that rats given an injection of busulfan late in pregnancy produced sterile offspring (Ogawa *et al.*, 1999a). Kemper CH and Peters PW discovered, nearly 2 decades ago that on postcoital day 13, almost all Primordial Germ Cells (PGCs) had reached the well developed genital ridges in the rat (Ma *et al.*, 2011). Treatment of pregnant female rats with a single dose of busulfan at 13.5 days of gestation resulted in the live birth of male progeny that were permanently infertile (Kemper and Peters, 1987).

In the earlier studies, histological and morphological methods have usually been used to evaluate the effects of treatment. More exact testing technologies are required. In the present study, researchers utilized special cell marker genes to evaluate the effects of the treatment. CDH1 (E-cadherin) has been identified and has greatly facilitated the study of undifferentiated spermatogonia

during spermatogenesis (Zhang *et al.*, 2011; Tokuda *et al.*, 2007). SCP3 is a special gene expressed in the nuclide of the spermatocytes (Schalk *et al.*, 1998; Scherthan and Schonborn, 2001) and TNP2 is expressed specifically in spermatid cells (Marret *et al.*, 1998). WT1 is expressed specifically in sterol cells. Researchers detected the relative quantities of these four genes to test the relative numbers of undifferentiated spermatogonia, germicides, spermatids and sterol cells.

Dose response relationships for busulfan have been examined only in mice (Wang *et al.*, 2010; Moisan *et al.*, 2003; Zohni *et al.*, 2011), therefore the aim of the present study was to find a suitable dose of busulfan for depleting the seminiferous tubules of germ cells in male rat offspring which was determined by measuring the mortality rates at the various doses and by assessing testicular mass, morphology and expression of spermatogonial cell specific genes over the time period critical for transplantation as well as the fertility of the male offspring.

MATERIALS AND METHODS

Animals: The 26 female and 26 male Wistar rats (8-10 weeks old) were used in this research. The rats were obtained from the Inner Mongolia University Laboratory Animal Center (Hohhot, China) and the protocol for animal use in the present investigation was approved by the university's institutional animal care and use committee. The rats were maintained in a germ free isolation facility at 22±1°C with 70% humidity under a light: dark cycle of 12:12 h. The food and water were autoclaved and were available *ad libitum*.

Busulfan administration: Female rats were placed separately in boxes and were paired with fertile males. The day that spermatozoa were found in the vaginal smear was regarded as day 0.5; the males were then removed and the females were allowed to proceed to term. Groups consisting of at least six animals were given injections of busulfan on day 13.5 postcoitum. The control animals (6 female rats) received a single i.p. injection or were given different doses of busulfan (B-2635; Sigma-Aldrich, St. Louis, MO, USA). The rats received a single i.p. injection of 7.5 mg busulfan per kg (6 female rats), 10 mg busulfan per kg (6 female rats) or 12.5 mg busulfan per kg (8 female rats) busulfan. At 1 and 2 months post partum, at least one F1 male litter from each female was sacrificed for analysis. The remaining males were kept alive to maturity when their fertility was assessed by mating with females of proven fertility. After mating, the male rats were

sacrificed at 6 month and the testes were examined. The survival rates of the animals were calculated at the end of the 4th week after treatment.

Testicular mass and histomorphology: Testes were collected and weighed after removing the epididymis and surrounding fat and then one testis from each rat was fixed in 10% neutral buffered formalin for 24 h at 4°C. The testes from each mouse were embedded in paraffin and 5 µm thick sections were cut perpendicular to the long axes of the testes. Three non-adjacent sections were selected from each testis at an interval of no <100 µm and were stained with hematoxylin eosin. A total of 4 testes from 4 different rats in each group were analyzed at each time point. About >80 seminiferous tubules were scored per section. The seminiferous epithelia of the tubules were classified as follows: no spermatogenesis (only one layer of cells), partial spermatogenesis (depletion of the spermatids in the innermost layer) or full spermatogenesis (showing a complete seminiferous epithelium with spermatids and spermatozoa).

Real time PCR: At 1, 2 and 6 months post partum, total RNA was extracted from the testis tissue, using the Raze Mini kit (Qiagen), according to the manufacturer's instructions. Total RNA was transcribed with the Takara M-MLV. Real time PCR reactions were carried out according to the manufacturer's instructions in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). The following primer sequences were used for PCR: GAPDH, forward 5'-gcaagttc-aacggcacag-3'; reverse 5'-gccagtagactccacgacat-3'; CDH1, forward 5'-gatcctggccctcctgat-3'; reverse 5'- tcttgaccaccgttctct-3'; SCP3, forward 5'-aacagcaaaagattttcagca-3'; reverse 5'-tctctc-cacatcctccaac-3'; TNP2, forward 5'-gcactctcgttactcacctca-3'; reverse 5'-ctcttgctcacttccctcc-3' and WT1, forward 5'-agatctgcgggaatcctgtga-3'; reverse 5'- catccggcgacatagtga-3'. The samples were heated for 30 sec at 95°C followed by 40 cycles of 15 sec at 95°C and then 31 sec at 60°C. Each sample and the negative controls (RT-PCR without cDNA) were run in triplicate for every primer combination.

Fertility studies of male offspring: The treated animals were paired individually with proven fertile females until mating occurred indicated either by the presence of spermatozoa in the vaginal smear or the occurrence of pseudopregnancy smears where the males were aspermic.

Statistical analysis: ANOVA was used to test for differences among groups in the litter weight weight,

spermatogenesis of the seminiferous epithelia and fertility rate of the F1 males. A Kaplan-Meier survival curve was used in the survival functions analysis. One way ANOVA was used for all treatment versus control pairwise comparisons.

RESULTS AND DISCUSSION

Dose of the busulfan treatment affecting the birth rate of the pregnant rats and survival rate of the male offspring : The birth rates of the pregnant rats are shown in Fig. 1. The doses of 7.5 and 10 mg busulfan per kg had no significant effects on birth rate although the 12.5 mg busulfan per kg group experienced a 75% abortion rate. The offspring were considered to have survived if they lived through the 1st week. The survival rates of the rats 1 week after birth are shown in Fig. 2. The doses of 7.5 and 10 mg busulfan per kg ad no significant effects on

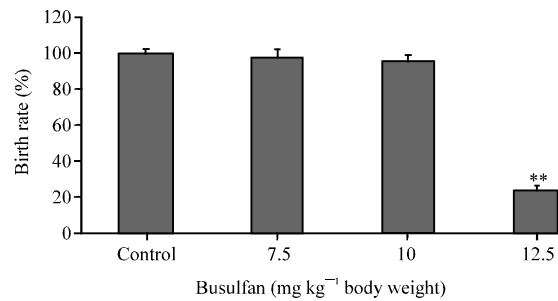


Fig. 1: Birth rates of female rats after the administration of busulfan at 13.5 dpc. *p<0.05, compared with the control group. **p<0.01, compared with the control group

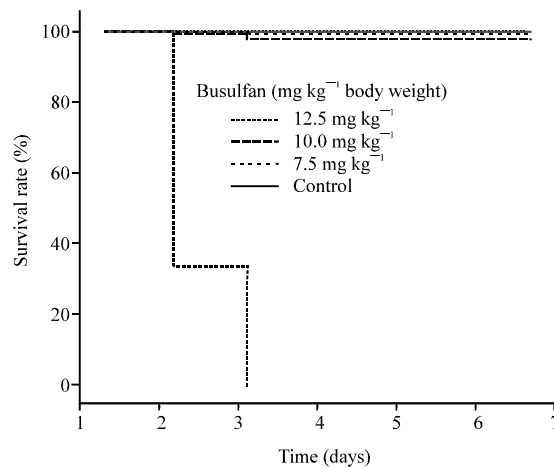


Fig. 2: The survival function of the offspring within the 1st week after birth

survival and the 12.5 mg busulfan per kg dose was 100% lethal within the 1st week. Owing to the high death rate, the rats already treated with 12.5 mg busulfan per kg usulfan were omitted from further studies.

The body weights of both the control and the busulfan treated rats slowly and steadily increased with time during the 6 months after the injections. In general, both the dose of 7.5 and 10 mg busulfan per kg busulfan had no obvious effects on the time dependent body weight increase.

Decrease of the testis mass of the male offspring: At 1, 2 and 6 months after birth, the testis mass of the 7.5 and the 10 mg busulfan per kg group was significantly lower than that of the control group. Testicular mass increased significantly in the control animals from 1-6 months after birth. The change in testis mass was limited from 2-6 months in both the 7.5 and the 10 mg busulfan per kg group (Fig. 3).

Morphological change in the male offspring testis: In the control group at 1 month after birth, most of the seminiferous tubules showed a thick wall with no lumen (Fig. 4A). The wall consisted of several layers of

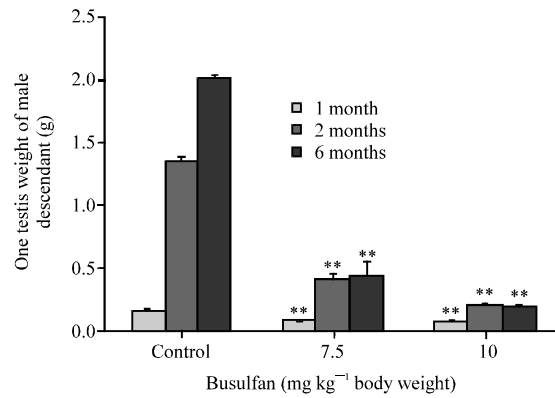


Fig. 3: Changes in the testicular mass of rat offspring over time after birth. * $p < 0.05$, compared with the control group. ** $p < 0.01$, compared with the control group

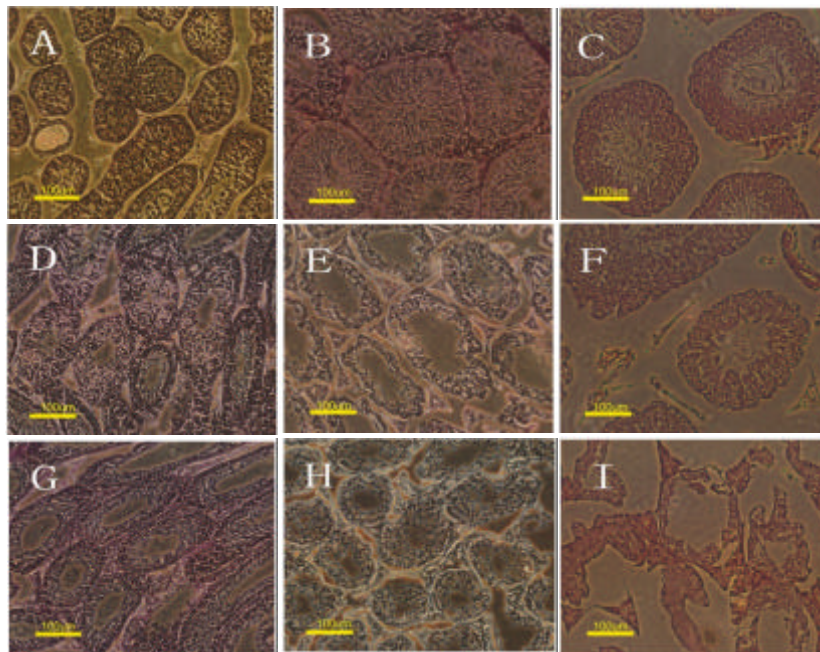


Fig. 4: Photomicrographs of the testes of the offspring of busulfan-treated male rats at 1, 2 and 6 months. A) Microscopic cross-section of a control rat at 1 month. B) Microscopic cross-section of a control rat at 2 months. C) Microscopic cross-section of a control rat at 6 months. D) Microscopic cross-section of an offspring of the 7.5 mg busulfan per kg group at 1 month. E) Microscopic cross-section of an offspring of the 7.5 mg busulfan per kg group at 2 months. F) Microscopic cross-section of an offspring of the 7.5 mg busulfan per kg group at 6 months. G) Microscopic cross-section of an offspring of the 10 mg busulfan per kg group at 1 month. H) Microscopic cross-section of an offspring of the 10 mg busulfan per kg group at 2 months. I) Microscopic cross-section of an offspring of the 10 mg busulfan per kg group at 6 months. Bar = 100 μm

seminiferous epithelial cells with the spermatogonia in the outer layer, the spermatocytes in the middle and the spermatids in the innermost layer; this is defined in the present study as a normal seminiferous tubule. In the control group at 2 and 6 months after birth, most of the seminiferous tubules showed a thick wall with a very limited lumen (Fig. 4B and C). The wall consisted of several layers of seminiferous epithelial cells with the spermatogonia in the outer layer, the spermatocytes in the middle and the spermatozoa and spermatids protruding toward the lumen; this is defined in the present study as a fully spermatogenic seminiferous tubule.

At 1 month after administration of 7.5 mg busulfan per kg busulfan, the walls of the majority of the seminiferous tubules became thinner due to the depletion of the spermatids in the innermost layer, a typical feature of partially spermatogenic tubules (Fig. 4D) and nearly 84% of the tubules observed were partially spermatogenic. At 2 months, the walls of the majority of the seminiferous tubules were thinner due to the depletion of both the spermatids and the spermatozoa in the innermost layer (Fig. 4E); 87% of the tubules observed were partially spermatogenic at 2 months (Fig. 5). At 6 months, majority of the walls of the seminiferous tubules were almost recovered to the normal level (Fig. 4F).

In animals treated with 10 mg busulfan per kg busulfan, even thinner walls with only one layer of cells seminiferous tubules (Fig. 4G) at 1 month; at this time

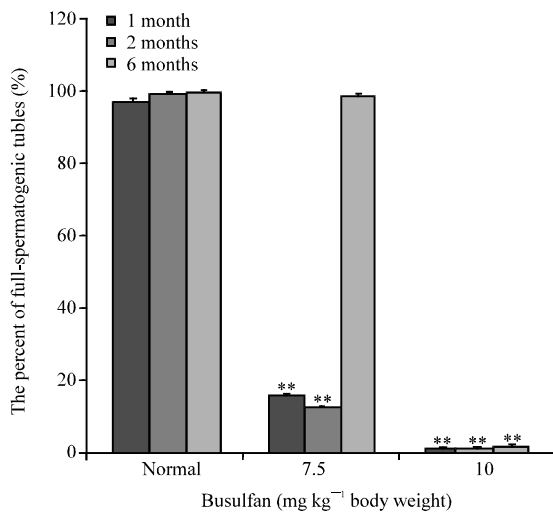


Fig. 5: The percentage of fully spermatogenic tubules of male offspring in the normal group, 7.5 and 10 mg busulfan per kg group at 1, 2 and 6 months. *p<0.05, compared with the control group. **p<0.01, compared with the control group

point, the percentage of non-spermatogenic tubules was 8.59%. At 2 and 6 months, walls with only one layer of cells (non-spermatogenic tubules) were maintained (Fig. 4H and I) in pproximately 98% of the spermatogenic tubules (Fig. 5).

Change of the spermatogenesis and Sertoli cells in the male offspring:

Researchers next wished to examine the degree of genes specifically expressing different types of spermatogonial cells. To this end, researchers performed quantitative RT-PCR experiments on RNA prepared from total testis tissue. The expression of CDH1 decreased significantly at 1 month after birth in the two treatment groups; however the level of expression increased to >2 times that of the normal group at 2 and 6 months when treating the pregnant females with the 7.5 and the 10 mg busulfan per kg group decreasing to 65.55 and 25.1% of the normal group, respectively (Fig. 6). The expressions of SCP3 and TNP2 decreased significantly at 1, 2 and 6 months after birth (Fig. 7 and 8). At 1 month, the expression of WT1, compared with the normal group is 56 and 74% in 7.5 and 10 mg busulfan per kg group, respectively. At 2 months the expression degree of WT1 in both treatment groups is nearly the degree of the normal group. At 6 months, the degree decreased to 67 and 82% in 7.5 and 10 mg busulfan per kg group, respectively (Fig. 9).

Sterile male offspring produced by busulfan treated pregnant rats:

The results of the fertility tests are shown in Fig. 10. From the occurrence of pseudopregnancy smears in the females and the absence of spermatozoa in the vaginal smears, it could be concluded that aspermic

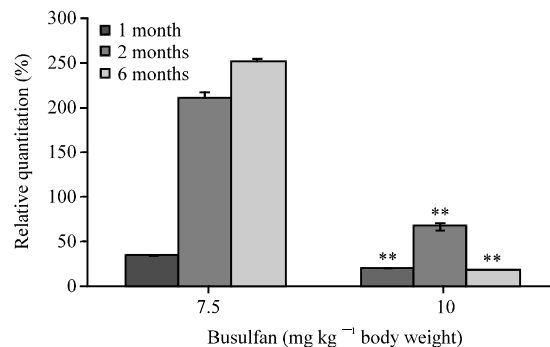


Fig. 6: The relative quantities of CDH1 in the 7.5 and 10 mg busulfan per kg group. The expression level of the normal group was regarded as 100%. *p<0.05, compared with the control group. **p<0.01, compared with the control group

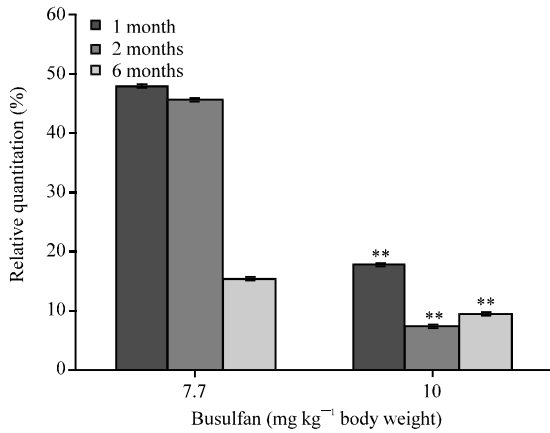


Fig. 7: The relative quantities of SCP3 in the 7.5 and 10 mg busulfan per kg group. The expression level of the normal group was regarded as 100%. *p<0.05, compared with the control group. **p<0.01, compared with the control group

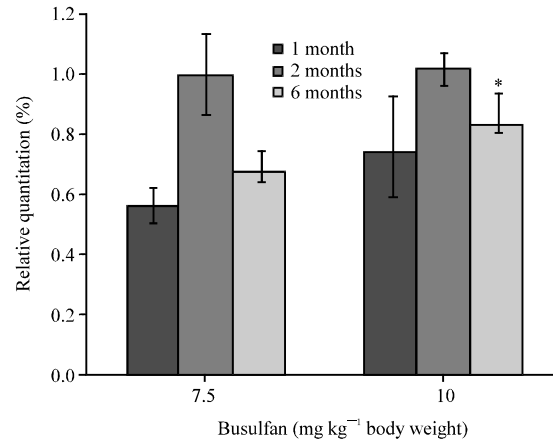


Fig. 9: The relative quantities of WT1 in the 7.5 and 10 mg kg busulfan group. The expression level of the normal group was regarded as 100%. *p<0.05, compared with the control group. **p<0.01, compared with the control group

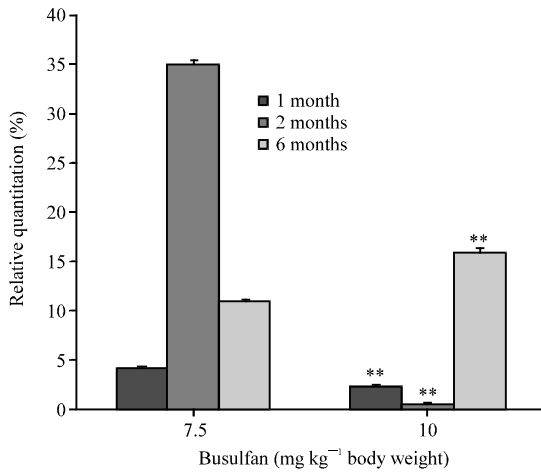


Fig. 8: The relative quantities of TNP2 in the 7.5 and 10 mg busulfan per kg group. The expression level of the normal group was regarded as 100%. *p<0.05, compared with the control group. **p<0.01, compared with the control group

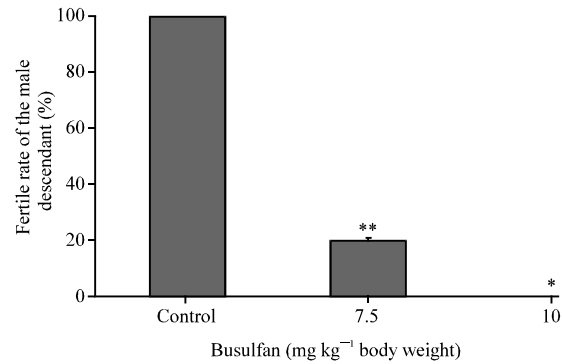


Fig. 10: The fertility rates of the male offspring. The male offspring were placed separately in boxes and were paired with three fertile female rats at 2 months after birth until the female rats were pregnant. If pregnancy did not occur by 6 months after birth, the male was regarded as infertile. *p<0.05, compared with the control group. **p<0.01, compared with the control group

copulation occurred. Following treatment with 7.5 mg busulfan per kg busulfan at 13.5 days of pregnancy, 20% of the F1 males were fertile. Following treatment with 10 mg busulfan per kg busulfan at 13.5 days of pregnancy, none of the F1 males was fertile.

Successful SSC transplantation is largely dependent on the preparation of the recipient. Few studies have been conducted however and chemotherapeutic preparation of adult and pup rat recipients is plagued by problems of high sensitivity to the toxic effects of busulfan and incomplete removal of endogenous spermatogenesis.

Experiments on mice have indicated that the treatment of pregnant females with busulfan as a means of obtaining germ cell depleted recipients may be warranted. Treatment of pregnant female rats with a single dose of busulfan between 13 and 18 days of gestation resulted in the live births of male offspring that were permanently infertile. Nearly two decades ago, Kemper CH and Peters PW discovered that on postcoital day 13, almost all PGCs had reached the well developed genital ridges in rats which indicated that 13.5 dpc may be a suitable point at which to treat pregnant rats with busulfan.

However, to obtain a suitable recipient, the dose of busulfan is important and in the present research, researchers compared 3 different doses to find a suitable dose for the preparation of rat recipients.

The present study showed that an injection of 12.5 mg busulfan per kg busulfan caused a death rate of 100% of the offspring, indicating that the dose of 12.5 mg busulfan per kg is higher than the 50% lethal dose of busulfan for rat offspring. However, for the doses of 7.5 and 10 mg busulfan per kg there were no significant effects on the survival rates of the offspring. The results indicated that female rats that received high doses of busulfan usually miscarried and they died during the 1st week after the injection suggesting that death was the result of lethal suffering from acute damage with the treatments of 7.5 and 10 mg busulfan per kg busulfan, no significant adverse effects on healthy rats were detected.

Research was conducted on the testis weight, histological examination, real time PCR of spermatogonial cell specific and Sertoli cell special genes and fertility rate of male offspring to detect the effects of 7.5 and 10 mg busulfan per kg busulfan on the functionality of the recipients. The testis mass decreased significantly in both the 7.5 and the 10 mg busulfan per kg group.

For the 7.5 mg busulfan per kg group, the walls of the majority of the seminiferous tubules became thinner than in the normal group due to the depletion of the spermatids in the innermost layer which appeared as a typical feature of partial spermatogenesis; the percentage of partially spermatogenic tubules was approximately 85% at 1 and 2 months. Most of the seminiferous tubules recovered to full spermatogenic tubules. Though the percentages of the germocytes and spermatids were significantly less than those in the normal group, the percentage of undifferentiated spermatogonia decreased to roughly 34% compared to the normal group and was eventually restored to >2 times that of the normal group. Sertoli cells play an important role in spermatogenesis and support the entire process of spermatogenesis (Sugimoto *et al.*, 2012), on the other hand, spermatogenic cells affect on the normal functions and existence of the Sertoli cells (Ryser *et al.*, 2011). The expression level of WT1 indicated the amount of the Sertoli cell in mg busulfan per kg group is 56% compared to the normal group at 1 month, the degree recovered to fair to the normal group at 2 months with the increase of the spermatogonial stem cell and decreased to 67% at 6 months because of the shortage of the spermatogenic cells. For the fertility test, 20% of the F1 males were fertile. From these analysis, researchers can conclude that following treatment of 7.5 mg busulfan per kg busulfan in pregnant rats at 13.5 dpc, the male offspring were not suitable for spermatogonial

transplantation because the percentage of undifferentiated spermatogonia was restored and the recovery of the endogenous spermatogenesis thus this environment did not constitute a conducive location for donor SSCs. For the 10 mg busulfan per kg group, even thinner walls with only one layer of spermatogonia (non-spermatogenic tubules) were found in most of the seminiferous tubules; the percentage of the non-spermatogenic tubules increased to roughly 98% at 1, 2 and 6 months. The percentages of undifferentiated spermatogonia, germocytes and spermatids cells were significantly less than those of the normal group. Sertoli cells play an important role in spermatogenesis and support the entire process of spermatogenesis (Sugimoto *et al.*, 2012) and on the other hand, spermatogenic cells affect on the normal functions and existence of the Sertoli cells (Ryser *et al.*, 2011). The expression level of WT1 indicated the amount of Sertoli cells in 10 mg busulfan per kg group is >74% compared to the normal group at 1 month, the degree recovered to fair to the normal group at 2 months with the increase of the spermatogonial stem cell and decreased to 82% at 6 months because of the shortage of the spermatogenic cells. In the fertility test, none of the F1 males was fertile. From the analysis, researchers can conclude that following treatment of 10 mg busulfan per kg busulfan in pregnant rats at 13.5 dpc, male offspring were suitable for spermatogonial transplantation because 98% of the seminiferous tubules maintained sterility for a long period of time and the percentage of undifferentiated spermatogonia remained low which provided an environment conducive to the development of a colony of donor SSCs and the male offspring maintained sterility for at least in 6 months.

CONCLUSION

The analysis suggests that the dosage of 12.5 mg busulfan per kg is too high for pregnant rats as it does not result in healthy offspring. For the 7.5 mg busulfan per kg group, the percentage of undifferentiated spermatogonia recovered to >2 times of the normal group which does not constitute a suitable environment for the transplantation of donor derived SSCs. The dosage of 10 mg busulfan per kg is suitable for ablative treatment in pregnant female rats at 13.5 days post-pregnancy to prepare the recipient rats because it can maximally delete endogenous spermatogonial stem cells with minimal effect on the recipient's health and niche accessibility. This dosage can create sufficient niches and maintain the microenvironment for at least 6 months, allowing the colonization of donor derived spermatogonial stem cells to take place.

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