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Radioprotective Effect of Mesna on Mouse Testis

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Mesna has been used with ifosfamide to prevent urotoxicity in the treatment of testicular cancers. This drug also protected the toxicities of adriamycin without compromising cytostatic activity. With an idea of radioprotective role of sulfhydryl group of radioprotectors and of mesna decreasing the toxic effect of adriamycin which produces free radicals, mesna and radiation were administered to mice to study the protective effect of this drug and to identify the difference in regenerative capacity of the germ cells in the testis between radiation-treated and both mesna- and radiation-treated groups. The shape and numbers of spermatogenic cells in the seminiferous tubules were examined every week after irradiation. In both groups, initial reduction and later recovery in germ cell numbers and shape was observed.

The lowest germ cell number was found around three weeks after irradiation. Mean germ cell number of the mesna-treated group was significantly higher than radiation-treated group at all observed periods ($p<0.05$). More competent regeneration was present in mesna-treated group. These results suggest that mesna protect the testis from radiation injury. Further study will be necessary to identify whether mesna protects other tissues from radiation and it does not hamper tumor control.

Key Words: Mesna, Radioprotector, Spermatogenic cells, Testis

INTRODUCTION

It is known that mesna (2-mercaptoethane sulfonic acid), after intravenous injection, is rapidly oxidized to its only metabolite dimesna. This disulfide is physiologically inert and rapidly constitutes more than 75% of the dose injected[1]. Mesna is relatively nontoxic compared to N-acethylcysteine, and no teratogenic or mutagenic effect have been identified[2]. The drug is administered with ifosfamide or cyclophosphamide to eliminate urotoxicities.

It is clear that hemorrhagic cystitis can almost completely be prevented by the use of mesna[1~4]. In addition, administration of mesna may affect the biotransformation of adriamycin and result in the decrease in toxic side effect and no apparent reduction in their antitumor effectiveness[5]. Since hydroxyl free radical generated during radiotherapy is responsible for the tissue damage, the reaction of this radical with sulfhydryl group of mesna was investigated to observe radioprotective effect of this drug.

Investigators have reported that focal regeneration of spermatogenic epithelium in several different mammalian species after severe radiation injury[6~8]. We have observed the cells in the testis capable of sustaining spermatogenesis - that is, of the stem cells. It has been assumed, according to Withers et al[9], that one surviving stem cell can regenerate a focus of spermatogenic epithelium, and we have used the number of regenerating foci as a measure of stem cell survival. Of the numerous organs in the animal, the testes were chosen because they express the radiation effects more rapidly than any other tissues.

The purpose of the current study is to 1) observe the radiation effects of the testis, 2) evaluate the radioprotective effect of mesna, and 3) identify the difference in regenerative capacity of the testis between the radiation-treated group and the radiation-and mesna-treated group.

MATERIALS AND METHODS

Mice: Healthy mice, weighing 20~25 gm, were obtained from animal facilities of Kyungpook National University Hospital and used in this current experiment. The mice were divided into three groups; control, only radiation-treated, and both mesna- and radiation-treated group, which included the animals of 5, 45, and 45, respectively.

Drug: Mesna was obtained from Sigma Chemical Co., U.S.A. Ten milligram of mesna was solved in 1 ml of normal saline and the aliquot of 0.1 ml (50

Table 1. Criteria by Which Spermatogenic Index was Assessed

Spermatogenic Index	Histologic findings
1	No evidence of active spermatogenesis
	Presence or absence of spermatogonia
2	Presence of primary spermatocytes
3	Presence of primary and secondary, or secondary spermatocytes
4	Evidence of spermatid production. Immature spermatids
5	Complete spermatogenesis with mature spermatids of spermatozoa

mg/kg) was intraperitoneally injected two times into the mouse 24 hours and 30 minutes prior to irradiation.

Irradiation: Each mouse was irradiated with single dose of 6 Gray on whole body, which is the $LD_{50/30}$ of rodents, under 6 MV linear accelerator, at 100 cm of SSD.

Autopsy: Five mice were scheduled to be killed and autopsied twice a week after irradiation. If the mice were dead spontaneously on unscheduled day, we tried to find the cause of death and the changes of the tissues.

Microscopy: The sample tissues were fixed in 10% normal formalin solution immediately after tissue extraction, embedded in paraffin, and then stained with hematoxylin and eosin for light microscopic examination.

Objectives: We observed the various different-aged germ cells, Sertoli and Leydig cells in the seminiferous tubules, and interstitial cells between the tubules to see the cellular morphology. The slides representative of the whole of both testes were studied at a magnification of X200 and X400, and from these slides 50 tabules were studied. Each field was a carefully selected median cross-seition of a tubule measured as 180 ± 20 μm in diameter, which was the same as the diameter of seminiferous tubules in control mice. We counted the primitive germ cells such as the spermatogonia, spermatocytes, and early spermatids at each autopsied mouse testis and the mean was calculated and statistical difference was computed by means of Student's t-test.

To estimate the regenerative abilition of the stem cells in the tubule, spermatogenic index was determined according to Atkinson's method[10], which is illustrated in Table 1. The spermatogenic activity was graded from 1 to 5 in 50 tubules, and mean score was calculated.

Table 2. Change of Germ Cell Number After Treatment

Weeks after radiation	Radiation + mesna	Radiation only
Control	148 ± 17	
1	129 ± 13	110 ± 11
2	80 ± 17	55 ± 15
3	45 ± 12	25 ± 8
4	54 ± 11	31 ± 10
5	79 ± 12	60 ± 11
9	91 ± 19	--
10	141 ± 21	--

$p < 0.05$

RESULTS

1. Control Group

Five untreated animals were used as a set of controls. The mean germ cell number in the control testes was 148 ± 17. Control mice showed essentially the same average incidence consisting all the different cells and stages of spermatogenesis, that is, spermatogonia, spermatocytes, spermatids, and sperms. Because the various types of germ cells are in a continuous process of differentiation, discrimination of each cell type was very difficult and this method of evaluation does not seem to provide a strictly quantitative analysis. So we counted all the germ cells present in the seminiferous tubules except late spermatids and mature sperms released into the lumen.

2. Radiation-treated Group

At the end of the first week after irradiation, many spermatogonia were missing at first. But the more matine cells, spermatocytes and spematids,

were unchanged to be in normal appearance. At the second week, there severe reduction in spermatogonia and spermatocytes. Most of the cells remained in the tubules were spermatids. The size of the spermatogonia has become smaller than control. At the third week, the nucleus and cytoplasm of spermatogonia became smallest among all the observed periods. All the types of the cells were severely depleted, especially in spermatids. Spermatogonia were the most radiosensitive cell and spermatocytes, spermatid, in decreasing order of radiosensitivity. The period of the lowest germ cell number (25 ± 8) was at the end of the third week after irradiation. But regenerative activity in spermatogonia were found consisting of one layer of cells on the basement membrane. At the end of the fifth week, there was marked regeneration in both spermatocytes and spermatogonia. The cellular column consisted of several layers on the basement membrane. But the spermatids were not left at this time. After this period, because all the mice were dead, we were not able to proceed more observation in this group. Change of the germ cell numbers were shown in Table 2 and Fig. 1.

3. Radiation- and Mesna-treated Group

A few spermatogonia were missing at the end of the first week after treatment. More mature cells were unchanged and remained intact. At the end of the second week, marked reduction in the number of all germ cells, especially spermatogonia and spermatocytes was noted. Unlike radiation-treated group, spermatids became smaller in their number and size. At the end of the third week, there showed regenerating cells in both spermatogonia and spermatocytes, which consisted of cell columns of at least two layers on the basement membrane. The number of germ cells (45 ± 12) was lowest at this time. These findings were different from the radiation-only-treated group. At the end of the fifth week, marked repopulation and differentiation in all the types of germinal cells were observed, consisting of cell columns of numerous layers. This was in sharp contrast with radiation-treated group. Thereafter, the cells were much increasing in number and size progressively to be near-normal at the end of the tenth week after treatment. Normal sperms were evident in seminiferous tubules at this period. These changes were illustrated in Table 2 and Fig. 1, comparing with the radiation-treated group. The difference in germ cell numbers between the two groups was statistically significant ($p<0.05$).

Leydig cells, Sertoli cells, and connective tissue were not affected by radiation and showed no difference at all observed periods in both groups.

4. Regenerative Capability

Grades according to the spermatogenic index in table 1 were determined at each observed period. This index was used to study the changes in regenerative ability and formation of sperms. The scores of the spermatogenesis were revealed in table 3. In mesna-treated group, the score of spermatic regeneration was increasing at the fifth week, to 4.0, which means the appearance of spermatid production. The score approached 5.0 at the tenth week.

This means that normal sperms are present in the seminiferous tubules. The score became the same as the control at the tenth week, when the

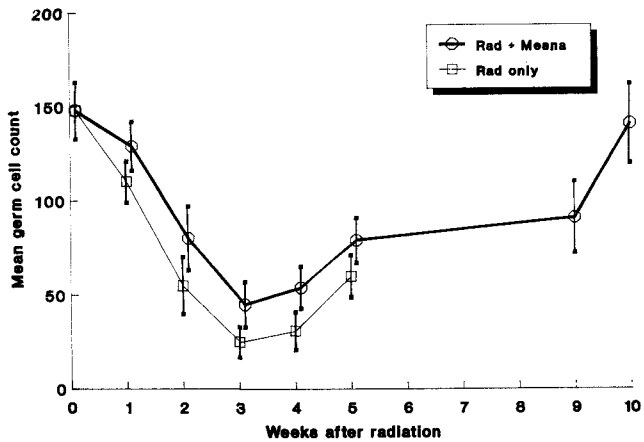

Fig. 1. Germ cell number changes after treatment.

Table 3. Score of Spermatogenic Index

Weeks after radiation	Radiation + mesna	Radiation only
Control	5.0	
1	4.0	5.0
2	4.0	4.0
3	2.0	4.0
4	2.0	2.0
5	4.0	2.0
9	4.5	–
10	5.0	–

number and shape of the germinal cells were near-normal as described above. This is in contrast when compared with radiation-treated group. Though we were not able to study further after five weeks due to expiration of all the mice and therefore could not accurately explain the trend, the score of spermatic index in radiation-treated group was not elevated, that is, 2.0 (presence of only primary spermatocytes), until the end of the fifth week. Maybe the score would have been increased after that time as evidenced by the tendency of increase in germ cells as depicted in figure 1. Althouth it was not statistically significant, the difference in regenerative capability between the two groups were present indicating that there was more rapid and powerful regeneration and differentiation in mesna-treated group.

DISCUSSION

Rapid progession of recent technology of radiation therapy has greately promoted the long-term survival of many cancer patients. As the result, the sequelae of cytotoxic effect of radiation, especially infertility, has become an important clincal issue in several cancers such as leukemia, Hodgkin's disease, testicular cancers, and so on. Main testicular change after cytotoxic therapy is the decrease in the number of reproductive cells[11~13]. There were atrophy of seminiferous tubules, fibrous infiltration in the connective tissue around seminiferous tubules, and loss of spermatogenic cells. Sertoli cells and Leydig cells were maintained normal. Clinically these changes appear as reduction in the size of the testis, oligo- or aspermia, and infertility[14]. Male fertility depends on the regeneration capability of the stem cells for sustained spermatogenesis. The development of infertility by cytotoxic agents is due to the fatal damage of the differentiating spermatogenic cells. Recovery of the fertility is determined by the number and regenerative ability of the stem cells. Thus, to estimate the extent of damage to the ability of sperm production, it is desirable to observe the changes of the stem cells directly. However, because the stem cells are scanty in the seminiferous tubule and they appear as so various shapes, obsevation of the stem cells is very difficult. The number of stem cells can be estimated from the count of repopulated and nonrepopulated tubules[15,16]. Through the sperm count in the testis and epididymis, the ability of regeneration of survived stem cells could also be estimated.

Roweley et al[17] found, in the study of graded radiation doses on the human testis, that spermatogonia was the most radiosensitive cell type. Spermatocytes were overtly damaged at doses of 200~300 rad by their inability to complete maturation division resulting in decrease in the number of spermatids. Spermatids showed no overt damage, however, after 400~600 rad, the resultant spermatozoa were significantly decreased in number suggesting covert spermatid damage. Withers et al[9] found that cell proliferation was evident by the appearance of colonies of spermatogenic cells, which were readily visible microscopically 28 days after irradiation. Moreover, Oakberg[18] had also shown that normal kinetics were reestablished within 8.5 days of irradiation. The cells seen microscopically in the tubule colonies were, in general, not spermatogonia, but later form spermatocytes, confirming that both cell division and differentiation have occurred. He has explained the lack of increase in the stem cells that the stem cells resume their usual, preirradiation, steady-state proliferation-differentiation pattern for at least 14 days after exposure. Fogg and Cowing[19] also reported that the average numerical incidence of germinal elements was approached in 35 days after irradiation. In the current study, spermatogonia were the most radiosensitive and the first damaged cell type early in the second week after radiation, and spermatocytes the next. Spermatids were the least radiosensitive among the differentiating cells. Regenerative activity in spermatogonia and, in part, spermatocytes was evident at the end of the third week, though spermatids were still depleting. The number of germ cells was generally lovest at this period. Therafter the spermatogonic cells began to repopulate and differentiate into mature forms, which suggests that normal kinetics be reestabli-

shed. These results confirmed that the spermatogenic regeneration and maturation into spermatocytes were evident at three weeks after irradiation, as described by other investigatiors[9,17~21].

Mesna has the structural formula $[HSCH_2\ CH_2\ SO_3]^-Na^+$. It is a low molecular weight thiol compound devoid of aromatic amino acid and histidine. After intravenous injection, mesna is rapidly oxidized to dimesna, the only known metabolite. Within several minutes of injection, over 75 percent of the thiol is in the disulfide form. This disulfide is physiologically inert. The majoning of the injecled mesna and its metabolite recain in The vesclar scystem, as deiermined by Broch's expeiment using reotio laheled mesna[4]. The inected mesna rapidly undegoes glomenlar filtration. The plasma half-life in humans is approximately 1.5 hours[2]. About one third of the filtered dimesna is reduced to the thiol mesna by the renal tubular epithelium during urinary excretion[1]. The singular species of dimesna is active in binding acrolein, a toxic metabolite of cyclophosphamide derivatives, and preventing urotoxicity. The use of mesna with ifosfamide or other chemotherapeutic agents such as adriamycin showed marked protection from the urothelial damage and no diminution of cytostatic activity in animal tumor models and clinical trials[5,22~26].

Toxic effects of the mesna include nausea and vomiting, crampy abdominal pain, diarrhea, fatigue, and arthralgia at a dose of 60 mg/kg per day[23]. This dose was widely used in humans previously and clinical toxicity was considered to be minimal. Recent use of ifosfamide in continuous infusion and increase in the dose of mesna developed gastrointestinal toxicity[24]. No teratogenic and mutagenic effects have been reported.

With an idea of possible radioprotective role of sulfhydryl group of mesna, and of the fact that mesna decreases in the toxic effect of adriamycin which induces free radical production[5], we carried out this currnt experiment. Mesna-treated group revealed lesser cell damage than radiation-treated group stage by stage of differentiation. The pattern of initial reduction and later recovery in the number of germ cells were evident in both groups. But the quantity of depletion of the spermatogenic cells was significantly smaller in mesna-treated group than radiation-only-treated group, at all observed periods($p<0.05$). The spermatic regenerative activity was more competent in mesna-treated group. These facts indicate that full regeneration was accomplished in mesna-treated group. The more rapid regeneration and the lesser cell diminution in mesna-treated group suggest that mesna protect the cells from radiation injury. The mechanism of protection would be through scavenging of hydroxyl free radical by sulfhydryl group of mesna in the same manner with other raioprotectors[27].

More study will be necessary to know whether mesna protects the other tissues from radiation injury and it does not hamper tumor control probability.

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= 국문초록 =

Mesna의 쥐 고환에 대한 방사선 보호 효과

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고환암에서 mesna는 ifosfamide와 병용 투여하면 요관 계통의 부작용을 방지할 수 있으며, adriamycin과 같이 사용하는 경우 항암제의 세포증식 억제능을 감소시키지 않으면서 부작용을 경감시킨다. 방사선 보호제의 sulfhydryl기가 방사선에 의하여 발생하는 hydroxyl 유리기와 반응하여 조직을 보호할 수 있고, 역시 체내에서 유리기를 발생하는 adriamycin의 독작용을 musua가 감소시키는 사실에 근거하여, 쥐에 *mesna*와 방사선을 투여하여 *mesna*의 방사선 보호 작용을 관찰하였다.

본 연구에서는 방사선 단독 처치군과 mesna와 방사선 처치군에서 고환의 수정관 내의 배아세포의 수와 형태적 변화를 현미경 하에서 매주 비교하고 재생 능력의 차이를 확인하였다. 양쪽 군에서 공히 초기에 세포 수가 감소하고 후기에 다시 세포가 증식 하였으며, 가장 세포 수가 적게 관찰된 시기는 방사선 조사 후 3주 째였다. 모든 관찰 기간에서 mesna 처치군의 평균 배아 세포수가 방사선 단독 처치군보다 통계적으로 유의하게 많이 관찰되었고($p<0.05$), 또한 *mesna* 처치군에서 더 유효한 세포 재생 능력이 있음이 관찰되었다. 이는 mesna가 방사선 상해로부터 고환의 수정관 내 배아세포를 보호하고 있음을 입증하는 것으로 사료된다. Mesna가 고환 이외의 다른 조직을 방사선으로부터 보호할 수 있는지의 여부와 종양의 관해율을 저해하지 않을지에 대하여는 더 이상의 연구가 필요할 것으로 생각된다.