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# Life span, testis damage and immune cell populations of spleen in C57BL mice with neutron irradiation by lying flat pose

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## Abstract

This study deals with the biological effects of black mouse (C57BL) irradiated with neutron irradiation by using Boron Neutron Capture Therapy facility in HANARO reactor. These include mortality, body wt., hair color, testis volume, sperm count and immune cell populations in mouse spleen after 80 days later by thermal neutron irradiation. Six week old C57BL male mice were irradiated with neutron irradiation for 1 hr or 2 hrs(flux: 1.036739E+09). These irradiation doses estimated 15Gy and 30Gy, respectively. Survival days and hair color in mice was checked. On day 80 after irradiation, testis were taken for volume and sperm count. Also Spleen was taken for FACS and spleen cells were isolated and discarded RBC by treating with lysising solution. These cells were placed on ice and immunofluorescence staining was performed. Phycoerythrin(PE)-anti-CD3e, fluorescein isothiocyanate(FITC)-anti-CD4, and FITC-anti-CD8 were added, then the immunostaining cells were incubated on ice for 40 min. The resulting cells were washed with a PBS buffer 3 times and analyzed using a Flow cytometer. All experimental animals survived over 90 days but in case of 30 Gy neutron irradiation, black mice hair were changed white color on the center of the back. Neutron irradiation of black mice show similar in damage of spleen immune cells by subpopulation of T helper and T cytotoxic cells compared to the control non-irradiated group. These results show that treatment of neutron irradiation without boron compounds for 2 hrs in mice can survive over 90 days with hair color change from black to white. Damaged spleen cells recover after long time by irradiation but testis volume and no. of sperm are not recover compared to the normal group in response to neutron irradiation.

## 1. Introduction

BNCT facility at HANARO reactor was completed in our country last year by means of peaceful use of atomic energy and so animal study of neutron irradiation started at BNCT facility in our country. Gamma-ray irradiation including neutron has been used widely cancer therapy and diagnosis in the medical science. BNCT is a binary form of an experimental treatment modality based on the reaction occurring between the non-radioactive isotope 10B and thermal neutrons. A low energy neutron is captured by the 10B-mucleus, which disintegrates into a Li- and a He-nucleus, two densely ionising particles with high biological effectiveness and short range in tissues. A selective targeting of this reaction to tumour cells would lead to a highly effective treatment while sparing healthy tissues, resulting in a 'targeted and timed cell surgery'. BNCT takes place in a nuclear research reactor and requires a strict quality management of safety. Before preclinical study to patient, biological effects of experimental aniamals by neutron irradiation provide good informations to progress BNCT to patient treatment. As the first step of animal study, black mice were not administrated boron compounds and laid flat in front of beam entrance and irradiated at the direction of the front with neutron and then investigated the body weight, survival days, hair color, testis volume and sperm count and immune cell population of spleen on the day 80 after neutron irradiation for chronic effect of biological damage with neutron irradiation. Spleen is the most important organ of the immune systems. This organ also is the largest lymph one among circulatory systems and consist of many lymphs. Inside spleen have many phagocytic cells and protect invested microorganisms from blood. Also this organ destroy the red blood cells.[1] Due to these functions. this is one of the most important organs in human beings. Therefore, the present study was designed and peformed to obtain the damage levels of immune system on the population change of the immune cells in spleen after 80 days by neutron irradiation.

## 2. Materials and methods

### 2.1. Animals and maintenance

Six week-old male C57BL mice weighing about 22g were obtained from Biolinks company, Umsung, Korea. The mice were raised in a 22 °C-controlled animal care room with the light/dark cycle of 12/12h. The animals had free access to tap water and commercial chow during the experiments kept in an environment complying the NIH guidelines for the care and use of laboratory animals.[2]

## 2.2. Neutron irradiation and sacrifice of animal

The neutron irradiation was carried out with BNCT facility in HANARO reactor at Korea Atomic Energy Research Institute(KAERI). At first, twenty mice were anesthetized with Fluka and laid flat in front of the beam entrance and then irradiated with neutron (flux : 1.036739E+09) for 1 hr or 2 hrs. Experimental mice were investigated the survival days for life span with checking the body weight and body appearance like hair color change and later four mice on day 80 were sacrified for taking testis and spleen.

## 2.3 Measurement of testis wt., volume and sperm count

Right and left of Testis were measured by balance( OHAUS Corp. U.S.A ) and length and width of testis were measured with a rule( Vernier Caliper, Mitutoyo Corp.) for calculating the testis volume. In case of sperm count, epididymis put into the eppendorf tube and homogenized with 1ml of 1% sodium citrate and then standed for 30 minutes and counted with hemocytometer by microscope( Canon, Japan).

## 2.4 Preparation of spleen cell suspension

Spleens were collected aseptically and pressed on the 100 mesh sieve by piston of syringe. Pressed tissue were moved the 15 ㎖ conical tube(Becton dickinson) and stand for about 5 min. After precipitation of pressed tissue, supernatants were taken and washed 2 times in PBS. Splenocytes suspension from 5 mice in each group were pooled and RBC were lysed by an additional 5-min incubation with 0.83% $NH_4Cl$. After 2 additional washings in PBS saline solution containing 3% fetal bovine serum and 0.1% $NaN_3$, cell suspensions were diluted at 5 x $10^6$ cells/㎖ in RPMI-1640 medium plus 2% fetal bovine serum(sigma).

## 2.5 Cell immunofluorescence staining and flow cytometric analysis

All steps of cell immunostaining were performed at 0 - 4 °C. Splenocytes were washed 3 times with PBS and moved the 5 ㎖ FACS tube added with 0.3 ㎖ staining buffer. After vortexing, this was centrifuged for 5 min, 1300 rpm at room temperature. For each one-step staining reactions, 100 ㎕ of antibody culture supernatants were placed on ice and reactioned for 40 min. After 3 additional washings in PBS, antibody were added 50㎕ of dilution solution (100X) of goat anti-rat immunoglobulin -fluorescenisothiocyanate (FITC) and incubated on ice for 40 min. The resulting cells were washed with a PBS buffer 3 times and resuspended with 0.3 ㎖ of staining buffer

and then analyzed using a Flow cytometer (Becton Dickinson, U.S.A). The data were collected by list mode about 5,000 cells per sample and analyzed using Consort 30 program. Data analysis was calculated population percentages of $CD4^+$ and $CD8^+$ cells among various cells divided into whole splenic cells, small lymphocyte or lymphoblast on a dot plot using dual parameter of forward scatter(FSC) and side scatter(SSC).

### 3. Results and discussion

After neutron irradiation, mice were checked the body wt. everyday for 1 week and then every 1 week. Table 1 shows the life span of mice with neutron irradiation. All experimetal mice in two groups were survived over 90 days but one experimental group of mice changed the hair color. Although these mice were irradiated with neutron at the direction of the front, life span of mice show no effect. Fig. 1 shows that hair color with high dose of neutron irradiation only were changed from black to white on the center of the back. Some high dose of neutron irradiation can change the hair color after long time by irradiation. Fig.2 shows that the body wt. of mice reduced rapidly on day 1 after irradiation and then increased gradually surviving over 90 days after irradiation. Neutron irradiation of mice induced shock and some biological damage by reducing body weight rapidly. Fig. 3 shows that testis weight were not reduced but testis volume were reduced a little according to the radiation dose after long time by neutron irradiation. Fig. 4 shows that sperm count were reduced a little compared to the normal group. In case of testis, testis volume and sperm count induced some damage after irradiation like reducing value compared to the normal group. Although all mice were survived for long time after irradiation, biological damage of some organs like hair and testis did not recover the normal value due to the chronic effect of neutron irradiation. Spleen is a lymphoid organ having a special function. This is made lymphocyte and destroyed red blood cells and protected from invested substances inside blood and stored the blood and so on. Lymphocytes are made at white pulp of spleen and red blood cells having with life-span of about 120 days are destroyed at spleen. Also this have defense functions because of B and T cells in it. Therefore spleen is an important biological organ as well being very sensitive to radiation. Fig. 4 shows the population of $CD4^+$ or $CD8^+$ cells in the splenic cells after 80 days by neutron irradiation. The population of $CD4^+$ in the non-irradiated control was 16.8%. Those by neutron irradiation for 1 hr were 13 and 19.1%, respectively. And those by neutron irradiation for 2 hrs were 20.3 and 13.7%, respectively. Also the population of $CD8^+$ in the non-irradiated control was 5.7%. Those by neutron irradiation for 1 hr were 3.7 and 5.9%, respectively. And those by neutron irradiation for 2 hrs were 6.8 and 5.0%, respectively. $CD3e^+$ cell in splenic cells divided two groups like $CD4^+$ and $CD8^+$ cells. $CD4^+$ cell is Th cell and $CD8^+$ is Ts/c cell. Increasement and reduction of $CD4^+$ cell mean that of antigen-antibody immune system. Also population of $CD8^+$ cell depend on the cell cytotoxicity and so it reduce when mice were damaged by chemicals and

irradiation. Fig. 5 shows the population of $CD3e^+$ or $NK1.1^+$ cells in the splenic cells after 80 days by neutron irradiation. The population of $NK1.1^+$ in the non-irradiated control was 1.23%. Those by neutron irradiation for 1 hr were 1.58 and 1.35%, respectively. And those by neutron irradiation for 2 hrs were 0.84 and 0.95%, respectively. Natural killer cell(NK cell) are immune cells killed with cancer cell and inflammation cell. Fig. 6 shows the population of $CD3e^+$ or B220 cells in the splenic cells after 80 days by neutron irradiation. The population of B220 in the non-irradiated control was 73.9%. Those by neutron irradiation for 1 hr were 75.1 and 73.9%, respectively. And those by neutron irradiation for 2 hrs were 73.5 and 78.2%, respectively. From these results, neutron irradiation without administration of boron compounds at the direction of the front after long time showed no damage of immune cells in spleen compared to the non-irradiated control group. Lim et al.[6] reported that the methanol extract of *Cordyceps hepialidicola* which are well-known and important ingredients in traditional oriental medicine enhanced the percentages of the $CD4^+$ and $CD8^+$ T cells in the healthy murine PBMCs. This result suggestes that certain factors in the fungal extracts induced the proliferation of T cells as a mitogen and/or the proliferation and activation of T cells via cytokine production as a polyclonal activator. Also Dreau et. al.[7] reported that the effects of 2-DG(2-Deoxy-D-glucose) were assessed by determining T splenocyte in vitro proliferation after stimulation with concanavalin-A and leukocyte subset distributions. Administration of 2-DG is associated with changes in the nervous and endocrine systems. In addition to metabolic and hormonal changes, 2-DG administration resulted in alteration of immune responses[8, 9, 10, 11, 12]. Dreau et. al. carried out that splenocytes were analyzed for the following cell-surface markers: CD3, TCR$\alpha/\beta$, CD4, CD8 and major histocompatibility complex(MHC) class II. In vitro proliferation of mature T splenocytes in the presence of concanavalin A was decreased in $BDF_1$ but not in BALB/c and C57BL/6 mice. In addition, in $BDF_1$ mice the decrease was highly correlated with a increase of $CD3^+$ and TCR $\alpha/\beta^+$ cells in the spleen. These results demonstrated high variability in the response of different mouse strains to 2-DG-induced stress. Like this, the subpopulation of T cells might be expressed the immune function. From these above results, neutron irradiation without administration of boron compounds at the direction of the front by lying flat pose showed no damage compared to the non-irradiated subpopulation of splenic immune cells and show the long life span over 90 days after irradiation. But black hair in mice with high dose of neutron irradiation were changed white color on the center of the back after long time by irradiation. In this study, normal mice were irradiated without administration of boron compounds and we found the chronic effect of some biological damage like hair and testis with neutron irradiation. Therefore, we need to study further about this with or without administration of boron compounds or cancer induced mice and so on.

## 4. References


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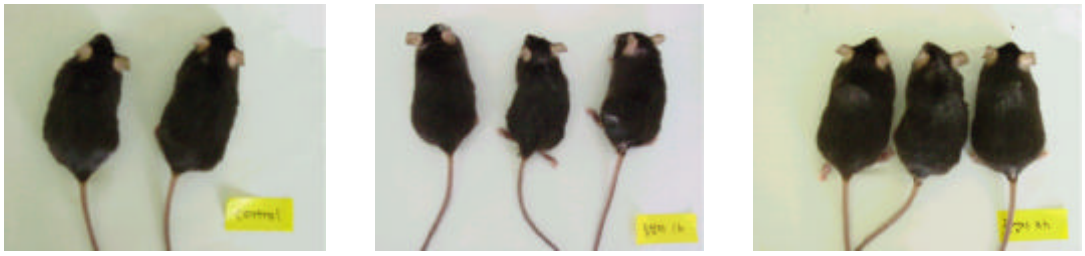
Table 1. Life span of black mice wih neutron irradiation by lying flat pose for 1 hr or 2 hrs.

| Irradiation time | No. of animals | Days of survival |
|---|---|---|
| Non-irradiated | 5 | 〉90 |
| 1 hour | 10 | 〉90 |
| 2 hours | 10 | 〉90 |



<control> < 1 hour-irradiated> <2 hours-irradiated>

Fig. 1. Change of hair color after long time( 2 months ) by neutron irradiation.

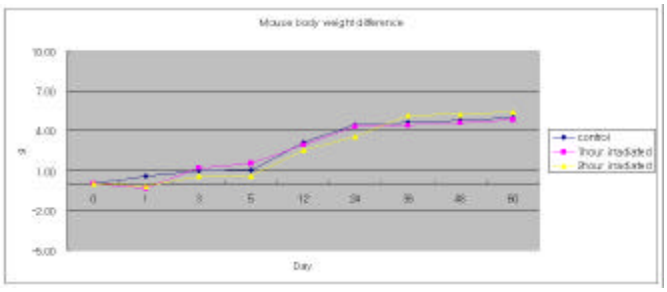



Fig. 2. Change of body weight according to the time after neutron irradiation.

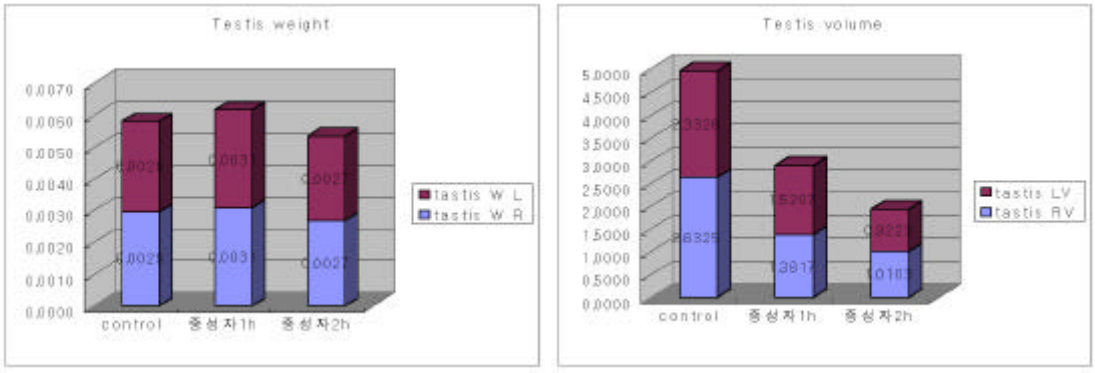



Fig. 3. Testis weight and testis volume after long time by neutron irradiation.

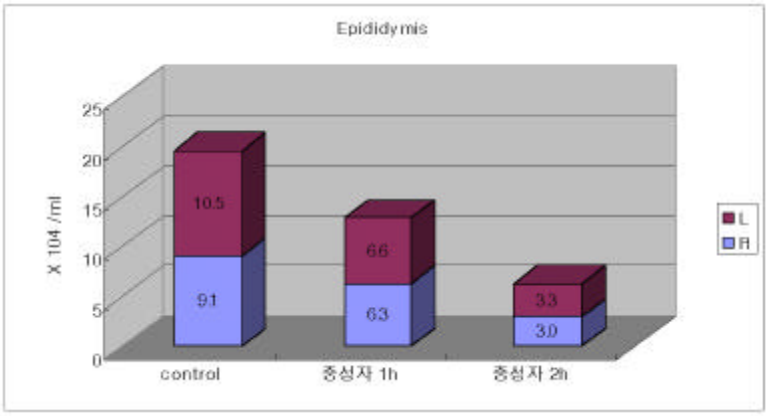



Fig. 4. Sperm count after long time by neutron irradiation.

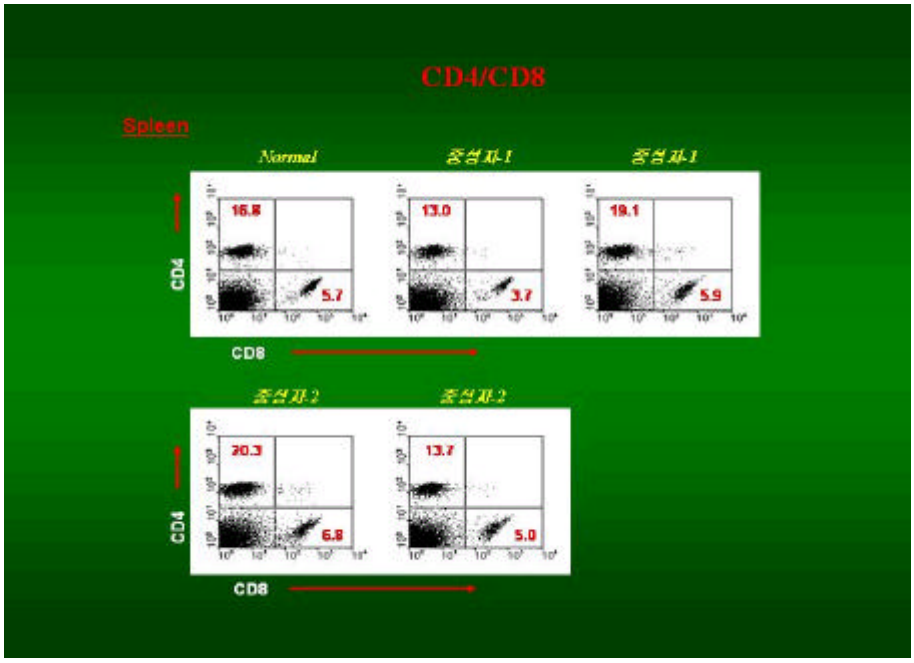



Fig. 5. CD4+ /CD8+ cell populations of mouse splenic cells after 80 days of neutron irradiation for 1 hr or 2 hrs.

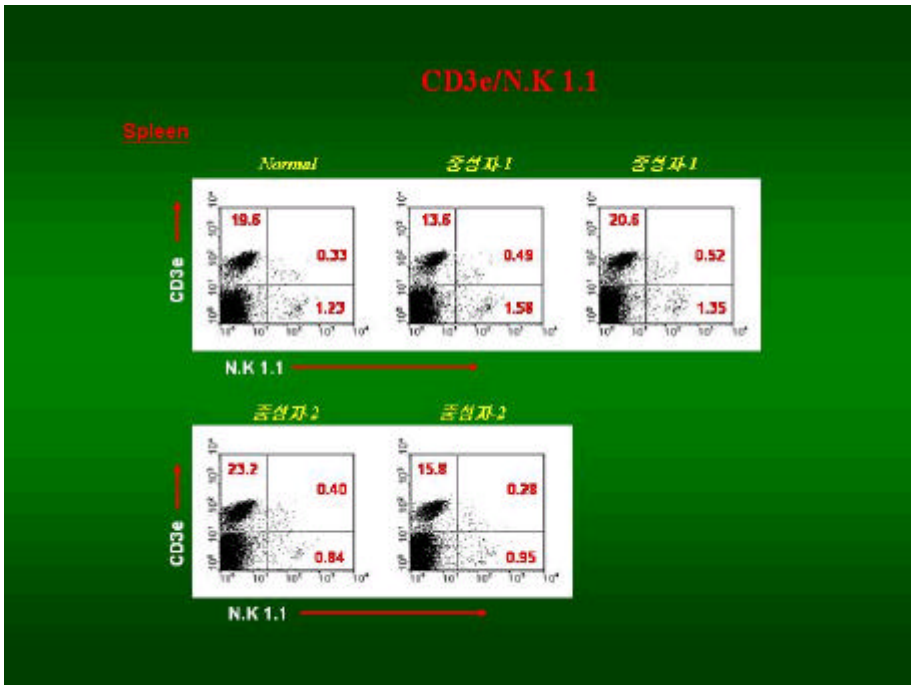



Fig. 6. CD3e+ /NK1.1+ cell populations of mouse splenic cells after 80 days of neutron irradiation for 1 hr or 2 hrs.

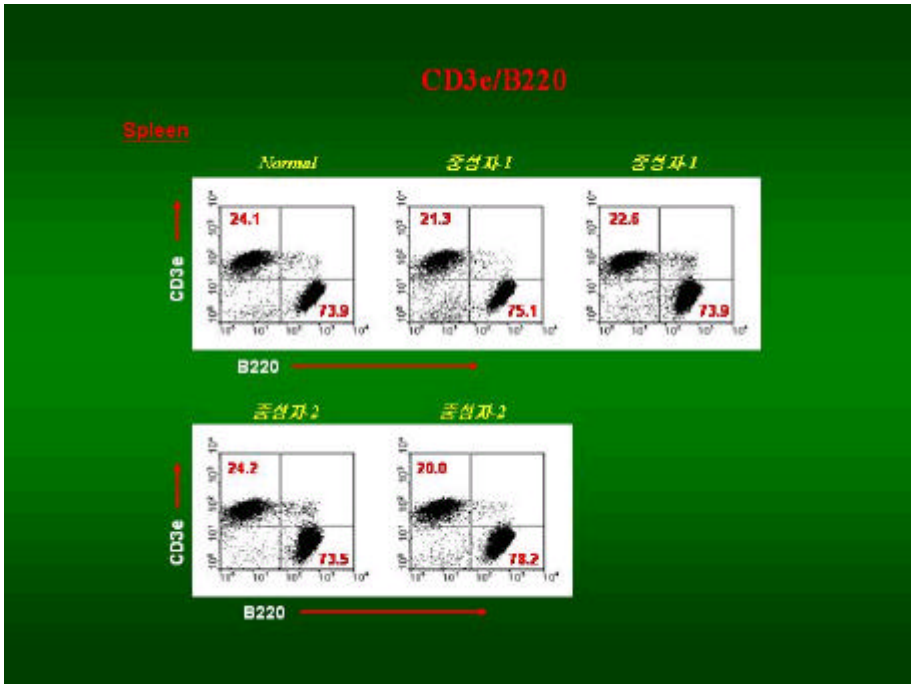



Fig. 7. CD3e+/B220 cell populations of mouse splenic cells after 80 days of neutron irradiation for 1 hr or 2 hrs.