in vivo *19*: 509-514 (2005)

Amifostine has an Inhibitory Effect on the Radiation-induced p53-branched Cascade in the Immature Mouse Ovary

YONG-DAL YOON[1], JI HYANG KIM[1,2], KYOUNG HEE LEE[1] and JIN KYU KIM[2]

[1]*Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791;*
[2]*Korea Atomic Energy Research Institute, 150 Deokjin-dong, Yuseong-gu, Daejeon 305-353, Korea*

Abstract. *The organic thiophosphate, amifostine, is a promising pharmacological compound showing selective protection in many tissues against the toxic side-effects of radiation and cytotoxic drugs. The aim of the present study was to assess the radioprotective effects of amifostine on ovarian follicles. Three-week-old female mice, with or without pretreatment with amifostine, were irradiated with 6.42 Gy of γ-ray. Reduced proliferation of granulosa cells was verified with BrdU staining and the incidences of follicular degeneration increased in ovarian follicles in the γ-ray-irradiated mice compared to that of the control or amifostine-treated group. Biochemical changes caused by γ-irradiation provoked a rise of p53 and Bax protein and a decline of the inactive form in caspase-3 and PARP protein. Caspase-3 and PARP cleaved into active peptides during apoptosis. This process was confirmed by the result of this study, which was that the amount of the stable form decreased immediately after irradiation. In the amifostine treatment group before irradiation, the increased rate of p53 and Bax was suppressed, particularly in the LD_5-treated group. The relationship between PARP and caspase-3 levels showed the effect of amifostine exposure before irradiation. In conclusion, amifostine had an inhibitory effect on ovarian programmed cell death induced by γ-ray, affecting the expression of apoptotic signaling molecules and the level of proliferation of the granulosa cells.*

Programmed cell death is the molecular mechanism by which more than 99% of ovarian follicles undergo atresia during their reproductive life (1), a process which principally involves the death of granulosa cells (2). Follicular atresia can occur normally during follicular growth and development (3) and, thus, follicles become atretic at any stage of their development by intrinsic or extrinsic detrimental signals. It is generally accepted that follicular atresia occurs by the mediation of apoptosis of the granulosa cells, either when the causing signals are spontaneous or induced (4). The first recognizable morphological evidence of follicular atresia is the appearance of pyknosis in the granulosa cells lining the antral boundary of the antral follicles and in those just external to the corona radiata of the secondary follicles (3). Apoptotic nuclear fragmentation was characterized by nuclear rupture and chromatin transformation into formless granules, often referred to as atretic (apoptotic) bodies, that were subsequently shed and eventually engulfed by the infiltrating macrophages or macrophage-like granulosa cells (3).

Since the status of atresia can only be detected after considerable changes of the follicular architecture, it is not easy to recognize the atretic process. Though morphological and biochemical transformations related to atresia have been well documented (5), the effect of radiation on atretic follicles is still far from being understood. Thus, we investigated the degenerative morphological changes in ovarian follicles, which presumably are the early stages of atresia, induced by whole-body irradiation.

It has been reported that radiation induces cell apoptosis (6) and impairs the ovarian functions. Jacquet *et al.* reported that the chromosomal damage of oocytes was induced by irradiation and that the affected oocytes eventually disappeared (7). The detrimental effect of radiation on cell physiology gives rise to primary lesions of DNA that trigger the apoptotic response (8). The DNA damage of p53-target molecules induces the gene expression of p53-target molecules involving p21 (WAF1), Gadd45, Bax, Bcl-2, poly(ADP-ribose) polymerase (PARP) and p53 itself (9). The p53 protein plays a pivotal role in the maintenance of genomic stability (10). An alarm from the p53-dependent mechanism activates caspases and the pathway of cell death.

The synthetic aminothiol, amifostine, also known as WR-2721 (S-2-(3-aminopropylamino) ethyl phosphorothioate),

Correspondence to: Jin Kyu Kim, Korea Atomic Energy Research Institute, 150 Deokjin-dong, Yuseong-gu, Daejeon 305-353, Korea. Tel: +82-42-868-2057, Fax: +82-42-868-2091, e-mail: jkkim@kaeri.re.kr

Key Words: Radiation, amifostine, apoptosis, ovary, mouse.

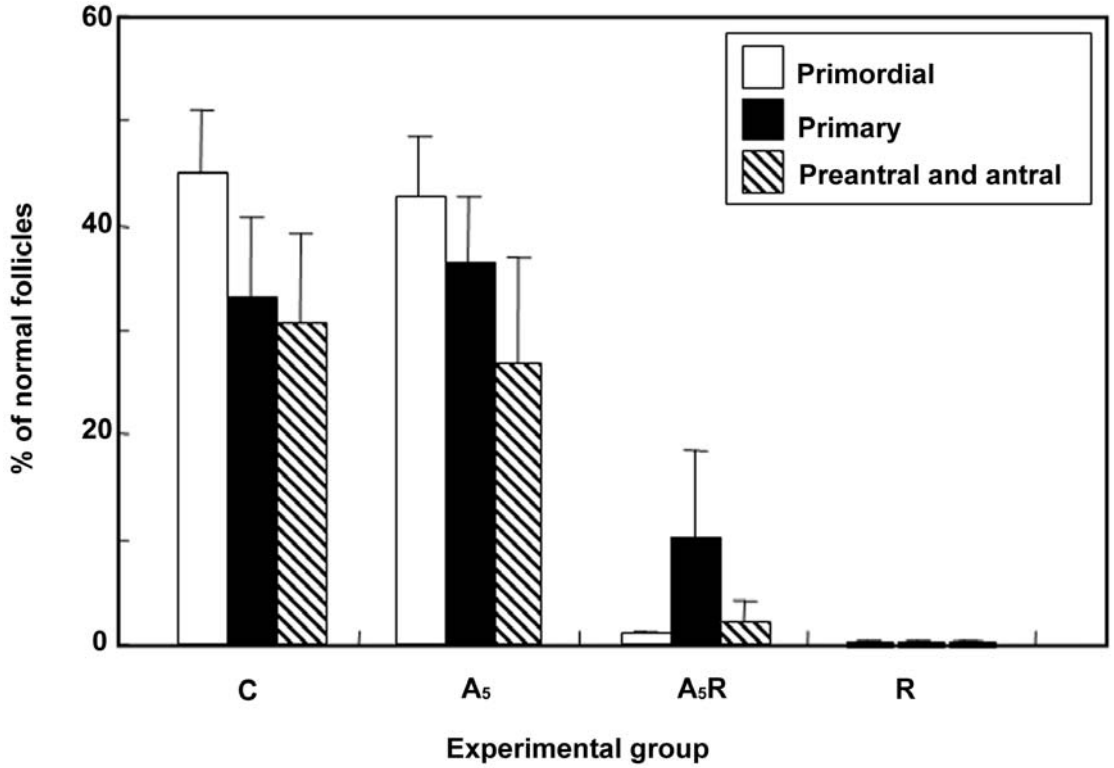

Figure 1. *The ratio of normal to total follicles of each developmental stage of oogenesis in irradiated mice. The largest cross sections of the whole ovarian slices were observed in the experiment. Abbreviations: C, the control group; A_5, the amifostine-treated group (LD_5, i.p. injection); A_5R, the irradiation group with amifostine pretreatment; R, the irradiated group without pretreatment.*

has been extensively used as a chemical radioprotector for normal tissues in cancer radiotherapy and chemotherapy (11). Protecting the body, especially in relation to tumor therapy, from the toxicities of ionizing radiation is a major concern. For that purpose, various chemicals have been synthesized and tested for their radioprotective effects. Among others, amifostine has been widely used in clinical trials. Amifostine was originally developed as a radioprotective agent under a classified nuclear warfare project by the Walter Reed Army Institute, and then it was approved as a new cytoprotective agent by the FDA in 1995 (12, 13). Despite a growing number of reports supporting amifostine's radioprotective efficacy in many tissues and the *in vitro* system, those related to the radioprotection of normal reproductive systems in prepuberty in the case of whole body irradiation are limited.

Therefore, the purpose of this study was to evaluate the effect of amifostine on the regulatory proteins and the granulosa degeneration related to the initiation of programmed cell death of the ovarian follicles in irradiated immature mice.

Materials and Methods

Treatment with amifostine and irradiation. Three-week-old female mice of the ICR strain were used. The animals were obtained from Daehan Biolink (Chungbuk, Korea) and housed under standard lighting conditions (12L:12D) and allowed food and water *ad libitum*. This experiment was approved by the Korean Laboratory Animal Care and Use Committee. All procedures were conducted in accordance with the ethical guidelines of the Committee. There were 6 experimental groups according to irradiation and/or amifostine (Sigma, MO, USA) exposure, each group composed of 5 mice. Amifostine dissolved in physiological saline was *i.p.* injected at a concentration of 70 mg/kg body weight (LD_5) 15 minutes before irradiation (14). The animals, either untreated or pretreated with amifostine, were whole body irradiated with 6.42 Gy, according to previous studies (15) of γ-rays from a ^{60}Co isotopic source (Panoramic Irradiator, AECL; source strength about 150 TBq). According to the experimental schedule, the animals were sacrificed by cervical dislocation and the ovaries were collected 0 hours after irradiation.

BrdU immunohistochemistry. To evaluate the proliferation of granulosa cells, BrdU (bromodeoxyuridine, Sigma) was injected *i.p.* 1 hour before irradiation. The excised ovaries (right side) were

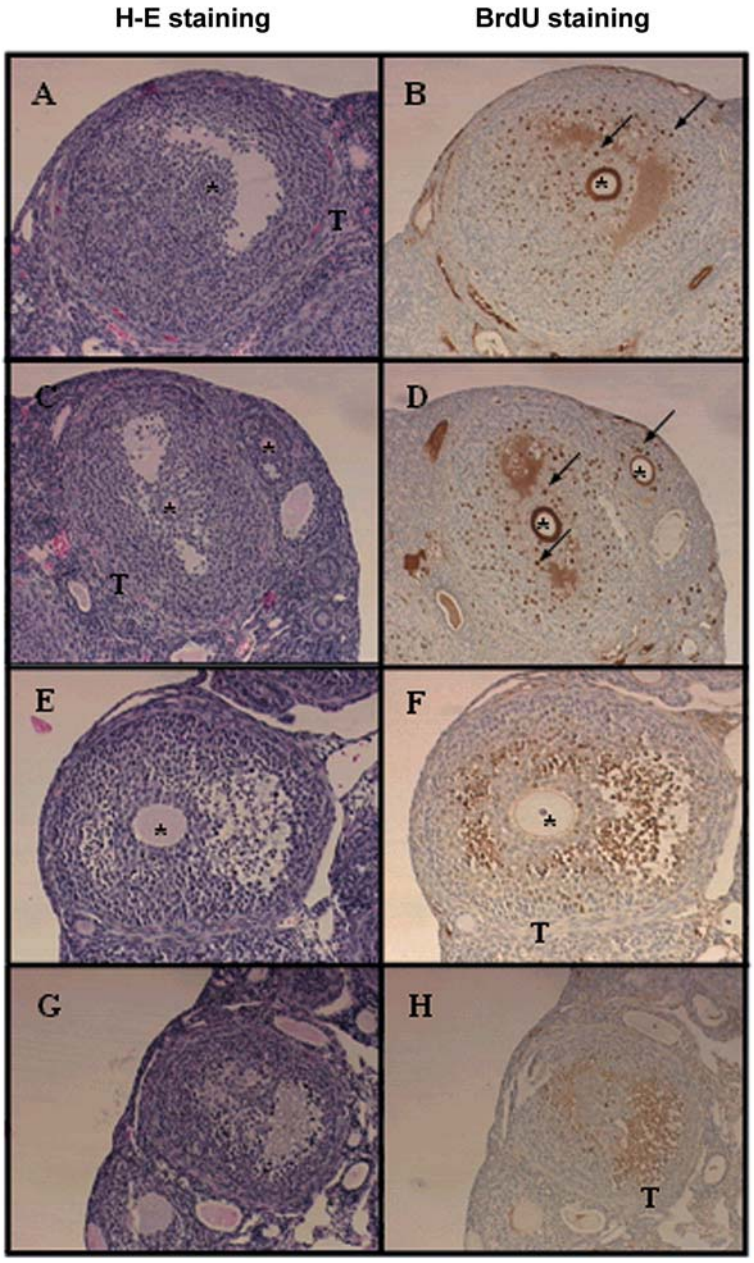

Figure 2. *Photomicrographs of ovarian follicles stained by BrdU immunohistochemistry in the right panel, while the left series of corresponding sections were stained by hematoxylin-eosin (H-E). A and B, the unirradiated saline-injected control group; C and D, the amifostine alone injected group; E and F, the irradiated group treated with amifostine; G and H, the irradiated group. Brown-colored cells (arrows) represent BrdU-positive granulosa cells. Asterisks and T represent the oocyte and theca layer, respectively. Original magnification was x400.*

fixed in 4% paraformaldehyde and embedded in paraffin. Cross sections of the ovary were cut 4-μm in thickness. The sections were deparaffinized, rehydrated in graded alcohols and treated with 2 N HCl to denature the DNA. This was followed by incubation for 30 minutes in 0.1% trypsin at 37˚C. Endogeneous peroxidase was inhibited with 3% hydrogen peroxide, and non-specific binding sites were blocked with normal goat serum. The sections were incubated with the primary antibody, monoclonal mouse anti-BrdU (Dako, Germany), followed by incubation with biotinylated goat anti-mouse antibody (Dako). The tissue sections were then incubated with streptavidin horseradish peroxidase. BrdU incorporation was localized by a final incubation with 3, 3'-diaminobenzidine tetrahydrochloride. The tissues were then counterstained with hematoxylin, dehydrated, coverslipped and studied by light microscope.

Western blot analyses. For protein immunoblotting, ovaries on the left side were collected, homogenized and disrupted in an extraction buffer consisting of 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF).

The samples were centrifuged at 1,000 xg for 20 minutes to remove debris. The supernatant was recentrifuged at 12,000 xg for 5 minutes. A part of the supernatant was taken for protein determination according to the Lowry method, while the remainder was immediately mixed (1:1) with 2 x SDS sample buffer and incubated in a heat-block for 5 minutes. Forty microgram of the total protein lysate was resolved onto 10 or 15% SDS polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF, Bio-Rad, CA, USA) membrane. The membranes were blocked by incubation with 3% BSA in Tween-Tris buffered saline (TTBS) for 1 hour at room temperature. The PVDF sheets were then incubated overnight at 4˚C in the primary antibody against each molecule diluted in 0.1% BSA-PBS (1:1, 000). Rabbit PARP antibody (Upstate, NY, USA), mouse p53 antibody (Santa Cruz Biotech, CA, USA), mouse bax antibody (Santa Cruz) and mouse caspase-3 antibody (Santa Cruz) were used as primary antibodies. After being rinsed with TTBS, appropriate horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, PA, USA) at 1:1,000 dilution for detecting polyclonal antibodies was added and incubated for 2 hours at room temperature. Immunoreative bands were visualized by the enhanced chemiluminescence (ECL) detection system, according to the manufacturer's protocol (Amersham Pharmacia Biotech, Sweden).

Statistical analysis. The results are expressed as the mean ± standard error of the mean (SEM). The means were compared using the Student's *t*-test assuming equal variances. A p value below 0.05 was considered significant.

Results and Discussion

Apoptosis is the physiological process of cell deletion and is essential in normal ovarian physiology. Follicular atresia can apparently occur at any point during follicular growth and development (3) and follicles become atretic at any stage of their development by intrinsic or extrinsic detrimental signals. In particular, apoptosis of granulosa cells is known to contribute to the regression of the ovary. Authors have reported that primordial follicular degeneration was induced by γ-irradiation (7). The morphological changes of primordial follicles with time after irradiation can be easily recognized by the presence of the apoptotic granulosa cells or degenerating oocytes (7, 16).

At the largest cross section of the ovarian slices, excised 6 hours after irradiation, the ratio of normal to total follicles was calculated at each stage of oogenesis (Figure 1). Primordial, primary, preantral and antral follicles in the irradiated group showed a remarkable degeneration, but, in the case of amifostine administration (LD_5) before γ-irradiation, a 10.3% of primary follicles was noted compared to that in the irradiated group.

In addition, BrdU staining was accomplished as a measure of proliferation inside the whole-body-irradiated ovary. Proliferation of granulosa cells reduced and the incidence of follicular atresia increased in the ovarian follicles of the γ-ray-irradiated mice compared to those of the control or amifostine-pretreated group (Figure 2). Therefore, pretreatment with amifostine may lead to a decrease in the irradiation-caused degeneration of the granulosa cells or ovarian follicles, especially the primary follicles.

It is well known that p53, a transcription factor, is involved in various cellular processes including cell regeneration, apoptosis, DNA repair, maintaining genomic stability and stem cell division kinetics (17-19). The p53 protein is induced by an exogenous stimulus (ionizing radiation, heat, pH, temperature and so on) and then provokes several molecules such as p21, Gadd45, Bax, Bcl-2 and PARP in a process characteristic of programmed cell death. The molecules are activated by proteolysis and changes occur in their cellular levels, corresponding to the internal signals (17).

An active caspase is activated by proteolytic cleavage to the large and small subunits. Procaspase-3 of 32 kD is cleaved to 17 and 12 kD-fragments during apoptosis. Activated caspase-3 (executioner) appears to be induced in the proteolysis of the PARP and Bcl-2 family. The elevated level of Bax as a member of the pro-apoptotic Bcl-2 family has been shown to enhance radiation-induced apoptosis, but only in the presence of functional p53 (20). PARP is a DNA nick sensor and uses βNAD^+ to form polymers of ADP-ribose, which are further bound to nuclear protein acceptors. PARP was cleaved and its upstream regulator, caspase-3, was activated (21, 22).

In the present study, marker proteins such as p53, caspase-3, Bax and PARP showed the representative radiation-induced alterative patterns 0 hours after γ-irradiation (Figure 3). However, the levels of p53 and Bax protein in the amifostine-pretreated irradiated groups showed a slight decline compared with the irradiated control as a result of amifostine pretreatment. Amifostine also caused a decrease of PARP proteolysis and the production of active caspase-3, especially in the A_5R group. Treatment with amifostine before γ-irradiation suppressed the p53-branched degenerative cascade *via* Bax and caspase-3 in the immature mice ovary. However, the rising activity of caspase-3 and Bax compared with the sham control in the amifostine-injected alone group is interesting. In the case of caspase-3, the antibody which we used was bound to the inactivated form of caspase-3. The elevated amount of caspase-3 in the amifostine-injected alone group in Figure 3 indicates the level of procaspase-3. Additional research is required to accurately understand these effects of amifostine on the activating process of caspase-3. The levels of caspase-3 in the irradiated groups did not show a remarkable difference (Figure 3), though it is clear that the levels of procaspase-3 in the unirradiated group were higher than in the irradiated groups. At the same time, the increased

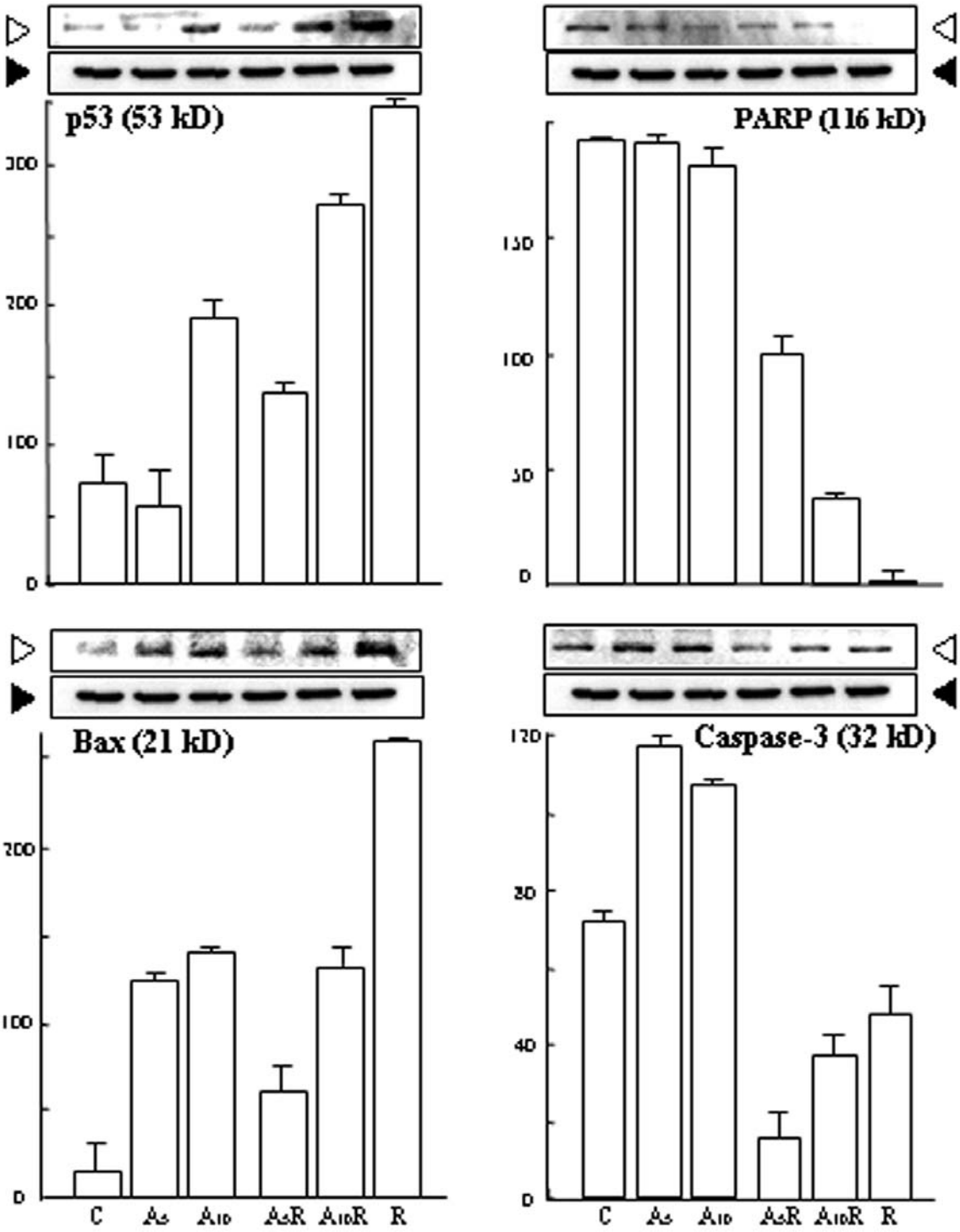

Figure 3. *Immunophoretograms of p53, PARP, Bax and caspase-3 protein in mouse ovaries 0 h after irradiation. Each 40 μg of supernatant protein from the homogenates was separated on a SDS-PAGE and immunoblotted with the respective antibodies. Actin protein (41 kD) was detected in the same membrane as the control. Relative levels are represented by the relative amounts of band density of each protein. The experimental groups are as follows: C, the unirradiated saline-injected sham group; A_5, the amifostine (LD_5)-injected group; A_{10}, the amifostine (LD_{10})-injected group; A_5R, the irradiated group treated with LD_5 amifostine; $A_{10}R$, the irradiated group treated with LD_{10}; R, the irradiated group. Open and closed arrow heads represent the desired protein and actin protein as a control, respectively.*

amounts of p53 and Bax and the extents of PARP indicate the effect of amifostine against whole-body γ-irradiation. It is clear that the caspase-3 and PARP interrelation indicates the effect of amifostine against irradiation. These findings suggest that amifostine has a protective effect on ovarian atresia induced by whole-body irradiation by interference in the degenerative processes.

Acknowledgements

This study was carried out under the Nuclear R&D Program of the Ministry of Science and Technology (MOST) of Korea, and supported by the research fund of the Korea Science and Engineering Foundation (ABRL: R14-2003-036-01001-0).

References

1 Tilly JL: The molecular basis of ovarian cell death during germ cell attrition, follicular atresia, and luteolysis. Front Biosci *1*: d1-d11, 1996.

2 Kim JH, Han JS and Yoon YD: Biochemical and morphological identification of ceramide-induced cell cycle arrest and apoptosis in cultured granulosa cells. Tissue Cell *31(6)*: 531-539, 1999.

3 Taylor AE, Schneyer AL, Sluss PM *et al*: Ovarian failure, resistance, and activation. *In:* Adashi EY and Leung PCK (eds.), The Ovary, pp.629-661. New York, Raven Press, Ltd., 1993.

4 Lee CJ, Park, HH, Do BR *et al*: Natural and radiation-induced degeneration of primordial and primary follicles in mouse ovary. Anim Reprod Sci *59*: 109-117, 2000.

5 Tilly JL: Apoptosis and ovarian function. Rev Reprod *1*: 162-172, 1996.

6 Kim JK, Lee CJ, Song KW *et al*: γ-radiation accelerates ovarian follicular atresia in immature mice. *In Vivo 13(1)*: 21-24, 1999.

7 Jacquet P, de Saint-Georges L, Buset J *et al*: Cytogenetic effects of X-rays in the guinea pig female germ cells: II. The maturing oocyte. Mut Res *391*: 193-199, 1997.

8 Allan DJ: Radiation-induced apoptosis: its role in a MADCaT (mitosis-apoptosis-differentiation-calcium toxicity) scheme of cytotoxicity mechanisms. Int J Radiat Biol *62*: 145-152, 1992.

9 Takahashi A, Ahnishi K and Ohnishi T: Radiation response of p53 and WAF1 accumulation after chronic or acute irradiation. *In:* Yamada T, Mothersill C, Michael BD and Poten CS (eds.), Biological Effects of Low Dose Radiation, pp. 59-66. Elsevier Science B. V., Amsterdam, 2000.

10 Fukasawa K, Wiener F, Vande Woude GF *et al*: Genomic instability and apoptosis are frequent in p53 deficient young mice. Oncogene *15*: 1295-1302, 1997.

11 Grdina DJ, Kataoka Y and Murley JS: Amifostine: mechanisms of action underlying cytoprotection and chemoprevention. Drug Metabol Drug Interact *16(4)*: 237-279, 2000.

12 Culy CR and Spencer CM: Amifostine: an update on its clinical status as a cytoprotectant in patients with cancer receiving chemotherapy or radiotherapy and its potential therapeutic application in myelodysplastic syndrome. Drugs *61(5)*: 641-684, 2001.

13 Nguyen NP, Levinson B, Dutta S *et al:* Amifostine and curative intent chemoradiation for compromised cancer patients. Anticancer Res *23*: 1649-1656, 2003.

14 Shaw LM, Bonner HS and Brown DQ: Metabolic pathways of WR-2721 (ethyol, amifostine) in the BALB/c mouse. Drug Metab Dispos *22(6)*: 865-902, 1994.

15 Kim JK, Kim JH and Yoon YD: Evaluation of caffeine as a radioprotector in whole-body irradiated male mice. In Vivo *17*: 197-200, 2003.

16 Kim JK and Lee CJ: Effect of exogenous melatonin on the ovarian follicles in γ-irradiated mouse. Mut Res *449*: 33-39, 2000.

17 Tilly JL, Tilly KI, Klenton ML *et al*: Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. Endocrinology *136*: 232-241, 1995.

18 Takahashi A: Pre-irradiation at a low dose-rate blunted p53 response. J Radiat Res *43*: 1-9, 2002.

19 Thornberry NA and Lazebnik Y: Caspases: enemies within. Science *281*: 1312, 1998.

20 Brady HJ, Salomons GS, Bobeldijk RC *et al*: T cells from baxalpha transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53 gene product. EMBO J *15*: 1221-1230, 1996.

21 Khan SM, Dauffenbach LM and Yeh J: Mitochondria and caspases in induced apoptosis in human luteinized granulosa cells. Biochem Biophy Res Comm *269*: 542-545, 2000.

22 Soldani C and Scovassi A: I. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis *7*: 321-328, 2002.

Received September 24, 2004
Accepted February 28, 2005