The Radioprotective Activities of Turpentine-induced Inflammation and $\alpha_2$-Macroglobulin: The Effect of Dexamethasone on the Radioprotective Efficacy of the Inflammation

LIJILJANA ŠEVALJEVIĆ1, SILVA DOBRIĆ2, DESANKA BOGOJEVIĆ3, MIODRAG PETROVIĆ3, GORAN KORICANAC1*, MOJCA VULOVIĆ1, DUŠAN KANAZIR1 and NEVENA RIBARAC-STEPIĆ1

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This work was aimed at the radioprotective efficacy of turpentine oil (TO), $\alpha_2$-Macroglobulin ($\alpha_2$-M), Amifostine (Ami) and/or dexamethasone (Dex). These agents were administrated, alone or in combination, prior to irradiation of rats with 6.7 Gy (LD$_{50}$/30). The survival was recorded daily for 4 weeks after irradiation and body weight, peripheral leukocytes and thrombocytes were measured. The plasma concentration of $\alpha_2$-M and other acute phase proteins were determined by crossed immunoelectrophoresis. All rats receiving $\alpha_2$-M and Ami alone or in combination survived the radiation injury, whereas the rate of survival of TO-treated rats was 90%. Radiation and therapy-induced changes in the expression of acute phase protein genes were atypical for the acute phase reaction. Dex alone was lethal for 45% and 55% of control and irradiated rats, respectively. Pretreatment with 1mg Dex reduced radioprotective efficacy of TO and Ami to 30% and 40%, respectively. Given together TO and Ami provided 70% protection to rats receiving Dex. The TO and $\alpha_2$-M enhanced the rate of survival from 50% to 90% and 100%, respectively. In the presence of 1mg Dex the TO-induced radioprotectors and Ami exhibited radiosensitizing rather than radioprotecting activities.

INTRODUCTION

The responses to ionizing radiation (IR) and acute tissue (AP) injuries share the capability to produce highly reactive oxygen intermediates which activate the second messenger system and elicit synthesis of three principal inducers of acute phase protein (APP) genes, namely IL-1, TNF$^1,2$ and IL-6$^3$. However, the pattern and timing of IR-induced APP synthesis$^4,5$ are different from those observed for AP response. Moreover, a multiple deleterious effects of IR on rapidly proliferating tissues (hematopoietic, gastrointestinal) render the clinical symptoms of the acute radiation disease and the acute response to inflammatory agents largely different. According to the established model of AP response a cascade of cytokines-mediated processes lead to activation of immune system and a concomitant synthesis of the counteracting glucocorticoids. The latter suppress cytokines production and actions, but synergize with them in the induction of synthesis of acute phase proteins (APP) which in concert with the inducers led to resolution of AP-response and recovery$^6,7$. In contrast to inflammatory agents, the primary effect of IR is on the rapidly proliferating hematopoietic elements of the bone marrow resulting in the destruction of hematopoietic components, depression of the immune response and enhanced susceptibility to infections$^8$. A large number of radioprotecting agents is presently used in radiotherapy (reviewed in Ref. 11$^{11}$). Of particular interest for the subject of this work were the findings that an inflammatory agent such as LPS$^1,2$ and two LPS inducible cytokines, the IL-1 and TNF$^13,15$ provide radioprotection from otherwise lethal dose of irradiation. Moreover, a major cytokines/glucocorticoids inducible APP, the $\alpha_2$-macroglobulin ($\alpha_2$-M), was found to provide protection against both, lethal scald$^{16}$ and lethal irradiation$^{17,19}$.

The $\alpha_2$-M is a tetrameric, disulfide bridges-containing plasma glycoprotein which as a multifunctional binding protein reacts with a wide range of proteases$^{20}$, as well as with numerous growth factors, cytokines and hormones, including the two principle AP-cytokines, the IL-1$\beta$ and TNF$\alpha$.$^{21,22}$ Glucocorticoid hormones have been identified as principal inducers of $\alpha_2$-M rat liver$^{23,24}$. Although the interactions of $\alpha_2$-M with various enzymes, inducers and mediators are considered to be a function of homeostasis restoration, the mechanisms underlying its protective effect from lethal injury are largely unknown.
In the present work we studied the radioprotective effect of α₂-M on the model of rats exposed to changes in the levels and mutual ratios of α₂-M and its inducers. An increase in the level of α₂-M was achieved either by i.v. injection of exogenous rat α₂-M or by induction of its synthesis in the liver of rats receiving 1 mg of dexamethasone and/or turpentine oil. The conditions differing in the levels of AP-cytokines and the ratios of glucocorticoids and AP-cytokines were created by exposure of untreated and Dex pretreated rats to inflammation.

The experiments were designed to provide answers to the following questions: (i) does the turpentine oil (TO)-induced inflammation has radioprotective effect; (ii) does the injection of α₂-M before irradiation enhance the rate of survival; (iii) does the α₂-M exert radioprotective effect if produced endogenously by injection of 1 mg dose of its inducer, dexamethasone; (iv) would the inflammation of Dex-pretreated rats, involving an α₂-M gene, provide radioprotective effects despite the Dex-induced inhibition of AP-cytokines.

The results presented in this work provide evidence for a high radioprotective effect of both, the administration of exogenous rat α₂-M and TO-induced inflammation. However, dexamethasone-induced α₂-M as well as TO and Amifostin given after dexamethasone, exerted radiosensitizing rather than radioprotective effect.

**MATERIALS AND METHODS**

**Chemicals**

Amifostine and dexamethasone were purchased from Sigma, St. Louis, USA.

**Animals**

We assure that experimental animals were used in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). These because, we have mainly used radioprotectors such as amifostine, α₂-macroglobulin and subletal inflammation (turpentine oil). For dexamethasone that we expected to have protective effect, the results showed, that in 1 mg dose it exerted some toxic effect.

The experiments were performed on adult male albino Wistar rats of weight 250–300 g. The animals were housed in cages at an experimental temperature of 20–22°C and fed with standard laboratory chow and water.

**Irradiation**

Rats were total body irradiated (Phillips linear accelerator SL 75–80, 8 MeV) with 6.7 Gy at the rate of 3.4 Gy/min. Each experimental group consisted of 10 animals placed in a plexiglass container divided into 10 chambers. The container was covered with 1 cm thick plexiglass sheet and rats were exposed to radiation at 100 cm distance from the source. The number of surviving rats was recorded daily for 30 days.

**Experimental protocol**

In one series of experiments amifostine, rat α₂-macroglobulin and turpentine oil were given alone or in combination before irradiation. In another series the groups receiving amifostine and turpentine oil were pretreated with dexamethasone. Those assigned for irradiation were exposed to 6.7 Gy as described above, whereas the non-irradiated groups were used as the controls. Each of the groups contained 10 to 30 rats.

Amifostine (WR 2721) was given i.p. 20 min before irradiation (300 mg/kg of body weight in 1 ml saline). The rats received s.c. injection of turpentine oil in the abdominal region (1 μl/g of body weight) and were exposed to irradiation 18 h later. The rats were injected with 4.5 mg α₂-macroglobulin in 0.7 ml saline in penile vein 30 min before irradiation. This amount was calculated to ensure that more than tenfold the basal value of the plasma α₂-macroglobulin concentration was achieved.

The group assigned for treatment with dexamethasone received i.p. 250 μl of 1% (v/v) ethanol in saline containing 1 mg of dexamethasone, 18 h before irradiation.

Body weight and the number of surviving rats were recorded daily for 4 weeks after irradiation. Blood samples for hematological and immunoelectrophoretic determinations were drawn from a fine incision in the tail vein one day before irradiation (basal values) and on days 1, 3, 7, 14, 21 and 28 after irradiation. Peripheral leukocytes and thrombocytes were measured by means of the apparatus Hemocount ERP-9 (Ortho Diagnostic Systems, Germany).

**Isolation of α₂-macroglobulin**

Rat α₂-macroglobulin was isolated from the blood serum collected 24 h after injection of turpentine oil (200 μl/rat). The pooled sera were sequentially fractionated with dextran sulphate, DEAE cellulose and gel filtration as described by Okubo et al. The purity of the isolated α₂-macroglobulin was tested by polyacrylamide gel electrophoresis. The preparations forming a single band upon electrophoresis were further processed for injection into rats.

**Determination of the plasma concentration of individual acute phase proteins**

The relative concentrations of α₂-macroglobulin (α₂-M), α₂-acid glycoprotein (AGP), haptoglobin (Hp) and thiostatin (TST) were determined by crossed immunoelectrophoresis according to the procedure of Garrot. For the preparation of polyspecific antiserum blood samples were taken 24 h after injection of turpentine oil. Serum aliquots (150 μl) were diluted with saline (350 μl), mixed with complete Freund’s adjuvant and administered subcutaneously into rabbits. Over a period of 2 weeks, three to five more injections were given with incomplete Freund’s adjuvant. The concentrations of acute phase proteins were established by quantification of the areas under the corresponding immunoprecipitation peaks and expressed as the ratio between the value obtained for the treated and control (100%) samples.
Statistical analysis

The results were analyzed by Anova followed by Tukey’s t-test. The criterion for significance was set at $p < 0.05$.

RESULTS

Comparison of radioprotector efficacy of Amifostine, $\alpha_2$-macroglobulin and turpentine-induced inflammation

The radioprotective effects of turpentine oil (TO)-induced inflammation and $\alpha_2$-macroglobulin ($\alpha_2$-M) were examined by measuring the survival time of rats receiving these agents 18 h and 20 min, respectively, before exposure to 6.7 Gy. The results of these experiments were recorded for 30 days and compared with the results obtained for the controls. The control groups consisted of nonirradiated rats receiving therapy. The survival rate of all of the control groups was 100%.

Figure 1 shows that all rats survived the first post-radiation week. Thereafter the number of survivors in the irradiated group receiving no therapy decreased with time reaching the lowest level of 50% by the end of the fourth week. Given 20 min before irradiation Ami provided a full protection. In the group receiving TO 18 h before irradiation 90% of rats survived the radiation injury. For TO administered rats which received Ami 20 min before irradiation the 30-day survival was 100%. Thus the radioresistance of rats exposed to radiation 18 h after the onset of the acute phase reaction to inflammation was found to be substantially increased. In this period of the acute phase response the increase in the plasma $\alpha_2$-M concentration can be achieved by injection of 4.5 mg of $\alpha_2$-M to control rats. Consequently, the group assigned for testing the radioprotective activity of $\alpha_2$-M received 4.5 mg of $\alpha_2$-M, 20 min before radiation exposure. The results revealed that $\alpha_2$-M pretreatment provided 100% protection against the lethality of 6.7 Gy dose of X-rays. Radioprotective efficacy of $\alpha_2$-M was thus found to be equal to that of Ami (WR 2721) which is one of the most promising compounds in clinical radiation therapy.

The effect of Ami, $\alpha_2$-M and TO therapy on the body weight and the number of circulating leukocytes and thrombocytes in irradiated rats

Figure 2 shows that rats used as controls gained about 20% in body weight during the 30-day period. In contrast to controls, the weight of irradiated rats decreased with time after irradiation reaching the highest reduction of 17% at the end of the second week. In the third week 50% of rats succumbed due to injury (Fig. 1), whereas the survivors tended to regain the initial body weight. Irradiated rats receiving Ami showed no significant change in body weight during the first two weeks. Thereafter, they rapidly gained weight, the increments registered for the second and third week being lower and for the fourth week equal to those of controls. Rats receiving $\alpha_2$-M showed a small but progressive weight loss during the first two weeks, the 14 day point even becoming significant. However, this phase failed to appear if $\alpha_2$-M was given in combination with Ami. The phase of weight loss in rats receiving $\alpha_2$-M alone was succeeded by a rapid increase in body weight, and the increments after the third and fourth week were higher than those observed in controls or in Ami treated groups. Unexpectedly, the radioprotective effect of TO-induced inflammation was found to exhibit a shorter period of decrease in weight and a consequent prolongation of the period of increase. The increments at the 7 and 14-day points were comparable to those observed in controls, whereas at the 21 and 28-day points they surpassed the values obtained for this or any other groups. Interestingly, rats receiving TO in combination with Ami gained weight at significantly lower rate than
Independent of therapy, the number of circulating leukocytes was 60% lower 24 h after irradiation. Ami alone or in combination with α2M prevented a further decrease in the leukocyte levels, whereas their number in the blood of unprotected and α2M treated rats continued to fall during the next 6 days to 7% and 17%, respectively. In the following three weeks a gradual restoration of the basal leukocyte level was recorded. At the 21-day point, the values for Ami and α2M administered rats were at basal level and 20% above, respectively. At the same time the number of circulating leukocytes in the blood of the survivors deprived of therapy or receiving Ami in combination with α2M was still about 50% below the basal level. At the end of the 30-day period the values obtained for all groups were 10–20% less than those recorded before irradiation.

The level of thrombocytes declined during the first post-radiation week but fell faster for unprotected than for protected rats (Fig. 3). On day 7 the thrombocyte counts reached the lowest level of 8% for the unprotected rats and 15–20% for the groups receiving therapy. Thus therapy slowed the rate of thrombocytes loss during the first post-radiation week. In addition, the recovery of thrombocytes during the second week proceeded faster for rats receiving Ami alone or in combination with α2M than the groups of unprotected or α2M treated rats. During the third week the level of thrombocytes in the blood of protected and unprotected rats increased to 50 and 20% of the basal value, respectively. At the 28-day point the thrombocyte counts for unprotected rats and for the group receiving Ami alone or in combination with α2M were about half of the basal level, but were higher in α2M administered rats.

These results indicated that administration of α2M stimulated the regeneration of bone marrow cells to an extent comparable to that observed for Ami.

Changes in the plasma acute phase protein concentrations in response to radiation injury and the applied therapy

In rats exposed to inflammatory agents the rate of acute phase protein (APP) synthesis increased with time after a 4 h lag period and reached the plateau level by the end of the first day. Thereafter it declined gradually reaching the control level after several days. For that reason the effect of radiation injury on the plasma APP concentration was measured at the 24 h and the 7-day points only.

Figure 4 shows that irradiated rats deprived of therapy exhibited at both points a twofold and 50% increase in the plasma α2M and AGP levels, respectively. These results differed greatly from the typical acute phase reaction where the plasma levels of these two major APP increased severalfold by the end of the first day and attained the control level on day 7. In contrast to α2M and AGP the plasma concentrations of three other APP, i.e hemopexin (Hx), haptoglobin (Hp) and thiostatin (TST), underwent no change during the first day. At the 7-day point the level of Hx remained unaltered, whereas the concentrations of Hp and TST were doubled. Thus the timing of increase in the Hp and TST levels in rats exposed to irradiation was found to be inversely
related to that observed after inflammation. The effect of therapy with Ami was the doubling of the plasma TST concentration, and an additional 50% increase in the AGP and Hp levels, at the 24 h point. At the 7-day point there was no further increase in the plasma TST level, whereas the Hx and Hp concentrations were more than twofold higher than in the controls. After injection of 4.5 mg of α₂-M the plasma concentration of α₂-M at the 24 h and 7-day points was more than tenfold the control level (Fig. 4). However, this therapy exerted a negligible effect on the levels of other APPs, except for Hx whose concentration on day 7 was twice higher than that observed for unprotected group. Thus Hx, TST and Hp were shown to be more sensitive to irradiation and therapy than the major APP, α₂-M and AGP. For that reason the effect of the combined therapy with Ami and α₂-M on the plasma Hx, TST and Hp levels were examined throughout the 30-day period.

Figure 5A shows that in rats receiving α₂-M in combination with Ami the plasma α₂-M level was enhanced tenfold during the first week. Thereafter it gradually decreased approaching the control value at the 28-day point. When acting together Ami and α₂-M promoted an increase in the Hp level ranging between 30 and 70%. At the 7-day point the magnitude of increase was even smaller than that observed for unprotected rats or the group receiving Ami alone (Figs. 5B and 4). However, the plasma Hx and TST concentrations increased gradually during the period from 14 to 28 days to values twice higher than in the controls.

These results indicated that radiation injury and the applied protective agents stimulated expression of the investigated APP, but preferentially of Hx, TST and Hp. However, neither the timing nor the level of individual APP expression were similar to those characterizing the acute phase reaction to acute tissue injury.

The effect of 1 mg dexamethasone dose on the 30-day survival of rats unexposed and exposed to irradiation

Dexamethasone (Dex) is a synthetic glucocorticoid which exerts a strong inhibitory action on the production and the activities of proinflammatory cytokines. When injected in 1 mg dose Dex was found to induce a several fold increase in the level of α₂-M gene expression in rat liver. In these experiments we asked whether α₂-M produced endogenously in response to an inducer which inhibits the production and actions of cytokines would have a radioprotective effect. Or, whether radioprotective activities of Ami, α₂-M and proinflammatory cytokines would be affected by an excessive concentration of glucocorticoids.

Figure 6A shows that all rats receiving 1mg Dex survived the first 7 days only. Thereafter the number of survivors fell to 76% at the 10-day point and to 64% at the 28-day point. Thus 1 mg...
Dex was found to exert toxicity corresponding to LD_{64/30} dose of a toxic agent. For that reason, a several fold increase in the level of rat liver $\alpha_2$-M gene expression which was observed 18 h after injection of 1mg Dex could be considered as an event elicited in response to a Dex-induced metabolic injury. Inflammation alone increased the 30-day survival of Dex-pretreated rats from 64% to 80%. However, Ami alone led to an additional 10% decrease in the number of survivors. Thus the survival of rats receiving Ami in the presence of an excessive concentration of Dex was reduced to 53%, whereas TO given alone or in combination with Ami enhanced the rate of survival to 80%.

Figure 6B shows the effect of irradiation on the survival time of Dex-pretreated rats deprived of therapy or protected with Ami alone or in combination with TO-induced inflammation. Rats receiving Dex 18 h before irradiation succumbed to injury at the rate comparable to that observed in non-pretreated rats exposed to 6.7 Gy (Fig. 1). However, the number of survivors in this group was 10% lower than that observed in Dex treated rats unexposed to radiation (Fig. 6A). Surprisingly, injection of Ami failed to protect Dex pretreated rats against the lethality of 6.7 Gy. Instead, the number of survivors receiving Ami was reduced from 53% to 45%. The radiosensitizing effect of TO was even more pronounced than that of Ami. Almost 70% of rats which received Dex before the exposure to TO-induced inflammation succumbed to radiation injury. However, given together, Ami and TO reduced the radiation-induced mortality of Dex-pretreated rats from 50% to 30%.

These results indicated that $\alpha_2$-M synthesized in response to...
1 mg Dex exerted no protective effect against the lethality of 6.7 Gy. In addition, Dex created conditions under which Ami or inflammation acted as radiosensitizers rather than radioprotectors. This effect of Dex was greatly reduced if the Ami and TO were given together.

**DISCUSSION**

In this study TO-induced inflammation and rat α2-M were shown to provide a 90% and 100% protection, respectively, against the lethality of 6.7 Gy dose of X-rays (LD50/30). The recovery was accompanied by the rise in the levels of circulating leukocytes and thrombocytes, as well as by the restoration of body weight. Both processes proceeded at the rates similar to those observed for one of the best radioprotector known to date, Ami.

The acute phase response to an agent is known to be achieved through the induction of proinflammatory cytokines. The radioprotective activity of an inflammatory agent such as bacterial endotoxin (LPS) were recognized more than forty years ago and confirmed more recently. Two LPS-induced cytokines, the IL-1 and the TNF, was also identified as efficient radioprotectors. The list of radioprotectors inducible by inflammatory agents was recently enlarged with the products of IL-1 mediated degradation of arachidonic acids, nitroxides, leucotrienes, and prostaglandins, all of which are known to be involved in the regulation of blood volume and pressure during the acute phase reaction. The activation of α2-M and other acute phase protein genes takes place in the aftermath of the early events such as the induction of cytokines, release of proteases, degradation of arachidonic acids and synthesis of glucocorticoids. In our experiments rats were irradiated 18 h after the onset of acute phase reaction when the rates of synthesis of IL-1, TNF, nitroxides, prostaglandins and leucotrienes were falling, while those of α2-M and other APP were approaching their maxima. The radioprotective efficacy which TO-induced inflammation exhibited at the 18 h point could, therefore, be related to the radioprotective activities of α2-M but also to the occurrence of the synergistic interactions between the early-synthesized radioprotectors and α2-M.

Radiation-induced release of proinflammatory cytokines, TNF, IL-1 and IL-6 and activation of APP genes in the rat liver were interpreted as the manifestation of the acute phase reaction. Our results showed that 24 h after irradiation with 6.7 Gy the plasma α2-M level was doubled, whereas the concentrations of Hp, AGP, TST and Hx were at the control level or slightly above. Seven days later when the expression of APP in irradiated rats was dissimilar from those operating in a typical acute phase reaction. The divergences were likely to arise from the deleterious effects of reactive oxygen radicals on the proinflammatory cytokines-mediated processes. The radioprotective efficacy of TO-induced inflammation might thus be explained by the protection of several radioprotectors synthesized during the acute phase reaction against the deleterious activities of oxygen radicals.

Our finding that pretreatment with α2-M provided a 100% survival of irradiated rats is in agreement with the previously reported radioprotective effects of the isolated 19S serum globulin fraction. The radioprotective activity of the serum 19S α2-globulin has been correlated with its stimulating effect on the regeneration of bone marrow cells and thymus and with its capability to inhibit lymphatic leukemia in irradiated mice. The more recent data also stress the regulatory role of α2-M in the maintenance of hemodynamic equilibrium. This is likely to rely on its capability to act in two ways, as a vasoconstrictor and vasodilator. It was reasonable, therefore, to assume that α2-M injected prior to irradiation or synthesized in the acute phase liver could achieve its radioprotective efficacy by dint of its involvement in the metabolic pathways of diverse cytokines and cytokines-derived biological mediators. Thiol-containing compounds have long time been known to possess radioprotective properties and it is likely that activation of thiol groups during these interactions confers radioprotective property to α2-M molecules.

The possible dependence of α2-M radioprotective activities on the presence of cytokines was assessed in the experiments based on the finding that potent inhibitors of cytokines production and action i.e., glucocorticoids, have been identified as principal inducer of α2-M gene in the acute phase rat liver. If the radioprotective activities of α2-M were not functionally linked to the production and the actions of proinflammatory cytokines, then the inhibition of these processes by an excessive dose of α2-M inducer, Dex, would not affect the radioprotective action of an inflammation. However our getting of a clear cut answer was hampered by the finding that all rats receiving 1 mg Dex survived for one week only. During the next three weeks more than one third of rats succumbed to the Dex-induced metabolic injury. A several fold increase in the α2-M mRNA level, and significant enhancement in the levels of AGP and Hp mRNAs which was observed 18 h after injection of Dex could, therefore, be ascribed not only to the inducer activity of Dex, but also to the acute phase reaction to Dex-induced metabolic injury. Inflammation in Dex-pretreated rats reduced their mortality rate from 36% to 20% (Fig. 6A). This finding suggested that some biological mediators counteracting the toxic effects of Dex were synthesized in response to inflammation. This suggests that metabolic injuries induced by 1 mg Dex or 6.7 Gy do cause some atypical acute phase reactions.

Irradiation of Dex-pretreated rats reduced the rate of the 30-day survival from 64% to 53% (Fig. 6B). This finding argued against the use of Dex, at least in such a large dose, as a therapeutic drug in the prevention of an uncontrolled rise of radiation-induced proinflammatory cytokines. Moreover, Dex-induced inhibition of proinflammatory cytokines production had greatly reduced the radioprotective efficacy of TO. Similar failure of
radioprotective efficacy has been observed following the inhibition of IL-1 and TNF-mediated effects of LPS\textsuperscript{15}. Even Ami was found to lose the radioprotective activity in the presence of Dex. Ami is a phosphorothiolate that is inactive until dephosphorylated by alkaline phosphatase to yield the radioprotective free thiol form. However, despite the inhibitory effect of Dex on the induction of proinflammatory cytokines, and the radioprotective activity of Ami, the cooperative actions of Ami and TO led to an enhancement of 30-day survival of irradiated rats from 50\% to 70\% (Fig. 6B). This coincided with an increase in the plasma Hx and TST levels during the third and the fourth weeks. Hemopexin is a heme binding protein mediating the uptake of heme by the liver. During the LPS-induced inflammation heme supplementation was found to stimulate the expression of $\alpha_2$-M gene and to affect the synthesis of P450 and several enzymes, including nitric oxide synthase\textsuperscript{60}. The significance of the increase in a cysteine protease inhibitor, such as TST, is unclear at present. However, overall results suggest that radioprotective efficacy of TO-induced inflammation, $\alpha_2$-M and Ami, are greatly influenced by processes involved in glucocorticoids signaling pathways. It is likely, therefore, that environmental conditions created by an excessive concentration of Dex prevented formation of active thiol groups in $\alpha_2$-M and Ami, facilitating thus their transition from antioxidative radioprotective to prooxidative radiosensitizing forms.

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