The effect of immunotherapy on the state of the antioxidant system in radiation damage in rats

Ruslan Gainullin1, Edie Plotnikova1*, Mulanur Shakurov1, Alexey Frolov1, Ekaterina Mayorova1, Elena Matveeva2, and Dina Sharifullina1

1Federal center for toxicological, radiation and biological safety, Nauchniy Gorodok Street 2, Kazan city 420075, Russia
2Kazan Innovative University named after V.G. Timiryasov, Moskovskaya Street 42, Kazan city, 420111, Russia

Abstract. Immunotropic drugs (such as sera, blood plasma and immunoglobulins) have polyfunctional activity with anti-infectious, anti-toxic, homeostasis-regulating properties. Based on this, we conducted the present study with the purpose to evaluate the therapeutic effect of anti-cadmium - anti-radiation serum (AARS) on the level of radiation-chemical damage. Modeling of experimental radiation-chemical damage was carried out by preliminary 4-time subcutaneous injection of cadmium chloride solution (CdCl2) at doses of 1/500 LD50, followed by radiation exposure of 90 outbred white rats at doses of 7.0 and 9.0 Gy (LD50 and LD100, respectively). It was found that a single subcutaneous injection of TDC at a dose of 25 mg/kg of live weight 24 hours after the combined radiation-toxic damage had a radioprotective and antitoxic effect, increasing the survival rate of animals affected by lethal doses of CdCl2 and γ-rays. Increase in the survival rate of animals treated with the test agent after exposure to two-factor damage was due to inhibition of the concentration of BFR-active toxic compounds and reciprocal increase in the synthesis of metallothioneins and SOD. Thus, the use of immunotropic agent of anti-cadmium anti-radiation serum (AARS) after exposure to two-factor combined radiation-chemical damage provided 90% survival rate by induction of increased formation of metallothioneins and SOD, inhibition of redox-cycled toxic radicals formation and neutralization of cadmium toxicity with the help of anti-cadmium antibodies. Keywords: therapeutic-decorporating composition, montmorillonite, metallothionein, superoxide dismutase.

1 Introduction

Technogenic impact on the biosphere has become one of the significant factors that determine the conditions of existence on our planet [1-3]. The main element in the pathogenesis of radiation and toxic damage is the activation of free radical processes and the failure of antioxidant defense system of the organism [4, 5]. Free radicals are chemical molecules containing one or more unpaired electrons that make these molecules highly reactive. Inducers of free radicals are UV-rays, X-rays, γ-rays, toxicants [6].
The harmful effect of free radicals is compensated by the action of antioxidant enzymes and non-enzymatic antioxidants. Due to the presence of a large number of SH-bonds, metallothioneins were shown to act as an active antioxidants under both single and combined effects of stress factors, in particular, cadmium and radiation damage [7].

Many scientists studied the role of metallothioneins in the radioprotection of muscle fibroblasts and human cell cultures. Metallothionein was shown to be 300 times more active than the antioxidant enzyme glutathione in relation to the superoxide radical induced by ionizing radiation. The antioxidant properties of metallothionein are caused by the high content of thiol (SH) groups and by their ability to activate antioxidant enzymes, such as superoxide dismutase and catalase [8].

An analysis of the literature data over the past 10 years allowed us to conclude that metallothioneins not only detoxify heavy metals, in particular cadmium, but also play an important role in the binding of free radicals induced by UV-rays and radiation exposure. The natural interest of radiobiologists is focused primarily on the protective role of metallothioneins during cytotoxic oxidative stress. They act as interceptors and extinguishers of free oxygen radicals, exerting a radioprotective and antitoxic (anticadmium) effect [9]. A relationship has been established between increased content of metallothioneins in the bone marrow and survival rate of mice exposed to radiation after the administration of heavy metal salts. It was also found that 4-time subcutaneous injection of cadmium chloride solution to mice (1 cm³ per animal) before exposure to radiation at a dose of 9.0 Gy caused a radioprotective effect, providing 40% protection from radiation death with 100% death of animals of lethal control group.

Considering the fact that inductors of metallothioneins have physical (UV-rays, γ-rays), chemical (cadmium, zinc, bismuth, mercury, etc.), biological (zinc-containing plant tissues – alfalfa, globulins, liver extracts, etc.) nature, and that chronic exposure to γ-rays and heavy metals (cadmium) in small doses has an activating effect on the body's metallothionein synthesis system, we conducted these studies with the purpose to evaluate the therapeutic effect of anti-cadmium anti-radiation serum.

2 Materials and Methods

The experiments were performed in accordance with the requirements of the "Rules for conducting work using experimental animals" (Order of the Ministry of Health of Russia No. 267 dated June 19, 2003) on white rats 14-15 weeks of age in a vivarium of Federal Center for Toxicological, Radiation and Biological Safety. The animals were kept on a standard diet, the live weight of rats was 200 g.

To simulate radiation-chemical damage, experimental animals (25 white rats) were subjected to oral administration of cadmium chloride at a dose of 1.5 mg/kg (5 MPC) for 5 days followed by radiation exposure at the "Puma" γ-installation at a dose of 7.0 Gr (LD50). Solution of cadmium chloride was administered intragastrically. Animals of the control group (25 rats) were not exposed to radiation and were not subjected to administration of cadmium chloride.

The selection of peripheral blood was carried out on the 5th day after radiation-chemical exposure. Serum was obtained from blood samples, globulins were isolated, standardized to 10% concentration, sterilized with γ-rays at a dose of 20 kGy. The content of metallothioneins (MT) was determined in the sera from experimental and control animals by enzyme immunoassay using a kit for the quantitative determination of total metallothioneins in the blood manufactured by Cusabio (China) and containing plates with immobilized antimetallothionein antibodies. The measurements were performed on a Microelisa Autorecedez MR 580.
Oxidative stress caused by a two-factor effect (γ-rays + CdCl₂) on the body of rats was assessed by changes in the activity of toxic radicals - redox-cycled quinones (QH-radicals) in BFR test using an antibody bentonite diagnosticum by determining the concentration of BFR-active compounds according to R. R. Gainullin (2002), as well as by changes in the content of the key enzyme of antioxidant protection - superoxide dismutase. SOD activity was determined by the method of I. Fridovich, based on the inhibition of quartzetin oxidation in the presence of N,N,N,N-tetramethylethylenediamine. The amount of protein that caused 50% inhibition of quartzetin oxidation was taken as a unit of SOD activity. In our experiments, one unit of activity corresponded to 7.6 + 0.5 mg of the SOD from bovine erythrocytes.

The therapeutic effect of anti-cadmium - anti-radiation serum (AARS) was tested on white rats affected by lethal doses of γ-rays (9.0 Gy) and cadmium chloride (18.74 mg/kg-1/5 LD₅₀).

Efficiency of the test agents was assessed by 30-day survival rate of animals exposed to lethal doses of radiation and subjected to CdCl₂ administration, as well as by changes in the concentration of BFR-active toxic compounds, metallothionein and SOD.

The results were subjected to statistical processing using conventional parametric methods. Statistical significance of revealed differences between the compared indicators was assessed using "one-wag ANOVA" test.

3 Results and Discussion

The survival of rats depended on the type of exposure to the stress agent, on its dose and also on the nature of the combination of stressors. So, with a 5-time intake of CdCl₂ at a daily dose of 18.74 mg/kg (total dose - 93.7 mg/kg, LD₅₀), the survival rate was 90%, which was 40% higher than with a single intake of the toxicant at a dose of 1 LD₅₀.

Isolated γ-radiation of rats at a semi-lethal dose (7.0 Gy) caused 55% death of animals within 12-14 days after exposure. The combined effect of the factors used at doses that individually cause 10% mortality with 5-time use of 1/5 LD₅₀ CdCl₂ and 55% mortality from γ-rays at a dose of 7.0 Gy led to a significant increase in the lethal effect, causing the death of 90% of rats exposed to radiation and poisoned with CdCl₂ in the period from 3 to 14 days.

Considering that serum preparations (serum, plasma, globulins) obtained from peripheral blood have the ability to inhibit oxidative reactions in the body, the following series of experiments was carried out on 40 white outbred rats with a live weight of 180.0-200.0. Animals were divided into four groups of 10 animals each.

Animals of the first group were subcutaneously injected with a solution of cadmium chloride 4-times daily at a dose of 0.2 mg/kg (0.04 mg per animal), followed by radiation exposure at a dose of 9.0 Gy (24 hours after the last injection of CdCl₂); animals of the second group were once subcutaneously injected with a solution of CdCl₂ at a dose of 1 LD₅₀ (93.7 mg/kg - 16.74 mg/animal) followed by exposure to radiation at a dose of 9.0 Gy; animals of the third group were subjected to a similar combined two-factor exposure and subsequent treatment by a single subcutaneous injection of anti-cadmium-anti-radiation serum at a dose of 25 mg/kg of body weight 24 hours after exposure; animals of the fourth group were not exposed to radiation, CdCl₂ and were not injected with the drug (control).

The animals were observed for 25 days, the number of dead rats in each group was registered daily. On the 7th day after radiation exposure (the peak period of acute radiation sickness (ARS), peripheral blood samples were taken from the tail vein of all animals. Serum was obtained from the blood samples, and the content of metallothioneins, BFR-active compounds (quinoid radiotoxins - redox-cycled toxic radicals) and concentration of superoxide dismutase were determined.
It can be seen that preliminary 4-time administration of CdCl$_2$ solution at a dose of 0.2 mg/kg (0.002 LD$_{50}$) and subsequent radiation exposure at lethal dose (9.0 Gy) had a radioprotective effect, providing a 40% survival rate for lethally irradiated animals. An increase in the dose of the toxicant to 1 LD$_{50}$ (93.7 mg/kg) with radiation dose of 9.0 Gy led to increased damaging effect of $\gamma$-radiation, accelerating the death of animals: 80% death of rats occurred in the first 9-10 days after the combined exposure to pathological factors, and the death of all animals occurred on the 12-16th day after two-factor exposure.

The use of immunotropic drug anti-cadmium - anti-radiation serum on the background of two-factor combined (cadmium-radiation) damage led to a modification of the combined pathology, providing 90% survival rate.

Parallel immunochemical studies (BFR-test) for the indication of redox-cycled radicals (semiquinones - radiotoxins), ELISA for determination of metallothioneins concentration and biochemical studies (determination of the concentration of the antiradical enzyme - superoxide dismutase) showed that an increase in the survival rate of animals subjected to two-factor damage was due to inhibition of BFR-active toxic compounds (*QH) synthesis, activation of metallothioneins synthesis and prevention of inactivation of the key antioxidant enzyme, superoxide dismutase (Table 1).

**Table 1.** Content of BFR-active compounds of metallothioneins and superoxide dismutase in the blood serum of white rats on the 7th day after the combined exposure to CdCl$_2$, $\gamma$-radiation and treatment with AARS.

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Concentration of BFR-active toxic compounds, log$_2$</th>
<th>Content of metallothioneins, ng/ml</th>
<th>Activity of SOD, U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, 4-time injection of CdCl$_2$ at a dose of 0.2 mg/kg and subsequent $\gamma$-radiation at a dose of 9.0 Gy</td>
<td>4.3±0.7*</td>
<td>28.2±2.1**</td>
<td>9.61±0.75*</td>
</tr>
<tr>
<td>Group 2, single subcutaneous injection of CdCl$<em>2$ at a dose of 1 LD$</em>{50}$ (93.7 mg/kg) and subsequent $\gamma$-radiation at a dose of 9.0 Gy after 24 hours</td>
<td>9.5±2.0*</td>
<td>6.1±1.1*</td>
<td>5.7±0.41*</td>
</tr>
<tr>
<td>Group 3, single subcutaneous injection of CdCl$_2$ at a dose of 93.7 mg/kg + treatment with AARS at a dose of 25 mg/kg 24 hours after radiation exposure</td>
<td>3.5±0.9*</td>
<td>41.7±2.5***</td>
<td>17.49±0.38**</td>
</tr>
<tr>
<td>Group 4, control</td>
<td>0.05±0.07</td>
<td>9.7±1.5</td>
<td>8.23±0.41</td>
</tr>
</tbody>
</table>

* - P<0,05; ** - P<0,01; *** - P<0,001.

It can be seen from the table that 4-time injection of CdCl$_2$ at a dose of 0.2 mg/kg (1/5 LD$_{50}$) followed by lethal exposure to radiation induced 8.6-fold increased in the content of BFR-active radiotoxic compounds in the blood serum of rats. It was found that radiotoxinemia of the organism (blood serum) was accompanied by a 2.9-fold increase in metallothioneins content and 1.17-fold increase in SOD content. With such a change in the content of radiotoxins, metallothioneins and SOD on the background of 4-time injection of CdCl$_2$ before exposure of rats to lethal doses of radiation, we observed a radioprotective effect, which was expressed in the protection of 40% of animals from radiation death (competition of toxic agents).
The combined radiation-chemical effect of CdCl₂ at a dose of 93.7 mg/kg followed by γ-radiation at a dose of 9.0 Gy led to mutual enhancement of the damaging effects of toxic and radiation factors, which was accompanied by more pronounced clinic manifestation of combined pathology and death of animals in the period from 9 to 15 days. At the same time, the development of combined pathology was accompanied by hyperantigenemia (toxinemia) of blood serum, 150-fold increase in the concentration of toxic BFR-active compounds (quinoid radiotoxins - radicals of semiquinones and ubiquinones (*QH), 1.59-fold decrease in the content of metallothioneins and 1.44-fold decrease in the content of superoxide dismutase. These biochemical changes led to 100% death of rats affected by CdCl₂ and γ-rays (synergism of damaging agents).

Single subcutaneous injection of AARS at a dose of 25 mg/kg 24 hours after exposure to damaging factors had a radioprotective and anticadmium effect on animals affected by both pathogenic agents (CdCl₂ + γ-rays), preventing the death of 90% of animals. Increase in the survival rate of animals subjected to two-factor exposure was accompanied by inhibition of antigenemia (radiotoxemia), decrease in concentration of BFR-active toxic compounds in the blood serum by 2.71 times in comparison with the of animals of the group 2 subjected to combined two-factor cadmium-radiation damage.

The used therapeutic agent induced increase in metallothioneins synthesis (by 4.29 times compared with the control and by 6.24 times compared with the affected and untreated animals), increase in superoxide dismutase activity by 2.12 times compared to the control and by 3.07 times compared with that of animals of the group 2, affected by two factors and untreated.

The indicated biochemical changes in the animals treated with the test agent on the background of two-factor lethal injury led to the modification of radiation and cadmium injury, ensuring 90% survival rate.

Analysis of the data in the table showed that two-factor radiation-chemical effect induced an imbalance of homeostasis which was expressed in the form of oxidative stress, accompanied by an increase in the synthesis of radioinduced toxic radicals in the form of BFR-active compounds (ubiquinone radical cation - *QH), and caused inhibition in SOD activity (group 2), while the use of the AARS drug led to inhibition of radiotoxins synthesis with increase in SOD activity (group 3).

For the integral assessment of radio- and chemo-induced oxidative homeostasis in rats, we used the oxidative stress coefficient (antioxidant-prooxidant index, API), which expresses the ratio of superoxide dismutase activity to the concentration of BFR-active toxic compounds (ubiquinone radical cation - *QH).

According to the presented data, the ratio of superoxide dismutase activity to the concentration of BFR-active compounds remained unchanged in intact animals during the experiment, while in rats of the experimental groups it tended to decrease with the rate depending on the nature of the exposure. So, starting from the first day of the experiment with combined exposure of animals to CdCl₂ and γ-rays in lethal doses, sharp (1.38 times) decrease in the SOD/BFR coefficient followed, which lasted up to 7 days of the experiment, when the value of this ratio was 27 and 3 times below the control level. At that time, the death of experimental animals occurred in the period from 9 to 14 days.

A single subcutaneous injection of the test AARS to rats affected by lethal doses of CdCl₂ and γ-rays 24 hours after exposure had a significant effect on SOD/BFR coefficient, the values of which were characterized by a slow and insignificant decrease, differing insignificantly from the control values. A more pronounced decrease in [SOD/BFR] coefficient was observed on the 7th day, when the value of this indicator was less than the control by 3.2 times. On the 10th day, it increased significantly, and then a tendency to stabilization was established.
The results of our experiments showed that 4-time subcutaneous injection of CdCl2 solution at a dose of 0.2 mg/kg (1/5 LD50) before γ-radiation at a dose of 9.0 Gy (LD100) had a radioprotective effect, causing a 40% survival rate of animals subjected to combined radiation-chemical effects. The observed increase in the survival rate of rats on the background of preliminary intake of CdCl2 before γ-radiation correlated with a significant (2.9-fold, P<0.01) increase in the level of metallothionein (MT) in the blood serum of animals. Such a relationship, expressed in decrease in the mortality of rats exposed to lethal doses of γ-radiation on the background of preliminary intake of CdCl2, suggests the presence of radioprotective properties of MT. This assumption is supported by available literature data on the radically induced formation of MT [10, 11].

The results of the present study allowed us to suggest possible mechanisms for the development of the observed effects of cadmium and γ-radiation combined action. Probably, the radio-protective properties of MTs were induced by the ability to trap free radicals. Increase in the survival rate of animals exposed to lethal radiation on the background of preliminary administration of CdCl2 before radiation exposure was accompanied by inhibition of semiquinone and ubiquinone radicals synthesis (BFR-active toxic compounds) with significant increase in metallothioneins concentration and SOD activity involved in the implementation of the body's protection against oxidative stress caused by exposure to a powerful stress agent - ionizing radiation. The study of the mechanism of MT antioxidant action in vitro and in vivo showed that MT lost zinc under oxidative stress due to the oxidation of sulfhydryl groups with the formation of a disulfide bond. The disulfide bonds of MT were probably intramolecular with alpha-beta domains involved in the formation of disulfide bonds. The results suggested that the formation of intermolecular disulfide bonds in MT was activated during maintenance of zinc homeostasis under physiological conditions. Additional zinc release from MT under conditions of oxidative stress was accompanied by the formation of a large number of disulfide bonds in MT. It is known that antioxidant properties of MT are caused by the high content of thiol (sulfhydryl) groups and the ability to activate SOD. The first mechanism was supported by studies that showed the antioxidant activity of MT in mice lacking Cu/Zn-SOD (Chostal and Majumder, 1999). Studies of MT effect on the antioxidant protection enzymes, e.g. Cu/Zn-SOD, catalase and peroxidase, found that SOD activity increased in the presence of MT and decreased in the presence of Zn-deprived MT.

Similar expression of SOD was revealed in response to the introduction of exogenous metallothioneins to cows (Zhang et al., 2007). Obviously, SOD activation was induced by Zn release from MT, since Zn ions are necessary for the formation of the tertiary structure of the SOD molecule, which determines its activity. The foregoing was confirmed by experiments that showed increase of MT antioxidant properties in the presence of zinc [12].

Considering the results of studies showing SOD activation in the presence of MT which caused the body's resistance to stress, we conducted this research to assess the possibility of reducing the radiation-toxicological combined pathology by using a radioprotector based on anti-cadmium - anti-radiation serum. During the experiments, we took into account the fact that the intensity of radio- and chemo-induced oxidative stress and the possibility of its leveling can be controlled or tracked using the oxidative stress coefficient.

Combined radiation-chemical (γ-rays + CdCl2) oxidative stress led to decrease in SOD activity and increase in the concentration of BFR-active oxidation products (radiootoxins-radicals of semiquinone and ubiquinone *QH). The coefficient expressing the ratio of SOD activity to the concentration of BFR-active compounds is the antioxidant - prooxidant index, reflecting the imbalance in redox processes (in the absence of a corrective, leveling agent), the launch of reparative processes when applying radio- or chemoprotectors.

During the combined exposure of the animal to lethal doses of CdCl2 and γ-rays, we observed a sharp decrease (by 1.38 times, P<0.05) in the [SOD/BFR] coefficient, which
lasted up to 7 days of the experiment, when the value of this indicator was 27.2 times less than the control level. At this point, the death of all animals affected by lethal doses of CdCl2 and γ-rays occurred in the period from 9 to 14 days.

The use of a potential radiochemoprotector (AARS) had a significant effect on the value of the [SOD/BFR] coefficient, which slowly and slightly decreased up to the 5th day of the experiment; more intensive decrease in the coefficient followed on the 7th day, then a tendency to stabilization was established.

These biochemical changes in animals affected by two stress factors and subjected to immunotherapy with the test agent (AARS) indicated a modification of radiation and cadmium damage, providing a 90% survival rate of affected animals.

In addition to antioxidant protection of metallothioneins, increase in the survival rate of animals subjected to two-factor environmental damage on the background of immunotropin agent use was also caused by the fact that the toxic agent (cadmium) binds to the external functional albumins and globulins groups of anticadmium - anti-radiation serum. Then the metal-protein complex is transported to the liver, where rearrangement occurs with the formation of a complex of heavy metals with metallothioneins. This helps to reduce the toxic effects of heavy metals. Further, the metal-metallothionein complex enters the kidneys and is destroyed in the lysosomes of the epithelium of the proximal renal tubules [13, 14].

Another important mechanism for protecting of animals subjected to radiation-chemical exposure on the background of immunotropic AARS is the presence of specific anti-cadmium antibodies that form a response to the introduction into the body of a low molecular weight compound (CdCl2) as an inducer of an immune response to xenobiotic [15].

4 Conclusion

Combined radiation-chemical pathology is a multi-step process that includes pathophysiological and pathobiochemical changes caused by a chemical and gamma radiation. As shown in Table 1, MT can play an important role as a radioprotective factor in chemical and radiation injury. Probably, the radioprotective properties of MT are determined by the ability to trap free radicals and modulate activity of antioxidant defense enzymes (SOD).

An increase in the survival rate of lethally radiated animals that previously received 4-time CdCl2 in small doses (1/5 LD50) is induced by the antagonistic effect of toxic and radiation factors (competition effect); with an increase in the dose of a toxic nature (cadmium) on the background of lethal exposure, that leads to increase of the damaging effect of pathological agents (additive, synergistic effect) and acceleration of death rate of animals subjected to two-factor exposure.

The use of the immunotrophic agent anti-cadmium anti-radiation serum (AARS) on the background of two-factor combined radiation-chemical injury provided 90% survival, inducing increase in the formation of metallothioneins and SOD, inhibiting redox-cycled toxic radicals formation (BFR-active compounds), neutralizing the toxicity of cadmium with the help of anti-cadmium antibodies and accelerating the removal of the xenobiotic from the body with the help of a nanosorbent - bentonite nanoparticles.

A single subcutaneous injection of AARS 24 hours after the combined exposure to the toxicant (CdCl2) and γ-rays had radioprotective and antidote effect, increasing the survival rate of animals up to 90%, restoring the balance of the oxidative stress coefficient and reducing the development of combined radiation-chemical pathology by triggering the mechanism for damage reparations.
References

9. V. Dogra, C. Kim, Front Plant Sci 10, 1640 (2020)
13. K.K. Kavindra, K.Jha. Niraj, Molecular and Integrative Toxicology 1, 394 (2021)