

Remote Combined Effects of Coal Dust and High-Dose γ -Irradiation on Immune System Changes

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Abstract

The objective of this research is to assess the effect of combined exposure of a high dose gamma radiation (6Gy) and coal dust on the immune system values in the long-term period.

The study was carried out on 40 male Wistar rats divided into 4 groups: group I, intact animals; group II, exposure to coal dust; group III, exposure to 6Gy γ -irradiation; group IV, combined exposure to sublethal irradiation and coal dust. In groups II and IV, anthracosis was caused by exposure to coal dust at a concentration of 50 mg/m³, using an exposure chamber for 90 days after irradiation. The animals of groups III and IV were irradiated once in dose 6Gy prior to the study, with the TERAGAM CO60 gamma-therapeutic device (ISOTREND spol. s.r.o., Czech Republic).

The study revealed a significant immunotoxicological shift induced by coal dust and high dose of radiation separately and in combination. The combined effect of factors exhibited an obvious immunodeficiency, as compared to the separate exposure. The combined exposure to the studied factors resulted in deterioration of T-cell immunity and disruption of cytokine production accompanied by reduced resistance to neoplasms, increased tendency to allergic diseases, and weakened immunological response of the organism.

Key-words: Coal Dust, High Dose Radiation, Cytokines, T-lymphocytes, B-lymphocytes.

1. Introduction

Nuclear tests at the Semipalatinsk Test Site, East Kazakhstan Region, caused deterioration of the environmental conditions in the most of the territories of Kazakhstan, and the inhabitants of these regions suffered external and internal irradiation. Health effects in the radiation-exposed human population include radiation-induced changes leading to socially significant diseases. Moreover, the

public health threat is aggravated by the combined impact of the industrial factors that the humans are exposed to throughout their entire lives. One of such factors is the coal dust from the coal mines located near the Semipalatinsk Test Site.

In this connection, the objective of this research is to assess the long-term effects of chronic combined exposure to high-dose radiation (6Gy) and coal dust and to study the immunogenic response in experimental rats.

Exposure to radiation, often described in terms of dose-dependence, dose-response, dose-reaction relationship and advanced risks, entails radiation-induced changes in the immune system and immune response. Generally, the radiation effect on immune parameters is dependent on the total dose absorbed, while the acute consequences of irradiation at high doses are cell death, inflammation and mutation (UNSCEAR 2006 Report, 2009).

High doses of radiation reduce the cell viability, promote tumour cell growth (Bang, 2016), and result in the excess risk of oncologic and other significant diseases in atomic bomb survivors; there was also life expectancy shortening (Duple, 2011). The long-term effect of high dose radiation was the immune suppressive action, causing quantitative abnormalities in the T-cell immunity, as confirmed by epidemiological studies (Akiyama, 1995).

Coal dust is classified as polluting mineral particles. It consists of more than 50 chemical elements and their compounds, some of them having genotoxic and carcinogenic properties. Continuous inhalation of coal dust causes significant respiratory disorders, such as chronic bronchitis, pneumoconiosis, silicosis, and lung cancer (Schins, 1999; Castranova, 2000; Bennett, 1979).

Dust particle induced pathogenesis results in the amplification of inflammatory cells, multiplication of fibroblasts, and degradation of the extracellular matrix components. The main mediators of the coal dust are cytokines, which participate in the intensification or suppression of the immune response to the inflammatory reactions.

Cytokines are released by the functional subpopulations of CD4⁺ T cells (Th1 and Th2 helper cells) and CD8⁺ T cells (T_C1 and T_C2 cytotoxic lymphocytes), which are mainly responsible for allergic reactions, in particular those caused by chemical substances and resulting in adaptive immunity after long exposure. Th1 cells express IFN- γ , IL-2 and TNF- α , and only Th2 cells express IL-4, IL-5, IL-6, etc.

Cytokines are especially important in the immune response regulation, inflammatory reactions and hematogenesis. They are produced by immune cells acting at a short distance and lasting a short amount of time. Cytokines are significant inflammatory mediators of toxic effects in mineral dusts

exposure; they generalize from macrophages and monocytes in inflammation as their content in blood increases(Akiyama, 1995; Ates,2011).

2. Materials and Methods

Experimental Animals

For the experimental purposes, 40 white laboratory male Wistar rats, weighing 220 ± 20 g, were randomized into 4 groups: group I, intact animals for reference; group II, exposure to coal dust at the average concentration of 50 mg/m^3 daily (4 hours) for 12 weeks; group III, exposure to γ -irradiation once in dose 6Gy; group IV, combined exposure to sublethal irradiation (once in dose 6Gy prior to the study) and coal dust (exposure to coal dust after irradiation at a concentration of 50 mg/m^3 daily (4 hours) for 12 weeks, using an exposure chamber).

Gamma Irradiation

The animals received a single 6Gy dose of radiation 90 days before the study with the TERAGAM Co⁶⁰ gamma-therapeutic device (produced by ISOTREND spol. s.r.o., Czech Republic). Animal experiments were in compliance with the Geneva Convention (1990), the 1964 Declaration of Helsinki and its later amendments, and Ethical Guidelines of the Local Ethics Committee (Protocol of the Local Ethics Committee of Semey State Medical University, Kazakhstan, No.2 dated 18.11.2016).

Prior to the exposure, there was topometric-dosimetric preparation of the experimental animals. To this end, the object was placed on an isocentric therapeutic desk of Terasix X-ray simulator (Czech Republic), which is similar to the therapeutic desk of the γ -irradiator by its construction and parameters. After displaying on a screen, the image slices of the irradiated animals were directly input in the planning system through a computer network connection using a digitizer. Isodoses were calculated using the PlanW-2000 planning software, and the topometric-dosimetric map with technical parameters and planned radiation doses was obtained. The animals underwent exposure to a single whole-body dose of γ -radiation (6Gy): SSD 97.2 cm, SAD 100.0 cm, 40×40 cm field, $t=352$ sec (SSD is the distance from the source of ionizing radiation in the apparatus to the conditional centre of the irradiated abnormal focus; SAD is the distance from the ionizing radiation source to the nearest surface of the irradiated object). During the exposure, animals were placed in a specially engineered organic glass cage, each rat in an individual compartment.

Exposure to Coal Dust. Inducing anthracosis in rat models using an exposure chamber was successful. The animals were placed into cone-shaped compartments, with their vertices attached to the side walls of the exposure chamber. The inhalation exposure device allowed to uniformly disperse the coal dust in the breathing area and maintain the required dust concentration in the chamber with the help of an automatic analyzer.

Coal dust used in the experiment was preliminary comminuted on a vibratory disintegrator. The final disintegration to the fractions, similar to aerosol dispersion in the working zones air, was performed manually in an agate mortar.

Measurement of Lymphocyte Subpopulation

In all experimental animals, the total number of leukocytes and lymphocytes in the peripheral blood was determined. The quantity of B- and T-lymphocytes and their subpopulations were determined by immunofluorescence staining using FITC-conjugated antibodies. GALTAG Laboratories (USA) was the supplier of monoclonal antibodies CD3+, CD4+, CD8+, CD20+ FITC. Investigators determined the leukocyte migration inhibition reaction to phytohemagglutinin (LMIR to PHA) and concentration of circulating immune complexes (CIC).

Measurement of Cytokine Profile

The concentration of cytokines (interleukin-2 (IL-2), interleukin-6 (IL-6), tumour necrosis factor (TNF- α), γ -interferon (IFN- γ)) was determined in the serum by the immunoenzyme method using the Uniplan immune enzymometric analyzer (Russia). BioChemMack, ZAO assisted in purchasing of the Bender MedSystems cytokine assay kit (specifically for rats: rat IL-2, rat IL-6, rat TNF- α , rat IFN- γ). To calculate the cytokines concentrations, the investigators used standard calibration dilutions and software, supplied by Bender MedSystems (Flow Cytomix) in the kit.

The obtained data underwent statistical analysis: differences were estimated by Student's t-Test was performed using GraphPad Prism (6.01)

3. Results

The authors studied the lymphocyte subpopulation and cytokine production in the peripheral blood of rats after the combined and separate long-term exposure to coal dust and sublethal γ -ionizing

radiation. The functional state of the immune system demonstrated changes in the parameters that indicated the disturbances resulting from the exposure to the studied environmental factors.

Effect of Coal Dust and High-Dose γ -Radiation on Lymphocyte Subpopulation

The dust coal exposed animals had a significant increase in the total number of leukocytes and lymphocytes (Tab. 1). At the same time, there were changes in the cellular differentiation markers, as compared to the intact animals.

Long-term exposure to coal dust gave a slight difference in the absolute number of CD4+ T cells (Th) and a 15.5% reduction of CD4+ circulation ($p < 0.05$) in the peripheral blood, as compared to the intact animals. CD4+ T cells are important T-lymphocytes, specific in their adaptive immune response to various allergens by activation of particular cytokines (Luckheeram, 2011).

In contrast to T-helpers, the percentage and absolute number of CD8+ T cytotoxic/suppressor cells increased. The percentage of CD8+ cells was 16.3% higher ($p < 0.05$), as compared to 11.3% in the reference group; there was a 24% increase in absolute terms. CD8+ T cells play a significant pathological role in the immune defence as they destroy various pathogens and tumour cells by activating cytokines, TNF- α and IFN- γ (Iannacone et al., 2006). Thus, it should be noted that the T helper/T suppressor lymphocyte ratio was reduced from 1.39 in the reference group to 1.21 in the animals exposed to the toxic agents.

In respect to the physiological norms, CD3+ lymphocyte percentage showed a doubtful decrease in the coal dust exposed animals after 90 days; there was a 19.5% increase ($p < 0.05$) in absolute terms. Exposure to organic toxic agents leads to an inflammatory response that manifests itself in the increase in CD3+ T cells (Poole, 2009; Lund, 1999).

Therefore, the radiation and radiation-dust factors impaired the immunogenic response in the animals. The number of leukocytes statistically and significantly reduced in both groups, causing leucopenia. It indicated the immune deterioration and the hematologicalmal function due to the continuous impact of high-dose irradiation. It proved that the studied contaminant caused intoxication (Dainiak, 2003; Goans, 2005). However, the lymphocytes showed an opposite result, as compared to the reference group; here, the sublethal radiation significantly activated the lymphocytes. It happened in response to allergens and malignant changes. A similar result was noted after the continuous contact with coal dust. Absolute lymphocytopenia was there after the combined exposure to harmful factors, such as radiation, unhealthy working conditions, and intoxication (Ray-Coquard, 2009).

The lymphocyte subpopulation in the animals of groups III and IV showed deteriorating results as compared to the intact animals. Exposure to high-dose ionizing radiation and coal dust triggered a two-fold decline in the percentage and absolute number of CD3+, CD4+, CD8+, which was respectively accompanied by a lowered immunoregulatory index (IRI) in the studied animals.

The influence of coal dust, radiation and their combination made the LMIR increase, as compared to the physiologically normal state, where an increase in LMIR indicated deterioration of the leukocytes' functional activity. A more significant inflammatory activity was reliably revealed after the combined exposure to coal dust and γ -radiation. It proved that the coal dust toxicity was intensified by radiation.

After exposure to the harmful factors in combination and separately, the level of CD20+ B-lymphocytes in the experimental animals increased in absolute terms and in percentage, as compared to the physiologically normal state. Doubling of CD20+, observed in the high-dose radiation exposed animals, appeared more reliable. A decreased level of CIC was found in the animals exposed to radiation and combined effect of two factors. At that, the observed effect was stronger in the latter case: a 2.4 time lower CIC content could be reliably observed, as compared to the reference group. On the contrary, in the animals exposed to coal dust, there was a reliable increase in the CIC in the blood serum.

Table 1- Immunological Response in Experimental Rats (10 rats in each group) Exposed to High Dose Radiation (6Gy γ -Rad), Coal Dust (CD) and a Combination of both Harmful Factors (6Gy γ -Rad+CD) for 90 Days

	Immune parameters	Group I intact animals		Group II exposure to coal dust		Group III exposure to 6Gy irradiation		Group IV combined exposure to 6Gy irradiation and coal dust	
		Absolute number	%	Absolute number	%	Absolute number	%	Absolute number	%
1	Leucocytes in 1 μ l	6.59 \pm 0.18	-	8.0 \pm 0.59***	--	4.92 \pm 0.19**	-	4.91 \pm 0.23 ***	--
2	Lymphocytes in μ l	2.85 \pm 0.12	39.11 \pm 3.25	4.32 \pm 0.37***	48.96 \pm 2.86**	4.10 \pm 0.32*	50.37 \pm 4.28*	2.62 \pm 0.21	41.22 \pm 3.54
3	CD20+	0.43 \pm 0.03	7.31 \pm 0.60	0.57 \pm 0.05*	8.77 \pm 0.62	0.64 \pm 0.04***	13.92 \pm 1.22**	0.49 \pm 0.03	9.21 \pm 0.84
4	CD3+	1.54 \pm 0.09	31.91 \pm 2.42	1.84 \pm 0.10 *	29.53 \pm 3.41	0.71 \pm 0.06**	12.71 \pm 1.09***	0.51 \pm 0.05***	16.31 \pm 1.14***
5	CD4+	0.79 \pm 0.05	21.02 \pm 1.42	0.91 \pm 0.06	15.53 \pm 0.99*	0.38 \pm 0.04**	11.27 \pm 1.33***	0.34 \pm 0.03 ***	10.15 \pm 0.87***
6	CD8+	0.57 \pm 0.03	11.34 \pm 0.43	0.75 \pm 0.08*	16.27 \pm 2.16*	0.36 \pm 0.03**	6.38 \pm 0.82***	0.33 \pm 0.03 ***	9.74 \pm 0.86
7	IRI	1.39 \pm 0.11	-	1.21 \pm 0.08*	--	1.05 \pm 0.10 *	-	1.03 \pm 0.11 *	--
8	LMIR	0.89 \pm 0.05	-	1.32 \pm 0.15*	--	1.61 \pm 0.15**	-	1.98 \pm 0.17 ***	--
9	CIC	1.37 \pm 0.03	-	1.84 \pm 0.19*	--	0.62 \pm 0.10**	-	0.58 \pm 0.04 ***	--

Note: Differences from group I are statistically reliable: * p <0.05; ** p <0.01; *** p <0.001.

Effect of Coal Dust and High-Dose γ -Radiation on Cytokine Production

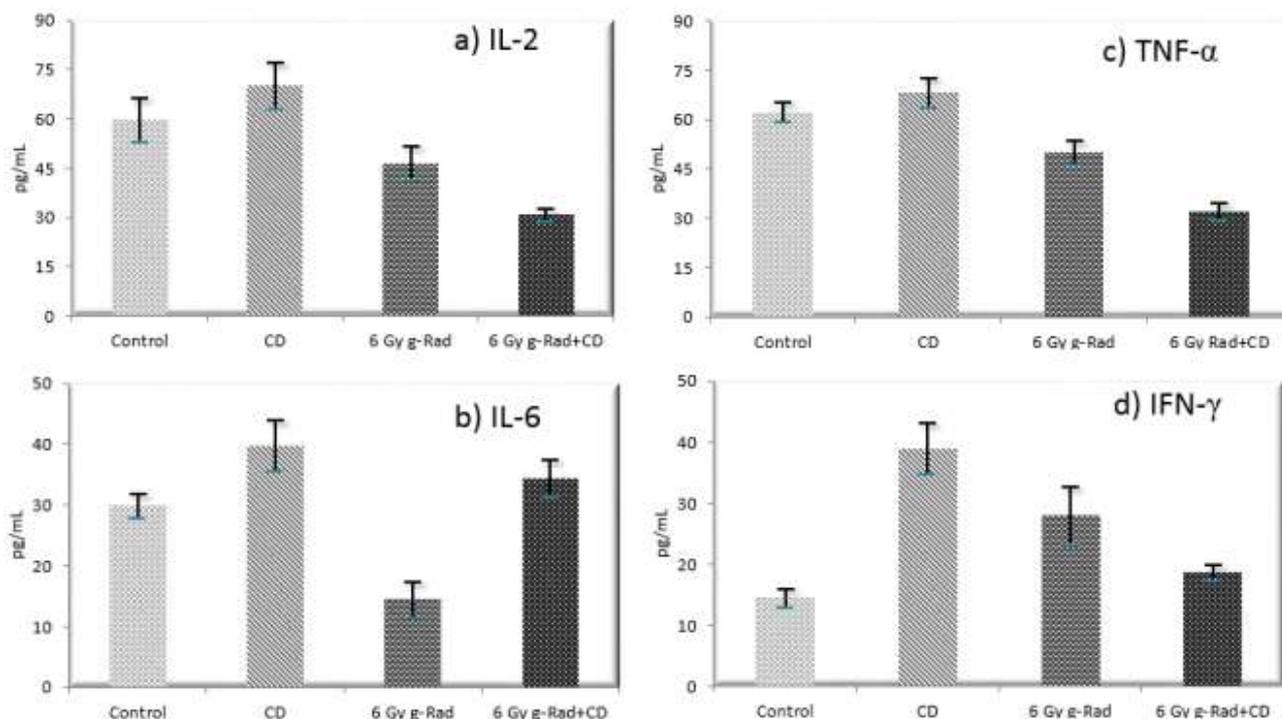
All the experimental animals chronically exposed to coal dust and high-dose radiation in combination and separately in the long-term period, showed obvious inflammatory responses with the release or suppression of cytokines (Fig. 1). Cytokines can significantly affect the internal cellular radiosensitivity, frequency and radiation-induced complications, side effects, genomic instability and tumour growth (Fig. 1). Dust particles can be detected by epithelial cells of the respiratory tract, activate macrophages, dendritic cells and innate immune cells, and then initiate a response in different populations of the immune cells, such as T-helpers, cytotoxic T cells and B cells. Initiation of inflammatory responses, activation of the immune cells and release of cytokines, chemokines and other inflammatory molecules have a different pathological effect on the lungs in respiratory diseases (Esmail, 2014).

In the peripheral blood of the experimental animals, IL-2 expression (Fig. 1a) increased by 17,5% under the influence of coal dust. It may prove the intensity of the immune response to dust as an allergic toxicant, where IL-2 activates and regulates the immune status, stimulates the proliferation and differentiation of T cells, and is involved into activation of the cytotoxic cells that induce cell death, thereby causing apoptosis (House, 2007). Single 6Gy γ -irradiation leads to a 22% decrease in the IL-2 level after a long period of time. These data suggest that high doses of radiation disturb the T-cell balance, leading to chronic inflammation as a result of the immune system aging (Kusunoki, 2008).

The combination of the sublethal dose of ionizing radiation and coal dust inhalation gave a two-fold decrease in IL-2, as compared to the intact animals. Insufficient level of IL-2 causes dysregulation of the immune system and suppresses the proliferation process in the cellular and humoral immunity.

After long-term inhalation of coal dust, IL-6 content (Fig. 1b) rises to 33,2%. In case of tissue injury or in the presence of extraneous agents, interleukin-6 usually exhibits an immune response, especially in the inflammatory processes and multiple tumors; in particular, high IL-6 level is a marker of such diseases (Hong DS et al., 2007; Ates et al., 2011). The ionizing radiation had a depressing effect on IL-6 and reduced its level to 52% ($p < 0.01$), manifesting itself as an inhibiting factor. The IL-6 decrease in the peripheral blood illustrates the violation of its induction by T cells and macrophages in the direction of depression; the organism becomes susceptible to many pathogens, for example, the decrease of IL-6 showed low resistance to some fungal infections (Diehl, 2002; Basu, 2008).

Figure 1- Level of cytokines: (a) IL-2, b) IL-6, c) TNF- α , d) IFN- γ in experimental rats (10 rats in each group) exposed to high-dose radiation (6Gy γ -Rad), coal dust (CD) and a combination of both harmful factors (6Gy γ -Rad+CD) for 90 days, as compared to the intact animals (control)



There was a slight growth in IL-6 (15,2%) after the combined exposure to radiation and coal dust, which could lead to various autoimmune diseases, such as rheumatoid arthritis, osteoporosis. It also appears to suppress the TNF- α content, which can be confirmed by inhibition of TNF- α by ~50% ($p < 0,001$) in case of dust-radiation exposure. Since IL-6 is a strong growth and differentiating factor for B cells, its activation stimulates the growth of tumor cells (Rose-John, 2012; Kaneko, 2000; Schindler, 1990, Chun, 2009).

Expression of tumor necrosis factor TNF- α (Fig. 1c) was detected as a response to the mineral dust action. The TNF- α content varies analogously to the IL-2 level, and in case of coal dust exposure it showed a slight increase. Being a macrophage cytokine, TNF- α is plays an important role at inhalation of the mineral particles. The long-term inhalation of coal dust results in the chronic cytokine release and fibrosis formation; accordingly, this causes pneumoconiosis in miners. Ates et al. showed that TNF- α is a marker for the severity of pneumoconiosis (Ates, 2011). Radiation creates a decreasing tendency of TNF- α activation (to 20%), whereas the combined effect of radiation and coal dust halved the TNF- α production. TNF- α is mainly produced by macrophages; it is an important antitumor cytokine, involved in apoptosis and cell survival.

As for the interferon level, all groups of exposed animals (Fig. 1d) demonstrated an increase in the INF- γ expression, starting from 1.3 times. In the case of the combined coal dust and radiation exposure, there was an increase up to 2.7 times as compared to the reference group.

4. Discussion

At the long-term exposure of coal dust, the effector functions of T-lymphocytes were partially preserved or generated. The regulatory functions shifted towards the predominance of T-helpers over T-suppressors. It results in the autoimmune and allergic reactions, which can be confirmed by an increase in pro-inflammatory T-helper cytokines in the coal dust exposed animals. The changes in the lymphocyte subpopulation and chronic cytokines release, both induced by coal dust, can cause the autoimmune and inflammatory diseases, such as pneumoconiosis.

Ionizing radiation at high doses plays a crucial role in the deterioration of T cells immunity observed in animals exposed to sublethal doses of radiation in the long-term period. In addition, a decrease in the T helper cytokines percentage composition in the peripheral blood leads to immunological modifications; it may also be associated with radiation damage and a higher risk of various diseases.

These studies of the combined effects of coal dust and a sublethal dose of ionizing radiation demonstrated significant, unconditional changes in the immune status of the experimental animals in the remote period. The observed amplification of IL-6 and INF- γ , secreted by the macrophage-lymphocytic infiltration, maintains the inflammation. In turn, IL-2 and TNF- α showed a weakening degree; the predominantly pronounced change was observed in IL-2, showing a two-fold declined result. There was also suppression of CD4+ T-lymphocytes. This leads to suppression of Th1 dependent cytokines (IL-2 and TNF- α) and can initiate genetic damages. Inhibition of Th1 cells results in suppression of the cellular immune response, while the dominance of cytokine controlled (IL-6) Th2 cells also contributes to this process, as confirmed by the increase in IL-6 level. At that, the humoral immunity generalizes, as confirmed by the increase in the CD20+ B lymphocytes level. Activation of humoral immunity response follows the increase in the immunoglobulin M level (IgM), which provides first-set reaction to infections, inflammation of the respiratory system and malignant neoplasms (Ouchida, 2012).

Excessive IL-6 production damages tissue as a result of an autoimmune reaction that can cause lung, bone marrow, etc. lesions in the long term. This is the remote effect of continuous exposure to coal dust on a living organism that was previously weakened by radiation.

The decrease in the TNF- α level can be a consequence of the suppression of macrophage activation and inhibition of CD4+ T cell. Such a condition was observed in fibrosis caused by ionizing radiation that increased the risk of tumour growth (Howard, 2009). In this regard, the two-fold inhibition of TNF- α intensifies the prognosis for cancer; even more so in case of the combined exposure to coal dust and radiation, as compared to the exposure to radiation only.

On the other hand, the findings point to a significant increase in the INF- γ level. A range of some in vitro and in vivo tests appears to prove that IFN- γ enhances the activation of Th1 cells yet inhibits the generation of Th2 cells, and intensifies the production of chemokines that promote Th1 immunity (House, 2007).

The increase in the INF- γ level demonstrates an immunomodulatory effect that seems possible in the long-term period after the radiation damage and anthracosis, as the body probably seeks to improve the immune status. Unfortunately, high doses and long-term effect of toxic substances cannot compensate the deterioration of the immune status, especially in case of the combined exposure to both high-dose radiation and coal dust.

5. Conclusion

The studies revealed a notable immunotoxicological shift induced by a separate and combined exposure to coal dust and high-dose radiation. However, the combination of these factors exhibits a more obvious effect on the immune deficiency, as compared to the effect of each harmful factor separately.

The registered immunological indicators make it possible to predict formation of chronic inflammations accompanied by intoxication, suppressed proliferation of immunocompetent cells (in particular, lymphocytes), and an increased risk of immunopathological reactions based on significant diseases, such as tuberculosis, rheumatoid arthritis, cancer, etc.

Thus, the combined effect of coal dust and high-dose radiation leads to the deterioration of T-cell immunity and cytokine production, that will possibly can lead to reduced resistance to tumours, high tendency to allergic diseases and weaker antiviral activity. This can provoke lung tissue damages and genetic disorders in the animal body. In this regard, further histopathologic and cytogenetic studies are necessary to identify possible pathogenetic events induced by the combined exposure to coal dust and radiation.

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