Journal of
APPLIED
BIOMEDICINE

J Appl Biomed. **9**: 219–224, 2011
DOI 10.2478/v10136-011-0008-1
ISSN 1214-0287

ORIGINAL ARTICLE

The nonlinear dependence between administered pro-oxidant doses and intensity of free-radical processes observed in rats

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Received 24th March 2011.
Revised 19th May 2011.
Published online 4th July 2011.

Summary
The influence of iron, copper and nitrate ions on free-radical processes in rats and the dependence between dose and effect of pro-oxidants were studied. Rats were divided into 14 groups and administered differing concentrations and combinations of chemicals with drinking water. Concentrations of iron, copper and nitrate in the water were 1, 0.5 and 0.33 of maximum permissible concentrations (MPCs) for every chemical. The action of the investigated pollutants on the intensity of free-radical processes was estimated by the determination of conjugated dienes in liver homogenate and the intensity of Fe^{2+}-induced chemiluminescence of the blood serum. It is estimated that chemicals entering the organism in doses that do not exceed their MPC lead to an increase in free-radical oxidation in comparison to the controls. A maximal effect of iron on the concentration of conjugated dienes was observed in a dose equal to 0.33 MPC, while copper and nitrate possess maximal activity in concentrations of 0.5 MPCs. Fast flash amplitude of chemiluminescence in serum was not dose-dependent in rats obtaining iron and copper, while nitrate had a reverse dose-dependent effect. Total luminosity was maximal in doses of chemicals equal to 0.33 MPCs. The combined action of pollutants was more evident in comparison to isolated chemicals in doses equal to 1 MPC.

Key words: iron; copper; nitrate; oxidative stress; rats

INTRODUCTION

It is well known that many pollutants possess a significant pro-oxidant effect, but the intensity of this effect during the administration of chemicals to the organism is insufficiently studied. In particular, the relation between the intensity of free-radical oxidation and the administered pollutant dose remains unknown. The investigation of this problem is not only theoretically significant, but is also useful practically, as the ability of some chemicals to induce free-radical oxidation is the basis of their toxicity (Valko et al. 2005, Flora et al. 2008). For this reason, the purpose of our investigation was to study the influence of iron, copper and nitrate ions on free-radical processes in the organism and the dose-dependent effect of pro-oxidants.

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MATERIALS AND METHODS

The current research was approved by local Ethics Committee. 105 female Wistar rats divided into 14 groups were used. The control group contained the maximum number of rats in order to estimate the

basal level of free-radical processes in animals. The animals were given free access to the drinking water and a standard diet. The light and dark cycle in the animal room was 12 hours. The animals were acclimatized for one week to the laboratory conditions before the study. The experiment lasted for 28 days. On the 29th day the animals were sacrificed by decapitation.

All groups of animals except the control ones received pollutants Fe^{2+}, Cu^{2+} and NO_3^-. Concentrations of chemicals used in the current study were calculated on the base of maximum permissible concentrations (MPC) for these pollutants accepted as per Russian norms. MPCs for single chemicals Fe^{2+}, Cu^{2+} and NO_3^- are 0.3; 1.0 and 45.0 mg per litre of drinking water respectively. For the adequate dispensing of the pollutants, we recalculated the MPCs for the salts used in the experiment. The animals received the investigated chemicals in the form of $FeSO_4 \cdot 7H_2O$; $CuSO_4$ and $NaNO_3$. The recalculated MPCs for these salts in the drinking water were 1.5; 2.44 and 61.6 mg/l respectively.

Animals of the first group (n=11) received high-quality drinking water (general mineralization < 250.0 mg/l) and served as a control group. Rats from 2 (n=9), 3 (n=5), and 4 (n=5) groups received drinking water containing $FeSO_4 \cdot 7H_2O$ in doses equal to 1; 0.5 and 0.33 MPC respectively. The animals from 5 (n=9), 6 (n=5), and 7 (n=5) groups obtained $CuSO_4$-containing drinking water in doses corresponding to 1, 0.5 and 0.33 MPC respectively. In groups 8 (n=9); 9 (n=5) and 10 (n=5) animals received drinking water with $NaNO_3$ in doses of 1; 0.5 and 0.33 MPC respectively. As for animals in 11 (n=9), 12 (n=9), and 13 (n=9) groups, they received water containing the following combinations of chemicals respectively: $[Fe^{2+}+Cu^{2+}]$; $[Fe^{2+}+NO_3^-]$ and $[Cu^{2+}+NO_3^-]$. According to principles of hygiene the sum of concentrations of all chemicals in water should not exceed 1MPC:

$$C_1/MPC_1 + C_2/MPC_2 + \ldots + C_n/MPC_n \leq 1$$

For that purpose the concentration of each compound from the two-component combination was equal to its 0.5 MPC. Finally, animals from the 14th (n=10) group were given drinking water containing the three-component combination of $[Fe^{2+}+Cu^{2+}+NO_3^-]$, where every chemical had a concentration of 0.33 MPC and the total concentration of the combination also did not exceed 1 MPC. It is important to mention that the groups of rats given isolated chemicals in concentrations equal to its 0.5 and 0.33 MPCs were also used as inner controls for groups obtaining different mixtures.

All groups of rats were given the abovementioned doses of chemicals daily. Water consumption was measured by daily weighing of the water bottles. The subsequent analysis has showed that there were no significant differences in water consumption by rats from the different groups.

The action of the investigated pollutants on the intensity of free-radical processes was estimated in liver homogenates and blood serum.

The level of free-radical oxidation in liver homogenates was estimated by the concentration of conjugated dienes. Liver homogenates were mixed with heptane-isopropanol solution. The mixture was centrifuged for 5 minutes at 5000 g. The supernatant was further used for determination of optical density at 233 nm. The final concentration of conjugated dienes in liver homogenates was measured in units of optic density per mg of protein (D/mg protein) (Placer 1968). Protein determination in liver homogenates was carried out using the method of O. Lowry (Lowry et al. 1951). Bovine serum albumin was used as a standard.

The intensity of free-radical oxidation in the serum was estimated on the base of the parameters of iron-induced chemiluminescence by using "Chemiluminomer-003" (Ufa, Russia). After the addition of serum into the device and stabilization of the signal, the inductor $FeSO_4 \cdot 7H_2O$ was entered into the system. After the induction of free-radical processes in the system fast flash amplitude and general luminosity were recorded. Fast flash amplitude characterizes the intensity of reactive oxygen species generation as a response to Fe^{2+} addition and indirectly shows the concentration of hydroperoxides in the serum. General luminosity serves as an indicator of free radical oxidation of biomolecules in the serum. The intensity of chemiluminescence was expressed in conventional units (c.u.) of luminosity (Lopukhin et al. 1983).

Data were expressed as mean values ± SEM and evaluated using Mann-Whitney U-test at the significance level $2\alpha=0.05$.

RESULTS

The data obtained are shown in Table 1 and indicate that intake of any of the investigated pollutants led to an increase in the concentration of conjugated dienes in rats. After administration of iron in a dose equal to its maximum permissible concentration, the level of conjugated dienes was enhanced by 1/3 in comparison with the controls. The intake of $FeSO_4$ in concentration of ½ MPC led to significantly increased

Table 1. **Concentration of conjugated dienes in liver homogenates** (D/mg protein) and intensity of serum chemiluminescence (c.u.) in rats obtaining different doses and combinations of investigated pollutants.

No	Chemicals	Dose of each chemical (MPC)	Conjugated dienes	Fast flash amplitude	General luminosity
I	Control group	–	0.24±0.02	0.32±0.05	1.47±0.17
II	Fe^{2+}	1	0.33±0.03	0.38±0.05	1.48±0.15
III	Fe^{2+}	0.5	0.43±0.05 [a]	0.42±0.07	1.68±0.19
IV	Fe^{2+}	0.33	0.56±0.07 [a, b]	0.39±0.11	1.72±0.20
V	Cu^{2+}	1	0.32±0.03	0.49±0.06 [a]	1.67±0.15
VI	Cu^{2+}	0.5	0.47±0.05 [a]	0.33±0.04	1.64±0.21
VII	Cu^{2+}	0.33	0.43±0.04 [a]	0.53±0.05 [e]	2.56±0.11 [a, b]
VIII	NO_3^-	1	0.35±0.03	0.37±0.04	1.31±0.12
IX	NO_3^-	0.5	0.55±0.06 [a, d]	0.41±0.02 [a]	1.88±0.17 [a, d]
X	NO_3^-	0.33	0.44±0.04 [a]	0.52±0.10 [a, d]	2.12±0.27 [a, d]
XI	Fe^{2+} + Cu^{2+}	0.5	0.50±0.04 [a, b, c]	0.35±0.05	1.68±0.18
XII	Fe^{2+} + NO_3^-	0.5	0.44±0.03 [a]	0.38±0.05	1.67±0.18
XIII	Cu^{2+} + NO_3^-	0.5	0.47±0.04 [a]	0.39±0.05	1.76±0.10 [d]
XIV	Fe^{2+} + Cu^{2+} + NO_3^-	0.33	0.47±0.03 [a, b, c, d]	0.54±0.08 [a, d]	1.81±0.19 [d, f]

[a] statistically significant versus control group
[b] statistically significant versus group receiving Fe^{2+} in 1MPC
[c] statistically significant versus group receiving Cu^{2+} in 1MPC
[d] statistically significant versus group receiving NO_3^- in 1MPC
[e] statistically significant versus group receiving Cu^{2+} in 0.5 MPC
[f] statistically significant versus group receiving Cu^{2+} in 0.33 MPC

levels of conjugated dienes; nearly 2-fold as compared with controls. Finally, after administration of water containing 1/3 MPC of iron, the concentration of this product of lipid peroxidation was elevated 2.3-fold (statistically significant).

Nonlinear dependence between the dose of incoming pollutants and the intensity of lipid peroxidation, estimated on the base of conjugated dienes in liver homogenates, was observed in rats receiving Cu^{2+} and NO_3^- with drinking water. The maximal effect of single chemicals was observed in concentrations equal to 0.5MPC (statistically significant).

The data from the table 1 also show that the effects of different pollutants acting in combinations were not cumulative in relation to their pro-oxidant action. At the same time the concentration of conjugated dienes in the liver homogenates was significantly higher than in the case of the isolated action of pollutants in a dose of 1 MPC.

A similar dependence between the intensity of free radical processes and concentration of investigated pollutants was observed in the rats' serum. After administration of iron and copper, the concentration of lipid hydroperoxides as estimated by the amplitude of fast flash did not depend on dose. In groups of animals obtaining different concentrations of nitrates, this dependence had an inverse character.

A significant increase of fast flash amplitude was observed after administration of three-component combination of pollutants.

It can also be seen from Table 1 that the intensity of free-radical oxidation of biomolecules in serum as estimated by general luminosity was maximal after intake of pollutants in doses equal to 1/3 MPC. In the case of combinations, no increase in pro-oxidant action was observed, though the general luminosity in these groups was higher than in the controls.

DISCUSSION

The data obtained allow us to make the following conclusions. First of all, chemicals entering the organism in doses that do not exceed their maximum permissible concentrations are able to induce oxidative stress. The observed pollutant-dependent activation of free-radical processes is a consequence of their pro-oxidant features. As redox metals, iron and copper have the same mechanisms of pro-oxidant action taking part in the generation of superoxide anion (Haber and Weiss 1934), Fenton-reaction (Fenton 1894) and lipid peroxidation (Valko et al. 2005). Moreover d-metals can also cause the depression of antioxidant enzymes (Ribarov et al. 1982).

The activation of free-radical oxidation in rats receiving $NaNO_3$ with drinking water is possibly a consequence of nitrate reduction to nitrite. It is known that nitrite uptake by erythrocytes can cause oxidative stress (May et al. 2000). It is possible that such an effect may be connected with the further reduction of nitrite to nitric oxide by different macromolecules (Gladwin et al. 2005). This hypothesis is confirmed by the data showing that inorganic nitrate can be a source of nitric oxide (Lundberg and Govoni 2004). NO·, except when serving as a signalling molecule, can interact with superoxide and nitrite forming peroxynitrite and N_2O_3 respectively (Wink and Mitchell 1998).

Secondly, there is an inverse dependence between pro-oxidants entering the organism and intensity of free-radical oxidation. This thesis can be supported by earlier data indicating that an increase in iron concentration leads to depression of chemi-luminescence *in vitro* (Suslova et al. 1968). It was supposed that high doses of iron *in vitro* enhance the amount of lipid peroxidation chains, and their length seems to be significantly reduced (Vladimirov and Archakov 1972).

At the same time the more expressed pro-oxidant action of pollutants in relatively low doses can be connected with the better gastrointestinal absorption of these concentrations.

It is known that copper bioavailability decreases with increasing amounts of dietary copper (Turnlund et al. 1989). There is also data indicating that a high-copper diet stimulates metal excretion (Turnlund et al. 2005). Such effect can be a consequence of the regulatory interaction of copper and transport proteins during absorption. It is known that high doses of copper lead to inhibition of apical copper transporters DMT1 and Ctr1 (Tennant et al. 2002, Petris et al. 2003, Wu et al. 2009) and Menkes ATPase that acts as a basolateral transporter (Monty et al. 2005).

A similar inhibitory action of high doses is observed in iron. The only known apical transporter of iron is DMT which can be inhibited by high doses of iron acting by different mechanisms (Sharp et al. 2002, Zoller et al. 2002, Mena et al. 2008). Duodenal reductase b (Dcytb), taking part in apical iron absorption, is also down-regulated by iron (Frazer et al. 2002). Iron transport through the basolateral membrane by IREG1 also seems to depend on incoming iron concentration (Zoller et al. 2002, Di Domenico et al. 2007). There is some poor data on reverse dependence between dose and bioavailability of nitrates. It is supposed that nitrate is rather inert in the organism and its active form is nitrite, which can be formed by bacterial reduction of nitrates in the gastrointestinal tract. In this connection data indicating that the reverse dependence between bacterial reduction and the dose of incoming nitrate (Harada et al. 1975) can support our hypothesis.

Thirdly, the combined action of pollutants leads to a slight inhibition of pro-oxidant features in comparison with the isolated action of these pollutants. According to our hypothesis this effect can be a consequence of the interaction between pollutants during absorption. There are a number of works indicating a decrease in absorption of iron and copper during combined administration (Arredondo et al. 2003, 2006). It is believed that such an effect occurs because of competition between these metals for DMT1. Despite the absence of data on inhibition of iron and copper absorption by nitrates there is a theoretical basis for such action. It is shown that NO· stimulates an NF-κB-mediated decrease in DMT1 transcription (Paradkar and Roth 2006). At the same time inhibition of Fenton-mediated free-radical oxidation by NO· was shown earlier (Gupta et al. 1997). These data can also explain the observed inhibition of pro-oxidant activity of combinations $[Me^{n+}+NO_3^-]$.

In conclusion it is important to note that the observed activation of free-radical processes by low doses of chemicals can play a part in the development of a number of diseases known to be associated with oxidative stress.

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