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RESEARCH ARTICLE

Sulfur mustard causes oxidative stress and depletion of antioxidants in muscles, livers, and kidneys of Wistar rats

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Abstract
Sulfur mustard (SM) is a chemical warfare agent with cytotoxic effect and a tight link to oxidative stress (OS). Depletion of antioxidants is considered as a cause of detrimental consequence and belongs to the important steps leading to cell death. The oxidative injury appearing after SM exposure is not well understood. Nevertheless, identification of the pathological processes would be a good opportunity to establish an efficient therapy. Here, we focused our effort on an estimation of reactive oxygen species homeostasis and apoptotic processes in Wistar rats exposed to 0–160 mg/kg of SM. We assayed antioxidant activity, thiobarbituric acid reactive substances, reduced glutathione/oxidized glutathione, metallothionein, glutathione reductase, glutathione peroxidase, glutathione *S*-transferase, caspase 3, and glucose in the livers, kidneys, and muscles of the animals. Significant OS, depletion of low-molecular-mass antioxidants, increase in caspase activity, and some other processes related to SM action were determined. Moreover, we infer a principal role of OS in the tested organs.

Keywords: Sulfur mustard, oxidative stress, antioxidant, apoptosis, glutathione, TBARS, FRAP

Introduction

Sulfur mustard (SM), a chemical warfare agent with the proper name of bis(2-chloroethyl)sulfide (CAS No.: 505-60-2), is a cytotoxic compound with a not well-understood mechanism of effect on an organism. SM was misused for military purposes several times. It can be even considered as the most frequently used chemical warfare because it was used in World War I, Italian raids in Africa, the Iran-Iraq War, and some other war events (Ghabili et al., 2011). No antidote or prophylaxis for SM exists, which is probably caused by limited knowledge about the molecular mechanism of SM's action. Research is complicated by the fact that SM toxicity manifestation can differ for furred and nonfurred animals (Paromov et al., 2007). As a result of the proven pathologies, oxidative stress (OS) and apoptosis play a crucial role in the toxicity mechanism (Ghanei and Harandi, 2011). Inflammation follows exposure to SM; hence, its implication in SM-induced detrimental consequences is also considered by many scientists (Yazdani et al., 2011). The inflammation role is supported by findings of cyclooxygenase 2, tumor necrosis factor alpha, and inducible nitric oxide synthase in tissues penetrated by SM (Malaviya et al., 2010). SM acts also as an alkylating agent. Alkylation can cause not only the arising of novel epitopes, but also the damaging of DNA. Hyperactivation of poly(ADP-ribose) polymerase is triggered by DNA alkylation and subsequent depletion of nicotinamide adenine dinucleotide (NAD) and then the adenosine triphosphate cell pool (Hinshaw et al., 1999). As the cell energy homeostasis is disrupted, cells do not have sufficient ability to cover reactive oxygen species (ROS) detoxification, and necrotic processes also contribute to the aggravation (Laskin et al., 2010).

The present article is devoted to the assessment of OS in SM-exposed rats. As reported in the references, the role of OS in the SM toxicity mechanism is not

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successfully answered (Shakarjian et al., 2010). It is not clear how OS can prevail upon antioxidant barriers and be implicated in other detrimental processes. For this reason, markers, such as uncovered OS, selected antioxidants, and apoptosis markers, are considered to be assayed in organs that are sensitive to the effect of SM. Liver, kidney, and muscle tissue were chosen for the aim of the experiment.

Methods

SM

Distilled SM was provided by the Military Technical Institute of the former Czechoslovakia Army. Stockpiling and manipulation was permitted by the Czech Republic government institution, the State Office for Nuclear Safety, the official representative of the Organization for the Prohibition of Chemical Weapons in the Czech Republic. Tools with residual content of SM were decontaminated by 2% (w/w) calcium hypochlorite in 0.5% (w/w) detergent overnight.

Animal model, organ collection, and processing

A total of 30 male Wistar rats were purchased from Velaz Corp. (Prague, Czech Republic). Animals' weight of 190 ± 8 g was determined at the beginning of the experiment. The whole experiment was supervised and permitted by the ethics committee of the Faculty of Military Health Sciences of the University of Defense (Hradec Kralove, Czech Republic). Animals were manipulated in compliance with permission and the appropriate laws. For the whole period of keeping animals in menagerie as well as experimental performance, animals were kept in an air-conditioned room with stable temperature (22 ± 2°C), humidity (50 ± 10%), and a light period 12 hours per day. Animals had free access to feed and water.

Animals were divided in five groups with each comprised of 6 animals. The first group was the control and received saline only. The other four groups were exposed to SM in doses of 20, 40, 80, and 160 mg/kg applied on a closely clipped intercapular region (4 cm^2). The upper dose responded to median lethal dose for a poisoning lasting 1 day (196 mg/kg) (Husain et al., 1996). In total, 100 μL of SM solution in saline was spread over the region and covered with a sheet plaster and allowed to penetrate into the body. Two hours after the poisoning, animals were narcotized with carbon-dioxide narcosis and euthanized by heart puncture. Livers, kidneys, and semitendinosus muscle were collected.

Samples of organs (livers, kidneys, and muscles) were frozen to disrupt cells. The mixture was prepared using a hand-operated ULTRA-TURRAX T8 homogenizer (IKA, Konigswinter, Germany) at 25,000 rpm for 3 minutes. Homogenate was transferred to a new test tube and diluted with phosphate buffer in a ration of 1:10. The mixture was further homogenized by shaking on a Vortex-2 Genie (Scientific Industries, New York, New York, USA) at 4°C for 30 minutes. Homogenate was centrifuged (14,000 rpm) for 30 minutes at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Before analysis, the supernatant was filtered through a membrane filter (0.45-μm Nylon filter disk; Millipore, Billerica, Massachusetts, USA).

Markers assay

Ferric reducing antioxidant power (FRAP)

The previous protocol was slightly adapted (Hoesseinzadeh et al. 2007; Sochor et al., 2010). First, 2.5 mL of 10 mmol/L of 2,4,6-tris (2-pyridyl)-s-triazine in 40 mmol/L of hydrochloric acid was poured with 2.5 mL of 20 mmol/L of ferric chloride and 25 mL of 0.1 mol/L of acetate buffer (pH 3.6). Next, 200 μL of mixture, 30 μL of sample (or saline solution as blank), and 770 μL of deionized water were injected into a plastic tube after the mixture incubation at 37°C for 10 minutes. After another 10 minutes of incubation, the mixture was spun at 10,000×*g* for 10 minutes. Absorbance of the supernatant was measured at 593 nm against the blank.

Glutathione reductase (GR)

A disposable cuvette was filled with 100 μL of 10 mmol/L oxidized glutathione (GSSG), the same volume of 1 mmol/L nicotinamide adenine dinucleotide phosphate (NADPH), and 650 μL of phosphate-buffered saline (PBS). The reaction was started immediately by the injection of 50 μL of the sample. Absorbance at 340 nm was measured for 120 seconds.

Glutathione S-transferase (GST)

The method is based on the GST-catalyzed reaction between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). The GST-catalyzed formation of GS-DNB produces a dinitrophenylthioether, which can be detected spectrophotometrically at 340 nm. A 180-μL volume of reactants consisting of 2 mmol/L of CDNB and PBS (1.4 mmol/L of NaH_2PO_4 and 4.3 mM of Na2HPO4; pH 7.4) (1:19, v/v; 37°C) was added to sample in a plastic microtube. Further, 12.5 mM of GSH (30 μL) in 0.1 mol/L PBS (pH 7.4) was added. A wavelength of 340 nm was used for the determination of GST activity.

Glutathione peroxidase (GPx)

A Glutathione Peroxidase Cellular Activity Assay Kit (CGP1; Sigma-Aldrich, St. Louis, Missouri, USA) was used for GPx assay. For determination of GPx activity, a BS 400 automated spectrophotometer (Mindray, Shenzhen, China) was used. A 260-μL volume of reagent R_1 (0.3 mmol/L of NADPH in GPx buffer) was pipetted into a plastic cuvette with the subsequent addition of 10 μL of sample, and after mixing, a 30-μL volume of reagent R_2 (3 mmol/L of *tert*-butyl hydroperoxide) was added to the cuvette, which started the reaction. The decrease in absorbance was measured at 340 nm using a kinetic program for 2 minutes 6 seconds. The spectrophotometer calculated GPx activity according to a calibration curve.

Thiobarbituric acid (TBA) reactive substances (TBARS)

TBARS was assessed in compliance with previously optimized and published methods (Pohanka et al., 2011a, 2011b). In the first step, 67 mg of TBA were mixed with 1 mL of dimethyl sulfoxide (DMSO) and then with 9 mL of deionized water. The sample (200 µl) was purified from proteins by precipitation with 400 µL of 10% trichloroacetic acid. After centrifugation at 3,000×*g* for 15 minutes, 400 µL of supernatant or 400 µL of saline solution (blank) were injected into a plastic tube, together with the same volume of the above-prepared mixture, and were heated at 100°C for 10 minutes. Absorbance was measured at 532 nm against the blank.

Tissue caspase 3 activity

Determination of caspase was based on the hydrolysis of the peptide substrate acetyl, Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. The pNA has a high absorbance at 405 nm. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm.

Total protein in tissue homogenates by Bradford's method were assayed with total protein kits (Sigma-Aldrich), in compliance with the attached protocol. Human serum albumin was used as a standard. Absorbance was measured at 595 nm using disposable plastic cuvettes.

Metallothionein (MT) assay

Tissue content of MT was assayed using an electrochemical instrument 747 VA Stand connected to a 746 VA Trace Analyzer and a 695 Autosampler (Metrohm AG, Herisau, Switzerland) and a hanging mercury drop electrode with a drop area of 0.4 mm^2. First, tissue samples were processed in compliance with the reference and 100× diluted in extraction buffer (100 mM of potassium phosphate, pH 6.8) before electrochemical measurements (Petrlova et al., 2006). Electrochemical assay was performed in compliance with the previously published protocol (Fabrik et al., 2008).

Glucose level in tissues

First, 200 µL of mixture containing 0.1 M of PBS (pH 7.5), 0.75 mmol/L of phenol, 0.25 mmol/L of 4-aminoantipyrin, glucose oxidase ≥15 kU/L, and peroxidase ≥1.5 U/L were poured with 20 µL of sample. Absorbance at 505 nm was measured at 37°C at the beginning of the experiment and after 10 minutes.

GSH assay

GSH and GSSG were assayed by high-performance liquid chromatography (HPLC) with electrochemical detection, based on two solvent delivery pumps (Model 582; ESA Inc., Chelmsford, Massachusetts, USA), a reaction coil (1 m), a Metachem Polaris C18A reverse-phase column (150.0 × 2.1 mm, 5 µm particle size; Varian, Inc., Palo Alto, California, USA), and a CoulArray electrochemical detector (Model 5600A; ESA). In total, 5 µL of sample was injected using an autosampler (Model 540 Microtiter HPLC; ESA). Gradient profile started at 100:0 (80 mmol/L of trifluoroacetic acid/methanol) and was kept constant for 9 minutes, then decreased to 85:15 for 1 minute, then kept constant for 8 minutes, and finally increased linearly up to 97:3 from 18 to 19 minutes, a mobile phase flow rate of 0.8 mL/min, and a column temperature of 40°C, with a working electrode potential of 900 mV.

Data processing and statistical analysis

All markers were calculated per gram of protein. Origin 8 SR2 software (OriginLab Corporation, Northampton, Massachusetts, USA) was used for significance testing by Bonferroni's test (named also Bonferroni's correction, in compliance with some literature sources). Both $P = 0.05$ and $P = 0.01$ probability levels were calculated considering group size 6 specimens. Each marker was calculated for one experimental group as mean ± standard error of mean (SEM).

Results and discussions

SM causes various adverse effects once penetrated into the body. Gastrointestinal, hematologic, and neurological diseases, accompanied with headache, fatigue, sickness, and convulsions, can be expected (Ghabili et al., 2011; Somani and Babu, 1989; Elsayed and Omaye, 2004). The role of OS in SM toxicity has been discussed, and some opposing conclusions can be found in scientific reports. We aimed our effort to the evaluation of OS in the most endangered organs for this reason.

Low-molecular-mass antioxidant GSH plays an important role in fighting with OS and detoxifying pathways in animals as well as microorganisms (Mukherjee et al., 2009). It also has an undisputed role in protection from an SM mimetic, bis(betachloroethyl) sulphide, in cell lines (Gentilhommme et al., 1992). In our experiment, GSH was significantly decreased in all processed organs (livers, kidneys, and muscles) after SM application (Figure 1). GSH level was depleted in a dose-dependent manner up to half the level of controls in animals that received SM in a dose higher than 20 mg/kg. Depletion had a reciprocal dose-dependent manner (range, 0–40 mg/kg). The doses of 80 and 160 mg/kg did not cause any further depletion of the GSH level. GSH level was not significantly different, when compared to the group exposed to 40 mg/kg and the two groups exposed to 80 and 160 mg/kg of SM. We infer that the depletion can be limited by solubility of SM in water conditions. The highest administered doses of SM probably do not cause an increase of SM because it is deposited in lipid compartments and SM particles (Benson et al., 2011). Depletion of GSH was reported by Shohrati et al. (2009) as well. They proved a significant decrease of GSH level in the blood of Iran-Iraq war victims poisoned by SM during war events. Beside GSH, we assessed GSSG as a second marker. However, GSSG has no significant alteration (data not shown), and the

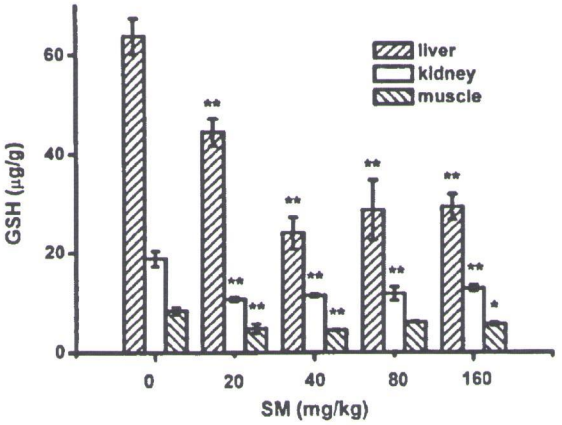

Figure 1. Alteration in GSH level in SM exposed animals. Significance versus control at $^{*}P = 0.05$ and $^{**}P = 0.01$, respectively. Error bars represent SEM for $n = 6$ specimens.

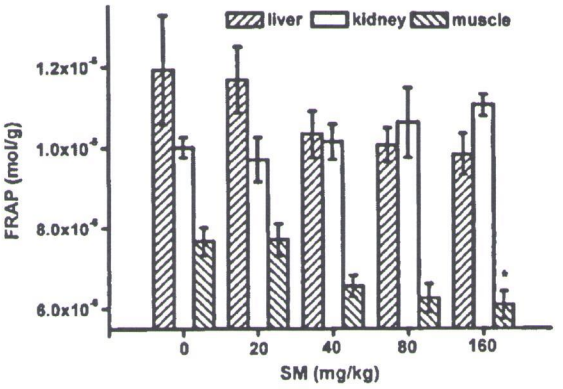

Figure 2. Alteration in FRAP level in SM-exposed animals. Description is the same as in Figure 1.

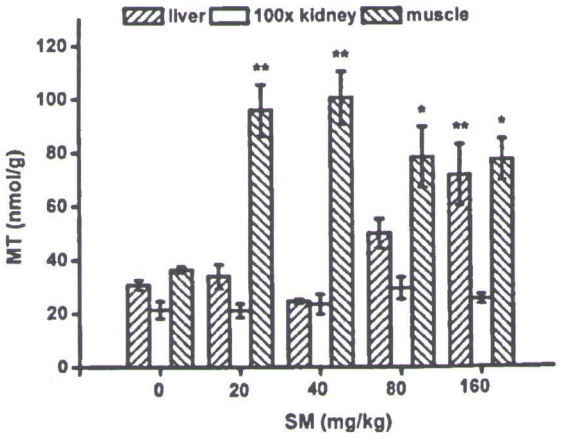

Figure 3. Alteration in the MT level in SM-exposed animals. Description is the same as in Figure 1.

levels of GSSG were fluctuated randomly. In an *in vitro* experiment, GSH depletion was revealed as one of the detrimental consequences of poisoning and the application of exogenous GSH was potent enough to ameliorate the effect in the both JB6 and HaCaT cells (Tewari-Singh et al., 2011).

Similarly to GSH, antioxidant activity was also decreasing in livers and muscles after SM application (Figure 2). Kidneys kept a stable level of low-molecular-mass antioxidants because antioxidant activity was unchanged. The decrease in the livers and muscles was on a lower scale than in kidneys. It was approximately 20% for the upper dose of SM and it was significant ($P = 0.05$) only in the muscle from rats exposed to 160 mg/kg of SM. For muscle, antioxidant activity was decreased in a dose-dependent manner. Similar findings are reported in other articles (Laskin et al. 2010; Baradaran-Rafii et al. 2011; Pohanka et al. 2011a).

MTs are important proteins with a detoxification role and key role in protection from OS (Vasatkova et al. 2009). We noticed a significant increase ($P = 0.01$) in MT level in the liver from animals exposed to SM in doses of 80 and 160 mg/kg. Muscles had the increased MT level in all animals exposed to SM (Figure 3), but the highest increase was found for the dose of 20 mg/kg. Doses of 40–160 mg/kg did not cause any further increase of metallothionein level. MT level was not significantly different in muscles, when compared to animals poisoned with 20 mg/kg and the higher doses. The increase in MT level as a response to stress and/or toxic conditions was reported on by other investigators as well (Vasatkova et al. 2009). Here, MT level was unchanged in kidneys of all animals. On the other hand, MT content was approximately 100 times lower in kidneys, comparing to livers and muscles. Expression of MT in liver and muscles is probably in the course of OS. On the other hand, experiments aimed at investigation of a link between MT and SM poisoning revealed that the SM effect is more complicated (Nourani et al., 2011). It is not clear why SM induces MT expression in a dose-dependent manner in the liver and not in the muscles. We can hypothesize that MT reached a maximal expression rate in muscles after poisoning with SM at the dose of 20 mg/kg, and there was no capacity to produce a higher amount of MT. The fact that the final content of MT is equal in liver and muscles after application of SM at the dose of 160 mg/kg supports the idea.

GR had increased activity in livers and muscles in a dose-dependent manner (Figure 4). However, the increase was significant ($P = 0.05$) only in muscles of rats exposed to SM at a dose of 160 mg/kg. Alterations are in compliance with Gautam and Vijayaraghavan. (2007). On the other hand, Vijayarghavan et al. (2008) reported on a decrease in GR activity. However, the investigators applied upper doses than the ones used here, so pathological processes would be uncovered by antioxidants resulting from total depletion in cell metabolism. Fighting OS is also confirmed by the expression of GR in livers and muscles. GR plays a crucial role in the covering of OS, and its role in the defense against SM was reported on by Sharma et al.

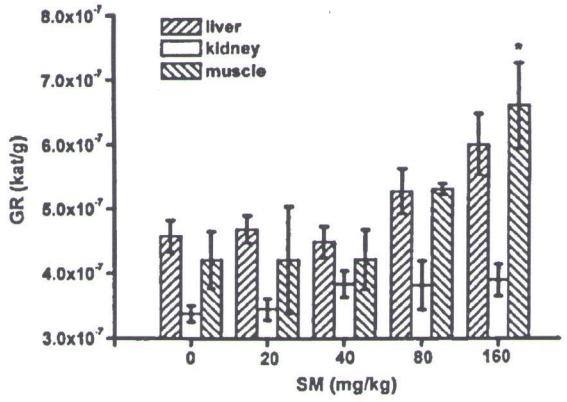

Figure 4. Alteration in GR activity in SM-exposed animals. Description is the same as in Figure 1.

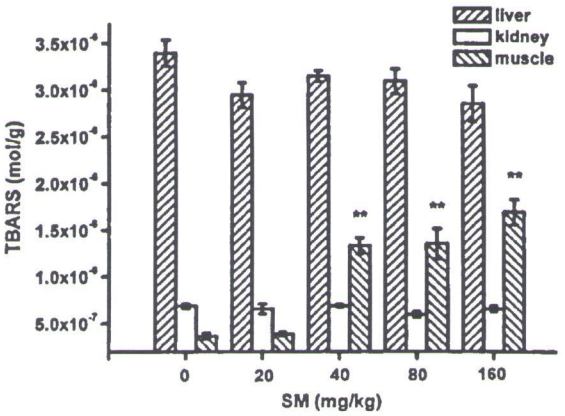

Figure 5. Alteration in TBARS level in SM-exposed animals. Description is the same as in Figure 1.

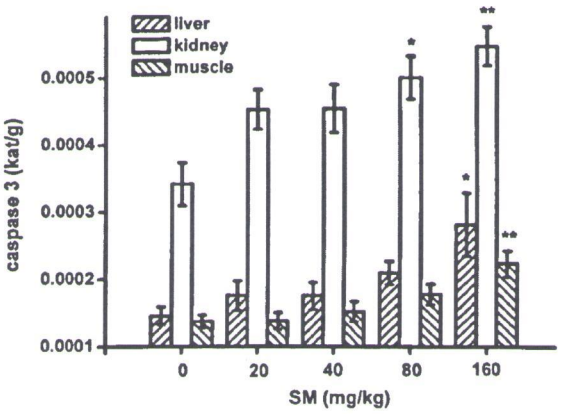

Figure 6. Alteration in caspase 3 activity in SM-exposed animals. Description is the same as in Figure 1.

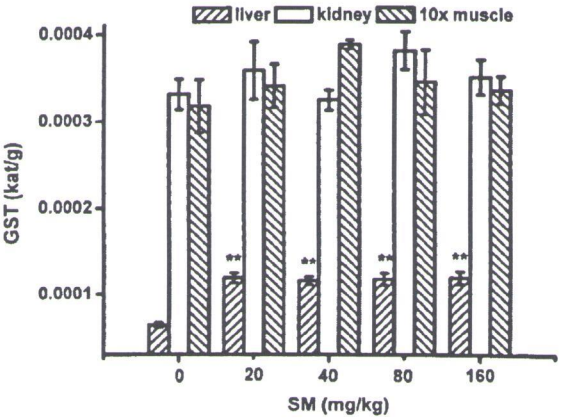

Figure 7. Alteration in GST activity in SM-exposed animals. Description is the same as in Figure 1.

(2009), too. The last assayed antioxidant marker, GPx, was not significantly altered (data not shown), and the level of GPx only randomly fluctuated with minor changes in its activity (changes less than 10%). A significant decrease in GPx activity was reported on by Husain et al. (1996) in the liver and spleen of SM-exposed rats. Contrary to the liver and spleen, the brain and kidney seemed uninfluenced.

TBARS level (Figure 5) represented the scale of lipid peroxidation (LPO) by assessment of malondialdehyde level. The level was not significantly altered in the liver and kidney. On the other hand, the level was increased in the muscle as a result of SM exposure in a dose-dependent manner. The increase was significant (P = 0.01) for SM doses 40–160 mg/kg, and the level was even 4.7 times higher in the animals receiving 160 mg/kg of SM, when compared to controls. Increase of TBARS and GST activity well correlate with investigation of the markers in patients performed by Jafari and Ghanei (2010).

Apoptosis was estimated by assay of caspase 3 activity in the examined organs (Figure 6). Caspase 3 activity was growing in a dose-dependent manner in the three organs. For the kidney, growth in caspase 3 activity was up to 40% and was significant for SM dose 80 mg/kg on a probability level of 0.05 and 0.01 for SM dose 160 mg/kg, respectively. Liver and muscle had significantly increased caspase 3 activities on probability levels of 0.05 and 0.01 for the upper dose of SM. GST activity (Figure 7) was not altered in the liver and kidney. Only random fluctuations of GST activity in samples were recognized because there was neither significance nor trend in the marker level. Contrary to the liver and kidney, the muscle's GST activities were nearly doubled after SM exposure and was significant on a probability level of P = 0.01. However, the increase was in a dose-dependent manner only for the lowest dose of SM. The further increase of SM dose did not cause an increase of GST level. The reason for such a process is probably the same as in the MT case. The activation could reach maximum and further increase of poisoning had no effect. Apoptosis processes followed the effect of SM, as can be inferred from the increased activity of caspase 3. Apoptosis is not surprising when considering reports of other scientists (Joseph et al., 2011; Weinberger et al., 2011). The increase in caspase 3 activity

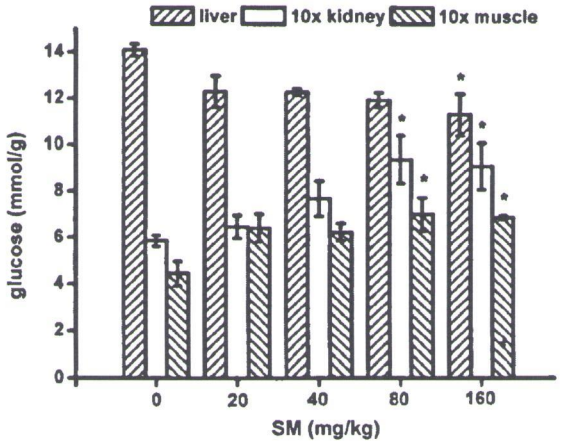

Figure 8. Alteration in glucose level in SM-exposed animals. Description is the same as in Figure 1.

well correlates with the decrease of GSH level. Both processes probably cohere, because of the biochemical link between GSH and apoptosis. GSH is considered as a regulator of apoptosis by *S*-glutathiolation of apoptosis factors (Dalle-Donne et al., 2011). Considering the previous literature, depletion of GSH can be inferred to be a factor enhancing apoptosis initiated by SM. Similar findings regarding GSH and caspase activity were done by Han et al. (2004) for an SM analog (2-chloroethylethyl sulfide). However, caspase 3 activation can be initiated by the immune system by the Fas receptor as well as by mitochondria and cytochrome c (Beere, 2005). Unfortunately, the assay used here does not provide information on how caspase 3 was initiated. An understanding of the caspase role in SM toxicity would be a good opportunity for the development of a suitable therapy in the future. Evidence of apoptotic processes and keeping of GSH level in the SM-poisoned body was extensively reviewed previously (Pohanka, 2012).

Glucose was the last parameter assayed in the organs (Figure 8). Its level was investigated as a parameter scoring basal homeostasis. Two opposite effects were recognized. First, glucose was decreasing in a dose-dependent manner in livers with a significant ($P = 0.05$) decrease for the upper dose of SM. Kidneys and muscles had increasing glucose level in a dose-dependent manner with significant ($P = 0.05$) alteration for the SM doses of 80 and 160 mg/kg. The increase of glucose level in the muscle and kidney can be attributed to the inhibition of glycolysis. The inhibition of glycolysis is considered as one of the SM toxicity pathways and would be related to depletion of cell energy (Martens and Smith, 2008). Glucose may be accumulated in the tissues because it is not metabolized. On the other hand, it is not clear why glucose level mitigates in livers. Some additional experiments will be needed on the issue of glucose fate in livers after SM exposure.

In compliance with expectations, SM had an unequal effect on the examined organs. It can be expected when considering known articles (Somani and Babu, 1989). As a result of OS, muscles seem to be more sensitive to SM effect because low-molecular-mass antioxidants were depleted and LPO was increased at the same time. The findings well correlate with our previous results (Pohanka et al., 2011c). As a result of the antioxidants and LPO scale, the upper vulnerability can be expected in muscles. Pertinent countermeasures, based on the application of exogenous antioxidants, would be effective for the therapy of OS-related pathologies in muscles (Bailey et al., 2011). Further, Livers and kidneys were sensitive to the depletion of low-molecular-weight antioxidants. However, the depletion was not followed by LPO, so lower vulnerability by OS can be expected. Considering all the OS markers, OS played a plausible role in SM toxicity in all of the tested tissues. Muscles had the lowest ability to resolve OS.

Conclusions

Here, considering the proven biological effects of SM, OS and depletion of low-molecular-mass antioxidants were plausibly proven. The other processes, such as apoptosis, follow the OS-caused injury. For the reason of oxidative insult, SM-exposed tissues would be protected by the application of low-molecular-mass antioxidants. Substitution of depleted GSH should be the main effort in subsequent research. When considering the time of the euthanasia of animals, antioxidant therapy should be provided early after SM exposure, if it should maintain effectiveness and protect from oxidative injury.

Declaration of interest

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