

Neurodegenerativne promjene u mozgu štakora izazvane sevofluranom i željezo-dekstranom

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University of Zagreb

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**NEURODEGENERATIVE CHANGES IN THE
RAT BRAIN INDUCED BY SEVOFLURANE AND
IRON-DEXTRAN**

Graduation Thesis

Zagreb, 2018.

Work for this thesis was done at Department of Animal Physiology, Faculty of Science, University of Zagreb, under supervisorship of Dr. Nada Oršolić, Prof. (Department of Animal Physiology, Division of Biology, Faculty of Science, University of Zagreb, 10000 Zagreb, Croatia). Thesis was submitted for evaluation to Division of Biology, Faculty of Science, University of Zagreb to achieve the academic degree Master of Experimental Biology (mag. biol. exp.).

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NEURODEGENERATIVNE PROMJENE U MOZGU ŠTAKORA IZAZVANE SEVOFLURANOM I ŽELJEZO-DEKSTRANOM

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Inhalacijski anestetici su lako hlapljive tekućine, a služe za uvođenje ili održavanje anestezije pri operativnim zahvatima nepredvidljivog trajanja. U ovom radu proučavan je utjecaj sevoflurana, samog ili združenog s željezo-dekstranom, na nastanak i razvoj neurodegenerativnih promjena na tkivu mozga štakora. Neurodegeneracija je mjerena: (i) promjenama oksidacijskog statusa mjerenjem razine malondialdehida (MDA), glutationa (GSH), te enzimatske aktivnosti katalaze (CAT) i superoksid dismutaze (SOD); (ii) (anti)genotoksičnog odgovora u limfocitima štakora, pokazujući razinu oštećenja DNA; (iii) procjenom razine neuroupale prema relativnoj težini mozga u odnosu na zdravu kontrolu; (iv) procjenom toksičnog učinka na hematološke i biokemijske odrednice u krvi, te osmotsku fragilnost eritrocita. Rezultati pokazuju da željezo-dekstran uzrokuje značajnu neuroupalu, te povećanu razinu oksidacijskog stresa: povećava razinu MDA, mijenja aktivnosti antioksidativnih enzima (SOD, CAT) i snižava GSH. Sevofluran u kombinaciji sa Fe-dekstranom pokazuje izraženi gubitak moždanih stanica i prividno niske razine MDA te antioksidansa, kao i značajne toksične učinke na krvne odrednice. Comet test pokazuje najveća oštećenja DNA limfocita pri izlaganju sevofluranu združenim sa željezo-dekstranom. Temeljem rezultata može se zaključiti kako jaka neuroupala i pojava lipidne peroksidacije u mozgu životinja obrađenih Fe-dekstranom ukazuju na neurotoksičnost, dok kombinacija sa sevofluranom uzrokuje najteža oštećenja mozga i trajno narušava homeostazu organizma.

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NEURODEGENERATIVE CHANGES IN THE RAT BRAIN INDUCED BY SEVOFLURANE AND IRON-DEXTRAN

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Inhalation anaesthetics, such as sevoflurane, are commonly used in modern medicine. They are volatile gases that easily diffuse through cell membranes, used as the introduction to anaesthesia or for maintaining it in procedures of unpredictable duration. Aim of this study was to study the effects of sevoflurane, alone or in combination with Fe-dextran, on development of neurodegenerative changes in the rat brain tissue. Neurodegeneration was evaluated by: (i) (anti)oxidative status of brain tissue, by measuring the malondialdehyde (MDA) and glutathione (GSH) concentrations, catalase (CAT) and superoxide dismutase (SOD) enzymatic activities; (ii) (anti)genotoxic response in rat lymphocytes, indicating extent of DNA damage; (iii) neuroinflammation, evaluated with relative brain weight compared to healthy control; (iv) toxic effects on haematological and biochemical blood parameters, and osmotic fragility of erythrocytes. Results show that Fe-dextran causes neuroinflammation and oxidative stress: it increases MDA concentration, SOD and CAT activity and decreases GSH. Sevoflurane combined with Fe-dextran causes extensive neuroapoptosis and seemingly low MDA and antioxidant concentrations, with the highest blood toxicity and DNA damage. In summary, severe neuroinflammation and lipid peroxidation in brain of Fe-dextran treated animals indicate neurotoxicity, whereas combination with sevoflurane causes severe neurodegeneration and permanently affects whole body homeostasis.

(55 pages, 16 figures, 7 tables, 50 references, original in: English)

Thesis is deposited in the Central Biological Library

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1 INTRODUCTION

1.1 Inhalation anaesthetics

Over the past few centuries, modern medicine grew into today's shape, part of it being advanced surgical procedures. Today, we can't imagine any operation without anaesthesia. Anaesthesia is derived from the Greek word *anesthesia* meaning "no feeling", defined as a state of reduced sensation or awareness, for specific period of time, induced by anaesthetics. General anaesthesia can be administered intravenously or by inhalation. Commonly used inhalation anaesthetics are halogenated ethers, isoflurane, sevoflurane and desflurane. Nature of these anaesthetics is volatile, meaning they easily evaporate and thus can be inhaled. They are small, liposoluble molecules that readily pass through cell membranes and rapidly induce unconsciousness as well as analgesia (Brozovic *et al*, 2006).

1.1.1 Mechanism of action

The mechanism of action can be analysed on macroscopic (brain and spinal cord), microscopic (synapses and axons) and molecular level (pre- and post-synaptic membranes). When the anaesthetic is inhaled into lungs, it diffuses through alveolar capillary network and enters the blood circulation, reaching all cells and tissues. At the macroscopic level, inhalation anaesthetics have effect on spinal cord, decreasing supraspinal arousal and reducing movement response to pain. In general, these agents increase cerebral blood flow and decrease cerebral metabolic rate and glucose metabolism, distinctly in thalamus and midbrain reticular formations. In synapses, inhalation anaesthetics inhibit excitatory presynaptic channel activity by acting on neuronal nicotinic, serotonergic and glutaminergic receptors. Further, they increase inhibitory post-synaptic channel activity of α -subunit of γ -aminobutyric acid (GABA_A) and glycine receptors. This combined effect reduces neuronal and synaptic transmission. GABAergic receptors are believed to have important role because inhalation anaesthetics prolong the GABA_A mediated chloride ion current. This hyperpolarizes the cell membrane and inhibits post-synaptic neuronal excitability. Recently, it was discovered how two-pore potassium channels are involved in mechanism of inhalation anaesthetics function. These channels are present pre- and post-synaptically in central nervous system (CNS) of mammals, and their two main characteristics are voltage independence with absent activation and deactivation kinetics. Background conductance or leaks in two-pore potassium channels

set the resting membrane potential of the cell. Sevoflurane and other inhalation agents enhance their activity, hence plasma membranes hyperpolarize and the generation of action potential in neurons decreases (Khan *et al*, 2014).

In order for anaesthetics to have clinical effect on the target tissue, the partial pressures of inhaled agent must be at equilibrium in alveoli, blood and brain. Pharmacokinetics of inhaled anaesthetics depends on numerous factors, but in general, alveolar concentration determines the uptake and release of anaesthetic, and its uptake by the pulmonary circulation (Khan *et al*, 2014). Inhaled anaesthetics are poorly metabolized and mostly eliminated from the body through respiratory system, in almost unchanged form. Smaller fraction is metabolized in liver by cytochrome oxidase P450 family and excreted via kidneys (Brozovic *et al*, 2006). Recently, these biometabolites became an issue in toxicology, since they cause acute and chronic toxicity.

1.1.2 Effects of sevoflurane

It is shown that inhalation anaesthetics have anti-inflammatory and antioxidative effect on all cells, except on the central nervous system cells. In CNS, inhaled anaesthetics exert neurotoxic effects by promoting oxidative stress and DNA damage. It is believed that these toxic reactions are connected with metabolism, moreover genetic damage has been shown in operating staff who has been exposed to the traces of leaking anaesthetic gases in operating theatre (Rozgaj *et al*, 2009). In addition, sevoflurane increases the permeability of blood brain barrier (BBB), therefore plasma influx increases. These structural changes are noticeable in older rats which fail to regain the barrier integrity within 24 h of exposure to sevoflurane (Acharya *et al*, 2015).

Sevoflurane or 1,1,1,3,3,3-hexafluoro-2-(fluoromethoxy)propane, is highly fluorinated methyl isopropyl ether with general anaesthetic property (Figure 1). Together with desflurane, it slowly replaces halothane and isoflurane in clinical practice, as it appears to be safer inhalation anaesthetic. Although its metabolic rate is ten times higher than that of isoflurane, thus concentration of metabolized inorganic fluoride is higher, it does not induce nephrotoxicity or hepatotoxicity in human studies. One of the main advantages of using sevoflurane is fast induction and fast recovery from anaesthesia in long procedures (Sakai *et al*, 2005). Sevoflurane, unlike isoflurane, is sweet-smelling so it is often preferred in pediatric

anaesthesia as less stressful choice for (already stressed out) young patients (Shen *et al*, 2013).

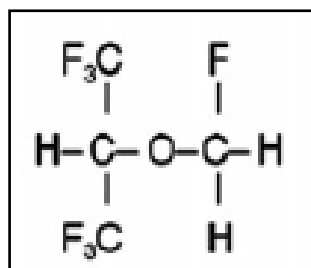


Figure 1. Molecular structure of sevoflurane (adapted from Khan *et al*, 2014)

However, studies have shown that in elderly patients sevoflurane can cause postoperative delirium (POD), which can lead to postoperative cognitive dysfunction (POCD). Postoperative delirium is characterised by agitation or acute attention disturbance in patients that have no prior records of cognitive disorders (Acharya *et al*, 2015). Also, studies in pediatric patients show either neutral or negative effects on postoperative cognition. Millar *et al.* (2006) confirmed impairment of children's cognitive function after brief sevoflurane-nitrous oxide anaesthesia in dental procedures. They described retrograde amnesia to pictorial stimuli shown to children before inducing anaesthesia, but their recognition memory was unimpaired.

Yet, Keaney *et al.* (2004) showed that when children have postoperative delirium after sevoflurane anaesthesia, it does not translate to longer negative behavioural changes. Still, these changes occur more frequently in children under the age of four. Also, there are significant spatial memory impairments in adult rats if they have been exposed to sevoflurane anaesthesia as pups. It appears that developing rat brain is particularly vulnerable, and the shorter the interval between two exposures, the greater the damage (Jevtovic-Todorovic *et al*, 2003; Shen *et al*, 2013). It is hard to decide the true significance of this data, because of the inconsistent conclusions given in these studies.

1.2 Iron

Heavy metals are known to humankind since the prehistoric age, yet only until recently have we been researching its compounds and their effect on the living beings. Nowadays, we know that metals are important in our physiology as part of enzymes in biochemical cycles, which enable all the crucial biometabolic processes in living cells. Iron is commonly present in normal, homeostatic conditions in physiology of all mammals. To better understand its biological importance, we must take a look at its chemical properties. Iron has incompletely filled "d" orbitals and exists in different oxidation states, two of them being ferrous [Fe (II)] and ferric [Fe (III)] forms. This property provides iron is excellent redox active cofactor: it binds to oxygen (O₂), transfers electrons and mediates catalytic reactions. Some of the iron-dependent enzymes are succinate dehydrogenase and aconitase in Krebs (citric acid) cycle. Further, iron is crucial component of heme in haemoglobin, myoglobin in muscles, cytochromes and iron-sulfur complexes in electron transport chain. Without iron there is no DNA synthesis, since it is required for the activity of rate-limiting enzyme ribonucleoside reductase. Also, iron is of crucial importance in central nervous system: oligodendrocytes utilise it for myelogenesis and myelin maintenance. It is necessary as cofactor in the synthesis of many neurotransmitters, such as serotonin, dopamine and norepinephrine. However, due to its reactivity, iron can be both useful and very toxic. It accumulates with age and can enhance oxidative stress, particularly in central nervous system (Udipi *et al*, 2012).

1.2.1 Iron metabolism

Great progress in understanding mammalian iron metabolism and its regulation has been made over the last few decades. Iron metabolism and transport is tightly regulated particularly because it can become toxic. Homeostasis is carefully maintained at systemic and cellular level, to secure optimal, nutritive amounts of iron at all times. In the human body, total quantity of iron is 4-5 grams in average, 65% of which is in the form of haemoglobin. Another 4% is in the myoglobin, 1% in the form of various heme compounds, 0.1% is combined with protein transferrin in the blood plasma and, finally, 15-30% is stored for later use. Iron is mainly stored in the reticuloendothelial system and liver parenchymal cells, generally as ferritin. Iron enters the body through digestion, and when it's absorbed from the small intestine, it combines with a β -globulin, apotransferrin, to form transferrin, which is transported in the plasma. Apotransferrin is secreted by liver into the bile, which gets to

duodenum through the bile duct. Transferrin readily binds to the receptors in the membranes of intestinal epithelial cells and it is absorbed by pinocytosis. From there, it can enter the blood circulation. Iron is loosely bound in the transferrin, which enables it to be released to any tissue cell in the body. In the cell cytoplasm, iron combines with protein, apoferritin, to create ferritin which can then contain small or large amount of iron. Another form of storage iron is in hemosiderin which serves as additional storage pool, when all of the apoferritin is saturated. If the plasma levels of iron are low, it is released from storage pool and transported as transferrin to the areas where needed. Also, hemoglobin released from senescent red blood cells is phagocytized by monocyte-macrophage cells and recycled iron is again stored mainly in ferritin pool (Guyton and Hall, 2006).

Compared to other metals, human body contains larger amounts of iron, and the excess iron is not actively excreted via kidneys. Daily, 0.6 mg of iron is excreted into the feces, and another 0.7 mg in menstrual blood loss in women. Therefore, regulation of total body iron depends almost entirely on the alteration in iron absorption rates. This is done by gene regulation at multiple steps, with iron regulatory proteins (IRP) as one crucial component. When iron levels are low, IRPs are activated to block the ferritin mRNA translation and as a result, transferrin receptors are upregulated, stimulating the acquisition of iron from plasma transferrin. Conversely, when iron levels are high, IRPs are inactivated thus ferritin mRNA is translated and excess iron is stored. Other limiting factor is peptide hormone, hepcidin. Hepcidin inhibits iron transport by binding to the iron export channel ferroportin in the enterocytes. If the iron levels are low, hepcidin is downregulated (Udipi *et al*, 2012).

Iron-dextran is a pharmaceutical drug used for treatment of patients with documented iron deficiency in whom oral administration is impossible or unsatisfactory (<https://pubchem.ncbi.nlm.nih.gov/compound/105075#section=Top>). It is dark red, sterile liquid complex of ferric hydroxide and dextran. Dextran is a complex branched glucan which has chains of varying lengths. The use of large *i.v.* doses of Fe-dextran may be associated with a range of adverse effects: moderate to high fever, dizziness, nausea, vomiting and exacerbations of existing inflammatory conditions (e.g. rheumatoid arthritis, lupus erythematosus). There have been fatalities following the administration of this drug, caused by anaphylactic-type reactions.

1.2.2. Iron in nervous system

In central nervous system, iron is stored in the form of ferritin. In physiological conditions, there is a delicate balance which ensures stability and non reactivity. It plays an important role in cell division of neuronal precursor cells, astrocytes, and oligodendrocytes. Areas of the brain with the largest amounts of iron are globus pallidus, substantia nigra, red nucleus, putamen and caudate nucleus. Normally, tight junctions in the blood brain barrier prevent iron from entering the brain and iron levels are balanced by cellular mechanisms. However, when the excess iron crosses blood brain and cerebrospinal barrier, it promotes oxidative stress in the brain. Stored iron can also be released from neurons in the event of brain damage (Udipi *et al*, 2012). In general, nervous system is the principal target for many toxic metals, and oxidative stress is one of the main mechanisms behind the toxicity (Clarkson, 1987).

1.3 Oxidative stress

About fifty years ago, scientist Denham Harman used his "free-radical theory" to hypothesise that endogenous oxidants are created in cells and are responsible for cumulative damage, resulting in ageing. It was too controversial at the time, to imagine that living in oxygenated environment implies damage to our cells, but about a decade later one of the antioxidative enzymes, superoxide dismutase was identified, making his hypothesis more plausible (Finkel and Holbrook, 2000).

1.3.1 Free radical reactions

Molecular oxygen is used in aerobic cell metabolism for generation of the energy. Its high electrochemical potential mediates the production of large quantities of ATP and free radicals are created as a consequence. Free radicals are molecules with one or more unpaired electrons in their outer shell, making them less stable and more reactive than non-radical species. Free radicals can be formed from molecules by breakage in the chemical bond so that each fragment keeps one electron, by cleavage of radical to give another radical and via the redox reactions. Collectively, free radicals and other non-radical derivatives are called reactive oxygen species (ROS) and reactive nitrogen species (RNS). In cells, formation of ROS and RNS can happen by enzymatic and non-enzymatic reactions. Enzymatic reactions generating free radicals are those in the respiratory chain, the phagocytosis, the prostaglandin

synthesis and the cytochrome P450 system. Otherwise, free radicals are created in non-enzymatic way by reactions of oxygen with organic compounds or those initiated by ionising radiations (Pham-Huy *et al*, 2008).

These reactive species can be generated from either endogenous or exogenous sources. Endogenous free radicals are produced from all homeostatic and allostatic processes that happen in organism: immune cell activation, inflammation, infection, ischemia, menstrual stress, excessive exercise, cancer or ageing. Exogenous sources include air or water pollution, cigarette smoke, alcohol, certain drugs (cyclosporin, tacrolimus etc.), industrial solvents, cooking (smoked meat, used oil), radiation and heavy metals (Cd, Pb, Hg, Fe, As). These compounds enter the body and metabolise or decompose to free radicals (Pham-Huy *et al*, 2008).

Generally, oxidative stress is defined as imbalance between oxidation and antioxidation. When the normal redox balance is disturbed and oxidants prevail, oxidative stress can be deleterious. Still, oxidative processes that are regularly going on in cell are essential for life and death of the cell. These free radicals are beneficial for normal functioning of cells. Some important processes which require ROS and RNS include maturation of cell structures and host defense system. In the immune system, free radicals in moderate concentrations serve as a weapon against pathogens. Phagocytes (neutrophils, monocytes, macrophages) release free radicals to destroy invading microbes as part of innate defense mechanism against disease. Also, ROS and RNS are necessary for cellular signaling system. The most important reactive oxygen species include hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\bullet-}$), singlet oxygen ($^1\text{O}_2$) and highly reactive hydroxyl radical (HO^{\bullet}). Some of the reactive nitrogen species are nitric oxide (NO), nitrogen dioxide (NO_2) and peroxynitrite (ONOO^-). Not all reactive species are necessarily free radicals, but they can easily lead to free radical reactions (Pham-Huy *et al*, 2008).

1.3.2 Iron in redox reactions

Iron acts as a pro-oxidant since it is involved in the production of ROS via Fenton and Haber-Weiss reactions. Free or poorly liganded ferrous ion [Fe(II)] reacts in the Fenton reaction with hydrogen peroxide, generating the highly reactive hydroxyl ions.



Figure 2. Fenton reaction (from Udipi *et al*, 2012).

Ferric ion [Fe(III)] created in this reaction can further react with superoxide anion in the Haber-Weiss reaction which recycles the iron to ferrous form.



Figure 3. Habber-Weiss reaction (from Udipi *et al*, 2012).

The toxic effects of free iron arise from these bio-catalytic properties, enhanced by excessive amounts of available metal. Substantially, Fenton reaction has its *in vivo* significance in the iron overloaded organism. The hydroxyl radical has a half-life in aqueous solution of less than 1 ns, and it reacts close to the site of its formation. Yet, it can cause DNA damage and initiate lipid peroxidation. In addition, the process of lipid peroxidation is catalyzed by iron and results in formation of peroxy radicals (ROO•) (Jomova and Valko, 2011). In study made by Piloni *et al.* (2016), sub-chronic doses of Fe-dextran (six doses of 50 mg/kg) triggered oxidative stress in brain and subsequent antioxidative mechanisms in rats.

1.3.3 Antioxidative mechanisms

However, body has its own antioxidative mechanisms to counterbalance possible negative effects of the ROS and RNS. These antioxidants can be enzymatic or non-enzymatic. The most significant antioxidative enzymes are superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT). Superoxide is primary ROS produced by various sources, so dismuting it to water and H₂O₂ is of great relevance to cells. There are three forms of SOD: CuZn-SOD, Mn-SOD and EC-SOD (extracellular SOD). Next, H₂O₂ is reduced to water by catalase and GSH-Px. Catalase is tetramer and each of four identical monomers contains heme group in the active site. Located in peroxisomes, it serves as cell protection from toxic concentrations of H₂O₂. Enzyme GSH-Px is also widely distributed tetrameric enzyme in cells, containing the unique amino acid selenocysteine within the active site. It uses glutathione (GSH) to reduce H₂O₂ to water, and lipid peroxides to their alcohols (Birben *et al*, 2012).

Non-enzymatic antioxidants are before mentioned GSH, vitamins such as ascorbic acid (vitamin C) and α -tocopherol (vitamin E), β -carotene etc. Glutathione is the most abundant antioxidant and it is soluble. Also, the ratio of GSH to glutathione disulfide (GSSG) is a major factor in analysing oxidative stress. GSH donates its electrons to detoxify hydrogen peroxide and it protects membrane lipids from oxidation by donating protons. GSH restores vitamins C and E into their active forms (Birben *et al*, 2012).

1.3.4 Why is brain vulnerable to oxidative stress?

For many reasons, the nervous system is especially vulnerable to oxidative stress. The neuronal network has distinctive anatomical, physiological and biochemical properties which may even support ROS-mediated damage. Brain is metabolically very active organ and its high energy needs, thus high O₂ consumption results in excessive ROS produced. At the same time, antioxidant defence mechanisms are scarce because the levels of CAT, GSH-Px and vitamin E are low. Further, neuronal membranes are rich in polyunsaturated fatty acids (PUFA), which are particularly sensitive to free radical attack. Along with it, neurons are non-replicating cells and this makes them even more sensitive to ROS. Their mitochondria generate O₂, cytochrome P450 produces ROS, and haemoglobin containing iron within neural tissue can promote oxidative stress. Brain is rich in iron, and brain damage releases iron ions involved in free radical reactions. All these reactive species then can downregulate proteins of tight junctions and weaken the blood brain barrier by indirectly activating matrix metalloproteinases (MMP). In general, the whole structure of neural network is susceptible to disruptions. The ratio of membrane surface area to cytoplasmic volume is high, axons of neurons have extended morphology, and the neuronal conduction and transmission depend on efficient membrane function. The main effects of oxidative stress on cells in CNS is through disruptions in signaling pathways rather than by causing nonspecific damage (Friedman, 2011).

1.4 Genotoxicity

Genotoxicity characterises a change in the genetic material of an organism induced by genotoxic agent. These agents are called genotoxins, and they can be chemical or radiation. Genotoxins can exert three primary effects on organisms: carcinogenic (causing cancer), mutagenic (causing mutations) and teratogenic (causing birth defects). Mostly, they act as

mutagens in various cells, causing mutations which can lead to a variety of diseases and of course, cancer. High concentrations of ROS are toxic, and since they are triggered by either chemicals or radiation, they can have genotoxic effects. For that reason, oxidative stress is a mechanism of indirect genotoxicity, as it can induce damage to DNA by modifying the base and altering DNA strands (Shah, 2012). As before mentioned, excessive iron is highly involved in reactions of oxidative stress, product of the Fenton reaction being genotoxic HO•. In addition, oxidative stress increases levels of Ca²⁺ by damaging proteins which hold these ions within cells. An excessive rise in intracellular Ca²⁺ can activate endonucleases and induce DNA fragmentation (Halliwell and Cross, 1994).

ROS-mediated DNA modifications are as relevant as they are common, reaching 10⁵ hits to DNA per cell per day. These modifications are numerous, including modifications of purines, pyrimidines, deoxyribose and single-strand breaks, as well as double-strand breaks and intra-strand crosslinks. All of them arise from ROS such as ¹O₂, O₂•⁻, H₂O₂, HO• and other species that are easily converted into radicals such as hypochlorite (HOCl) and peroxynitrite (ONOO⁻). The most frequent and the most mutagenic DNA modification described is hydroxylation of guanine in the 8-position. This 8-hydroxylation of guanine leads to misreading of the modified base and nearby residues, as well as wrong base pairing (Poulsen *et al*, 1998).

Among the other compounds, anaesthetic gases came to spotlight recently for their genotoxic effects. In mice, comet assay has shown that sevoflurane caused immediate DNA damage in leukocytes, and subsequent damage in the liver, kidney and brain cells, due to the toxicokinetics of anaesthetic. Another method, micronucleus assay, showed significant and time-dependent increase in micronuclei, which only confirms genotoxic/mutagenic effect of sevoflurane. Additionally, sevoflurane biometabolites interfere with DNA repair mechanisms so that broken DNA is not removed until after 24 hours of exposure. Also, ROS may be produced during the inhalation and potentiate the genotoxicity (Brozovic *et al*, 2010).

1.5 Neurodegenerative illnesses

It is estimated that hundreds of millions of people around world are suffering from neurological disorders. In numbers, there are 47.5 million people with dementia globally, with 7.7 million new cases every year - Alzheimer's disease contributing to 60-70% of cases. These numbers are projected to almost double by 2030 and to even triple by year 2050, with

estimated 132 million patients. So, the question arises: why is this the case? According to WHO (<http://www.who.int/features/qa/55/en/>), age is the strongest known risk factor for dementia, and it matches the demographic statistics of Europe, estimated to have one quarter of population over the age of 65 by year 2030. Well known risks include lifestyle risk factors, such as physical inactivity, obesity, unbalanced diet, alcohol or tobacco abuse, diabetes and middle-age hypertension. Other environmental factors are connected to socioeconomic status, e.g. low education, social isolation and cognitive inactivity.

Firstly, neurodegeneration defines a progressive loss of function and structure of neurons, often followed by their apoptosis. The nervous system, along with all of its synapses, is extremely plastic and delicate system. Since it is metabolically highly demanding, the chance of neurotoxic effects of various agents increases. Further, neurotoxicity is any structural or functional change in the nervous system induced by chemical, biological or physical agent. To understand neurotoxicity and neurodegeneration, it is necessary to take anatomy and physiology of the nervous system into consideration. Due to neurophysiological events in the developing brain, such as neurogenesis and synaptogenesis occurring at high rates, and neurotransmitters exhibiting sometimes contrasting effects (e.g. GABA acting excitatory) with functions other than neurotransmission, the developing brain is especially vulnerable to neurotoxicity. In addition, during development the BBB is not fully developed, which facilitates toxins passing into the brain. On the other hand, matured but old brain may be sensitive to these effects because of the loss of different neurotransmitters and receptors, as well as shrinkage of neurons with noticeably lower rate of neuro- and synaptogenesis. Also, toxic byproducts such as the AD peptide β -amyloid ($A\beta$) and lipid peroxidation-derived 4-hydroxynonenal (HNE) start to accumulate, and ability to compensate for all harmful events is limited (Sayre *et al*, 1997; Culley *et al*, 2007).

1.5.1 Alzheimer's disease

Some of the most prevalent neurodegenerative illnesses are Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS) etc. In this thesis, I will go into details of Alzheimer's disease, since Fe-dextran is used in the AD animal models to mimic pathohistologic AD-related changes in the brain. Alzheimer's disease is prevalent type of neurodegenerative disease with dementia as main symptom, and it is seventh main cause of death in the developed countries. According to Alzheimer's

Association (2014), it has seven stages, and some of the symptoms include difficulty remembering recent events or conversations, changes in mood or apathy, depression, confusion with time or place, poor judgement, behavioural changes, and ultimately, difficulty speaking, swallowing and walking.

The disease is characterised by progressive accumulation of insoluble protein filaments, which are stored in neurons and blood vessels, thus in brain tissue, in the form of extracellular plaques and intracellular tangles (Figure 4) (Brookmeyer *et al*, 1998). Proteins that play important role in AD occurrence are a) A β protein which is created from precursor APP, causing neurotoxicity by directly reacting with neural membrane and by acting on membrane receptors and intracellular signalling pathways; b) Tau protein phosphorylated by protein kinases which then pile up as aggregates of hyperphosphorylated insoluble Tau, neurofibrillary tangles (NFTs). These proteins promote further oxidative stress which can induce production of these proteins again, creating one vicious circle (Behl *et al*, 1994). In addition, APP upregulation happens in response to stress caused by disregulations in iron metabolism, ROS, cytokines or physical trauma (Mattson, 2004).

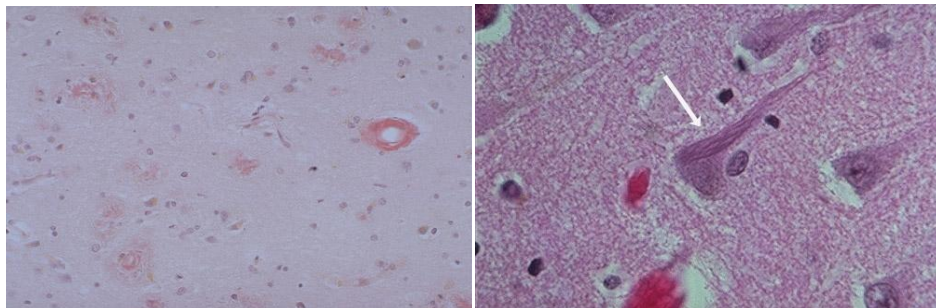


Figure 4. Amyloid plaques and neurofibrillary tangles (adapted from WebPath;
<https://library.med.utah.edu/WebPath/TUTORIAL/CNS/CNSDG.html#1>)

As said, onset of AD is strongly linked to the ageing processes. However, it is important to understand that, although changes in memory and thinking happen while ageing, they shouldn't affect daily functioning or the ability to live independently. So, AD is not a normal part of natural ageing process. It is incurable and today, all available medicines treat cognitive symptoms, including cholinesterase inhibitors and regulators of glutamate activity. There are some genetic aberrancies, which increase the chances for the Alzheimer's and its earlier onset. Some of the common risk genes are preseniline 1 and 2, as well as ApoE gene. The ApoE gene in its isoform ApoE4 promotes A β fibrillogenesis by binding to A β . In fact, neuropathological alteration in AD patients with this allele is significant increase in the

number of amyloid plaques (Yamada and Nabeshima, 2000). New study by Shi *et al.* (2017) shows ApoE4 noticeably affects neuroinflammation and neurodegeneration, exhibiting a 'toxic' gain of function. In individuals with AD who are positive for amyloid- β pathology and carry ApoE4 allele, disease progression rate is greater.

1.5.2 Environmental risks

Environmental exposure to heavy metals is one of the triggers in cognitive and neurological deficits. Some of the environmental sources of heavy metals are contaminated food, air, water or hazardous occupations. Even though levels of heavy metals contamination decreased recently in the developed world, the parts of world still in development have high levels of metal pollution. Moreover, humans are never exposed to a single heavy metal, but instead to heterogeneous metal mixtures (Neal and Guilarte, 2015). In air, heavy metals exist as fine particles which can be inhaled (Li *et al.*, 2015). Children are particularly sensitive to neurotoxic heavy metals, because of the developing nervous system.

Recently, neurotoxicity of general anaesthetics became an issue, with many different results *in vitro* and *in vivo*. Study by Xie *et al.* (2008) showed that common inhalation anaesthetic, isoflurane, is a powerful metabolic depressant and causes increased A β accumulation *in vivo*. In comparison to isoflurane, sevoflurane didn't alter the Bcl-2 to Bax ratio, implicating sevoflurane doesn't increase apoptosis. So, at equipotent concentrations, sevoflurane is not cytotoxic to brain cells *in vitro* (Wei *et al.*, 2005). Sevoflurane also seems to act as neuroprotectant in case of ischemic brain *in vitro*, and preconditioning before induced global cerebral ischemia in rat brain slices helps recovery of neuronal function after hypoxia (Kitano *et al.*, 2007).

However, there is substantial data suggesting inhalational anaesthesia plays a role in neurodegeneration and cognitive decline. Even at low concentrations, inhalation anaesthetics can cause short-term memory loss, which is probably caused by impairment of hippocampus function, major part in short-term memory processes. Also, they affect excitatory NMDA (N-methyl-D-aspartate) neurotransmitter receptors and inhibitory GABA receptors in hippocampus. The subsequent Ca²⁺ overload enhances oxidative stress and generation of neurotoxic ROS (Wang *et al.*, 2010). Further, the specific mechanism of POCD pathogenesis is very complicated and it seems to share certain pathological factors with the AD, like β -amyloid peptide accumulation, Tau phosphorylation and presence of astrocytic gliosis (Fodale

et al, 2010). Since POCD incidence in humans is higher in older age, it is important to keep the brain stimulated. Positive social interactions, mental activity as well as physical activity can support brain health. Healthy diet, particularly the one rich in antioxidants, prebiotics and probiotics can make a significant difference in AD prevention (Bhattacharjee and Lukiw, 2013). In the end, we can't yet cure neurodegenerative diseases, but we can do our best to support natural defense mechanisms.

1.6. Aim of this study

Following all the given data we can see that much on the effect of inhalational anaesthesia and heavy metals on neurotoxicity and neurodegeneration is not yet understood. Conflicts in experimental data and conclusions of given studies are not surprising, considering currently limited knowledge on the matter. Since iron-dextran and sevoflurane can have negative effects in mammals, it is interesting to see how will they affect the brain tissue individually but also in combination. In this study, I will be concentrating on the oxidative and genotoxic status of brain tissue cells because I reason that changes in these parameters could indicate clear neurotoxic effect and consequent neurodegeneration.

I hypothesised that sevoflurane alone or in combination with iron-dextran would promote oxidative stress and cause DNA damage, exerting even stronger negative effects when combined. To confirm that hypothesis I used different approaches. Finally, I want to find a connection between markers of oxidative stress, biochemical and haematological parameters, inflammatory reaction and DNA damage. Also, I want to broaden our knowledge about environmental risks of modern day life, taking into account that incurable neurodegenerative illnesses became not just health, but also socioeconomic issue.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

Present study was approved by the ethical committee (Faculty of Science, University of Zagreb, Croatia). Three months-old rats of both sexes from highly fertile Y59 strain (<http://www.informatics.jax.org/external/festing/rat/docs/Y59.shtml>), weighing 200 to 250 g, obtained from Department of Animal Physiology, Faculty of Science, University of Zagreb, were used in this study. The animals were kept in individual cages during the experiment under standard housing conditions (12 hours of light per day, 24 °C temperature and controlled humidity). They were fed a standard laboratory diet (4 RF 21, Mucedola, Settimo Milanese MI, Italy) and tap water *ad libitum*. This research was conducted on total of 24 Y59 rats, divided into four groups with six animals in each group (Table 1).

Maintenance and care of all experimental animals were carried out according to the guidelines in force in Republic of Croatia (Law on the Welfare of Animals, NN135/06 i NN37/13) and in accordance with EU Directive 2010/63/EU for animal experiments (reference: OJEU 2010) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123.

2.1.2 Chemicals

- 1) Saline solution (Natrii chloridi infundibile), provided by Pliva Croatia, LTD, Zagreb, Croatia.
- 2) Iron-dextran ($\text{FeH}_2\text{O}_4\text{S}$; Mr = 153.917 g/mol) provided by Santa Cruz Biotechnology, USA. Used in dose of 50 mg/kg animal body weight, as a solution of $\text{FeH}_2\text{O}_4\text{S}$ in injection grade water.
- 3) Sevoflurane (Sevoflurane 100%), provided by Baxter Inc., USA. Used as 1.5 % inhalation.
- 4) Narketan® (100 mg/mL), provided by Vetoquinol S.A. BP 189, Lure Cedex, France.
- 5) Xylapan® (20 mg/mL), provided by Vetoquinol Biowet Sp., Gorzow, Poland.

2.2 Methods

2.2.1 Experimental design

Patophysiological changes in rats were induced by intraperitoneal subchronical doses (50 mg/kg animal weight) of iron-dextran solution, and by sevoflurane (1.5 %) anaesthesia during the 21 day period (Table 1).

Experimental animals were divided into four groups with six animals in each. First was the control group which was treated with 0.9 % saline solution every other day during the 21 days. Second group was exposed for two hours to inhalation anaesthetic Sevoflurane (1.5 %) every other day, during the 21 days. For gas administration, rats were placed into an anaesthetic chamber, which was connected to an anaesthesia system. Carbon dioxide and sevoflurane concentrations were monitored on gas monitor, this way avoiding the rebreathing of CO₂ by adjusting the fresh gas flow. Third experimental group was injected with 50 mg/kg iron-dextran every other day, during the 21 days. Fourth group of animals was injected iron-dextran (50 mg/kg) intraperitoneally (*ip*), and two hours after that, it was exposed to inhalation anaesthetic Sevoflurane (1.5 %) in duration of two hours, every other day during the 21 days.

After the 21 day treatment period, animals were anaesthetised and analgesized by intraperitoneal injection of Narketan® and Xylapan® combination, and sacrificed afterwards to collect tissues and organs for further analysis.

Table 1. Experimental groups and treatment plan

Experimental group	Experimental chemicals	Dose	Administration	Treatment period (days)
0. Control	0.9 % NaCl	0.5 mL every other day	<i>ip</i>	21
1. Sevoflurane	Sevoflurane, inhalation anaesthetic	1.5 % every other day	inhalation	21
2. Fe-dextran 50 mg/kg	50 mg/kg FeH ₂ O ₄ S in 0.5 mL pro aqua	0.5 mL every other day	<i>ip</i>	21
3. Fe-dextran 50 mg/kg + Sevoflurane	50 mg/kg FeH ₂ O ₄ S in 0.5 mL pro aqua, in combination with Sevoflurane	0.5 mL Fe-dextran solution and 1.5 % inhalation anaesthetic, every other day	<i>ip</i> + inhalaton	21

2.2.2 Analysis of haematological and biochemical parameters

For the evaluation of haematological parameters, 1 mL of blood withdrawn from the abdominal aorta of experimental animals was taken in a test tube with a vacuum with the addition of anticoagulants K₃EDTA (Becton Dickinson, Plymouth, United Kingdom), and kept at the temperature from 4 °C in 2-4 hours to determine the haematological parameters. For the assessment of biochemical parameters, blood samples were collected in tubes without anticoagulant, the clotting of blood serum was used for the analysis of biochemical parameters. The coagulation of blood was centrifuged at 3000 rpm for 10 minutes and then serum was collected in 1.5 mL polypropylene tubes (Eppendorf AG, Hamburg, Germany) and stored at -20 °C until determination of biochemical parameters.

The haematological parameters that were determined were the number of erythrocytes (E), the average cellular volume of erythrocytes (MCV), haemoglobin (Hgl), haematocrit (Hct), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), total leukocyte count (L), and the mean volume of platelets (MPV). From the standard biochemical parameters were determined albumin (ALB), alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase (AMY), blood urea nitrogen (BUN), creatinine (CRE), blood

glucose levels, total bilirubin (TBIL), total protein (TP), sodium, potassium, calcium, phosphorus and globulin (GLOB).

The osmotic fragility of erythrocytes was determined using the modification of method described before (Ambali *et al*, 2011). A set of glass tubes was prepared, with 9 mL of 0.9 %; 0.8 %; 0.7 %; 0.6 %; 0.5 %; 0.4 %; 0.3 %; 0.2 %; 0.1 % i 0.0 % NaCl solution in each. Whole blood sample was mixed using glass stirring rod, to maintain homogenous cell suspension. Next, 0.1 mL of blood sample was pipetted into each tube in set of prepared NaCl solutions. Tubes were covered with parafilm, mixed turning upside-down couple of times and incubated at room temperature for 30 minutes. By visual examination, I inspected state of total, partial or no hemolysis. Tubes were centrifuged 10 min using 2000 rpm. Supernatant of each tube was transferred to cuvettes using Pasteur pipette. Spectrophotometer Libra S22 (Biochrom, UK) was set at 540 nm, absorbance of first cuvette containing blood in 0.9 % NaCl solution was measured, and set as blank solution (isotonic sample with no hemolysis). Then, absorbance was measured for all samples with NaCl solutions, from higher to lower concentrations of NaCl. The measuring method was repeated for each different sample.

Blood was analysed using standard laboratory methods. Haematologic parameters were analysed on veterinarian haematological counter ABX Micros (Horiba, France) and the biochemical using VetScan® *Comprehensive Diagnostic Profile reagent rotor* on apparatus VetScan® VS2 (Abaxis, UK). Differential blood analysis was done by counting 100 leukocytes manually, using Olympus BX41 microscope (Olympus Optical Co.).

2.2.3 Tracking changes in animal weights

Before starting the experiment, animals were individually marked and weighted, in order to form the experimental groups that contain animals of similar body weight (± 10 g). Also, the doses of experimental treatments were calculated based on the animal weight. The animals are weighed on digital scale ABS 220 – 4 (Kern & Sohn, Germany): i) in the beginning of the experiment; ii) during administration of iron-dextran and sevoflurane, iii) in the end of the experiment. From these data, the mean change in body weight was calculated. The maximum percentage of animal weight loss, was calculated for individual animals as:

$$\text{Percentage change in weight} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Final weight}} \times 100$$

2.2.3.1 Brain weight

The relative brain weight was expressed in g/100 g and was calculated as:

$$\text{Relative brain weight (g/100 g)} = \text{Total brain weight} \times 100 / \text{Final body weight}$$

2.2.4 Alkaline comet assay

To evaluate the impact of iron-dextran and sevoflurane on DNA molecule, the comet assay was carried out under alkaline conditions, basically as described by Singh *et al.* (1988). Fully frosted slides were covered with 1 % normal melting point (NMP) agarose (Sigma, St. Louis, MO). After solidification, the gel was scraped off the slide. The slides were then coated with 0.6 % NMP agarose. When this layer had solidified, a second layer containing the whole blood sample or liver and kidney cells mixed with 0.5 % low melting point (LMP) agarose (Sigma), was placed on the slides. After 10 min of solidification on ice, the slides were covered with 0.5% LMP agarose. The slides were then immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris-HCl, 1 % sodium sarcosinate (Sigma), pH 10) with 1 % Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Kemika, Zagreb, Croatia) to allow DNA unfolding. The slides were then placed on a horizontal gel electrophoresis tank, facing the anode. The unit was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH 13.0) and the slides were placed in this alkaline buffer for 20 min to achieve complete DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4 °C under dim light. Electrophoresis was carried out for 20 min at 25 V (300 mA). After electrophoresis the slides were rinsed gently three times with neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 µg/mL) and covered with a coverslip. The slides were then stored in sealed boxes at 4 °C until analysis. A total of one hundred randomly captured comets from each slide were examined at 250x magnification using an epifluorescence microscope (Zeiss, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). A computerised image analysis system was used to acquire images, compute the integrated intensity profile for each cell, estimate the comet cell components and evaluate the range of derived parameters. To quantify DNA damage, three comet parameters were evaluated: tail length, tail intensity (% of DNA in tail) and tail moment. Tail moment was calculated as (tail length x % of DNA in tail) / 100.

2.2.5 Evaluation of total oxidative stress

2.2.5.1 Tissue preparations

Portions of brain samples of 70-100 mg were homogenised in 1 mL of 50 mM phosphate buffer (pH 7.0) by ultrasonic homogenizer SONOPLUS Bandelin HD2070 (Bandelin, Germany) using probe MS73 (Bandelin, Germany) with a power of 10 %. Homogenates were centrifuged by Micro 200R centrifuge (Hettich, Germany) for 15 minutes at a speed of 15 000 x g at +4 °C. The supernatant was used for the measurements of glutathione and lipid peroxidation level, superoxide dismutase (SOD) and catalase (CAT) activity. All methods are described before. All parameters were normalised in relation to exact protein content.

2.2.5.2. Protein measurement by the Lowry

The protein content was determined following the protocol by Lowry *et al.* (1951). This method is based on reaction of proteins with Cu^{2+} ions and Folin reagent (commercial reagent dissolved by water; $M_r = 260.2$), which contains phosphowolframic and phosphomolybdic acid. Cu^{2+} ions are reduced to Cu^+ in reaction with peptide bonds in alkaline medium, forming the Cu^+ - protein complex. Folin reagent reacts with Cu^+ - protein complex, producing blue coloration. Samples are measured spectrophotometrically at 600 nm. An array of known concentrations of bovine serum albumin (BSA) was used for the creation of the calibration curve, using the same protocol as for homogenized samples. Concentrations of measured proteins were expressed in mg protein/ mL.

2.2.5.3 Lipid peroxidation

The presence of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA), the major products of lipid peroxidation, as described in Orsolic *et al.* (2016). Malondialdehyde reacts with thiobarbituric acid and produces a chromogen which can be measured spectrophotometrically. A total of 200 μL supernatant was mixed with 200 μL of 8.1 % aqueous sodium dodecyl sulfate (SDS), 1.5 mL of 20 % aqueous acetic acid (pH 3.5) and 1.5 mL of 0.81 % aqueous thiobarbituric acid. The mixture was heated for 60 minutes at a temperature of 95 °C. After the cooling of the samples on ice, the absorbance

was measured at 532 nm and 600 nm with Libra S22 spectrophotometer (Biochrom, UK). The total absorbance was determined using the formula $A_{\text{total}} = A_{532} - A_{600}$. An array of known concentrations of tetrametoxipropane was used for the creation of the calibration curve using the same protocol as for the homogenised samples. The concentration of lipid peroxides was expressed as mg MDA/mL/mg protein measured by the method of Lowry.

2.2.5.4 Glutathione assay

The glutathione assay is described before in paper (Oršolić *et al*, 2016). Briefly, 200 μL of 3 mM of 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) was added to 30 μL of sample supernatant. DTNB reacts with GSH to form the chromospheres, 5-thionitrobenzoic acid (TNB) and GS-TNB and the absorbance was measured at 412 nm. The results were calculated from the standard curve of reduced glutathione measured by the same protocol.

2.2.5.5 Measurement of total superoxide dismutase (SOD) activity

The measure of activity of SOD is calculated from the percentage of inhibition of the reaction of xanthine oxidation by xanthine oxidase (optimised reaction ratio $\Delta A / \text{min} \approx 0,025$), which creates superoxide anion as a substrate for SOD present in samples. The superoxide anion which is not used by the enzyme SOD oxidizes the cytochrome. For determination of SOD activity, 25 μL of undiluted sample was mixed with 1.45 mL of reaction mix (Cytochrome C, 248 0,05 mM; Xantin, 1 mM mixed in 10:1 ratio with addition of DTNB). In this mixture a 20 μL xantin oxidase 0.4 U mL^{-1} was added to start the reaction, which was measured over 3 minutes at 550 nm. The absorbance and percentage of inhibition were compared to the calibration curve created with different dilutions of SOD enzyme, values are presented as U/mg protein. This method is described by Flohé and Otting (1984).

2.2.5.6 Measurement of total catalase (CAT) activity

Catalase activity was determined by a spectrophotometric method described previously by Aebi (1984). In this method, catalase activity was estimated by decrease in absorbance of H_2O_2 at 240 nm. The reaction mixture of total volume of 1 mL contained 980 μL 10 mM H_2O_2 (in phosphate buffer, pH 7.0) and 20 μL PBS or sample. Catalase activity

was measured by the extinction coefficient of H_2O_2 ($\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$); the specific activity was calculated and was expressed as $\mu\text{moles/min/mg}$ of total protein.

2.2.6 Statistical analysis

The data was presented as mean \pm standard error (SE) of the representative experiment from three independent experiments. All data were analysed by Kruskal-Wallis ANOVA. Further analysis of the differences between the groups was made with Multiple comparisons of mean ranks for all groups. Statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA). The data were considered significant at $P < 0.05$.

3 RESULTS

3.1 Differences in body and organ weights

3.1.1 Body weight

Percentage of change in body weight of animals (Figure 5) shows that all treatment groups gained less body weight, compared to control. The biggest weight loss was in group treated with iron-dextran ($P < 0.001$), in comparison with control. However, there is no significant change ($P > 0.05$) in body weight of animals treated with sevoflurane, compared with control and group which had combined treatment with sevoflurane and iron-dextran.

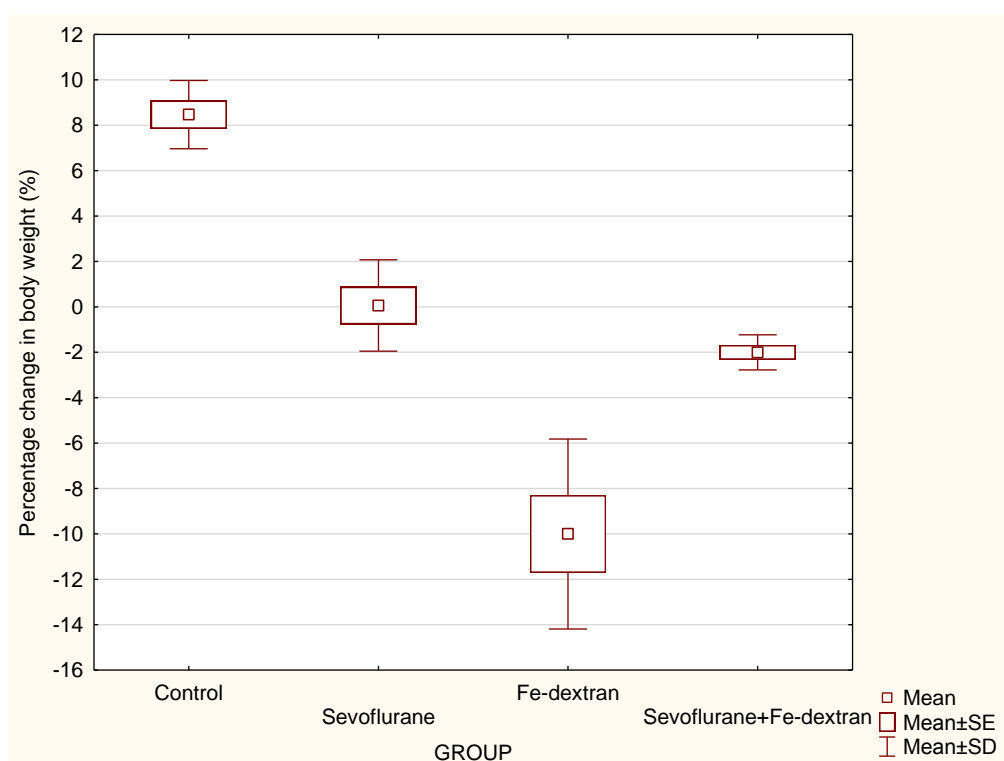


Figure 5. Percentage of change in body weight of Y59 rats treated with sevoflurane, iron-dextran and their combination. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Control vs Fe-dextran ($P < 0.001$); Control vs Sevoflurane + Fe-dextran ($P < 0.05$); Sevoflurane vs Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.1.2a Relative brain weight

To avoid false significance, loss or gain of body weight in each treatment group was taken into consideration, and relative brain weights were calculated based on 100 g of animal body weight (Figure 6). There are no significant changes ($P > 0.05$) in relative brain weights of any treatment group, in comparison with control group. Results show significant changes in iron-dextran treatment group when compared to sevoflurane treatment ($P < 0.01$), and when compared with combined treatment with sevoflurane and iron-dextran ($P < 0.05$).

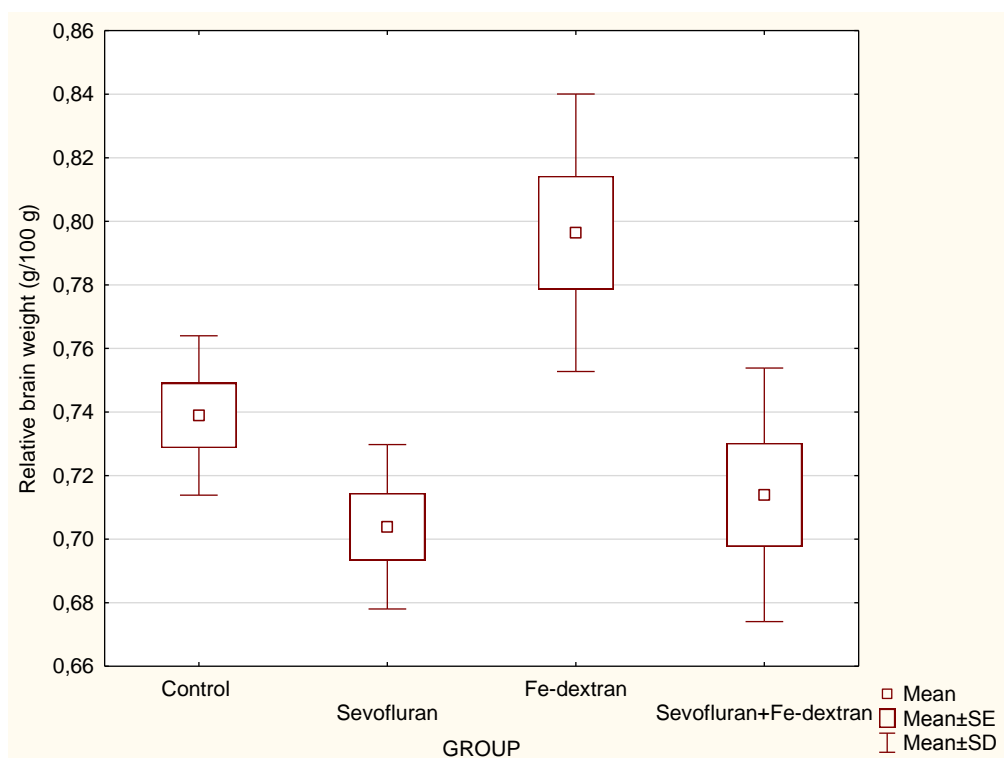


Figure 6. Relative brain weight of Y59 rats treated with sevoflurane, iron-dextran and their combination Rats (n = 6 per group) were treated with 1.5 % sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Sevoflurane vs Fe-dextran ($P < 0.01$); Fe-dextran vs Sevoflurane + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.1.2b Brain weight index

To increase precision of results, index of brain weight was calculated for every animal, as ratio of brain weight to body weight (Figure 7). There are no significant changes ($P > 0.05$) in brain weight index in any treatment group, compared with control animals. Results show significant changes in iron-dextran group when compared to sevoflurane treatment group ($P < 0.01$), and when compared with combined treatment with sevoflurane and iron-dextran ($P < 0.05$).

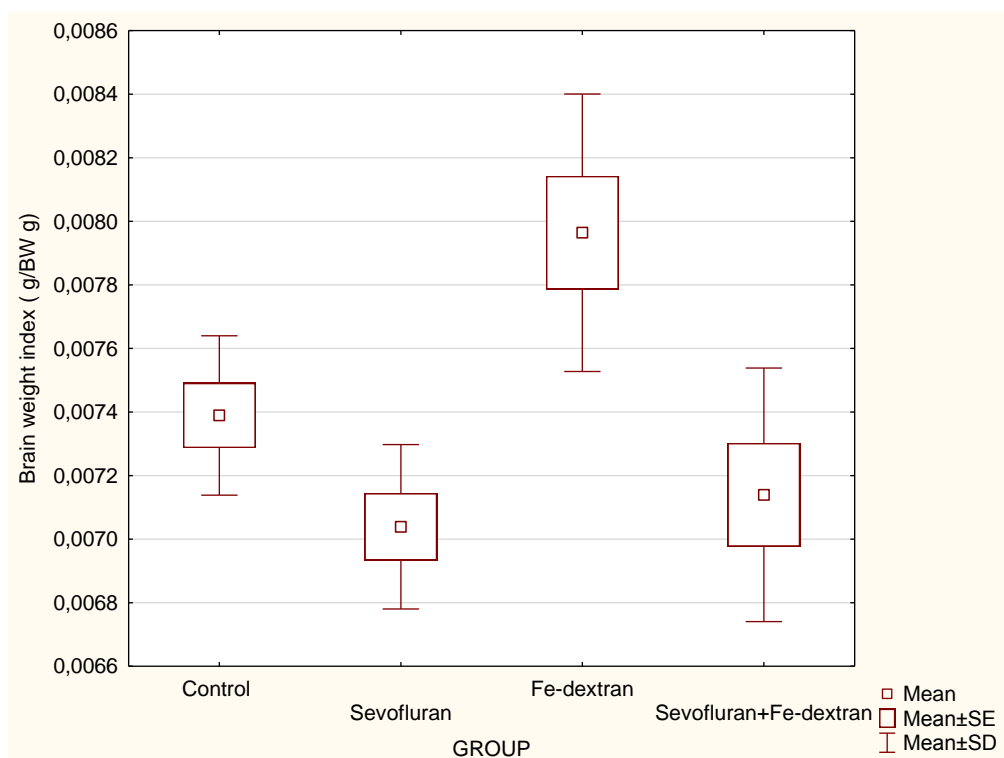


Figure 7. Brain weight index of Y59 rats treated with sevoflurane, iron-dextran and their combination Rats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Sevoflurane vs Fe-dextran ($P < 0.01$); Fe-dextran vs Sevofluran + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.2 Haematological and biochemical analysis of whole blood and serum

3.2.1 Differential blood analysis and total leukocyte count

Results of differential blood count are presented in Table 2. Total leukocyte count (Figure 8) shows significant changes in group treated with the combination of sevoflurane and iron-dextran, when compared with sevoflurane group ($P < 0.05$), and iron-dextran group ($P < 0.05$). There are no significant changes in differential blood analysis in any of the treatment groups in comparison with negative control ($P > 0.05$).

Table 2. Differential haematological analysis of peripheral blood.

Treatment ^a	Leukocytes ($\times 10^9/L$)		Differential analysis of peripheral blood ($\bar{X} \pm SE$)			
	($\bar{X} \pm SE$)	Min-Max	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
Control	5.26 \pm 1.42	1.60 \pm 9.40	30.33 \pm 2.66	68.00 \pm 3.05	2.00 \pm 0.57	1.00 \pm 0.00
Sevoflurane	1.96 \pm 0.08	1.70 \pm 2.10	38.33 \pm 5.28	60.66 \pm 5.61	-	1.50 \pm 0.28
Fe-dextran	1.96 \pm 0.08	1.70 \pm 2.10	38.33 \pm 5.28	60.66 \pm 5.61	-	1.50 \pm 0.28
Sevoflurane+ Fe-dextran	6.03 \pm 0.54 ^{♦◇}	4.80 \pm 7.70	37.00 \pm 1.59	63.00 \pm 1.59	-	-

^a Rats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE.

♦ Significantly different when compared with Sevoflurane (♦ $P < 0,05$); ◇ Significantly different when compared with Fe-dextran (◇ $P < 0,05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

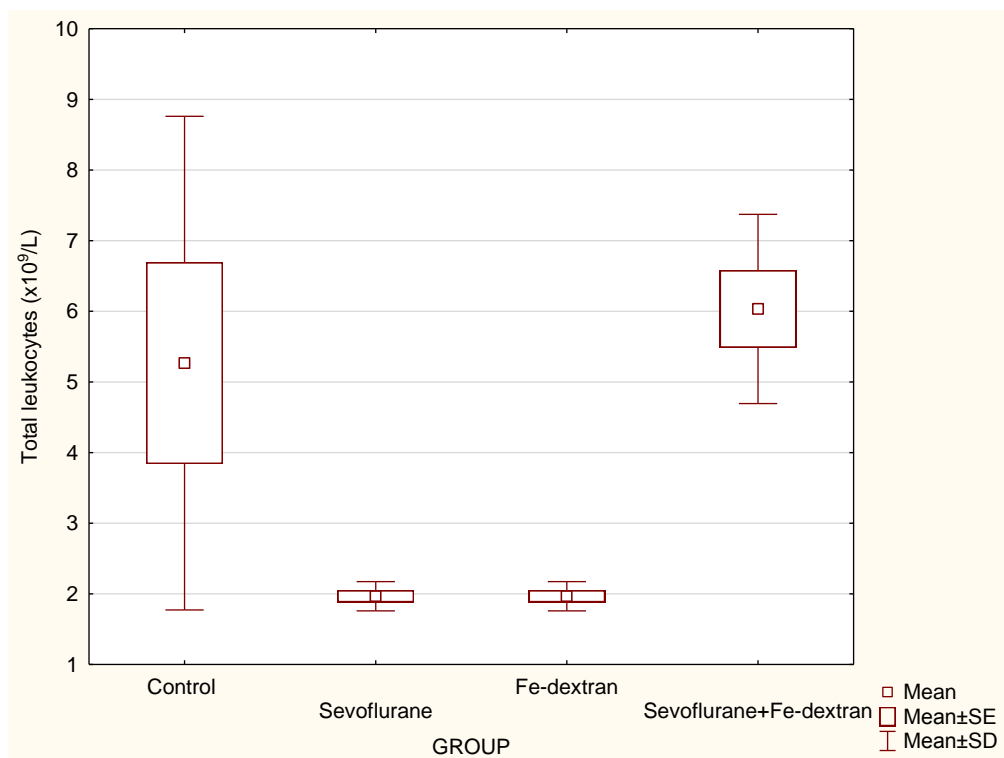


Figure 8. Total leukocytes count of Y59 rats treated with sevoflurane, iron-dextran and their combination Rats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Sevoflurane *vs* Sevoflurane + Fe-dextran ($P < 0.05$); Fe-dextran *vs* Sevoflurane + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.2.2 Values of haematologic parameters

Haematological analysis of rat blood samples was used to determine standard haematological parameters (Table 3). Additionally, the most significant changes are shown in Figures 9-10.

Table 3. Values of haematological parameters in rat blood samples.

Treatment ^a	Analysis of haematological parameters (X±SE)							
	Erythrocytes (x10 ¹² /L)	Hgl (g/L)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/L)	RDW (%)	MPV (fL)
Control	6.98±0.23	140.17±4.80	38.17±1.35	54.67±0.21	20.17±0.17	368.00±3.63	13.67±0.33	6.00±0.00
Sevoflurane	6.80±0.13	132.67±1.28	38.00±0.63	55.67±0.21	19.33±0.21	348.67±3.51	13.33±0.21	6.33±0.21
Fe-dextran	7.37±0.09	134.67±1.05	40.33±0.56	54.67±0.21	18.33±0.21 ^{♦♦}	333.33±1.48 ^{♦♦}	12.00±0.00 [♦]	6.33±0.21
Sevoflurane+ Fe-dextran	7.37±0.09	134.67±1.05	40.33±0.56	54.67±0.21	18.33±0.21 ^{♦♦}	333.33±1.48 ^{♦♦}	12.00±0.00 [♦]	6.67±0.21

^aRats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE.

♦ Significantly different when compared to control (♦ $P < 0.05$, ♦♦ $P < 0.01$).

Legend: Mean - mean values, SE - standard error.

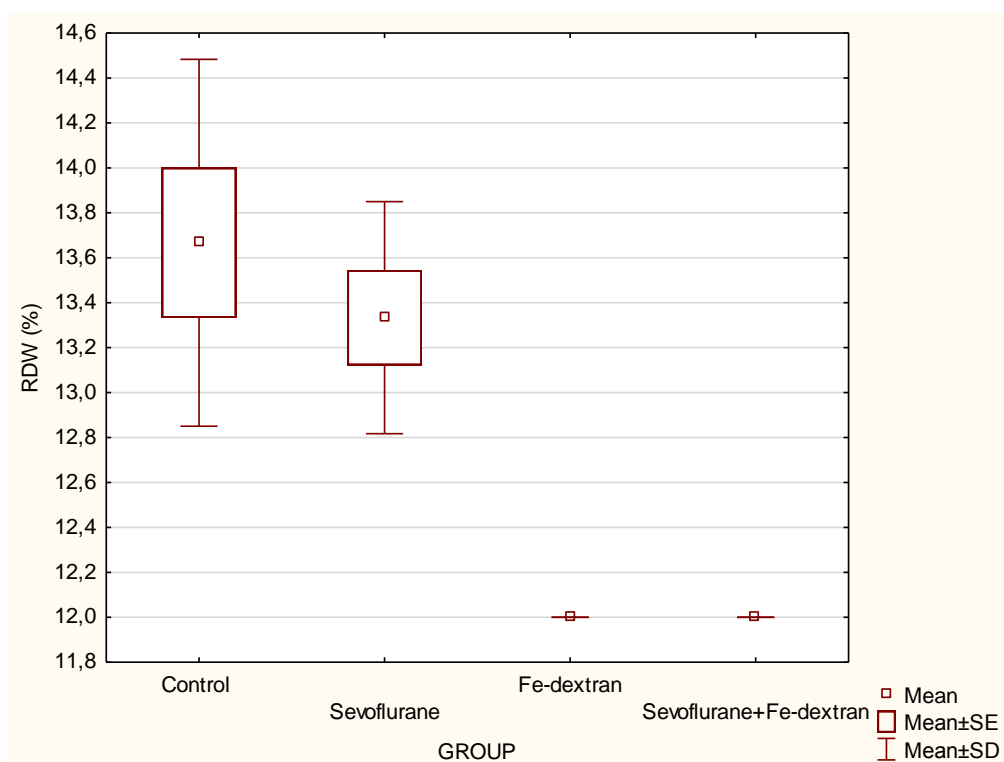
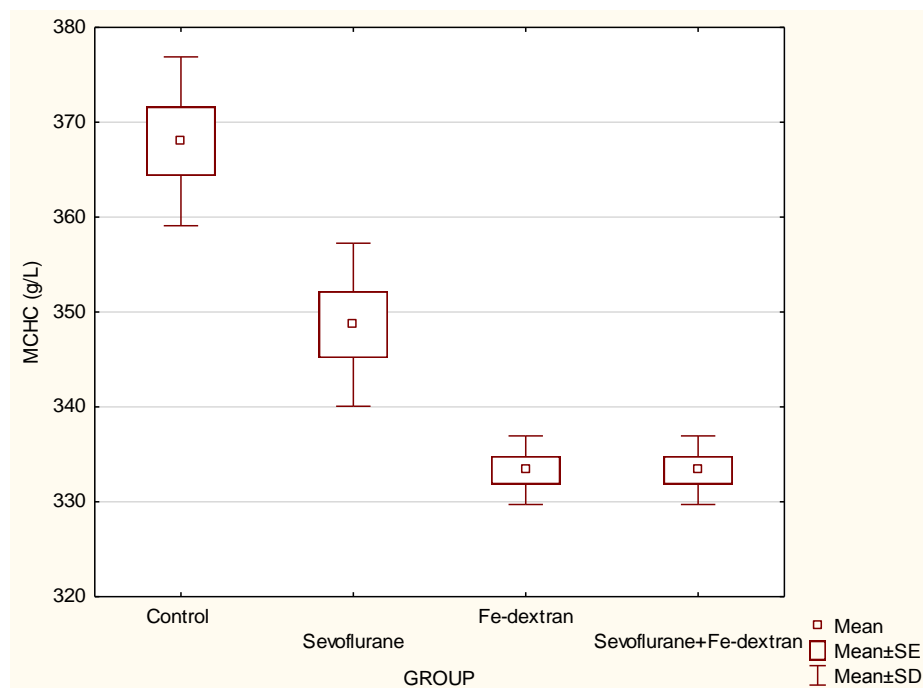


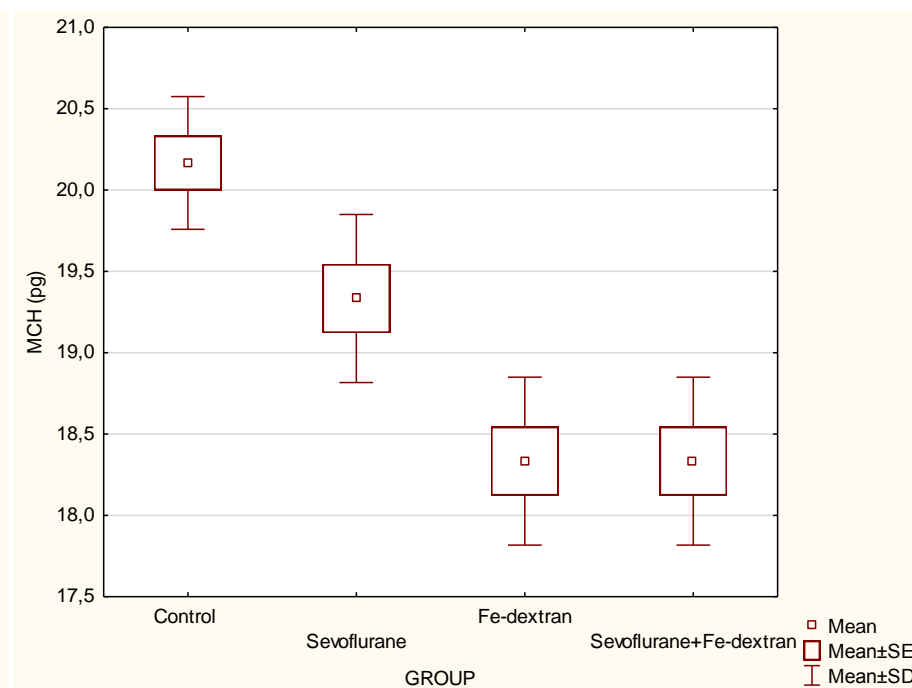
Figure 9. Changes in red blood cell distribution width of Y59 rats, treated with sevoflurane, iron-dextran and their combination. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Control *vs* Fe-dextran ($P < 0.05$); Control *vs* Sevoflurane + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.



a)



b)

Figure 10. Changes in MCHC (a) and MCH (b) in Y59 rats treated with sevoflurane, iron-dextran and their combination. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

a) Significantly different: Control vs Fe-dextran ($P < 0.01$); Control vs Sevoflurane + Fe-dextran ($P < 0.01$); b) Significantly different: Control vs Fe-dextran ($P < 0.01$); Control vs Sevoflurane + Fe-dextran ($P < 0.01$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.2.3 Osmotic fragility and hemolysis of erythrocytes

Results of erythrocyte osmotic resistance test (Figure 11) show 100 % of hemolysis at approximately 0.3 % NaCl concentration in all experimental groups. In control group, half of the erythrocytes are hemolyzed when NaCl concentration is approx. 0.5 %, whereas in other treatment groups 50 % hemolysis happened at lower NaCl concentrations.

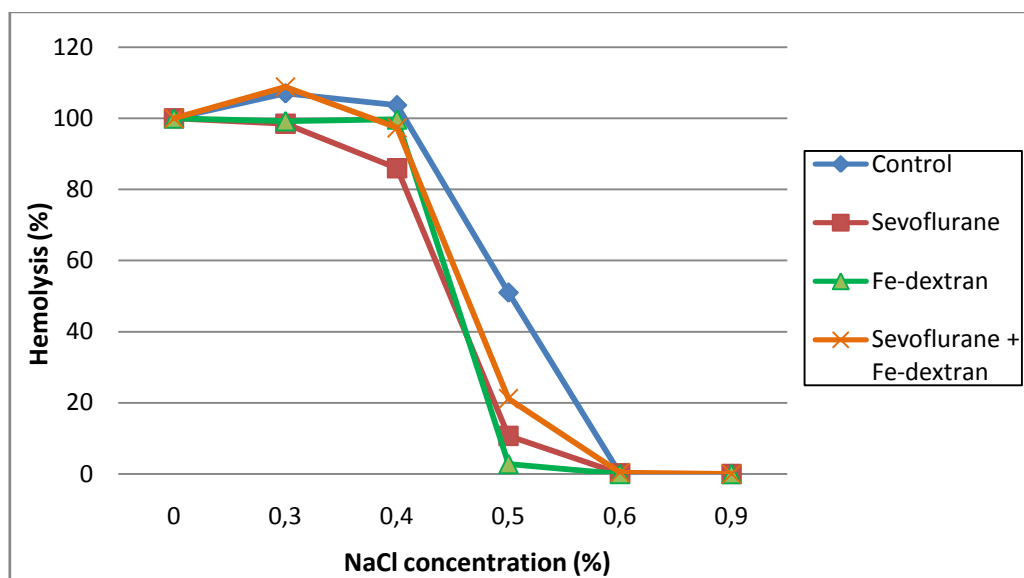


Figure 11. Changes in percentage of hemolysis in erythrocytes in Y59 rats treated with sevoflurane, iron-dextran and their combination. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.2.4 Values of biochemical parameters

Biochemical analysis of rat blood serum shows significant difference in enzymes and proteins (Table 4) for combined treatment group, when compared to control. Values of metabolites and substrates (Table 5) show the most significant changes in iron-dextran group, when compared to control and sevoflurane group. The most significant changes in biochemical parameters are presented in Figures 12-13.

Table 4. Values of enzymes and proteins concentrations in rat blood serum samples.

Treatment ^a	Analysis of biochemical parameters - enzymes and proteins (X±SE)					
	ALP (U/L)	ALT (U/L)	AMY (U/L)	TP (g/L)	GLOB (g/L)	ALB (g/L)
Control	194.00±20.00	43.80±1.02	691.20±74.60	68.60±0.81	13.20±0.49	55.00±0.63
Sevoflurane	134.33±8.66	31.33±0.76 [♦]	679.00±42.38	72.33±3.11	17.00±1.59	55.00±2.22
Fe-dextran	152.33±7.50	34.33±3.55	532.33±22.43	69.33±1.28	18.67±0.92 [♦]	50.67±0.42
Sevoflurane+ Fe-dextran	146.43±4.21 ^{♦♦}	33.67±1.17	524.33±37.41	69.33±0.92	19.33±0.21 [♦]	50.33±0.92

^aRats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE.

[♦] Significantly different when compared to control ([♦] $P < 0.05$, ^{♦♦} $P < 0.01$);

Legend: Mean - mean values, SE - standard error.

Table 5. Values of metabolites and substrates concentrations in rat blood serum samples.

Treatment ^a	Analysis of biochemical parameters - metabolites and substrates (X±SE)							
	GLU (mmol/L)	TBIL (umol/L)	BUN (mmol/L)	NA+ (mmol/L)	K+ (mmol/L)	CA (mmol/L)	PHOS (mmol/L)	CRE (umol/L)
Control	15.12±0.30	4.80±0.20	6.22±0.55	138.40±1.57	5.54±0.18	2.54±0.04	2.55±0.13	33.60±4,07
Sevoflurane	13.57±0.72	5.00±0.00	7.07±0.47	146.00±2.03	5.43±0.24	2.72±0.07	2.15±0.06	58.67±6,29 [♦]
Fe-dextran	14.23±0.70	4.67±0.21	5.30±0.24	133.67±0.21 ^{***}	4.53±0.11 ^{♦*}	2.46±0.02 ^{**}	1.95±0.18 [♦]	38.00±2,56
Sevoflurane+ Fe-dextran	9.93±0.33 ^{♦♦◇}	4.67±0.21	4.97±0.22 [*]	136.00±0.63	4.87±0.11	2.59±0.03	2.01±0.03 [♦]	43.33±1.69

^aRats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE.

♦ Significantly different when compared to control (♦ $P < 0.05$, ♦♦ $P < 0.01$); * Significantly different when compared to Sevoflurane (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$); ◇ Significantly different when compared to Fe-dextran (◇ $P < 0.05$, ◇◇ $P < 0.01$).

Legend: Mean - mean values, SE - standard error.

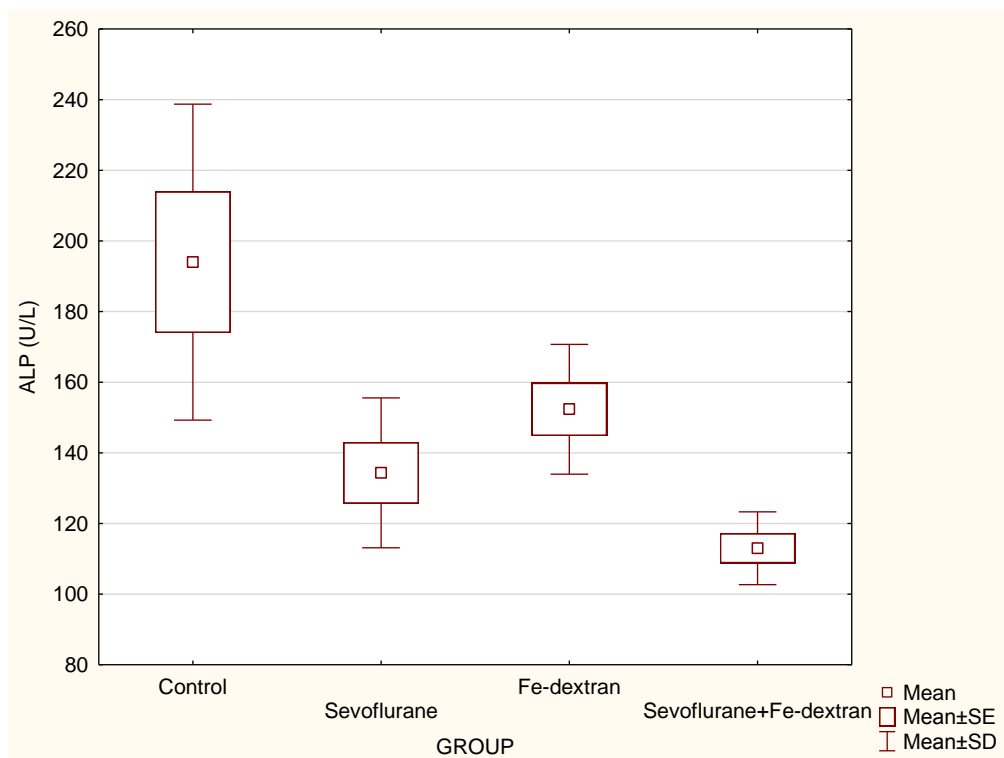


Figure 12. Values of ALP concentrations in blood serum of Y59 rats treated with sevoflurane, iron-dextran and their combination. Rats (n = 6 per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Control *vs* Sevoflurane + Fe-dextran ($P < 0.01$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

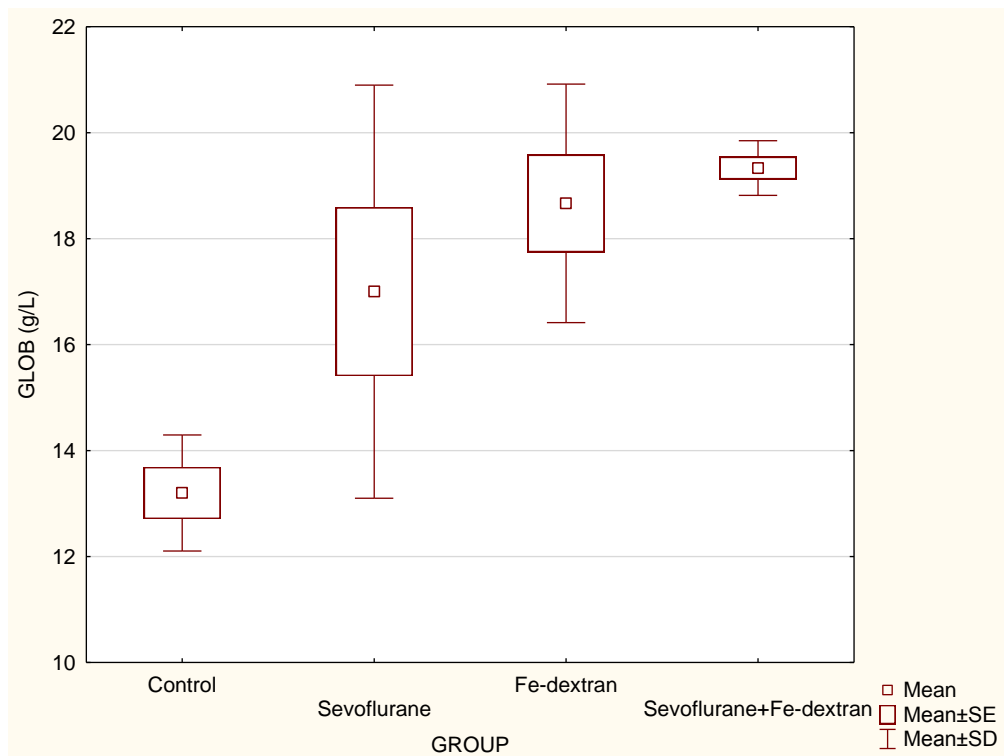


Figure 13. Values of GLOB concentrations in blood serum of Y59 rats treated with sevoflurane, iron-dextran and their combination. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Control vs Fe-dextran ($P < 0.05$); Control vs Sevoflurane + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.3 Parameters of oxidative stress

3.3.1 Concentrations of rat brain proteins

Results of brain protein measurements (Figure 12) indicate increased concentrations of brain proteins in combined treatment group. There are significant changes in group treated with both sevoflurane and iron-dextran, when compared to sevoflurane treatment group ($P < 0.01$), and also when compared with iron-dextran group ($P < 0.05$). There are no significant changes in total brain proteins in any of the treatment groups in comparison with negative control ($P > 0.05$).

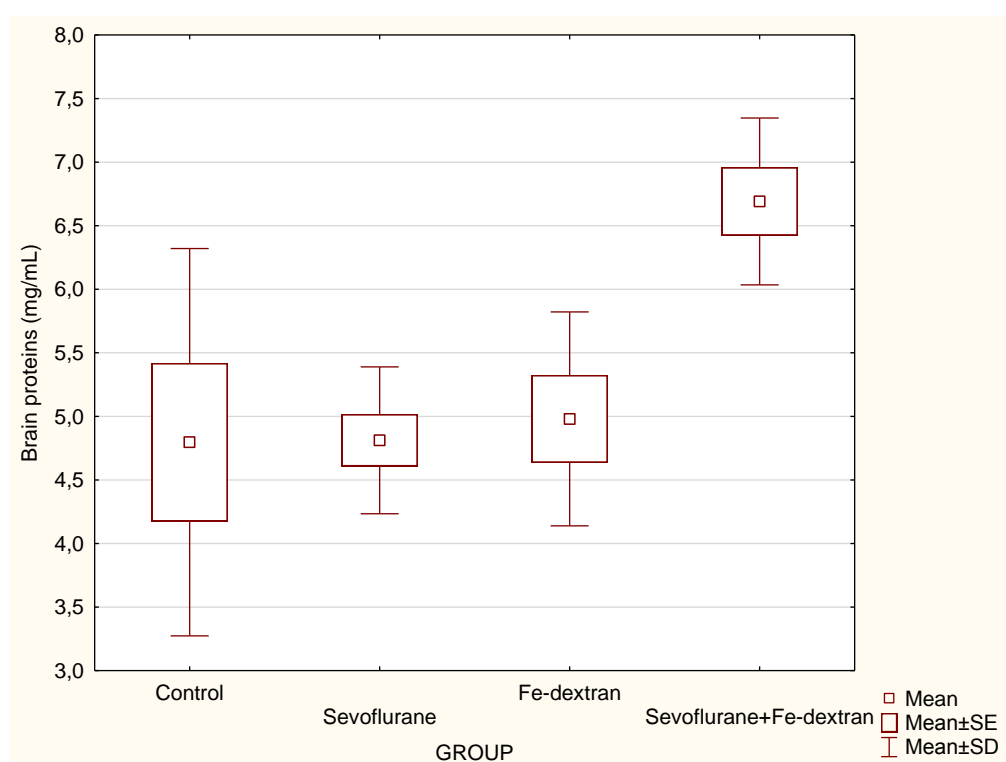


Figure 12. Effects of sevoflurane, iron-dextran and their combination on brain proteins concentrations of Y59 rats. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Sevoflurane vs Sevoflurane + Fe-dextran ($P < 0.01$); Fe-dextran vs Sevoflurane + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.3.2 Values of lipid peroxidation (MDA) in rat brain tissue

Results of MDA values in rat brain samples (Figure 13) show increased values in sevoflurane and iron-dextran treatment groups, in comparison with control, as well as when compared to combined treatment. There are significant changes in MDA concentrations between: control group and sevoflurane treatment group ($P < 0.05$); control group and Fe-dextran group ($P < 0.01$); sevoflurane group and combined treatment group ($P < 0.05$); Fe-dextran group and combined treatment with sevoflurane and iron-dextran ($P < 0.01$).

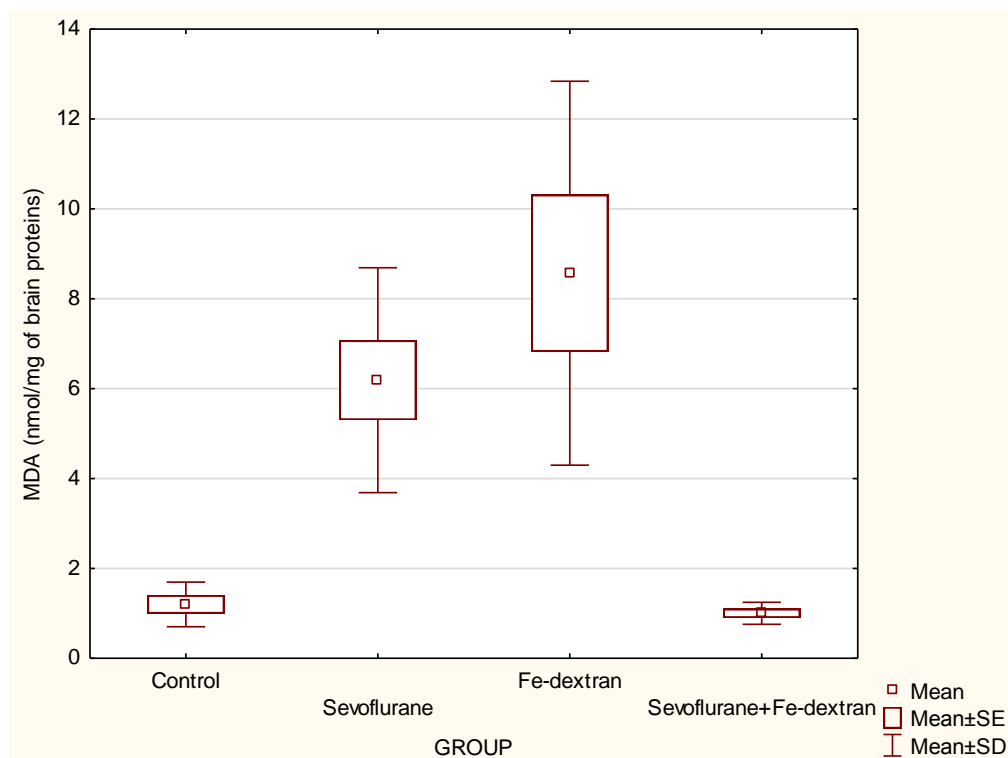


Figure 13. Effects of sevoflurane, iron-dextran and their combination on MDA concentrations in brain of Y59 rats. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Control vs Sevoflurane ($P < 0.05$); Control vs Fe-dextran ($P < 0.01$), Sevoflurane vs Sevoflurane + Fe-dextran ($P < 0.05$); Fe-dextran vs Sevoflurane + Fe-dextran ($P < 0.01$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.3.3 Values of catalase enzymatic activity (CAT) in rat brain tissue

Results of CAT concentrations (Figure 14) show lowest catalase activity in control group, as well as group treated with combination of sevoflurane and iron-dextran. There are significant changes in iron-dextran group, when compared with control ($P < 0.05$) and when compared with combined treatment group ($P < 0.01$); in sevoflurane group when compared with combined treatment group ($P < 0.01$).

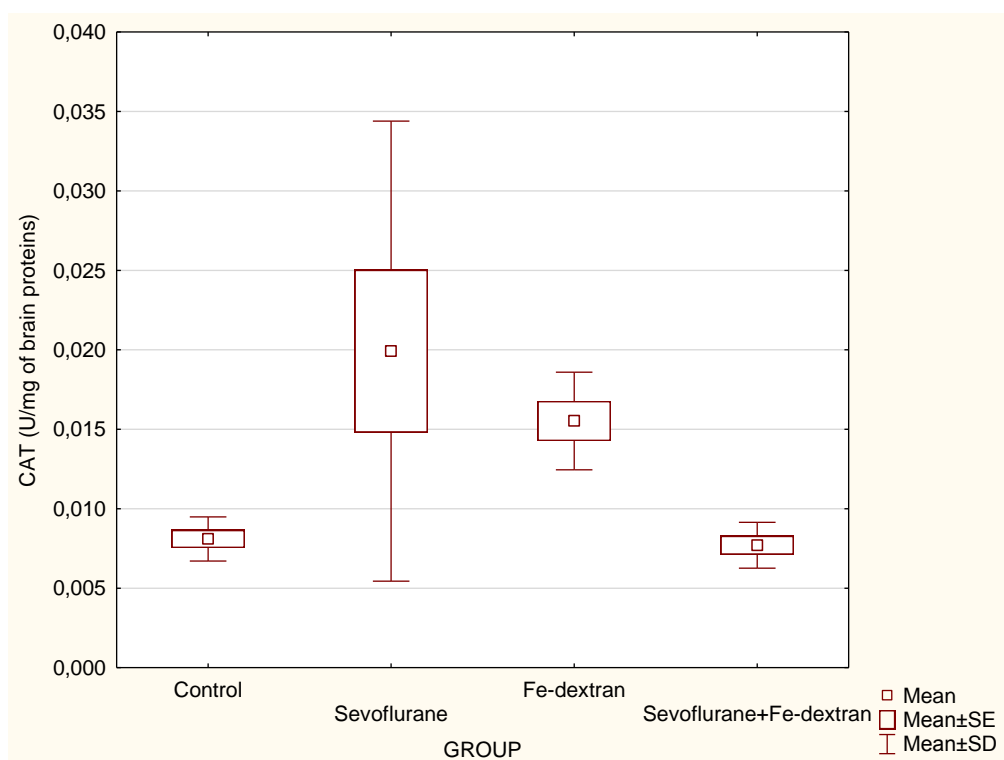


Figure 14. Effects of sevoflurane, iron-dextran and their combination on CAT concentrations in brain of Y59 rats. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Statistically significant: Control vs Fe-dextran ($P < 0.05$); Sevoflurane vs Sevoflurane + Fe-dextran ($P < 0.01$); Fe-dextran vs Sevoflurane + Fe-dextran ($P < 0.01$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.3.4 Values of superoxide dismutase (SOD) enzymatic activity in rat brain tissue

Results of SOD concentrations (Figure 15) show lowest values in group treated with combination of sevoflurane and iron-dextran, in comparison to all other groups. There are significant changes in combined treatment group when compared to: negative control ($P < 0.05$), sevoflurane group ($P < 0.01$), iron-dextran group ($P < 0.05$).

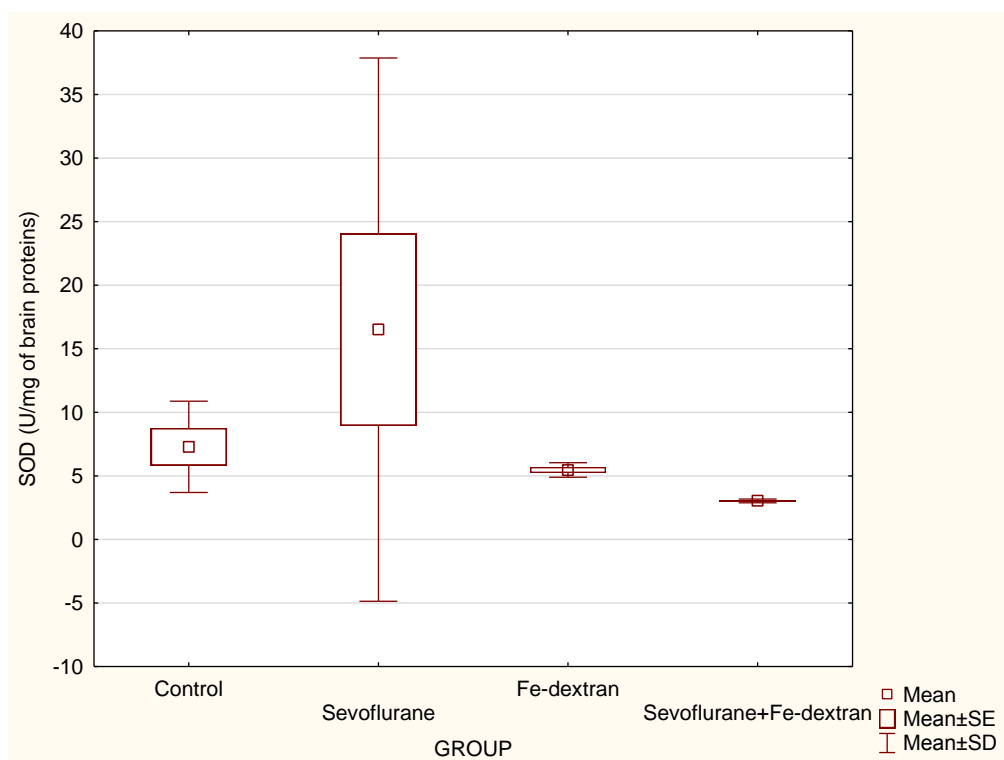


Figure 15. Effects of sevoflurane, iron-dextran and their combination on SOD concentrations in brain of Y59 rats. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Significantly different: Control *vs* Sevoflurane + Fe-dextran ($P < 0.05$); Sevoflurane *vs* Sevoflurane + Fe-dextran ($P < 0.01$); Fe-dextran *vs* Sevoflurane + Fe-dextran ($P < 0.05$).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.3.5 Values of glutathione concentration (GSH) in rat brain tissue

Results (Figure 16) show lower concentrations of glutathione in all treatment groups compared with control group, however, with no statistical significance ($P > 0.05$).

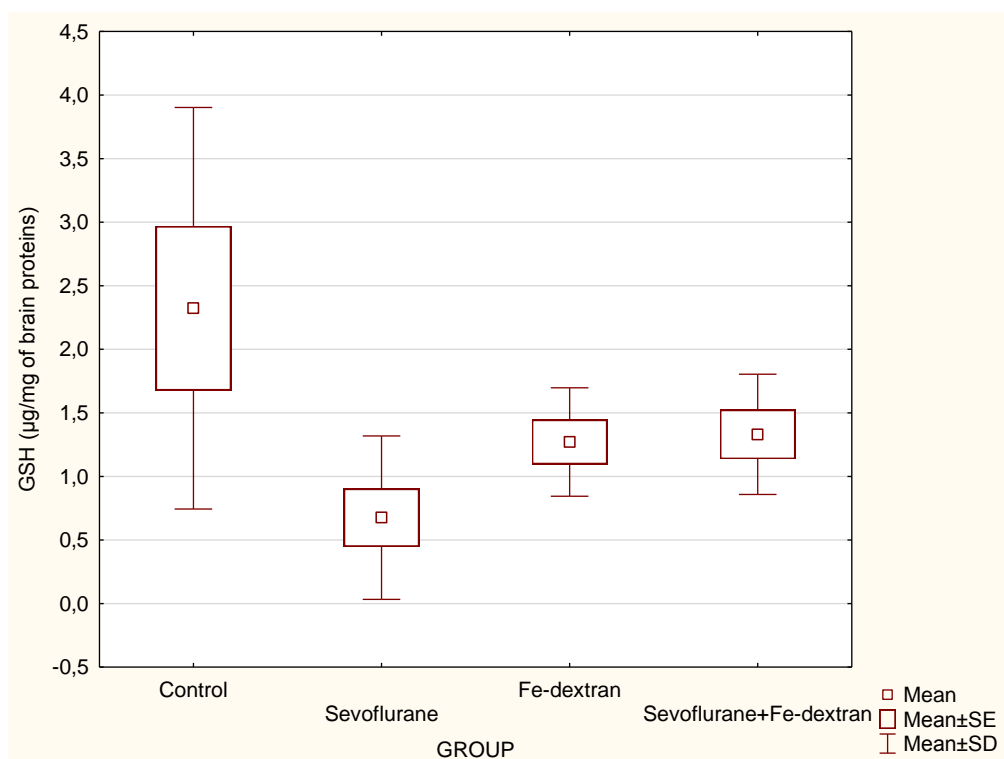


Figure 16. Effects of sevoflurane, iron-dextran and their combination on GSH concentrations in brain of Y59 rats. Rats ($n = 6$ per group) were treated with 1.5 % Sevoflurane inhalation, injected *ip* with 50 mg/kg Fe-dextran and with combination of 1.5 % Sevoflurane and 50 mg/kg Fe-dextran (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection). Control group had saline solution (0.9 % NaCl) injected intraperitoneally. Animals were treated every other day for 21 days. Results are expressed as Mean + SE (SD).

Legend: Mean - mean values, SE - standard error, SD - standard deviation.

3.4 Results of comet assay

Results of comet assay parameters are presented in Tables 6-7. Parameters of comet test indicate genotoxic effect on rat peripheral blood cells. Additionally, comet assay of brain tissue samples showed too many apoptotic cells and was not representative (data not shown).

After 7 days of treatments, tail length (distance of shortest broken DNA fragments) is lower in all treatment groups and the most significant changes are: iron-dextran when compared to control ($P < 0.001$); combined treatment group when compared to control ($P < 0.001$). Sevoflurane treatment group has significantly higher tail lengths when compared to iron-dextran treatment ($P < 0.01$). Tail intensity (the percentage of migrated DNA in tail) is highest after sevoflurane treatment, in comparison with control ($P < 0.05$). Tail intensity in combined treatment is significantly lowest when compared to sevoflurane treatment ($P < 0.01$). Tail moment shows lowest values in group treated with combination of sevoflurane and iron-dextran, when compared to sevoflurane treatment group ($P < 0.01$).

After 15 days of treatments, results show more changes in genotoxicity parameters. Values of tail length show lowest values in combined treatment group in comparison with control ($P < 0.001$), and in comparison with Fe-dextran group ($P < 0.001$). There are significant changes in sevoflurane treatment when compared to control ($P < 0.05$). Also, when compared with sevoflurane group, iron-dextran group has significantly higher tail length values ($P < 0.01$). Values of tail intensity show higher percentage of DNA in tails of iron-dextran group compared to control group ($P < 0.05$), and when compared with sevoflurane group ($P < 0.05$). Combined treatment group has lower tail intensity in comparison with Fe-dextran treatment group ($P < 0.01$). Measure of tail moment shows significantly higher value in iron-dextran group, when compared to control group ($P < 0.05$), and to sevoflurane group ($P < 0.05$). Tail moment in treatment with combination of sevoflurane and iron-dextran is significantly lower when compared to iron-dextran treatment ($P < 0.01$).

Table 6. Comet assay responses in lymphocytes of rats treated for 7 days with Sevoflurane, Fe-dextran and their combination.

Treatment ^a	Tail length (μm)				Tail intensity (% DNA)				Tail moment			
	Mean ± SE	M	25%	75%	Mean ± SE	M	25%	75%	Mean ± SE	M	25%	75%
Control	15.93±0.24 ^{♦♦♦}	15.38	13.46	17.94	2.78±0.45	0.50	0.00	2.36	0.39±0.06	0.07	0.00	0.30
Sevoflurane	15.62±0.25 ^{♦♦}	15.38	12.82	17.94	4.46±0.56 [*]	0.94	0.00	5.06	0.55±0.06	0.13	0.00	0.63
Fe-dextran	14.46±0.23 ^{***ΔΔ}	14.10	12.82	15.38	2.99±0.44	0.55	0.06	3.48	0.35±0.05	0.06	0.00	0.42
Sevoflurane+ Fe-dextran	15.14±0.12 ^{***ΔΔ}	14.74	12.82	17.30	1.95±0.27 ^{ΔΔ}	0.35	0.00	1.88	0.26±0.03 ^{ΔΔ}	0.04	0.00	0.24

^a Y59 rats were treated with Sevoflurane, Fe-dextran and their combination (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection) every other day for 7 days in the following doses: Sevoflurane 1.5 %; Fe-dextran 50 mg/kg.

^{*} Significantly different compared to control (^{*} $P < 0.05$; ^{***} $P < 0.001$); [♦] Significantly different compared to Fe-dextran ([♦] $P < 0.05$; ^{♦♦} $P < 0.01$; ^{♦♦♦} $P < 0.001$); ^Δ Significantly different compared to Sevoflurane (^{ΔΔ} $P < 0.01$).

Legends: Mean ± SE: mean values ± standard error; M: median; 25%: 25% percentile; 75%: 75% percentile.

Table 7. Comet assay responses in lymphocytes of the rats treated for 15 days with Sevoflurane, Fe-dextran and their combination.

Treatment ^a	Tail length (μm)				Tail intensity (% DNA)				Tail moment			
	Mean ± SE	M	25%	75%	Mean ± SE	M	25%	75%	Mean ± SE	M	25%	75%
Control	15.93±0.24 ^{ΔΔ}	15.38	13.46	17.94	2.78±0.45 [♦]	0.50	0.00	2.36	0.39±0.06 [♦]	0.06	0.00	0.30
Sevoflurane	15.08±0.19 ^{*♦♦}	14.10	12.82	16.02	2.54±0.28 [♦]	0.45	0.00	2.65	0.31±0.03 [♦]	0.05	0.00	0.34
Fe-dextran	16.38±0.28 ^{ΔΔ}	15.38	13.46	18.90	3.51±0.41 ^{*Δ}	0.82	0.06	3.87	0.45±0.05 ^{*Δ}	0.10	0.00	0.52
Sevoflurane+ Fe-dextran	14.94±0.30 ^{***♦♦♦}	13.46	12.17	16.66	2.13±0.31 ^{♦♦}	0.53	0.00	1.94	0.26±0.03 ^{♦♦}	0.06	0.00	0.27

^a Y59 rats were treated with Sevoflurane, Fe-dextran and their combination (Sevoflurane inhalation was administered 2 h after Fe-dextran *ip* injection) every other day for 15 days in the following doses: Sevoflurane 1.5 %; Fe-dextran 50 mg/kg.

* Significantly different compared to control (* $P < 0.05$; *** $P < 0.001$); ♦ Significantly different compared to Fe-dextran (♦ $P < 0.05$; ♦♦ $P < 0.01$; ♦♦♦ $P < 0.001$); Δ Significantly different compared to Sevoflurane (Δ $P < 0.05$; ΔΔ $P < 0.01$).

Legends: Mean ± SE: mean values ± standard error; M: median; 25%: 25% percentile; 75%: 75% percentile.

4 DISCUSSION

Results of this study indicate neurodegenerative changes induced by Fe-dextran and sevoflurane, correlated with excessive ROS production. Comparison of the relative and absolute brain weights values is consistent with parameters of oxidative stress (Figure 6 and Figure 7). The lowest absolute and relative brain indices in group exposed to sevoflurane and in combined treatment group indicates loss of brain cells. This is expected, since research suggests sevoflurane affects neuronal cells in neonatal brain by impairing the function of NMDA glutamate receptors, which results in increased neuroapoptosis (Jevtovic-Todorovic *et al*, 2003). In addition, prolonged exposure to sevoflurane alters the neonatal rat brain metabolism (glucose, aminoacids and lipid metabolic pathways) and causes changes in osmolyte system, intracellular antioxidants and cellular ultrastructure of cerebral cortex (Liu *et al*, 2015). On the other hand, treatment with Fe-dextran alone causes increase in these values, implying the development of neuroinflammatory reaction (Figures 6 and 7). Increased ROS production in brain leads to activation of inflammatory transcription factors such as NFκB which then readily binds to DNA, preventing damage made by ROS by upregulating catalase activity (Piloni *et al*, 2013). Further, increased ROS production enhances inflammatory response by attracting microglial cells, thus causing more oxidative stress (Droge, 2002; Lutsikii and Esaulenko, 2007). Results of increased brain weight indexes in iron-dextran group correspond with increased lipid peroxidation (MDA) and antioxidant defence (CAT).

In the same manner, the biggest changes in body weight loss occurred in Fe-dextran group compared to negative control (Figure 5). Also, animals treated with the combination of sevoflurane and iron-dextran gained less weight in comparison with control. Treatment with sevoflurane alone does not affect body weight in such distinct manner as other treatments.

Blood is one of the first tissues that comes in contact with toxic compounds and can be damaged by it. That is, naturally, due to physiological function of blood: it transports oxygen, nutrients, hormones and metabolites, circulating throughout body. For this reason, we can use haematological and biochemical parameters to evaluate how toxic some compound is. Sevoflurane, as volatile anaesthetic, is easily inhaled and its liposoluble molecules are diffused through alveolar capillaries. On the other hand, iron in blood is bound to plasma transferrin. When they enter the bloodstream, both chemicals can reach all tissues and organs,

including the brain. Experimental results show changes in haematological and biochemical parameters, indicating toxic effects.

Differential blood analysis shows lower total leukocytes count in sevoflurane and in Fe-dextran treatment, when compared to combined treatment (Table 2). Previous research by Brozovic *et al.* (2010) showed strongest and immediate DNA damage in leukocytes of mice exposed to sevoflurane. Iron in Fe-dextran can destroy white blood cells by triggering oxidative stress and the production of genotoxic hydroxyl radical (HO•). It initiates lipid peroxidation cascade that impairs cell structure by acting on organelles membrane fragility (Udipi *et al.*, 2012). Mentioned studies can explain overall leukocyte damage and subsequent lower counts in our experiment.

Haematological analysis (Table 3) shows no significant change in total erythrocytes count, however, it is slightly increased in iron-dextran and combined treatment group. Also, the most prominent changes in mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red blood cell distribution width (RDW) occur in mentioned treatment groups. Even though total haemoglobin is only non-significantly decreased in all groups compared to control, MCH clearly indicates decreased haemoglobin mass in average red blood cell of animals exposed to Fe-dextran and to the combination of sevoflurane and Fe-dextran (Figure10a). Consistently, MCHC indicates decreased amount of Hgl in average red blood cell relative to its size, in the same treatment groups compared to control (Figure 10b). In addition, values of red blood cell distribution width (RDW) follow the described pattern, and lower % RDW in Fe-dextran and combined treatment group suggests less variable, uniform erythrocyte volumes. When MCH and MCHC are taken into account, lower RDW indicates smaller erythrocyte volumes. Also, abnormally high number of polychromatic cells (immature red blood cells recognized by blue-gray stained cytoplasm) was present on blood smears. Given results suggest detrimental effects of treatment with Fe-dextran alone or in combination with sevoflurane, on erythropoietic homeostasis, haemoglobin synthesis and normal maturation of blood cells. Furthermore, these changes can be associated with mycrocytic anemia (<https://www.placebo.hr/lab/index.php>) caused by excessive ROS production and cell damage mediated by oxidative stress (Livrea *et al.*, 1996; Vehapoglu *et al.*, 2014).

Osmotic fragility (OF) of erythrocytes is used as a measure of stability of red blood cells in decreasing concentrations of saline solution (Amanullah *et al.*, 2013). It represents

index of cell surface-to-volume ratio. In hypotonic solution water osmotically enters the erythrocytes, causing them to swell and eventually burst. In other words, erythrocytes which can endure lesser changes in NaCl concentration without bursting are more fragile. Increased fragility reduces erythrocyte life span, leading to anemia, and some of the factors contributing to changed OF are red blood cell age, hemoconcentration and peroxidation of the erythrocyte membrane (Amanullah *et al*, 2013). Analysis of our results (Figure 11) shows negligible hemolysis in 0.8 % NaCl concentration, and significant percentage of hemolysis occurring at 0.5 % NaCl concentration in control group. Groups exposed to treatment chemicals have seemingly lower hemolysis in comparison to control, and 50 % hemolysis occurs around concentration 0.45 % NaCl. This would indicate that control group has less stable thus damaged erythrocytes. However, if haematologic analysis is taken into consideration (MCH, MCHC, RDW) it is clear that treatments with sevoflurane, iron-dextran and their combination destroyed mature erythrocytes thus young, immature cells are seemingly less fragile.

Biochemical analysis of blood serum confirms the toxicity of Fe-dextran, sevoflurane and their combination, disrupting metabolic pathways and whole body homeostasis. Combined treatment shows the most perturbations, including the lowest ALP, glucose levels, lower concentration of blood urea nitrogen and decrease in phosphates. All these parameters indicate malnutrition and disturbed kidneys and liver metabolism. Malnutrition is probably because of impairments in nutrient absorption in stomach and small intestines, and loss of necessary micronutrients (Zn, Mg). This corresponds with smaller body weights of these animals, compared with control. Additionally, increase in globulin concentrations, most likely γ -globulin, suggests activation of humoral immune response, or potentially autoimmune reactions. Animals treated with iron-dextran also show increase in globulin. Iron-dextran is responsible for disturbances in concentrations of important blood electrolytes, as there are decreased concentrations of sodium, potassium, calcium and phosphate ions. Therefore, overloading the body with iron causes malabsorption and changes in liver and kidney functions. Correspondingly, these animals had the lowest body weights. Animals exposed to sevoflurane inhalations have fewer changes in biochemical parameters including low ALT and increased creatinine concentrations, which suggest impairments of liver and kidney functions.

Finally, toxicity of sevoflurane, Fe-dextran and their combination is evaluated through analysis of genotoxicity on the rat lymphocytes. One of the most destructive ROS-mediated toxic effects is surely DNA damage, since DNA molecules are semi-replicable, so damage of

the DNA structure leads to increased genetic instability or cell apoptosis. Hydroxyl radical, the most potent radical, is able to react with purine and pyrimidine bases, and even with DNA backbone. These reactions lead to ROS propagation, creating sugar radicals which can react with bases. This causes loss of bases, DNA breakage and cross-linking of DNA with proteins. Analysis of comet assay after seven-days treatment already shows substantial DNA damage in group exposed to Fe-dextran alone or in combination with sevoflurane. This group has the smallest DNA fragments, based on tail intensity with similar tail length as other groups, which means extensive damage and rapid loss of DNA fragments with respect to high ROS activity. Following, second most genotoxic is sevoflurane. Comet assay analysis after 15-days exposure confirms these findings. Combined treatment is the most genotoxic, with smallest DNA fragments, indicating cell apoptosis induced by oxidative stress and ROS propagation. This is consistent with previous research (Brozovic *et al*, 2006; Brozovic *et al*, 2010).

In essence, Fe-dextran successfully caused AD-related neurodegenerative changes, in terms of promoting oxidative stress damage and causing activation of cellular antioxidative defense, though insufficient, leading to multiple impairments: changed structure and function of brain proteins, neuroinflammation with activation of microglia and astrocytes, and eventually, neuroapoptosis. This model proves that Alzheimer's disease is truly multiple organ systems disease, rather than being only neurodegenerative. Changes in brain redox status, with infinite loop of ROS production and inflammatory cascade perturb the whole body. It affects absorption of nutrients, kidney and liver metabolism, destroys both red and white blood cells and causes secondary anemia, leading to decay of the whole body. Further, inhalation anaesthetic sevoflurane triggers excessive ROS production, followed by weak antioxidative protection. Sevoflurane has neurotoxic effect on the rat brain cells, but it is also genotoxic and damages DNA of the rat lymphocytes. It also disturbs nutrient absorption and metabolism in kidneys and liver, however less than Fe-dextran. Their combination proved to be the most fatal, resulting in extensive neurodegeneration. Interestingly, parameters of oxidative stress were seemingly lowest, including MDA concentrations. Considering the high protein concentrations, this indicates toxic protein pile-up mediated by increased ROS production and attracting more microglial cells to deal with damage and extensive cell loss, hence remaining cells appear to be "stronger" or unimpaired. Combined treatment induced neurodegeneration by triggering oxidative stress with extensive neuroapoptosis, perturbing the metabolic pathways and impairing kidney and liver function, as well as having genotoxic effects, causing the greatest DNA damage to rat lymphocytes.

5 CONCLUSION

To conclude, in this study I demonstrated the following:

1. Iron-dextran causes significant damage to the rat brain and blood parameters compared to negative control:
 - a. Increases values of lipid peroxidation (MDA) and antioxidative response (CAT, SOD)
 - b. Causes neuroinflammation, confirmed by increase in absolute and relative brain weight
 - c. Causes changes of erythrocyte indices (MCH, MCHC, RDW), increases hemolysis and impairs haemoglobin synthesis
 - d. Decreases total leukocyte number
 - e. Causes malabsorption and nutrient deficiency related metabolic disturbances
 - f. Animal body weight is significantly decreased, due to malabsorption and malnutrition
2. Sevoflurane causes DNA damage, changes in the rat brain and the blood parameters:
 - a. Increases values of lipid peroxidation (MDA)
 - b. Decreases total number of leukocytes
 - c. Causes DNA damage in the rat lymphocytes
 - d. Causes malabsorption and nutrient deficiency related metabolic disturbances, to the smaller extent than Fe-dextran
 - e. Animal body weight is slightly decreased, probably due to malabsorption
3. Combination of sevoflurane and iron-dextran causes the biggest neurodegenerative changes, DNA damage and particularly toxic effects on blood:
 - a. Causes neuroapoptosis hence decreased brain weight indices
 - b. Seemingly lowest lipid peroxidation (MDA) and antioxidant defense (SOD, CAT) due to extensive neuroapoptosis
 - c. Causes the greatest DNA damage in the rat lymphocytes
 - d. Causes malabsorption and nutrient deficiency related metabolic disturbances
 - e. Induces changes in erythrocyte indices (MCH, MCHC, RDW) and impairment in haemoglobin synthesis
 - f. Animal body weight is decreased, due to malabsorption
4. Exposing the animals to sevoflurane in combination with Fe-dextran increases the iron toxicity and results in extensive neuroapoptosis induced by excessive production of ROS.
5. Neurodegenerative changes induced by sevoflurane and Fe-dextran mimic the pathophysiology of Alzheimer's disease.

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7 CURRICULUM VITAE

PERSONAL INFORMATION

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EDUCATION

2015 - to date: Graduate study programme in Experimental Biology - Physiology and Immunobiology, University of Zagreb, Faculty of Science, Croatia

2012 - 2015: Undergraduate study programme in Biology, University of Zagreb, Faculty of Science, Croatia

- BSc thesis: Odgovor organizama na izloženost elektromagnetskim poljima (Response of organisms to electromagnetic fields exposure); supervisor: Dr. Marko Miliša, Associate Professor

2008 - 2012: Secondary education, Gymnasium of Antun Vrančić, Šibenik, Croatia

WORK EXPERIENCE & INTERSHIPS

June 2016 - September 2016: Erasmus+ Internship in the Department of Animal Ecology, Faculty of Life Sciences, Vrije Universiteit Amsterdam, The Netherlands

- gained knowledge on behavioural ecology, sensory ecology of insects, experimental design
- supervisor: Dr. Wouter Halfwerk, Assistant Professor.

February 2016 - June 2016: Laboratory internship in the Department of Animal Physiology, Faculty of Science, University of Zagreb, Croatia

- gained knowledge on neurophysiology, rat brain, histological brain tissue preparation and imaging

- supervisor: Dr. Dubravka Hranilović, Professor.

September 2015 - February 2016: Student demonstrator in Vertebrates undergraduate course, Department of Zoology, Faculty of Science, University of Zagreb, Croatia.

March 2015 - July 2015: Laboratory internship in the Department of Zoology, Faculty of Science, University of Zagreb, Croatia

- gained knowledge on ecotoxicology, genotoxicity, comet assay

- supervisor: Dr. Göran Klobučar, Professor.

PERSONAL SKILLS

Languages: English C1, German A1

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Volunteering:

2012 - 2015: Active participant (workshops, lectures) at the annual event Noć Biologije (The night of Biology) for popularisation of science in organization of Division of Biology, Faculty of Science, University of Zagreb, Croatia.

CONFERENCES:

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PUBLICATIONS

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