ORIGINAL ARTICLE

Effect of Intramuscular Injection on Oxidative Homeostasis in Laboratory Guinea Pig Model

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Summary: In animal models, there was observed alteration of various physiological processes caused by microtraumas. Here reported experiment was aimed on the research of link between injection and development of an oxidative imbalance. Laboratory guinea pig was chosen as a suitable model for examining of the oxidative stress.
Markers indicating oxidative homeostasis were assayed in the frontal, temporal and occipital brain lobe, cerebellum, liver, kidney, spleen and heart one hour after an intramuscular injection. Common biochemical parameters were measured in plasma samples as well.
The most extensive effect was observed in the heart where the thiobarbituric acid reactive substances value was more than twice increased after the injection. The level of carbonylated proteins was significantly elevated in the kidney and ferric reducing antioxidant power value was increased in the brain compartments. The enzyme activities in the organs were not influenced except the activity of superoxide dismutase, which was moderately decreased in the brain. In the plasma samples, there was observed increase of the blood urea nitrogen.
The results showed significant the influence of the intramuscular injection on a development of an oxidative insult. The injection can be considered as an adverse effect with quite extensive stress consequences.

Keywords: *Injection; Oxidative stress; Antioxidant; Microtrauma; Animal model*

Introduction

Owing to many scientific reports, oxidative stress plays an important role in pathogenesis of various diseases (neurodegenerative disorders, cardiovascular diseases, cancer), in the processes linked to ageing and in adverse effects of some drugs (1–4). Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS), carbonylated proteins, superoxide dismutase (SOD) activity and glutathione reductase (GR) activity are markers indicating oxidative stress and oxidative homeostasis (5). The markers are often used for demonstration of detrimental or beneficial effects of various substances to laboratory animal models (6–8).

Guinea pig (*Cavia porcellus*) is not able to synthesize ascorbic acid, which ranks among the most important low molecular weight antioxidants. That is the main reason why guinea pig is widely used laboratory model for examining of the oxidative stress (9–11). The inability to produce endogenous ascorbic acid makes results from guinea pigs extrapolatable to humans (12–14). Except the inability to synthesize vitamin C there are other similarities like a similar response of lipid metabolism to dietary intervention (15).

Small rodents, including guinea pigs, are very sensitive to a way of handling and to living conditions. Stress caused by manipulation or inconvenient housing may lead to alteration of results in separate experiments. E. g., presence or an absence of huts can significantly influent a level of stress hormones in laboratory guinea pig (16).

However, psychical stress affects more processes than the secretion of stress hormones. In laboratory animals, experimentally induced stress elicits tachycardia and rise of body temperature (17, 18). Transient increase of body temperature after a stress stimulus is called stress-induced hyperthermia and it was observed in multiple laboratory animals. Measuring of the stress induced hyperthermia is a standard test for recognizing psychical stress in animals. After injection, a complication for example in testing of anxiolytic drugs was observed (19, 20). Alteration of various physiological processes caused by the injection stress was

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noticed in small laboratory rodents. Renaud et al. (21) observed facilitation of learning caused by adolescent injection stress in laboratory rat model. On the other hand, no significant alterations in behavior and in hematologic parameters and no elevation of fecal corticosterone level were found in mice undergoing multiple intraperitoneal injections (22).

Despite knowledge about injection stress, studies focused on the implication of the injection in the oxidative homeostasis have not been done yet. The objective of our experiment was to confirm or exclude implication of intramuscular injection (which is a common way of drug administration in experiments on laboratory animal models) in the markers of oxidative stress levels.

Material and methods

Animal exposure, sample collecting and sample preparation

The experiment was permitted and supervised by the Ethical Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic.

The male guinea pigs were purchased from the Velaz Company (Prague, Czech Republic). They were at the age of three months and their body weight was 250 ± 10 g. The animals were kept in the experimental facility (22 ± 2 °C, 50 ± 10% humidity, 12 hours light period per a day) and they were fed by a common food, supplemented with vitamins including vitamin C, and water *ad libitum*.

The guinea pigs were divided into two groups – injection and control group. Each group consisted of eight specimens. The saline solution in the amount of 100 µl per 100 g of body weight was intramuscularly injected into the pelvic limb of the animal in the injection group. The animals in the control group were exposed to the manual handling imitating the condition of the injection.

At the time of 1 hour after the injection, the animals were sacrificed in CO_2. The blood was collected by cutting carotide into tubes with lithium heparine and centrifuged at 1,000 × g. Separated plasma was transferred into a new tube, frozen immediately and stored at −80 °C. In a total, the liver, kidney, spleen, heart, cerebellum, frontal, temporal and occipital lobe were collected. After the collection, 100 mg of the tissue was mixed with 1 ml of phosphate buffer saline (Sigma-Aldrich, Saint Louis, USA) and mechanically homogenized by an Ultra-Turrax mill (Ika Werke, Staufen, Germany) for one minute. The homogenates were frozen immediately and stored at –80 °C as well.

Markers of oxidative stress and biochemical markers assessment

FRAP, TBARS, carbonylated proteins, caspase 3 (CASP3), SOD and GR were measured in tissue homogenates and levels of glucose, total cholesterol, HDL cholesterol, triglycerides (TG), blood urea nitrogen (BUN), creatinine (CRE), total bilirubin, total protein, albumin and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured in plasma samples.

FRAP is a spectrophotometric method based on reduction of ferric to ferrous ions. Concentration of malondialdehyde was assayed as TBARS by spectrophotometry using thiobarbituric acid. The FRAP and TBARS were done in compliance with reported paper (23). Carbonylated proteins were measured according to the protocol published by Cao & Cutler (24) with minor modifications which are mentioned in references (23). 2,4,6-Tris(2-pyridyl)-S-triazine, ferric chloride (Sigma-Aldrich, Saint Louis, USA) were purchased for FRAP, dimethylsulfoxide, trichloroacetic acid and thiobarbituric acid (Sigma-Aldrich) for TBARS and 2,4-dinitrophenylhydrazine, trichloracetic acid (Sigma-Aldrich), hydrochloric acid, ethanol and ethyl acetate (Penta, Prague, Czech Republic) for the assay of protein carbonyls.

The GR activity was measured as the Wartburg optical test. The assay was performed as described previously (25). Oxidized glutathione, NADPH, ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma-Aldrich) and phosphate buffer saline were purchased for the measurement. For the SOD and CASP3 activity assessment, the Sigma caspase 3 assay kit and the SOD assay kit (Sigma-Aldrich) were used. The SOD activity in the tissue homogenates was quite high, so it was need for dissolving the samples.

Biochemical parameters in plasma were assessed using an automated analyzer SPOTCHEM TM EZ SP-4430 (Arkray, Japan).

Statistical evaluation

Comparison of the data obtained from the control and injection group was done using one-way ANOVA with Bonferroni test (or Bonferroni correction in some sources). The analysis was processed using the statistical software Origin 8 SR2 (OriginLab Corporation, Northampton, USA).

Results

The most extensive effect of the injection on stress markers was observed in the heart where the TBARS value was more than twice increased 1 hour after the injection. However, no significant alterations of the TBARS value were found in the other organs. The level of carbonylated proteins was significantly elevated in the kidney one hour after the injection. On the other hand, the other organs had insignificant alteration in the level of protein carbonyls.

The FRAP value was significantly increased in the frontal lobe and in the cerebellum one hour after the injection. In the spleen, there was increased FRAP value as well. However, the increase was not significant. In the other organs including the temporal and occipital lobe, only slight changes in the FRAP value were observed.

Activities of the enzymatic markers in the tissue homogenates were quite stable. One exception was in the temporal and occipital lobe where moderate and statistically significant decrease of the SOD activity was proved. The CASP3 activity in the heart and in all of the brain compartments was too low for measurement by the assay. In other organs, we did not notice any significant change of the CASP3 activity.

In the plasma samples, levels of total and HDL cholesterol forms and creatinine were not detectable (total cholesterol bellow 1.3 mmol/l, HDL cholesterol bellow 0.26 mmol/l and creatinine bellow 27 μmol/l). Biochemical test proved statistically significant alteration in BUN level. The marker was elevated from 4.78 ± 0.29 mmol/l (control group) to 7.92 ± 0.44 mmol/l (1 hour after the injection). The other

Tab. 1: Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS) and carbonylated proteins in tissue samples ± standard errors of mean.

Assay	FRAP [μmol/g]		TBARS [μmol/g]		carbonylated proteins [μmol/g]	
	control	injection	control	injection	control	injection
liver	2.58 ± 0.14	1.86 ± 0.31	0.177 ± 0.007	0.185 ± 0.017	0.323 ± 0.008	0.362 ± 0.033
kidney	1.96 ± 0.14	1.84 ± 0.20	0.240 ± 0.018	0.205 ± 0.007	0.259 ± 0.015	0.327 ± 0.006**
spleen	1.90 ± 0.12	2.45 ± 0.19	0.102 ± 0.012	0.0732 ± 0.0085	0.249 ± 0.020	0.235 ± 0.018
heart	0.376 ± 0.017	0.484 ± 0.062	0.0738 ± 0.0036	0.189 ± 0.008**	0.266 ± 0.019	0.249 ± 0.020
frontal lobe	0.262 ± 0.015	0.336 ± 0.007**	0.295 ± 0.013	0.291 ± 0.007	0.249 ± 0.017	0.269 ± 0.010
temporal lobe	0.260 ± 0.016	0.273 ± 0.013	0.305 ± 0.015	0.323 ± 0.017	0.284 ± 0.018	0.228 ± 0.022
occipital lobe	0.225 ± 0.011	0.215 ± 0.025	0.253 ± 0.012	0.261 ± 0.011	0.253 ± 0.011	0.257 ± 0.015
cerebellum	0.184 ± 0.013	0.252 ± 0.005*	0.273 ± 0.013	0.292 ± 0.027	0.256 ± 0.015	0.265 ± 0.015

* $p < 0.05$, ** $p < 0.01$, n = 8 specimens in each group

Tab. 2: Glutathione reductase (GR), superoxide dismutase (SOD) and caspase 3 (CASP3) in tissue samples ± standard errors of mean.

assay	GR [nkat/g]		SOD [nkat/g]		CASP3 [pkat/g]	
	control	injection	control	injection	control	injection
liver	103 ± 7	126 ± 4	275 ± 0	274 ± 0	52.8 ± 7.1	46.0 ± 4.1
kidney	115 ± 5	116 ± 3	267 ± 1	267 ± 2	28.1 ± 4.1	26.8 ± 4.1
spleen	53.5 ± 4.8	76.2 ± 3.8	206 ± 3	208 ± 3	69.4 ± 14.9	62.4 ± 8.8
heart	18.0 ± 0.9	17.9 ± 2.2	161 ± 3	161 ± 5		
frontal lobe	63.4 ± 2.8	66.2 ± 5.4	205 ± 2	200 ± 4		
temporal lobe	62.4 ± 7.8	62.5 ± 4.1	216 ± 4	196 ± 5**		
occipital lobe	68.7 ± 8.9	57.8 ± 4.6	205 ± 2	188 ± 7**		
cerebellum	42.0 ± 5.9	62.0 ± 7.8	232 ± 2	223 ± 2		

** $p < 0.01$, n = 8 specimens in each group

Tab. 3: Biochemical marker assesment in plasma samples ± standard errors of mean. Abbreviations used in the table: AST – aspartate aminotransferase; ALT – alanine aminotransferase; LDH – lactate dehydrogenase; ALP – alkaline phosphatase; BUN – blood urea nitrogen; T-pro – total plasma protein; Alb – albumin; T-bil – total bilirubin; TG – triglycerides; GLU – glucose.

Assay	Control	Injection	Assay	Control	Injection
AST [μkat/l]	2.03 ± 0.35	1.31 ± 0.41	T-Pro [g/l]	39.8 ± 0.8	44.2 ± 2.0
ALT [μkat/l]	0.738 ± 0.064	0.817 ± 0.061	Alb [g/l]	20.7 ± 0.2	20.7 ± 1.3
LDH [μkat/l]	12.1 ± 3.45	4.88 ± 1.25	T-Bil [μmol/l]	5.67 ± 0.33	5.67 ± 1.28
ALP [μkat/l]	5.39 ± 0.47	4.25 ± 0.22	TG [mmol/l]	0.403 ± 0.081	0.362 ± 0.041
BUN [mmol/l]	4.78 ± 0.29	7.92 ± 0.44**	GLU [mmol/l]	9.02 ± 0.18	7.83 ± 0.25

** $p < 0.01$, n = 8 specimens in each group

biochemical markers assessed in the plasma were altered in a very low scale, except the AST and LDH activity, which were quite noticeably but not significantly declined – AST from 2.03 ± 0.35 μkat/l (control group) to 1.31 ± 0.41 μat/l (1 hour after the injection) and LDH from 12.1 ± 3.45 μkat/l (control group) to 4.88 ± 1.25 μkat/l (1 hour after the injection). Experimental data are summarized in tables 1 (oxidative stress markers), 2 (specific enzymatic markers) and 3 (biochemical markers).

Discussion

The TBARS value informs about a level of malondialdehyde which signifies lipid peroxidation and so oxidative damage of cell membranes (6, 26–28). In a similar way, protein carbonyls are products of an irreversible oxidative modification of proteins (6, 26, 29, 30). From the elevated TBARS value in the heart and the content of carbonylated proteins in the kidney of guinea pigs exposed to the injection stress, we can infer that the heart and kidney tissue were injured by the oxidative stress. These findings are in compliance with the observation of Reis et al. (31), who stated that psychical stress can contribute to the elevation of reactive oxygen, nitrogen and sulphur levels in the laboratory guinea pig model. However, other tissues seem to be more resistant to oxidative stress.

FRAP level corresponds to the concentration of the low molecular weight antioxidants (9). As the FRAP level was elevated, we judge that the injection stress caused activation of the antioxidant system in the cerebellum and the in the frontal lobe.

GR and SOD are enzymes participating in the antioxidant defense of the organism. GR catalyzes regeneration of reduced glutathione from its oxidized form. The task of SOD is to accelerate the transformation of superoxide anion, into hydrogen peroxide. Their increased activity occurs when the oxidative stress in the organism rises. On the other hand, when the oxidative stress is too high and the capacity of the antioxidant system is depleted, the activity of SOD and GR can decrease as well (6). We found slightly decreased SOD activity in the temporal and the occipital lobe. The results were quite surprising, because no other marker signs the oxidative stress exceeding the capacity of the antioxidant system in the temporal and occipital lobe. For the reason, depletion in the SOD level is not well understood.

CASP3 is an enzyme participating in the apoptotic cascade. Its activation is, besides other processes, directly linked to the oxidative stress caused by mitochondrial failure (32). As the CASP3 activity was not elevated in any of the examined organ, we infer that injection stress did not lead to the apoptosis.

The results of the plasma biochemical markers` assay were influenced by the injection stress as well. The BUN level was nearly twice increased one hour after the injection. The similar effect is often described in relation to the muscle injury caused by the intramuscular injection (33), to the kidney malfunction (34) or to the acute-phase proteins production (35). Owing to the selected biochemical markers indicating tissue damage, we demonstrated that the microtraumatic injury probably does not play a crucial role as both AST and LDH were not increased. Unfortunately, the assays were followed by quite extensive differences indicating high inter-individual variability. If the muscle is serious injured by the injection, the LDH activity will raise. The kidney is relatively susceptible to the oxidative damage which can be manifested by the increase of BUN (36). As was mentioned above, the kidneys of the animals in the injection group showed a marker of the oxidative injury (increased level of protein carbonyls in comparison to the control group). For the reason, the increase of the BUN level is probably linked to the oxidative kidney injury caused by the injection stress.

Rabe (37) established reference ranges for blood chemistry in guinea pigs by the use of a common dry chemistry blood analyzer. Considering the fact, that another analyzer was used for the measurement, the levels of biochemical markers found in the injection as well as the control group corresponded with the published data. Even the described BUN alteration did not deviate from the reference range. However, the difference between groups in BUN level is evaluated as statistically significant.

As was described previously, drug application using an injection is a very stressful moment for small laboratory rodents and the injection stress may alter a homeostasis of an experimental organism in a multiple way (19–21, 38). Our results show, that the oxidative homeostasis could be altered after the injection. The imbalance in oxidative homeostasis is higher when compared to the direct tissue damage indicated by the biochemical markers LDH and AST. Considering extrapolation of the results on humans, we can expect that repeated application of a drug using injection would initiate an oxidative insult even without a manifestation in standard biochemical markers.

In a conclusion, we proved significant link of injection on a development of an oxidative insult. Separate organs can be burdened by an oxidative stress insult shortly after an injection administration. The findings are in compliance with the other experimental works, as mentioned in the introduction. For the reason, it is always necessary to inject an appropriate volume of saline solution or similar solution to animals belonging to a control group. Moreover, the injection can be considered as an insult with quite extensive stress consequences.

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