

THE EFFECT OF DOXORUBICIN-INDUCED OXIDATIVE STRESS ON CITRULLINE CONCENTRATION IN THE SMALL INTESTINAL MUCOSA AND PLASMA BLOOD IN RATS WITH NON-ALCOHOLIC STEATOHEPATITIS

DOI: 10.36740/WLek202106105

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ABSTRACT

The aim: Is to investigate the effect of doxorubicin-induced oxidative stress on plasma citrulline and citrulline concentration in the small intestinal (SI) mucosa of rats with non-alcoholic steatohepatitis (NASH).

Materials and methods: The studies were carried out on 30 white non-linear adult rats, 15 (50%) males, 15 (50%) females, weighing 160-220 g. The rats were divided into three groups: I (n=10) – rats without NASH, received a standard diet, subsequently were injected with doxorubicin in cumulative dose of 15 mg/kg; II (n=10) – rats with NASH, received a high-calorie diet with doxorubicin injection similarly to group I; III (n=10) – control group, received a standard diet, were injected with placebo. In the SI mucosa, the levels of thiobarbituric acid reactive substances (TBARS), citrulline and catalase were determined. Also the level of plasma citrulline was analyzed.

Results: Doxorubicin injection resulted in induction of oxidative stress in rats of group I and II, which was characterized by 2.1 (p=0.002) and 1.4 times (p=0.0059) increase in TBARS and in 3.4 (p=0.002), and 5.2 times (p=0.002) decrease in catalase activity respectively comparing with the control group. Simultaneously plasma citrulline level in group I and II was in 1.4 (p=0.0039) and 1.5 times (p=0.0039) respectively lower, compared to the control group.

Conclusions: Doxorubicin-induced SI injury was associated with a significant decrease in plasma citrulline in rats regardless of NASH. The plasma citrulline could become an important marker in the assessment of the SI injury in clinical practice.

KEY WORDS: doxorubicin, non-alcoholic steatohepatitis, oxidative stress, citrulline

Wiad Lek. 2021;74(6):1317-1321

INTRODUCTION

Nowadays it is widely recognized that a wide spectrum of cytostatic agents, which are used in cancer therapy, induces painful inflammation and dysfunction of the entire gastrointestinal tract (GIT) [1]. Chemotherapy (CT) affects all cells in the human body, including the most susceptible tissues with a high proliferating rate. The small intestinal epithelium is one of the most rapidly proliferating tissues in the body, which makes enterocytes of the small intestine particularly susceptible to chemotherapy-induced damage. Indeed chemotherapy-induced small intestinal injury is reported in up to 40 % of patients who receive CT [2]. Doxorubicin (DOX) is an antibiotic anthracycline, clinically known as Adriamycin, is one of the most effective anti-tumour agents, which is used in the treatment of solid tumours as well as in the treatment of haematological malignancies, as Hodgkin's lymphoma and non-Hodgkin's lymphomas, acute and chronic leukaemia [3]. However, its clinical usefulness is limited by its toxicity. It has been reported that doxorubicin-induced GIT injury develops during the first 5-10 days after CT [4]. Cytostatic-induced intestinal injury is one of the dose-limiting side effects with vary variable clinical presentation: from asymptomatic

presentation to development of diarrhoea of all degrees of severity. Intestinal injury can be diagnosed by using histopathological examination after endoscopy or at the time of necropsy [5]. However, endoscopy is a traumatic procedure, thus, for patients undergoing CT treatment endoscopy is often contraindicated. Therefore, the discovery of non-invasive, simple and reliable biomarker of intestinal injury, which can be used in the settings of CT and can be effectively implemented in clinical practice has become a very important issue.

Although several potential biomarkers of intestinal injury such as diamine oxidase, calprotectin, miRNAs and citrulline have been reported, for the present day, we do not have any officially validated biomarkers of intestinal injury [6,7].

From our standpoint, plasma citrulline can serve as a non-invasive biomarker of intestinal injury. Citrulline is a non-protein amino acid, which is synthesized from glutamine. And the most important feature is that citrulline produced practically exclusively by enterocytes of the small intestine. Enterocytes in the small intestine produce a high amount of citrulline due to the very high activity of enzymes, which participate in citrulline synthesis such

as pyrroline-5-carboxylate synthase, and low activity of enzymes, which degrade citrulline as arginosuccinate synthase and arginosuccinate lyase [5, 8, 9].

Considering the unique metabolic features of citrulline, the determination of plasma citrulline can serve as a biomarker of intestinal injury, which can provide a non-invasive assessment of the quantity of enterocytes as well as their functional state [9].

One of the major mechanisms in the pathogenesis of doxorubicin-induced mucositis is oxidative injury [1]. From this standpoint, the comorbid pathologies in which oxidative stress also plays a major role in pathogenesis can aggravate the potential side effects of CT - in particular, chemotherapy-induced mucositis.

Over the last decade, the worldwide prevalence of obesity rapidly has increased, thus the prevalence of obesity-associated diseases correspondingly has increased, including non-alcoholic steatohepatitis (NASH) [10].

One of the major mechanisms in the pathogenesis of NASH is oxidative stress, which potentiates the effects of cytostatic agents, thereby increasing the incidence of chemotherapy-related complications [11].

Moreover, oxidative stress can be responsible for alterations in citrulline level in both plasma and enterocytes compartments. It has been reported that citrulline has potent antioxidant properties. Citrulline increases the expression of endothelial nitric oxide synthase, which in turn reduces the formation of reactive oxygen species (ROS). Also, citrulline, as amino acid, directly scavenges free radicals, thereby protecting DNA and enzymes from ROS attacks [12].

Thereby, the exact effect of doxorubicin-induced oxidative stress on citrulline level in both enterocytes and plasma requires further investigation. Although considering the common oxidative injury-related pathogenesis of NASH and doxorubicin-induced mucositis, the investigation of detrimental effects of NASH on chemotherapy-induced mucous injury presents as a particularly relevant problem.

THE AIM

The aim is to investigate the effect of doxorubicin-induced oxidative stress on the level of plasma citrulline and citrulline concentration in enterocytes of the small intestinal mucous membrane of rats with NASH.

MATERIALS AND METHODS

The studies were carried out on 30 white non-linear adult rats, including 15 (50%) males, 15 (50%) females, weighing 160-220 g. The study was conducted in two stages. At the first stage, the second group of animals (n=10; 5 males and 5 females) received a high calorie during 63 days, thereby NASH was modelled. The ration per one animal for NASH modelling included combination fodder-concentrate granulated (0.04 kg), 72.5 % dairy butter (0.01 kg), refined sunflower oil (0.01 kg), palm oil (0.01 kg). A 4% aqueous fructose solution was used as the sole source of fluid for rats.

Simultaneously, during 63 days, the other rats (n=20; 10 males and 10 females) received a standard vivarium diet,

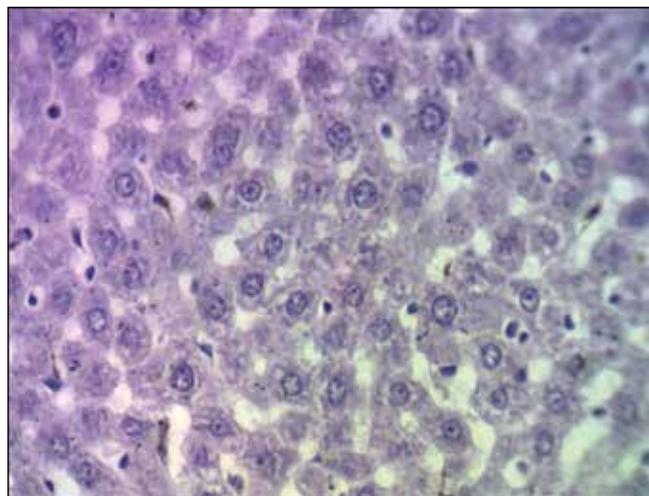


Fig. 1. Intermediate lobule of the liver of the rat with modelled NASH. Stained with haematoxylin-eosin: 1 – endotheliocyte nucleus; 2 – hepatocyte nucleus; 3 – hepatocyte; 4 – liver sinusoid.

containing combination fodder-concentrate granulated (0.04 kg), low-fat cheese (0.006 kg), carrots (0.02 kg), cabbage (0.015 kg) per one animal.

At the second stage, of the experiment rats underwent the modelling of doxorubicin-induced damage, which was carried out by injection of DOX for three consecutive days (from day 64 to day 66), according to which rats were divided into three groups:

Group I (n=10; 5 males and 5 females) rats received a standard vivarium diet from day 1 to day 63. Subsequently, from day 64 to day 66 they were injected with 5mg/kg/day DOX intraperitoneally (the cumulative dose of 15 mg/kg).

Group II (n=10; 5 males and 5 females) rats received a high-calorie diet, thus they were exposed to modelled NASH from day 1 to day 63. Subsequently from day 64 to day 66 they were injected with DOX similar to Group I.

Group III (n=10; 5 males and 5 females) rats received a standard vivarium diet from day 1 to day 63. Subsequently, from day 64 to day 66 they were injected with 0.9 % sodium chloride solution intraperitoneally at a dose of 1 ml.

Decapitation of rats was performed under thiopental anaesthesia on day 67 of the observation.

Liver tissue specimens were fixed in 10% neutral buffered formalin solution and then embedded into paraffin blocks. Liver morphological changes were examined by light microscopy by analysing paraffin blocks sections stained with haematoxylin-eosin.

Dissection of the animal's anterior abdominal wall was performed. The small intestine was taken out and cut lengthwise, subsequently washed with 0.9% sodium chloride solution and after mucous membrane was separated with a scalpel. Then the mucosa of the small intestine was homogenized in 0.9% sodium chloride solution in a ratio of 1: 5 at a speed of 3000 rpm in a homogenizer.

In the homogenate of the mucosa of the small intestine, the state of oxidative stress was determined by the concentration of thiobarbituric acid reactive substances (TBARS) and the state of antioxidant system by catalase activity. Also, the level of plasma citrulline and citrulline concentration in the mucous membrane of the small intestine was determined.

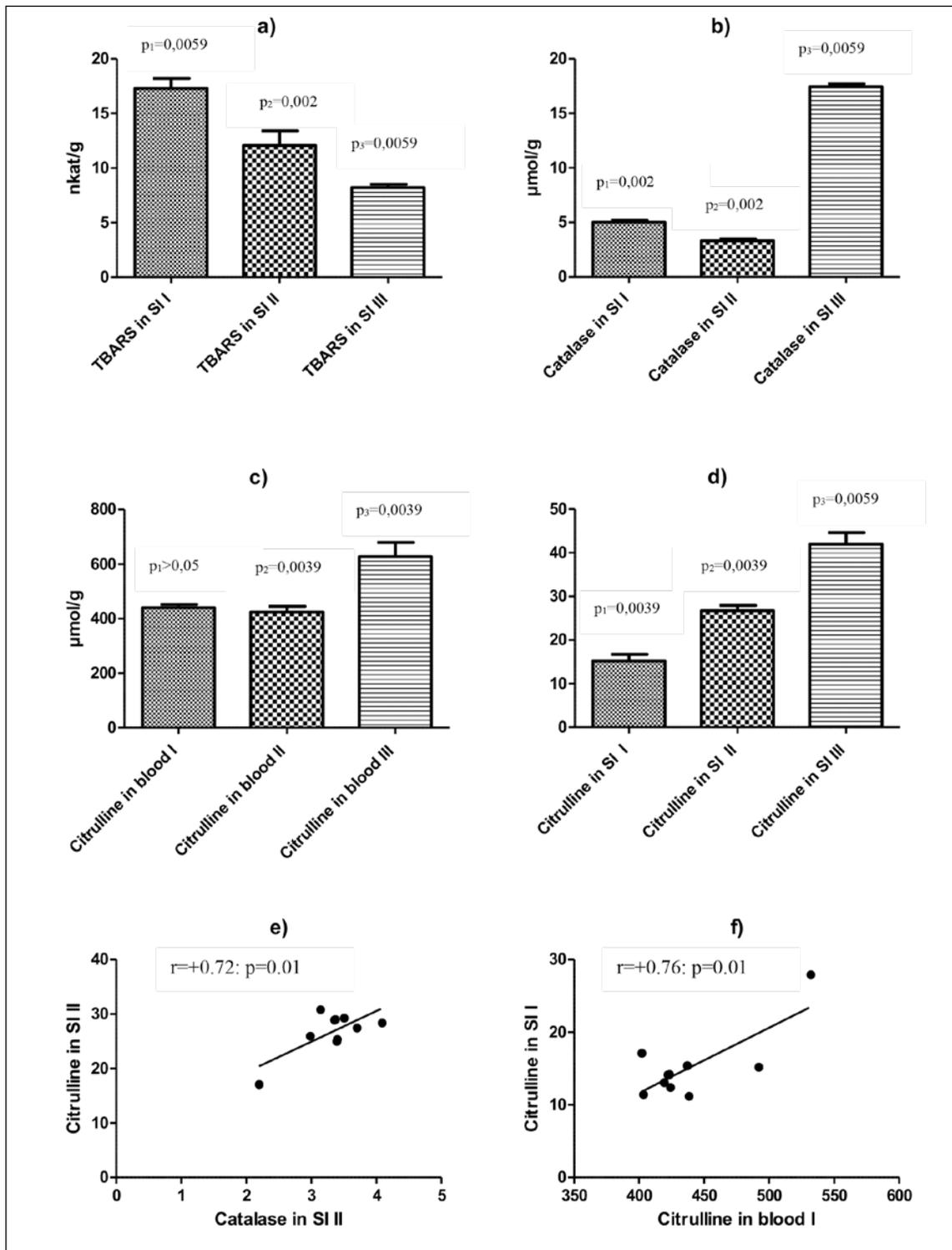


Fig. 2. a) the concentration of TBARS in the homogenate of the mucosa of the SI; b) the catalase activity in the homogenate of the mucosa of the SI; c) the concentration of plasma citrulline; d) citrulline concentration in the mucous membrane of the SI; e) the direct correlation between catalase activity and citrulline concentration in SI; f) the direct correlation between the level of plasma citrulline and citrulline concentration in SI. Note: SI – the small intestine; p_1 – significant difference I and II groups vs III group ($p<0,05$); p_2 – significant difference I vs II groups ($p<0,05$).

Statistical processing of research results was conducted using statistical software GraphPad Prism version 5.00 (GraphPad Software, Inc., San Diego, CA, USA), which allows parametric and non-parametric statistical analysis. With the normal data distribution, the results were presented as

arithmetic means (M) and their errors (m). The significance was determined by Student's t-test. Non-normal distributions paired nonparametric Wilcoxon and Mann-Whitney rank tests were used. The correlation was evaluated using the Spearman's correlation coefficient. $P<0,05$ was considered

significant. The research was carried out in accordance with the principles of the Declaration of Helsinki.

RESULTS

In rats in group II fed with a high-calorie diet for 63 days, on morphological examination the following pathological changes were found. Remodelling of liver lobules - a characteristic feature of moderate NASH. Fatty dystrophy in the central, intermediate, and peripheral lobule zones. In the intermediate lobule zones hepatocytes with karyopyknotic were found. Remodelling of hepatic lobules was associated with sinusoidal dilation of their irregular congestion (Fig. 1).

In rats in group I fed with a standard vivarium diet, injection of DOX in a cumulative dose of 15 mg/kg resulted in induction of oxidative stress, that was characterized by 2.1 times increase in TBARS (17.3 ± 0.91 vs 8.21 ± 0.29 ncat/g; $p=0.002$) compared to control group III. (Fig. 2; a).

The major pathological mechanism in NASH pathogenesis is oxidative stress, which potentiates the effect of DOX. In rats in group II, which received a high-calorie diet, the concentration of TBARS in the mucous membrane of the small intestine was 1.4 times higher compared to group III (12.07 ± 1.34 vs 8.21 ± 0.29 ncat/g; $p=0.0059$) and 1.5 times lower compared to group I (12.07 ± 1.344 vs 17.3 ± 0.9149 ncat/g; $p=0.0059$). (Fig. 2; a).

Doxorubicin-induced oxidative stress was associated with a decrease in the level of antioxidants in rats of both groups. In rats in group I fed with a standard vivarium diet, catalase activity was 3.4 times lower (5.01 ± 0.18 vs 17.42 ± 0.28 $\mu\text{mol/g}$; $p=0.002$), and in rats with modelled NASH in 5.2 times lower (3.32 ± 0.16 vs 17.42 ± 0.28 $\mu\text{mol/g}$; $p=0.002$) comparing with control group III. (Fig 2; b). It is important to mention that the catalase activity in the mucous membrane of the small intestine in rats with modelled NASH in group II was 1.4 times lower (3.32 ± 0.16 vs 5.01 ± 0.18 $\mu\text{mol/g}$; $p=0.002$) compared to rats with the previously intact liver (Fig 2; b).

Thus, administration of DOX in rats induced oxidative stress, which was characterized by marked disbalance between the generation of ROS and the activity of antioxidant enzymes in the small intestinal mucous membrane, which could potentially result in functional and morphological damage of enterocytes.

Plasma citrulline is considered to be a potential biomarker of small intestinal mucosal damage. According to recent reports, the concentration of plasma citrulline reflects intestinal enterocyte mass and also the functional state of enterocytes [9]. Therefore, the determination of plasma citrulline can provide insight into the functional state of the small intestine in the settings of doxorubicin-induced injury.

In rats in group I the level of plasma citrulline was in 1.4 times lower (439.4 ± 13.0 vs 627.8 ± 51.72 $\mu\text{mol/ml}$; $p=0.0039$) and in rats in group II in 1.5 times lower compared to the control group (423.4 ± 22.4 vs 627.8 ± 51.72 $\mu\text{mol/ml}$; $p=0.0039$) (Fig 2; c). In rats in group II, which were exposed to modelled NASH, the level of plasma citrulline didn't differ substantially from the rats in group I with normal body weight ($p>0.05$).

Such dramatic changes in plasma citrulline level were accompanied by a simultaneous decrease in citrulline concen-

tration in enterocytes of the small intestinal mucosa. In rats in both group I and group II citrulline level in small intestinal mucosa was in 2.8 times (15.2 ± 1.53 vs 41.99 ± 2.62 $\mu\text{mol/g}$; $p=0.0059$) and 1.6 times (26.72 ± 1.22 vs 41.99 ± 2.62 $\mu\text{mol/g}$; $p=0.0039$) lower, respectively compared to group III (Fig 2; d). In rats in group II with modelled NASH the direct correlation between catalase activity and citrulline concentration in small intestinal mucosa was found ($r=+0.72$; $p=0.01$) (Fig 2; e). The possible reason for a decrease in citrulline concentration in the small intestinal mucosa is the participation of citrulline in antioxidant defence, as a free radical scavenger [12].

The citrulline level in small intestinal mucosa in rats in group I, fed with a standard vivarium diet was in 1.76 lower compared to the rats in group II with modelled NASH (15.2 ± 1.53 vs 26.72 ± 1.22 $\mu\text{mol/g}$; $p=0.0039$) (Fig 2; b). Simultaneously, in rats in Group I with previously intact liver plasma citrulline level was positively correlated with citrulline concentration in the small intestinal mucosa ($r=+0.76$; $p=0.01$) (Fig 2; f). Therefore, administration of DOX induces massive oxidant stress, regardless of NASH, results in severe damage and dysfunction of enterocytes of the small intestinal mucosa.

DISCUSSION

The use of a high-calorie diet with a high percentage of fat for 63 days was associated with structural alterations in liver tissue, which was characterized by lobule remodelling with the development of fatty dystrophy, karyopyknotic of hepatocytes nuclei, sinusoidal dilation with their irregular congestion. The major factor in the pathogenesis of NASH is oxidative stress. Simultaneously one of the major mechanisms of doxorubicin-induced tissue injury is also oxidative stress. From this standpoint, it was particularly relevant to assess the activity of oxidative stress and the state of the antioxidant system in rats with and without NASH. Administration of doxorubicin-induced oxidative stress in both rats with and without NASH, that was characterized by a substantial increase in the concentration of TBARS and the activity of catalase in the mucous membrane of the small intestine. Also, it is interesting, that the extent of doxorubicin-induced oxidative stress was slightly higher in rats without NASH. This phenomenon can be explained by the so-called obesity paradox and requires further investigation [13, 14].

Administration of DOX led to a significant decrease in citrulline level in the mucous membrane of the small intestine in rats with and without NASH. The decrease in citrulline level in the small intestinal mucosa, can be explained by the participation of citrulline in antioxidant defence, as a free radical scavenger [12].

Doxorubicin-induced injury of the small intestinal mucosa was associated with a significant decrease in plasma citrulline in rats regardless of NASH. Because plasma citrulline reflects enterocytes mass in the small intestine and their functional state, a significant decrease in plasma citrulline in rats of both groups, may be the consequence of severe doxorubicin-induced injury of the small intestinal mucosa.

Because plasma citrulline level reflects the functional state of enterocytes in the small intestine, a decrease in plasma citrulline confirms doxorubicin-induced injury

of the small intestinal mucosa. The results of other studies demonstrate that chemotherapy-induced injury of the small intestine was characterized by villous atrophy and a decrease in plasma citrulline level. The results of our study correspond with this notion [7, 15]. Moreover, even irinotecan-induced small intestinal injury without villous atrophy led to a decrease in plasma citrulline level [5]. Also considering the difficulty in diagnosis of chemotherapy-induced GIT injury. Determination of plasma citrulline level can be a clinically important marker in the assessment of small intestinal injury in patients undergoing CT.

CONCLUSIONS

Administration of the doxorubicin in rats induced massive oxidative stress, which was characterized by an increase in TBARS concentration with a simultaneous decrease in catalase activity in the mucous membrane of the small intestine in both rats with NASH and rats with the previously intact liver.

Doxorubicin-induced oxidative stress led to a significant decrease in citrulline concentration in the mucous membrane of the small intestine in rats regardless of the NASH. The decrease in citrulline concentration in small intestinal mucosa can be explained by the participation of citrulline in the antioxidant defence as a free radical scavenger.

Doxorubicin-induced injury of the small intestine was associated with a significant decrease in plasma citrulline level in rats regardless of NASH. A decrease in plasma citrulline level is caused by doxorubicin-induced damage to the small intestinal mucosa. Considering the particular difficulty in diagnosis of chemotherapy-induced GIT injury, determination of plasma citrulline level can become an important marker in the assessment of the small intestinal injury in clinical practice.

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Current study was the part of research work of Department of Internal Medicine №1, Ukrainian Medical Stomatological Academy "Development of methods of prevention and treatment of drug-induced internal organs injuries" (number of state registration 0115U001087). The study has no additional financial support.

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Conflict of interest:

The Authors declare no conflict of interest.

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Received: 21.11.2020

Accepted: 17.04.2021