UDC 577.161.2+616.379-008.64

VITAMIN D_3 CONTRIBUTION TO THE REGULATION OF OXIDATIVE METABOLISM IN THE LIVER OF DIABETIC MICE

D. O. LABUDZYNSKYI, O. V. ZAITSEVA, N. V. LATYSHKO, O. O. GUDKOVA, M. M. VELIKY

Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv; e-mail: konsumemt3@gmail.com

This work is devoted to the study of the features of oxidative metabolism of hepatocytes in diabetic mice and those under the vitamin D_3 action. We found out that a 2.5-fold decrease of $25OHD_3$ content in the serum was caused by chronic hyperglycemia in diabetes. Intensification of the reactive oxygen species (ROS) and nitrogen monoxide (NO) production, protein oxidative modifications (detected by the contents of carbonyl groups and 3-nitrotyrosine), accumulation of diene conjugates and TBA-reactive products of lipid peroxidation, and the decreased level of free SH-groups of low molecular weight compounds in the liver were accompanied by development of vitamin D_3 deficient state. It was shown that there was a decrease in the key antioxidant enzymes activity (catalase, SOD), while the activity of prooxidant enzymes NAD(P)H:quinone oxidoreductase, xanthine oxidase and NAD(P)H oxidase was increased. The identified oxidative metabolism lesions caused the elevation of the hepatocytes necrotic death that was tested for the ability of their nuclei to accumulate propidium iodide. Prolonged vitamin D_3 administration (during 2 months) at a dose of 20 IU to diabetic mice helps to reduce the ROS formation and biomacromolecules oxidative damage, normalizes the antioxidant system state in the liver and increases survival of hepatocytes. The results suggest that vitamin D_3 is a key player in the regulation of the oxidative metabolism in diabetes.

K e y w o r d s: vitamin D_3, $25OHD_3$, oxidative stress, antioxidant protection, experimental type 1 diabetes.

Insufficiency of insulin production by β-cells of the pancreatic Langerhans islets caused by their destruction, which develops under the influence of environmental factors (viral infections, stress, certain nutritional components) and in the presence of genetic background (genetic mutations, autoimmune processes) underlies pathogenetic development of insulin-dependent diabetes [1]. The primary reason in the development of diabetes pathology is a hyperglycemia. When the glucose level is chronically elevated an excessive non-enzymatic glycosylation (glycation) of proteins that form cytotoxic advanced glication end products (AGEs) of protein-lipid complexes glycoxidation is observed. These AGEs can interact with appropriate receptors (RAGE) on the cell membrane and this leads to the intensification of prooxidant processes and increased synthesis of proinflammatory cytokines [2]. The development of oxidative-nitrosative stress (ONS) under diabetes increases metabolism and endocrine disorders caused by pathological changes in the insulin secretion, pathoimmune reactions and depletion of antioxidant reserves [3]. ONS is a universal non-specific mechanism of numerous morphological changes in the body. The antioxidant defense system of the cell gets damaged one of the first in the organism.

For a normal organism functional activity free-radical processes are maintained at a steady level that is ensured by the balance of the intracellular reactive oxygen species (ROS) formation rate and functioning of a complex tissue-specific system of radical traps and biomacromolecules peroxidation inhibitors. Permanent formation of prooxidants is balanced by their enzymatic and non-enzymatic detoxification by antioxidants [4]. Activity of the main enzymes that generate reactive oxygen and nitrogen (specific – NAD(P)H oxidase and NO-synthase, unspecific – NAD(P)H:quinones-oxidoreductase and xanthine oxidase) is significantly changed in diabetes [5].

At the molecular level, the pathological effects of excess ROS content appear as compartment-dependent changes in cell redox status and oxidative modifications of biomacromolecules (protein carbonyl level, lipid peroxidation, mutations in nucleic acids). Accumulation of nitrosylated and poly(ADP)

ribosylated proteins in different cell types indicates biochemical disorders in diabetes [6]. A key role in the intensification of nitrosylation and poly(ADP) ribosylation belongs to the activation of poly(ADP) ribosopolymerase (PARP), an enzyme whose main physiological function is the NAD-dependent reparation of single-stranded DNA breaks (SSBs) [7]. ONS plays a key role in the development of diabetic complications such as cardiovascular, microvascular, neuropathic, immune etc. [8].

Besides a well-known role of vitamin D_3 in the regulation of calcium and phosphorus metabolism and involvement of cholecalciferol in the bone remodeling process, it is also known that vitamin D_3 exhibits immunomodulatory, anti-inflammatory, anti-proliferative effects and is able to prevent transformation of tumor cells [9]. Molecular mechanisms of vitamin D_3 cytoprotective properties can be implemented both through genomic regulation which largely corresponds to the steroid hormone-like action and non-genomic effects, including its regulatory effect on the protein expression, cellular metabolism, inflammation and oxidative stress [10]. The present studies examine the effects of vitamin D_3 in cells as a biological antioxidant, consider its action mechanism in the context of structural similarity with cholesterol and ergosterol. Thus, it is associated with the ability of cholecalciferol to directly modify the physicochemical state of biological membranes [11]. In addition, the antioxidant activity of vitamin D_3 can be provided through its hormonally active forms on the genes expression level and regulation of pro-/antioxidant enzyme activity.

The aim of this work was to determine the vitamin D_3 role in the regulation of reactive oxygen species formation, processes of biomacromolecules oxidative modification, state of enzymatic antioxidant defense system in the mice liver and hepatocytes survival in experimental diabetes.

Materials and Methods

Studies were conducted on male mice C56Bl/J6 weighing 21 ± 3 g. Type 1 diabetes was caused with 5-fold administration of streptozotocin (STZ, Sigma-Aldrich, USA) at a dose of 40 mg/kg body weight of the animal. This type of administering STZ is commonly used for the induction of experimental autoimmune type 1 diabetes in mice. In this study the animals after 6 weeks of diabetes with blood glucose 20.4 ± 4.3 mmol/L were used. After development of stable hyperglycemia mice were injected with vitamin D_3 (DSM, the Netherlands) for 2.5 months in an aqueous suspension form (800 IU/kg body weight, *per os*). Control animals were kept on a complete diet in the vivarium. For the adaptation period (one week) and during the experiment animals were kept in the vivarium at a temperature of 18-22 °C, humidity 50-60%, natural light mode "day-night" in standard plastic cages with free access to their food and water and in compliance with *General Ethical Principles for Experiments on Animals* (Ukraine, 2001). Mice were decapitated, using diethyl ether for anesthesia. Selection of animals and the formation of groups were performed by the method of random numbers [12].

We evaluated vitamin D_3 availability in mice by the level of serum $25OHD_3$, which was determined by immunoenzyme technique (ELISA kit, Immunodiagnostic Systems Ltd., USA). This method is based on the competitive binding of serum $25OHD_3$ and $25OHD_3$-biotin with vitamin D_3 binding protein (VDBP), immobilized on immunological 96-well plate.

Hepatocytes were isolated after liver perfusion with phosphate-saline buffer through the portal vein. The rinsed liver was cut into thin slices, which were incubated with collagenase at 37 °C within an hour, followed by washing with phosphate-saline buffer containing 0.146 M NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2$, 0.7 mM $Na_2HPO_4 \cdot 12H_2O$, 0.7 mM KH_2PO_4, 1% albumin, pH 7.4 [13]. The number of cells was counted in a Goryaev cell chamber.

Fluorescent determination of the reactive oxygen and nitrogen content were performed with DCFH-DA (2′,7′-Dichlorodihydro fluorescein diacetate) [14]. The amount of 1 µl of DCFH-DA solution (2.5 mM) was added to 100 ml of mice isolated hepatocytes suspension ($0.5 \cdot 10^6$ cells) and incubated for 30 min at 37 °C in the dark. Samples were centrifuged at 300 g for 10 minutes and washed 2 times with a PBS solution (pH 7.4), and the final precipitate of hepatocytes was resuspended in 0.5 ml of PBS. Right after preparation, the samples were analyzed using flow cytometer COULTER® EPICSTM XLTM. The fluorescence of the DCF-DA oxidized form (Dichlorodihydro fluorescein diacetate) was measured at λ_{ex} = 488 and λ_{em} = 540 nm.

Fluorescent determination of NO content was carried out with DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) [15]. DAF-FM (1.5 mM) in the amount of 1 µl was added

to 100 µl of the suspension of isolated mice hepatocytes ($0.5 \cdot 10^6$ cells) and incubated for 30 minutes at 37 °C in the dark and then resuspended in 0.5 ml PBS. Subsequently, the samples were analyzed using flow cytometry (COULTER® EPICSTM XLTM). The fluorescence of oxidized forms of DAF-FM DA was measured at λ_{ex} = 488 and λ_{em} = 540 nm.

The level of hepatocyte death (necrosis) was estimated using flow cytometry by the number of cells that could accumulate propidium iodide [16].

The level of oxidative modification of proteins (OMP) was evaluated by the content of carbonyl derivatives of proteins (ketone-2,4-dinitro-phenylhydrazone) in the presence of electron donors and metals with variable valences, especially copper or iron ions. Changes in absorbance were recorded using automatic microspectrophotometer µQuant (Biotek, USA) [17]. Determination of lipid peroxidation products was carried out by reaction with thiobarbituric acid [18], and the content of low molecular weight compounds free SH-groups – by reaction with ortho-phthalaldehyde [19] in the liver homogenates.

The activity of antioxidant enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (EC 1.11.1.6) and glutathione reductase (EC 1.8.1.7) was determined using previously described methods [20-22].

The activity of NAD(P)H:quinone oxidoreductase (EC 1.6.5.5) was determined in liver homogenates in a reaction mixture containing NADPH-generating glucose-6-phosphatedehydrogenase system, menadione (2-methyl-1,4-naphthoquinone) and MTT [3-(4,5-dymetyltiazo-2-il)-2,5-diphenyltetrazolio bromide]. NAD(P)H:quinone oxidoreductase catalyzes NADPH-dependent reduction of menadione to menadiol. The following formation of formazan that appeared as a result of non-enzymatic MTT reduction due to menadiol, was detected on a microplate reader at the wavelength range of 550-640 nm [23].

The activity of xanthine oxidase (EC 1.17.3.2) was determined in the reaction of ethanol oxidation by hydrogen peroxide (which was formed due to substrate hypoxanthine oxidation by xanthine oxidase) into acetaldehyde, which was cleaved by NAD-dependent aldehyde dehydrogenase. Reduction of coenzyme NAD^+ was detected with spectrophotometry (λ = 334 nm) [24].

The activity of NAD(P)H:oxidase (EC 1.6.3.1) was determined in liver homogenates spectrophotometrically by the rate of NADPH to $NADP^+$ oxidation in parallel samples without addition and with the addition of NAD(P)H oxidase specific inhibitor - apocynin (10 mM) [25].

The level of nitrozylated proteins was determined by Western blotting [26]. The intensity of the signals on X-ray film was evaluated using GelPro32 [27].

Statistical analysis of the data was performed using the computer program (Microsoft Excel). Results were presented as means of 6 independent determinations ± standard error. The assessment of statistical significance of distinction averages were performed using a standard Student *t*-test for non-correlated samples [12].

Results and Discussion

The most objective and informative measure of vitamin D_3 availability is $25OHD_3$ content in serum, which is in the range of 100-150 nmol/L (40-100 ng/mL) in human organisms. Reduction of serum $25OHD_3$ level below 75 nmol/L shows the development of D_3-hypovitaminosis [28]. The results shown in Table 1 indicate that in terms of diabetes the level of serum $25OHD_3$ decreases to 2.52 times as compared to control animals, reflecting a lack of vitamin D_3 availability and inhibition of its biologically active hydroxylated forms synthesis. The serum level of glucose in the animals with diabetes

Table 1. Content of $25OHD_3$ and glucose in serum of mice in experimental diabetes and with the vitamin D_3 administration (M ± m, n = 6)

Experimental groups	Content of $25OHD_3$		Glucose concentration, mmol/L
	nmol/L	ng/mL	
Control	85.6 ± 4/11	34.2 ± 1.64	5.2 ± 1.1
Diabetes	33.9 ± 1.91*	13.56 ± 0.76*	20.4 ± 4.3*
Diabetes + D_3	75.4 ± 5.21#	30.16 ± 2.02#	14.5 ± 3.2#

Here and in Table 1-3 *the difference compared with the control is reliable ($P < 0.05$); #the difference compared with the group "Diabetes" is reliable ($P < 0.05$)

significantly increased to 20.4 ± 4.3 mmol/L compared to control – 5.2 ± 1.1 mmol/L.

Production of free radicals, their neutralization and free radical oxidation are normal physiological processes that ensure the regulation of cellular activity and there is a balanced equilibrium among them. However, overproduction of ROS and accumulation of lipid peroxidation products (LPO) under pathological conditions can lead to inactivation of cytosolic and membrane-bound regulatory proteins, alterations of their physicochemical properties, biological membrane permeability and functional genome integrity [29].

Pathologies that are accompanied with the tissue hypoxia development (including diabetes) are characterized by a significant imbalance between the rate of free radical species production and antioxidant enzyme activity (AOP), resulting in the accumulation of excess peroxide compounds, and oxidative stress progression [30]. Firstly, we studied the intensity of bio-oxidants production (reactive oxygen and nitrogen species), the level of cellular macromolecule oxidative modifications and the efficiency of the antioxidant defense system in the liver to determine the role of ROS-mediated processes under chronic hyperglycemic condition (experimental type 1 diabetes).

In accordance with the data obtained using ROS-sensitive fluorescent probe DCFH-DA, we have found out that chronic diabetic hyperglycemia stimulates formation of reactive oxygen metabolites in isolated hepatocytes, increasing the intensity of fluorescence by 90.4% ($P < 0.05$) compared with control animals (Fig. 1). Similarly, a significant increase by 73.1% ($P < 0.05$) in fluorescence of DAF- FM DA, a probe specific for the nitric oxide (II) molecule, was shown for hepatocytes from diabetic mice (Fig. 2). It should be noted that the results of recent year studies confirm the direct effect of insulin signaling on the regulation of nitric oxide synthesis [31]. On the other hand NO exhibits some insulin-like effects such as stimulating glucose transport and oxidation [32]. Due to impairment of insulin-producing system in type 1 diabetes we can suggest that activation of NO production in tissues (especially in the liver) may be a regulatory mechanism of partial compensation for the lack of insulin in diabetic organisms.

It is known that excessive production of reactive oxygen metabolites is one of the key mechanisms leading to cell dysfunction and death. Cytotoxic and mutagenic action of ROS is caused by increased free radical (peroxidative) oxidation of physiologically important macromolecules [33]. The results of biochemical analysis of changes in integrated indicators of free radical oxidation of biomolecules evidence for a significant intensification of the oxidative process under conditions of chronic hyperglycemia. This applies to the content of TBA-reactive products (predominantly MDA) and dien conjugates (DC) in particular, which are the end products of lipid peroxidation (LPO) and can cause damage of biological membranes, proteins and nucleic acids. It was established that the level of TBA-reactive products and DC exceeded 2.5 and 1.9 times, respectively, in the experimental type 1 diabetic mice compared with control group of mice (Table 2).

It is important that in conditions of chronic hyperglycemia, ROS also causes protein oxidative degradation. It is believed that protein oxidative modifications play a key role in the molecular mechanisms of oxidative stress and may trigger damage to other cell biomolecules (lipids, DNA) [34, 35]. The degree of protein oxidative modifications was evaluated by the content of carbonyl groups (>C=O), which are formed in protein molecules mainly due to the direct oxidation of some amino acid radicals (especially Pro, Arg, Lys), as well as due to the interaction with lipid peroxidation products or reducing sugars.

Table 2. Content of free radical oxidation products and free SH- groups compounds in the liver of diabetic mice and with vitamin D_3 administration (M ± m, n = 6)

Index	Control	Diabetes	Diabetes + D_3
TBA-reactive products, nmol/mg of protein	0.6 ± 0.02	1.5 ± 0.05*	1.0 ± 0.03#
Dien conjugates, nmol/mg of protein	15.7 ± 1.8	29.8 ± 2.3*	22.4 ± 1.7#
Protein carbonyl groups, nmol/mg of protein	0.8 ± 0.03	1.3 ± 0.,04*	0.9 ± 0.04#
Free SH-groups of low molecular weight compounds, nmol/mg of protein	5.6 ± 0.18	3.3 ± 0.11*	4.9 ± 0.15#

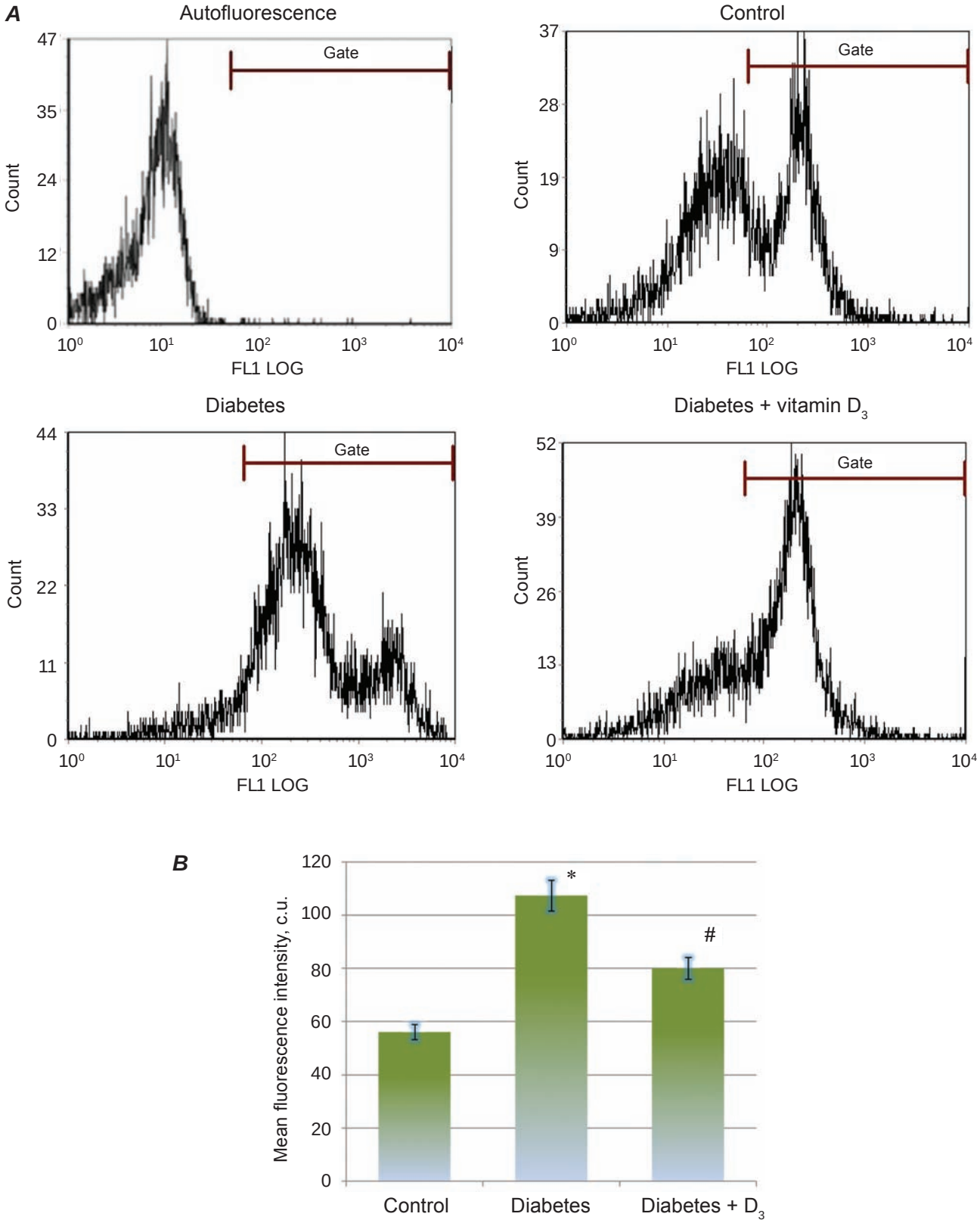

*Fig. 1. The rate of ROS formation in hepatocytes of diabetic mice and with vitamin D_3 administration. (A) Fluorescence cytofluorograms of DCF-DA (count – the number of events; FL1 LOG – fluorescence intensity) and (B) mean fluorescence intensity. *P < 0.05 compared with control, #P < 0.05 compared with diabetes group (M ± m, n = 6)*

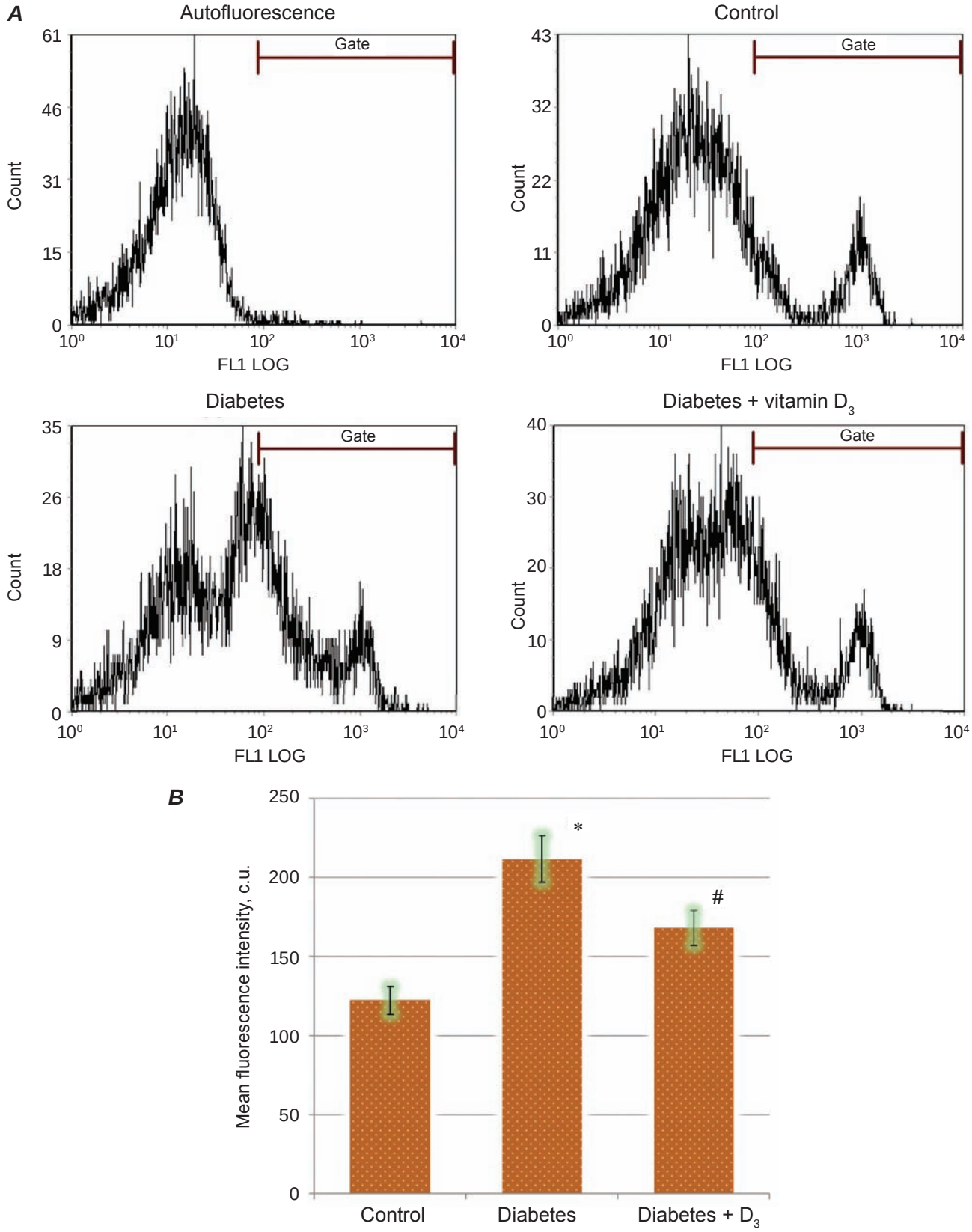

*Fig. 2. The rate of nitric oxide (II) in hepatocytes of diabetic mice and with vitamin D_3 administration. (A) Fluorescence cytofluorograms of DAF-FM DA (count – the number of events; FL1 LOG – fluorescence intensity) and (B) mean fluorescence intensity. * $P < 0.05$ compared with control, # $P < 0.05$ compared with diabetes group ($M \pm m$, $n = 6$)*

Since amino acid residues normally contain a small amount of carbonyl groups, even little increase of their content per unit of protein can be considered as a result of oxidative stress, which occurs because of an imbalance of ROS-generating and degradation processes. Moreover, determination of carbonyl groups in proteins has significant advantages compared to the detection of lipid peroxidation products because of the relative stability of oxidized proteins and their formation at the beginning of oxidative stress development [34]. It was established that the content of carbonyl groups in the studied samples of liver is 1.65 times higher compared to the control group (Table 2). Among various types of macromolecules damage, mainly oxidative modification of cellular proteins often leads to loss of their function and then such molecules are to be selectively degraded. Despite the fact that moderate oxidative modification makes proteins more accessible for many proteases which play an important role in protein degradation, the increased level of modified proteins (particularly enzymes) under conditions of oxidative stress may harm cellular metabolism.

As was mentioned above, chronic diabetic hyperglycemia can lead to ROS overproduction in various tissues, especially superoxide anion, which interacts with other active forms of oxygen and can be converted to hydroxyl radical, hydrogen peroxide and peroxynitrite ($ONOO^-$). Reactive nitrogen-containing compounds, including peroxynitrite play a crucial role in the pathogenesis of diabetes and its complications [36]. The growth of peroxynitrite content, which today is recognized as the number one oxidant in biological systems leads to the development of oxidative/nitrosative stress, accompanied by protein nitrosylation, lipid peroxidation, DNA breaks, changes in cell signaling, activation of poly(ADP-ribose)polymerase-1 (PARP-1), induction of necrosis and apoptosis. High level of nitrosylated proteins (estimated by 3-nitrotyrosine content) is one of the earliest markers indicating the beginning of chronic complications in diabetes [36]. It was shown (due to immunoblotting analysis) that there is a 1.72-fold increase ($P < 0.05$) of nitrosylated proteins in the liver lysates compared with the control group of mice (Fig. 3). It should be noted that the increase in nitrosylated protein content correlated with high NO production by hepatocytes (Fig. 2), which actually is one of the peroxynitrite precursors. This growth of NO concentration can be explained by inducible form of nitric oxide synthase (iNOS) overexpression in the liver under diabetes [37].

A thiol-disulfide system is an important component of oxidative homeostasis in cells and tissues. It includes low molecular weight thiols, including glutathione, SH- and SS-groups of proteins, the level of which is regulated by glutathione cycle enzymes [38]. Cyclic oxidation/reduction of amino acids thiol groups serves as a kind of trap that captures ROS, protecting these proteins from irreversible, more

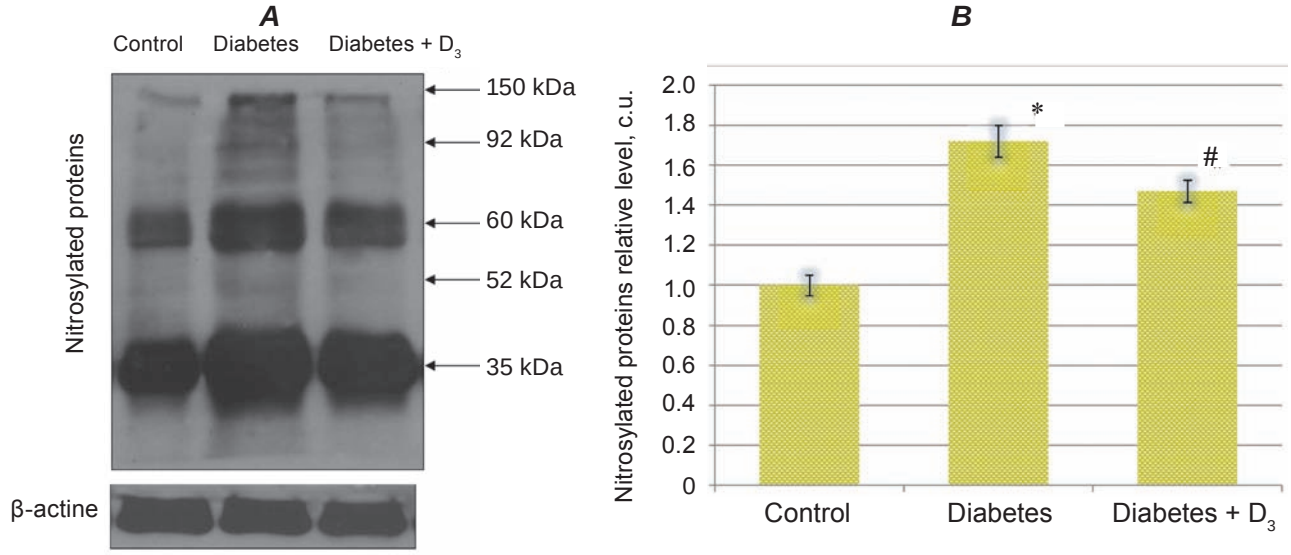

*Fig. 3. Western blot analyses of nitrosylated proteins in the liver of diabetic mice and with vitamin D_3 administration (A). Graphical representations are expressed as means ± SD (B). *P < 0.05 compared with control, # P < 0.05 compared with diabetes group (M ± m, n = 6)*

harmful oxidative modifications. Changes in the redox state of GSH/GSSG are able to regulate the reversible formation of mixed disulfides between protein thiol groups and glutathione. However, a decrease of free SH-groups content under different forms of stress and pathologies, formation of mixed disulfides in proteins may prevail over their recovery process and lead to an increase of the disulfide bonds number and the rigidity of the protein conformational structures. These changes modify cell membrane state, its permeability and adhesive properties, besides having negative influence on enzymes activity and cell survival [34, 38]. A disturbance in thiol-disulfide system was observed in type 1 diabetes, and these changes were traced in a decreased level of free SH-groups of low molecular weight compounds in the liver to 41.1% ($P < 0.05$) compared with controls (Table 2). This shows a significant suppression of antioxidant protection adaptive mechanisms. Reduction of free SH-groups number in SH/SS system under conditions of oxidative stress promotes the accumulation of lipid peroxidation products and oxidative protein damage and can be an additional factor for further enhancement of the oxidative stress.

Significant reactivity of ROS and macromolecules peroxidation products necessitates continuous activity of antioxidant protection (AOP) specific mechanisms, which are important components of antioxidant enzymes. The latter along with non-enzymatic antioxidants perform a cytoprotective function reliably limiting the pro-oxidant process at all stages, starting from the stage of reactive oxygen species formation. Weakening of any AOP element may contribute to the development of oxidative stress [33, 39]. Pro-oxidant processes activation within diabetes may result from the disruption of coordinated system mechanisms of enzymatic and non-enzymatic control over ROS and free radical oxidation products content. Furthermore alterations in enzyme activity in diabetes may be due to the post-translational modifications especially by glycosylation.

Intensification of protein oxidative modification processes, lipid peroxidation and descension of thiols antioxidant capacity in diabetes correlates with changes in the AOP enzymes activity – superoxide dismutase (SOD), catalase and glutathione peroxidase (GPO). These enzymes complement each other and play a key role in oxidation/reduction processes regulation in the liver. The most powerful natural antioxidant is SOD enzyme, which catalyzes the reaction of superoxide anion radical dismutation and converts them into less reactive molecule – H_2O_2. Therefore, changes in the SOD activity characterize the depth of tissue destruction and metabolic changes caused by oxidative stress. The studies of SOD activity (Table 3) indicate that enhancement of free radical processes in diabetes is accompanied with a significant decrease in liver enzyme activity by 34.5% ($P < 0.05$) compared to control. Changes in SOD activity may be caused by modifying influence of reactive oxygen species, whose content increases significantly under chronic hyperglycemia. The presence of metals with variable valences associated with the imidazole group of histidine residues in the active Cu, Zn-SOD, makes this enzyme particularly sensitive to high concentrations of both reactive oxygen species (superoxide anion radical, hydroxyl radical, hydrogen peroxide) and a number of lipid peroxidation intermediates (hydroperoxides of un-

Table 3. Activity of pro- and antioxidant enzymes in the liver of diabetic mice and with vitamin D_3 administration ($M \pm m$, $n = 6$)

Enzyme	Control	Diabetes	Diabetes + vitamin D_3
Catalase, μmol H_2O_2/min per mg of protein	188 ± 10	137 ± 8*	160 ± 9#
Superoxide dismutase, IU/mg of protein	381 ± 20	250 ± 12*	309 ± 16#
Glutatione peroxidase, nmol GSH/min per mg of protein	130 ± 11	220 ± 18*	170 ± 14#
NAD(P)H:quinonreductase, nmol of reduced MTT/min per mg of protein	22.5 ± 2.3	49.1 ± 3.3*	29.92 ± 1.8#
Xanthine oxidase, IU/mg of protein	2.07 ± 0.09	2.95 ± 0.08*	2.61 ± 0.10#
NADPH-oxidase, nmol NADPH/ min per mg of protein	5.93 ± 0.34	9.72 ± 0.15*	7.42 ± 0.23#

saturated fatty acids, aldehydes, ketones, etc.) [40]. Cu, Zn-SOD may also interact with H_2O_2 and act as a pro-oxidant agent, initiating the formation of superoxide anion radicals and hydroxyl radicals.

The next step of the antioxidant protection system includes catalase and glutathione peroxidase which eliminate hydrogen peroxide and lipid hydroperoxides. Catalase which decomposes hydrogen peroxide is a hem-containing enzyme localized mainly in peroxisomes. Simultaneously, catalase may be a source of active oxygen metabolites production because approximately 0.5% of O_2 is formed by the decomposition of H_2O_2. Our study showed that the catalase activity in the liver is decreased by 27.2% ($P < 0.05$) in diabetes compared with the control group (Table 3). This result can be explained by degradation of plasma and peroxisome membranes where this enzyme is mainly localized. Glutathione antioxidant system includes glutathione peroxidase, glutathione reductase, glutathione-S-transferase, glucose-6-phosphate dehydrogenase and cofactors – glutathione and NADPH. Protective functions of this system are determined by specific partial contribution of each individual component during oxidative stress. Glutathione forms a reserve of cellular mobile SH groups, provides recovery and isomerization of disulfide bonds in proteins, and is involved in the eicosanoids and xenobiotics metabolism, reparative processes and adaptation to the stress factors in particular. Selenium-dependent glutathione peroxidase catalyzes reduction of hydrogen peroxide and organic hydroperoxides (linoleic, linolenic, arachidonic acid, cholesterol, corticosteroids) to their hydroxylated forms using glutathione [41, 42]. The activity of glutathione peroxidase (GPO) – an enzyme that uses GSH, increased by 69.5% ($P < 0.05$) in diabetes compared with controls (Table 3). This significant increase suggests that GPO is the major enzyme which can provide antioxidant protection under oxidative stress conditions that develops in experimental diabetes in the liver cells.

It should be noted that the investigated antioxidant protection enzymes – SOD, catalase and glutathione peroxidase form not only a complete enzymatic antioxidant system of cells, but also directly protect each other from the inhibitory effects of reactive oxygen species. In particular, SOD removes their negative effects on the catalase active center while eliminating superoxide anion radicals. However, the GPO and catalase protect SOD against oxidative modification by decomposing H_2O_2. Therefore, the combined effect of antioxidant enzymes under oxidative stress conditions provides not only the greatest manifestation of their biological activity, but also protection against reactive oxygen species. Conversely, impairment of this cooperativity by reducing of at least one antioxidant enzyme activity due to oxidative modification alters the functioning of the whole antioxidant defense system. Therefore, the obtained results about the changes in the activity of catalase and glutathione peroxidase may also be explained by the increased activity of SOD, which catalyzes the reaction of superoxide anion conversion to hydrogen peroxide – the only substrate of catalase [43]. It is known that GPO is more effective at low H_2O_2 concentrations, and also neutralizes various organic peroxides [44].

Hyperglycemia-induced changes in the structural organization and functional status of hepatocytes may be associated with disturbances in the biotransformation systems of xenobiotics and endogenous compounds, including the processes of their enzymatic oxidation. NAD(P)H:quinone oxidoreductase (NQO) related mechanisms of xenobiotics biotransformation are not fully clarified. It is known that the development of xenobiotic-induced oxidative stress impairs the function of mitochondria oxidative phosphorylation system. Hydrophilic quinones can partially counteract negative effect in these conditions. NQO in the presence of NADH (which is over-accumulated in these conditions) reduces hydrophilic quinones to hydroquinones. Then these molecules are oxidized in mitochondria respiratory chain complex III, subsequently forming a shunt that provides a bypass of complex I [45]. In the case of prolonged hyperglycemia a significant increase (2.2 times) in the liver NQO activity (Table 3) was found. According to literature data, the expression of this enzyme increases dramatically as a response to the oxidative stress and xenobiotics influence in diabetes [46]. These results evidence that hyperglycemia-induced reduction of quinones to dyhydroquinones under the effect of NQO can be used for electron transport in the respiratory chain to restore the cell function. Although NQO as inducible antioxidant enzyme prevents the ROS formation, the formed dyhydroquinones themselves may be an additional source of ROS, as shown by N. Watanabe et al [47]. Thus, the observed intensification of free radical processes in diabetes may partly be connected with rising of this enzyme activity.

Functional disorders in enzymatic systems of metabolites biotransformation are linked with the ac-

cumulation of the toxic catabolism products, which can greatly enhance the process of oxidative stress and disrupt intracellular processes. From this viewpoint, it is important to study the liver xanthine oxidase (XO) activity that is involved in the oxidation of hypoxanthine and xanthine to uric acid. It is important that purine oxidation reaction is coupled with the oxygen reduction to singlet oxygen. Thus, XO is a potent ROS producer, especially in various diseases [48]. We have shown that there is an increase in the liver xanthine oxidase activity in diabetic animals to 1.43 times ($P < 0.05$) compared with control (Table 3). It can be assumed that the increased activity of XO caused by pathological hyperglycemic conditions will result in the accumulation of purine degradation products. This can be considered as an additional factor enhancing oxidative stress and reduction of reparative processes in the liver.

Functional activity of ROS generation elements and antioxidant system are largely dependent on the cellular NADPH pool. Interdependent redox transformation of important antioxidants, in particular, like glutathione and ascorbic acid (the latter recovers tocopherol radicals and various polyphenols) occurs due to NADPH. The observed decrease in the hepatocytes antioxidant capacity could be a result of intensified competitive NADPH use in the NAD(P)H oxidase catalyzed reaction. This multi-oxidase complex provides ROS production mainly in phagocytic blood cells and resident macrophages in peripheral tissues. Moreover, it also plays a key role in the inflammatory process within Kupffer cells in the liver [49]. The main product of NAD(P)H oxidase is a superoxide anion radical, which has bactericidal action and is an essential component of nonspecific immune defense in the organism. In addition, NAD(P)H oxidase is expressed in the hepatocytes and other liver cells [50]. We have found out that the activity of this enzyme in liver homogenate of diabetic mice increase 1.64 times ($P < 0.05$) compared with control (Table 3). This data is consistent with the generalized inflammation state that is characteristic of diabetic liver tissue.

Oxidative modifications of biomacromolecules and cellular structures largely determine the cytotoxic effects of free radical oxygen and nitrogen species under conditions of their excessive production. It was important to investigate the relation between prooxidant processes, functional activity and survival of hepatocytes in diabetes. The oxidative stress induced by chronic hyperglycemia can cause mitochondrial dysfunction with subsequent initiation of hepatocytes damage and death. Our research has shown that intensification of free radical processes correlated with an increase in the proportion of dead (necrotic) cells in the isolated hepatocytes culture to 9.3% in diabetes compared with 5.8% in control ($P < 0.05$), Fig. 4. The destruction of parenchymal liver cells potentially can trigger the activation of other cell populations that are able to initiate inflammatory and/or adaptive immune responses and inhibit liver regeneration.

Diabetic disorders that were unveiled in the research were observed on the background of vitamin D_3 deficiency in the organism of experimental animals, and it was evidenced by more than 2.5-fold $25OHD_3$ reduction in the serum of mice compared with control (Table 1). One of the reasons for the cholecalciferol decreased level under chronic hyperglycemia can be an observed reduction in vitamin D_3 25-hydroxylase expression in diabetes [26]. These results indicate changes in vitamin D_3 metabolism and we can suggest that the increase in prooxidant status of the liver may be associated with vitamin D_3 deficiency in chronic hyperglycemia.

Vitamin D_3 administration in physiological doses (10 IU of D_3 for 45 days) was accompanied by 2.2-fold growth of $25OHD_3$ content compared to its level in the animals with experimental diabetes (Table 1). Investigation of biochemical markers of free radical processes, lipid peroxidation products content and protein oxidative modifications in the liver under vitamin D_3 therapeutic administration, showed a decrease in the intensity of DCF-sensitive ROS and NO formation by 25.1 and 20.6%, respectively (Fig. 1 and 2); TBA-reactive compounds, diene conjugate and protein carbonyl groups content by 33.3, 24.8 and 30.8%, respectively (Table 2); nitrotrozylated proteins by 14.5% (Fig. 3); and increase of the content of thiol compounds by 32.7% (Table 2) compared to animals with diabetes ($P < 0.05$). Inhibition of lipid and protein peroxide modifications and increase of thiols antioxidant capacity under cholecalciferol administration correlated with partial or complete normalization of detoxification enzymes which can enhance prooxidative processes. With the vitamin D_3 treatment, NQO, xanthine oxidase and NADPH-oxidase activities were reduced by 39, 11.5 and 23.7%, respectively, compared with diabetes group ($P < 0.05$), Table 3. The activity of antioxidant enzymes SOD and catalase increased by 19.1 and 14.4%, respectively, while GPO activity

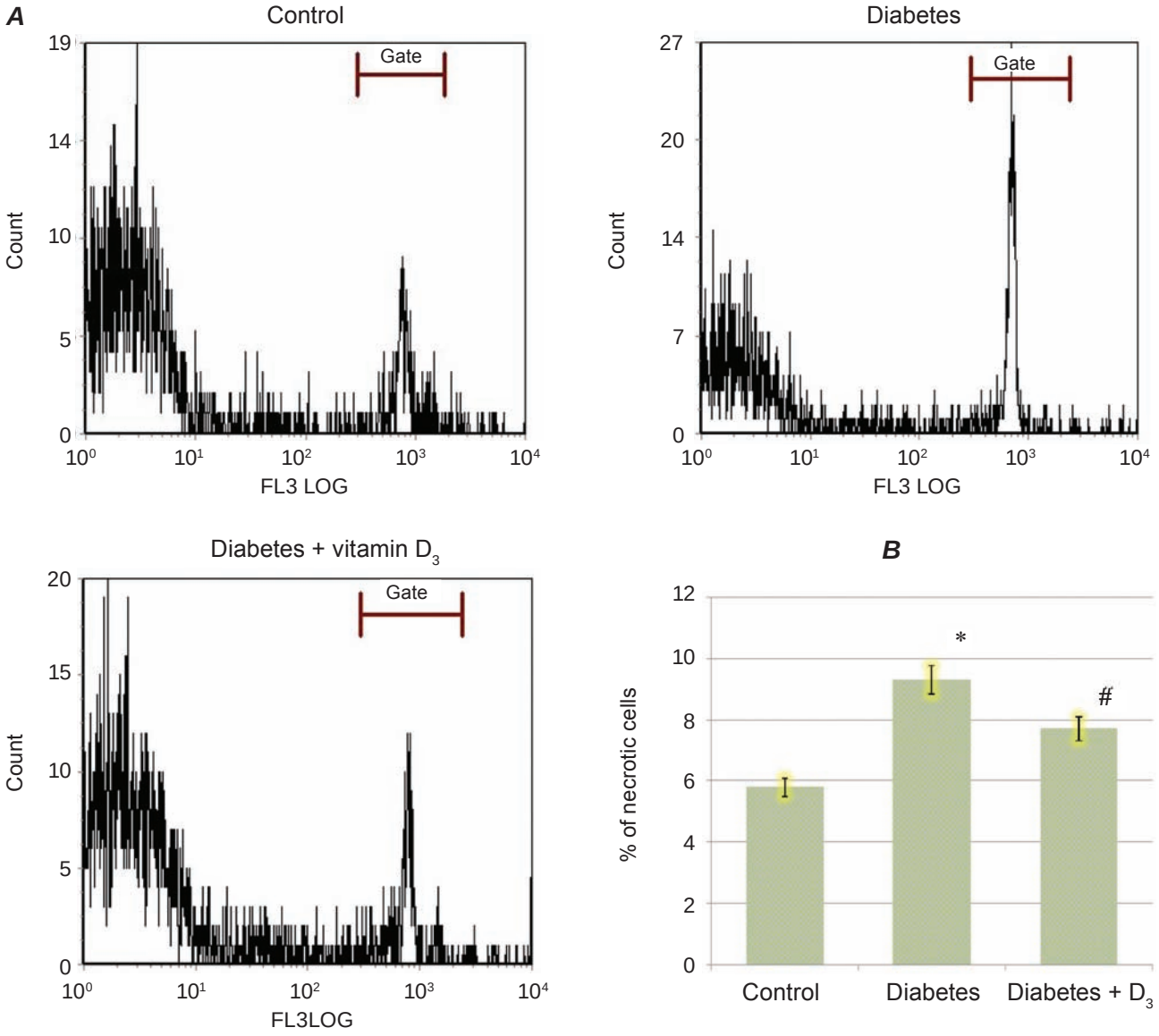

*Fig. 4. The cytofluorograms of necrotic hepatocytes death using propidium iodide dye. (A) Fluorescence cytofluorograms of propidium iodide accumulation (count – the number of events; FL1 LOG – fluorescence intensity) and (B) data quantitative analysis. *P <0.05 compared with control, #P <0.05 compared with diabetes group (M ± m, n = 6)*

level decreased by 32% compared to diabetic mice ($P < 0.05$). It was found out that vitamin D_3 administration significantly increased the hepatocytes survival, reducing the percentage of dead cells, which may be due to inhibition of free radical compounds formation and biomolecules oxidative modification.

These results demonstrate that vitamin D_3 prevents the oxidative stress development in liver cells caused by chronic hyperglycemia. First of all, antioxidant effects of vitamin D_3 are mediated by hormonally active metabolite of cholecalciferol – $1.25(OH)_2D_3$. This multifaceted regulation of oxidative metabolism with vitamin D_3 is caused by complex interactions between several nuclear co-activators or co-repressors that mediate the regulation of gene transcription at the level of their interaction with receptors of hormonally active form of cholecalciferol (VDR). When binding receptors, vitamin D_3 enhances signaling and effectively controls the rate of free radicals formation in mice liver cells [9, 51]. As a natural ligand of VDR, vitamin D_3 influences the development of proinflammatory and prooxidant processes through the gene expression regulation of numerous important cytokines, and

therefore its deficiency increases the risk of many multifactorial polygenic diseases [52].

However, there are data in literature that indicate a significant antioxidant effect of cholecalciferol in mature erythrocytes without nucleus. These findings confirm that vitamin D_3 has the antioxidant activity [10]. It is believed that cholecalciferol may act as a direct membrane antioxidant, stabilizing and protecting them from lipid peroxidation through interaction with their hydrophobic areas [11]. Data on the antioxidant activity of cholecalciferol correlate with the results obtained in several experimental works, which indicated that vitamin D_3 is able to slow down the lipid peroxidation process and enhance the SOD activity in the liver and other organs of mice [51]. The ability of cholecalciferol to protect prostate cells from oxidative stress-induced death through cell damage inhibition associated with increased formation of ROS was also demonstrated [53]. According to the literature, calcitriol (hormonally active form of vitamin D_3) may enhance the pathway of reactive oxygen and nitrogen species elimination, increasing the intracellular pool of reduced glutathione, partially through upstream regulation of glutamate-cysteine ligase (GCL) and glutathione reductase (GR) genes expression [54]. This correlates with the data established in this research that there is increased content of low molecular weight thiols (mostly glutathione) in the liver under the cholecalciferol influence. In addition, vitamin D_3 anti-inflammatory effects can be mediated through inhibition of NF-kB-dependent activation of numerous pro-inflammatory cytokines gene transcription induced by hydroxyl radicals [55].

Thus, the results of experimental studies demonstrate that chronic hyperglycemia conditions are accompanied by the oxidative-nitrosative stress development, increased content of metabolites of oxygen and nitrogen, products of biomacromolecules oxidative modification and decreased activity of antioxidant protection. All these diabetes-induced injuries correlated with lack of adequate vitamin D_3 supply, which is confirmed by a significant decrease in the content of $25OHD_3$ in serum. Cholecalciferol administration to experimental animals greatly inhibits the formation of reactive oxygen and nitrogen in hepatocytes, normalizes metabolic and antitoxic function of liver cells and provides them with better survival. It was established that vitamin D_3 level supply is an essential factor in the normalization of structural and functional abnormalities in hepatocytes caused by prolonged hyperglycemia, which is consistent with the results of the leading laboratories and demonstrates a significant therapeutic potential of vitamin D_3.

УЧАСТЬ ВІТАМІНУ D_3 У РЕГУЛЮВАННІ ОКИСНОГО МЕТАБОЛІЗМУ ПЕЧІНКИ МИШЕЙ ЗА ЦУКРОВОГО ДІАБЕТУ

Д. О. Лабудзинський, О. В. Зайцева, Н. В. Латишко, О. О. Гудкова, М. М. Великий

Інститут біохімії ім. О. В. Палладіна НАН України, Київ;
e-mail: konsument3@gmail.com

Робота присвячена вивченню особливостей окисного метаболізму гепатоцитів мишей за цукрового діабету та дії вітаміну D_3. Встановлено, що хронічна гіперглікемія за цукрового діабету зумовлює зниження у 2,5 раза вмісту $25OHD_3$ у сироватці крові та компенсаторне зростання вітамін D_3 25-гідроксилазної активності гепатоцитів. Розвиток вітамін D_3 дефіцитного стану супроводжується інтенсифікацією продукції активних форм кисню (АФК) та монооксиду азоту (NO), окисної модифікації протеїнів (за вмістом карбонільних груп та 3-нітротирозину), накопиченням дієнових кон'югатів та ТБК-активних продуктів пероксидації ліпідів і зниженням рівня вільних SH-груп низькомолекулярних сполук у печінці. Показано зниження активності ключових ензимів системи антиоксидантного захисту (каталази, СОД), водночас як активність прооксидантних ензимів NAD(P)H:хінон-оксидоредуктази, ксантиноксидази і NAD(P)H оксидази зростала. Виявлені порушення окисного метаболізму обумовили посилення процесу некротичної загибелі гепатоцитів, тестованої за здатністю їхніх ядер до накопичення пропідію йодиду. Тривале введення (протягом 2 місяців) вітаміну D_3 у дозі 20 МО тваринам із цукровим діабетом супроводжується зниженням рівня утворення АФК та окисного ушкодження біомолекул, нормалізує стан системи антиоксидантного захисту в печінці та підвищує виживання гепатоцитів. Результати роботи свідчать про залучення вітаміну D_3 у регулюванні окисного метаболізму за цукрового діабету.

Ключові слова: вітамін D_3, $25OHD_3$, оксидативний стрес, антиоксидантний захист, експериментальний цукровий діабет 1-го типу.

УЧАСТИЕ ВИТАМИНА D_3 В РЕГУЛЯЦИИ ОКИСЛИТЕЛЬНОГО МЕТАБОЛИЗМА ПЕЧЕНИ МЫШЕЙ ПРИ САХАРНОМ ДИАБЕТЕ

Д. О. Лабудзинский, Н. В. Латышко, О. А. Гудкова, О. В. Зайцева, Н. Н. Великий

Институт биохимии им. А. В. Палладина НАН Украины, Киев;
e-mail: konsument3@gmail.com

Работа посвящена изучению особенностей окислительного метаболизма гепатоцитов мышей при сахарном диабете и действии витамина D_3. Установлено, что хроническая гипергликемия при сахарном диабете вызывает снижение в 2,5 раза содержания $25OHD_3$ в сыворотке крови и компенсаторный рост витамин D_3 25-гидроксилазной активности гепатоцитов. Развитие витамин D_3 дефицитного состояния сопровождается интенсификацией продукции активных форм кислорода (АФК) и монооксида азота (NO), окислительной модификации протеинов (по содержанию карбонильных групп и 3-нитротирозина), накоплением диеновых конъюгатов и ТБК-активных продуктов пероксидного окисления липидов, а также снижением уровня свободных SH-групп низкомолекулярных соединений в печени. Показано уменьшение активности ключевых энзимов системы антиоксидантной защиты (каталазы, СОД), в то время как активность прооксидантных энзимов NAD(P)H: хинон-оксидоредуктазы, ксантиноксидазы и NAD(P)H оксидазы возросла. Выявленные нарушения окислительного метаболизма обусловили усиление процесса некротической гибели гепатоцитов, тестируемой по способности их ядер к накоплению пропидий йодида. Длительное введение (в течение 2 месяцев) витамина D_3 в дозе 20 МЕ животным с сахарным диабетом сопровождается снижением уровня образования АФК и окислительного повреждения биомолекул, нормализует состояние системы антиоксидантной защиты в печени и повышает выживаемость гепатоцитов. Результаты работы свидетельствуют об участии витамина D_3 в регуляции окислительного метаболизма при сахарном диабете.

Ключевые слова: витамин D_3, $25OHD_3$, оксидативный стресс, антиоксидантная защита, экспериментальный сахарный диабет 1-го типа.

References

1. Salsali A., Nathan M. A review of types 1 and 2 diabetes mellitus and their treatment with insulin. *Am. J. Ther.* 2006;13(4):349-361.
2. Jakus V., Rietbrock N. Advanced glycation end-products and the progress of diabetic vascular complications. *Physiol. Res.* 2004;53(2):131-142.
3. Pazdro R., Burgess J. R. The role of vitamin E and oxidative stress in diabetes complications. *Mech. Ageing Dev.* 2010;131(4):276-286.
4. Shymanskyy I., Kuchmerovska T., Donchenko H., Veliky M., Klymenko A., Palyvoda O., Kuchmerovskyi M. Oxidative stress correction by nicotinamide and nicotynol-GABA in diabetic neuropathy. *Ukr. Biokhim. Zhurn.* 2002;74(5):89-95.
5. Desco M. C., Asensi M., Márquez R., Martínez-Valls J., Vento M., Pallardó F. V., Sastre J., Viña J. Xanthine oxidase is involved in free radical production in type 1 diabetes: protection by allopurinol. *Diabetes.* 2002;51(4):1118–1124.
6. Obrosova I. G., Drel V. R., Pacher P., Ilnytska O., Wang Z. Q., Stevens M. J., Yorek M. A. Oxidative-nitrosative stress and poly(ADP-ribose) polymerase (PARP) activation in experimental diabetic neuropathy: the relation is revisited. *Diabetes.* 2005;54(12):3435–3441.
7. Schreiber V., Dantzer F., Ame J.C., de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. *Nat. Rev. Mol. Cell Biol.* 2006;7(7):517-528.
8. Giacco F., Brownlee M. Oxidative stress and diabetic complications. *Circ. Res.* 2010;107(9):1058-1070.
9. Bouillon R., Lieben L., Mathieu C., Verstuyf A., Carmeliet G. Vitamin D action: lessons from VDR and Cyp27b1 null mice. *Pediatr. Endocrinol. Rev.* 2013;10(2):354-366.
10. Wolden-Kirk H., Gysemans C., Verstuyf A., Mathieu C. Extraskeletal effects of vitamin D. *Endocrinol. Metab. Clin. North Am.* 2012;41(3):571-594.
11. Wiseman H. Vitamin D is a membrane antioxidant. Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and

relevance to anticancer action. *FEBS Lett.* 1993;326(1-3):285-288.
12. Lapach S. N., Chubenko A. V., Babich P. N. Statistical methods in biomedical studies using Excel. K.: Morion. 2000; p.320 (In Russian).
13. Seglen P. Preparation of isolated rat liver cells. In Methods in cell Biology. Ed. D. M. Prescott. New York: Academic press. 1987; P. 29-83.
14. Das J., Ghosh J., Manna P., Sil P. C. Taurine provides antioxidant defense against NaF-induced cytotoxicity in murine hepatocytes. Pathophysiology. 2008;15(3):181-190.
15. Gracia-Sancho J., Laviña B., Rodríguez-Vilarrupla A. Increased oxidative stress in cirrhotic rat livers: A potential mechanism contributing to reduced nitric oxide bioavailability. *Hepatology.* 2008;47(4):1248-1256.
16. Ning B., Bai M., Shen W. Reduced glutathione protects human hepatocytes from palmitate-mediated injury by suppressing endoplasmic reticulum stress response. *Hepatogastroenterology.* 2011;58(110-111):1670-1679.
17. Zaitseva O. V., Shandrenko S. G. Modification of spectrophotometric method of determination of protein carbonyl groups. *Ukr. Biokhim. Zhurn.* 2012;84(5):112-116. (In Ukrainian).
18. Janero D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Rad. Biol. Med.* 1990;9(6):515-540.
19. Hissin P. J., Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* 1976;74:214-226.
20. Eriksson U. J., Borg L. A. Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformations in vitro. *Diabetologia.* 1991;34(5):325-331.
21. Beers R. F., Sizer I. W. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 1952;195(1):133-140.
22. Moin V. M. A simple and specific method for determining glutathione peroxidase activity in erythrocytes. *Lab. Delo.* 1986;12:724-727. (In Russian).
23. Petrova G. V., Donchenko G. V. Cytotoxicity of troglitazone, a structural analogue of α-tocopherol is mediated by inhibition of NAD(P)H:Quinone oxidoreductase. *Ukr. Biokhim. Zhurn.* 2009;81(4):105-111. (In Russian).
24. Heinz F., Reckel S., Kalden J. R. A new spectrophotometric assay for enzymes of purine metabolism. Determination of xanthine oxidase activity. *Enzyme.* 1979;24(4):239-246.
25. Murillo M. M., Carmona-Cuenca I., Del Castillo G., Ortiz C., Roncero C., Sánchez A., Fernández M., Fabregat I. Activation of NADPH oxidase by transforming growth factor-beta in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands through a nuclear factor-kappaB-dependent mechanism. *Biochem. J.* 2007;405(2):251-259.
26. Labudzynskyi D. O., Shymanskyy I. O., Riasnyi V. M., Veliky M. M. Vitamin D_3 availability and functional activity of peripheral blood phagocytes in experimental type 1 diabetes. *Ukr. Biochem. J.* 2014;86(2):107-118.
27. Harper D. R., Murphy G. Nonuniform variation in band pattern with luminol/horseradish peroxidase western blotting. *Anal. Biochem.* 1991;192(1):59-63.
28. Pramyothin P., Holick M. Vitamin D supplementation: guidelines and evidence for subclinical deficiency. *Curr. Opin Gastroenterol.* 2012;28(2):139-150.
29. Rehman A., Nourooz-Zadeh J., Möller W., Tritschler H., Pereira P., Halliwell B. Increased oxidative damage to all DNA bases in patients with type II diabetes mellitus. *FEBS Lett.* 1999;448(1):120-122.
30. Wolff S. P. Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. *Br. Med. Bull.* 1993;49(3):642-652.
31. Rajwani A., Ezzat V., Smith J., Yuldasheva N. Y., Duncan E. R., Gage M., Cubbon R. M., Kahn M. B., Imrie H., Abbas A., Viswambharan H., Aziz A., Sukumar P., Vidal-Puig A., Sethi J. K., Xuan S., Shah A. M., Grant P. J., Porter K. E., Kearney M. T., Wheatcroft S. B. Increasing circulating IGFBP1 levels improves insulin sensitivity, promotes nitric oxide production, lowers blood pressure, and protects against atherosclerosis. *Diabetes.* 2012;61(4):915-924.
32. Kahn N. N., Acharya K., Bhattacharya S., Acharya R., Mazumder S., Bauman W. A., Sinha A. K. Nitric Oxide: The "Second

Messenger" of Insulin. *IUBMB Life*. 2000;49(5):441-450.
33. Varvarovská J., Racek J., Stetina R., Sýkora J., Pomahacová R., Rusavý Z., Lacigová S., Trefil L., Siala K., Stozický F. Aspects of oxidative stress in children with type 1 diabetes mellitus. *Biomed. Pharmacother.* 2004; 58(10):539-45.
34. Gubskiy U. I., Belenichev I. F., Pavlov S. V. Toxicological effects of oxidative modification of proteins in various pathological conditions (review). *Sovr. Probl. Toks.* 2005;3:20-26. (In Russian).
35. Tang V. M., Young A. H., Tan H., Beasley C., Wang J. F. Glucocorticoids Increase Protein Carbonylation and Mitochondrial Dysfunction. *Horm. Metab. Res.* 2013;l45(10):709-715.
36. Drel V. R., Pacher P., Vareniuk I., Pavlov I., Ilnytska O., Lyzogubov V. V., Tibrewala J., Groves J. T., Obrosova I. G. A peroxynitrite decomposition catalyst counteracts sensory neuropathy in streptozotocin-diabetic mice. *Eur. J. Pharmacol.* 2007;569(1-2):48–58.
37. Ingaramo P. I., Ronco M. T., Francés D. E., Monti J. A., Pisani G. B., Ceballos M. P., Galleano M., Carrillo M. C., Carnovale C. E. Tumor necrosis factor alpha pathways develops liver apoptosis in type 1 diabetes mellitus. *Mol. Immunol.* 2011;48(12-13):1397-1407.
38. Gérard-Monnier D., Chaudiere J. Metabolism and antioxidant function of glutathione. *Pathol. Biol. (Paris).* 1996;44(1):77-85.
39. Fiorentino T., Prioletta A., Zuo P., Folli F. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Curr. Pharm. Des.* 2013;19(32): 5695-5703.
40. Yim M. B., Chock P. B., Stadtman E. R. Copper, zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. *Proc. Natl. Acad. Sci. USA.* 1990;87(13):5006-5010.
41. Dalle-Donne I., Rossi R., Giustarini D., Colombo R., Milzani A. S-glutathionylation in protein redox regulation. *Free Rad. Biol. Med.* 2007;43(6):883-898.
42. Murphy M. Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. *Antioxid. Redox Signal.* 2012;16(6):476-495.
43. Domínguez L., Sosa-Peinado A., Hansberg W. Catalase evolved to concentrate H_2O_2 at its active site. *Arch. Biochem. Biophys.* 2010;500(1):82-91.
44. Thomas J. P., Maiorino M., Ursini F., Girotti A. W. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *J. Biol. Chem.* 1990;265(1):454-461.
45. Lind C., Cadenas E., Hochstein P., Ernster L. DT-diaphorase: purification, properties, and function. *Methods Enzymol.* 1990;186:287–301.
46. Cheng Q., Aleksunes L. M., Manautou J. E., Cherrington N. J., Scheffer G. L., Yamasaki H., Slitt A. L. Drug-metabolizing enzyme and transporter expression in a mouse model of diabetes and obesity. *Mol. Pharm.* 2008;5(1):77-91.
47. Watanabe N., Dicknson D. A., Liu R. M., Forman H. J. Quinones and glutathione metabolism. *Methods Enzymol.* 2004;378:319-340.
48. Nomura J., Busso N., Ives A., Matsui C., Tsujimoto S., Shirakura T., Tamura M., Kobayashi T., So A., Yamanaka Y. Xanthine oxidase inhibition by febuxostat attenuates experimental atherosclerosis in mice. *Sci. Rep.* 2014;4(4554):1-9.
49. De Minicis S., Brenner D.A. Oxidative stress in alcoholic liver disease: role of NADPH oxidase complex. *J. Gastroenterol. Hepat*ol. 2008;1:98-103.
50. Crosas-Molist E., Bertran E., Sancho P., López-Luque J., Fernando J., Sánchez A., Fernández M., Navarro E., Fabregat I. The NADPH oxidase NOX4 inhibits hepatocyte proliferation and liver cancer progression. *Free Radic. Biol. Med.* 2014;69:338-347.
51. Zhong W., Gu B., Gu Y., Groome L. J., Sun J., Wang Y. Activation of vitamin D receptor promotes VEGF and CuZn-SOD expression in endothelial cells. *J. Steroid Biochem. Mol. Biol.* 2014;140:56-62.
52. De Borst M. H., de Bore R. A., Stolk R. P., Slaets J. P., Wolffenbuttel B. H., Navis G. Vitamin D deficiency: universal risk factor for multifactorial diseases? *Curr. Drug Targets.* 2011;12(1):97-106.
53. Bao B. Y., Ting H. J., Hsu J. W., Lee Y. F. Protective role of 1α, 25-dihydroxy vitamin D_3 against oxidative stress in nonmalignant

human prostate epithelial cells. *Int. J. Cancer.* 2008;122(12):2699-2706.

54. Jain S., Micinski D. Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes. *Biochem. Biophys. Res. Commun.* 2013;437(1):7-11.
55. Luong K. V., Nguyen L. T. The role of vitamin D in autoimmune hepatitis. *J. Clin. Med. Res.* 2013;5(6):407-415.

Received 02.12.2014