ORIGINAL ARTICLE

ARGININE/CITRULLINE CYCLE CHANGES IN DIET-INDUCED RAT MODEL OF NON-ALCOHOLIC FATTY LIVER DISEASE

DOI: 10.36740/WLek202006101

Igor M. Skrypnyk, Ganna S. Maslova, Roman I. Skrypnyk, Oleksandr F. Gopko, Tetiana V. Lymanets
UKRAINIAN MEDICAL STOMATOLOGICAL ACADEMY, POLTAVA, UKRAINE

ABSTRACT

The aim: to study the Arginine / Citrulline-cycle features on a diet-induced rat model of non-alcoholic fatty liver disease (NAFLD).

Materials and methods: The studies were carried out on 20 white non-linear adult rats, including 10 (50%) males, 10 (50%) females, weighing 160-220 g. NAFLD was modeled by a 9-week fast food diet. The level of arginine and citrulline, and the arginase activity were investigated in the animals' liver homogenates and in the blood. The morphological analysis of liver tissues changes was done.

Results: NAFLD modeling using a 9-week fast food diet resulted in maximum weight gain in male rats ($p<0.05$). Female rats had 3 times more accumulation of intra-abdominal fat than male rats in the main group ($p<0.05$). Histopathologic liver examination confirmed NAFLD development in rats on a fast food diet during 9 weeks ($p<0.05$). NAFLD led to an increment of arginine level in the blood and liver homogenate in the main group compared to controls ($p<0.05$). NAFLD development was accompanied by a decrease in arginase activity and citrulline level in the blood and liver homogenate compared to control ($p<0.05$).

Conclusions: The experimental rat model of NAFLD showed the Arginine / Citrulline cycle disorders, that were characterized by an increased arginine level, a decreased arginase activity and citrulline concentration in the blood and liver tissues.

KEY WORDS: non-alcoholic fatty liver disease, arginine, arginase, citrulline

Wiad Lek. 2020;73(6):1087-1092

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most pressing medical and social problems, due to its high prevalence worldwide, especially in economically developed countries [1, 2]. NAFLD includes a range of diseases from liver steatosis and non-alcoholic steatohepatitis (NASH) to cirrhosis and hepatocellular carcinoma. Nowadays, the leading mechanisms of lipid accumulation in hepatocytes, the role of oxidative stress and mitochondrial dysfunction in the NASH progression, the development of liver fibrosis and cirrhosis have been identified [1, 3, 4].

The arginine amino acid has a variety of biosynthetic functions due to its participation in protein synthesis, urea cycle, synthesis of polyamines, creatine and nitric oxide (NO) [5, 6, 7, 8]. In terms of the pathogenesis of obesity, metabolic syndrome, as well as the mechanisms of NASH development, the reaction of arginine conversion to NO and citrulline catalyzed by the NO-synthase (NOS) system, and the reaction of arginine metabolism into ornithine with the arginase participation, are of particular importance [9]. Citrulline is an amino acid precursor to arginine. At the same time, the citrulline can itself be converted to arginine, closing the Citrulline / NO-cycle or Arginine / Citrulline-cycle. The processes of arginine conversion are competitively affected by NOS and arginase systems [5, 7, 10, 11, 12, 13]. Arginase is a regulatory enzyme that determines the arginine bioavailability for NO, polyamines, agmatine, proline, and glutamate synthesis [7, 14, 15]. Experimental studies have shown that arginase deficiency contributes to the NASH development against a high-calorie diet [15]. From this point of view, the Arginine / Citrulline cycle may be very important in the study of the main mechanisms of NAFLD.

THE AIM

The aim – to study the Arginine / Citrulline-cycle features on a diet-induced rat model of NAFLD.

MATERIALS AND METHODS

The studies were carried out on 20 white non-linear adult rats, including 10 (50%) males, 10 (50%) females, weighing 160-220 g. All experimental animals were divided into two series of experiments. The first series of experiments included 10 rats (5 males and 5 females) with NAFLD modeling. The second series – included 10 intact animals (5 males and 5 females) that made up the control group. The experimental animals were divided into two groups:

I (n=10) – main group, with NAFLD modeling;

II (n=10) – control group, fed with a standard vivarium diet;

Additionally, experimental animals were divided into subgroups according to gender: subgroup A – males; subgroup B – females.

Table I. Anthropometric indices of rats after 9 week NAFLD modeling, (M±m)

Studied groups	Weight, g	Height, cm	BMI, kg/m2	VF, g
I-A (n=5)	275.2±3.86	22.9±0.36	5.38±0.15	3.07±0.02
p	p_1=0.0003 p_3=0.0001	p_1>0.05 p_3>0.05	p_1=0.0004 p_3>0.05	p_3=0.0095
I-B (n=5)	217.8±6.86	21.1±0.29	5.03±0.11	9.25±1.32
p	p_2=0.01	p_2>0.05	p_2=0.0002	-
II-A (n=5)	216.6±6.82	22.8±0.37	4.1±0.09	-
p	p_2>0.05 p_4=0.01	p_2>0.05 p_4>0.05	p_2>0.05 p_4>0.05	-
II-B (n=5)	186.2±5.93	21.3±0.37	4.08±0.09	-

Notes: VF –visceral fat,
p_1 – significant difference between indicators in I-A and II-A subgroups;
p_2 – significant difference between I-B and II-B subgroups;
p_3 – significant difference between I-A and I-B subgroups;
p_4 – significant difference between II-A and II-B subgroups.

The experiment lasted for 9 weeks. The control group rats received the standard vivarium diet throughout the observation period, which included the following components based on 1 animal per day: granulated feed concentrate 0.04 kg, fat-free cheese 0.006 kg, carrots 0.02 kg, cabbage 0.015 kg. The energy value of the standard diet was 93.1 kcal.

NAFLD was modeled in rats by fast food, using a high-fat diet and 4% aqueous fructose solution. Vegetables were excluded from the standard diet. A high-calorie diet was used, which, per animal, included a granulated feed concentrate 0.04 kg (calorie content 19.6 kcal per 0.01 kg), butter 72.5% 0.01 kg (calorie content 66.2 kcal per 0.01 kg), refined sunflower oil 0.01 kg (calorie content 89.9 kcal per 0.01 kg), palm oil 0.01 kg (calorie content 89.9 kcal per 0.01 kg). The energy value of the diet for NAFLD modeling was 324.4 kcal. A 4% aqueous fructose solution was used as the sole source of fluid for rats. The experimental animals were weighed 10 times: before being included in the experiment and once a week (every Tuesday) throughout the observation period. Weight and height of the animals were measured, and the body mass index (BMI) was calculated using the following formula: BMI = weight (kg) / height (m^2).

Decapitation of rats was performed under thiopental anesthesia after 9 weeks of observation. The visceral fat mass was evaluated. These indicators were compared between the main and control groups regarding to gender characteristics. 10% liver homogenate was prepared. The blood sampling was performed. The levels of arginine, citrulline, and arginase activity in the liver homogenate and blood were determined in tested animals. Liver morphological changes were examined by light microscopy by analyzing paraffin blocks sections stained with hematoxylin-eosin.

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.

RESULTS

At baseline the weight in the comparison groups of males and females did not differ significantly (p>0.05). Rats' weight in subgroup I-A was 212.8±8.5 g, in subgroup I-B – 183.1±6.7 g, in subgroup II-A – 193.8±7.2 g, in subgroup II-B – 170.8±2.5 g. After NAFLD modeling using fast food diet in males in subgroup I-A weight gain was 62.3±5.4 g, in females in subgroup I-B – 29.3±5.9 g. In rats of the control group, fed with standard vivarium diet, in subgroup II-A weight gain was 19.2±7.1 g, in subgroup II-B – 14.8±4.7 g. Thus, in subgroup I-A weight gain 3.2 times exceeded this indicator in subgroup II-A (p <0.05). The weight of rats in subgroup I-A was 1.3 times greater than in subgroup II-A (p<0.05) (Tab. I).

Weight gain in subgroup I-B was 2 times greater than in subgroup II-B (p<0.05). There was no significant difference between the weight ratios of female rats in subgroups I-B and II-B (p>0.05) (Tab. I). Significant differences were found in terms of weight between male and female rats of the main group. The weight in subgroup I-A was 1.26 times higher than the weight in subgroup I-B (p <0.05) (Tab. I). Thus, maximum weight gain and weight were observed in male rats receiving 9-week fast food diet.

BMI of male rats in subgroup I-A was 1.3 times higher than in subgroup II-A (p<0.05) (Tab. I). BMI of female rats in subgroup I-B exceeded 1.2 times (p<0.05) the subgroup II-B indicator (Tab. I). Visceral fat was only found in group I animals. In female rats, visceral fat weight was 3 times higher than in male rats (p<0.05) (Tab. I).

Morphologically NAFLD development was confirmed in rats of the main group, fed with 9-week fast food diet (Fig. 1).

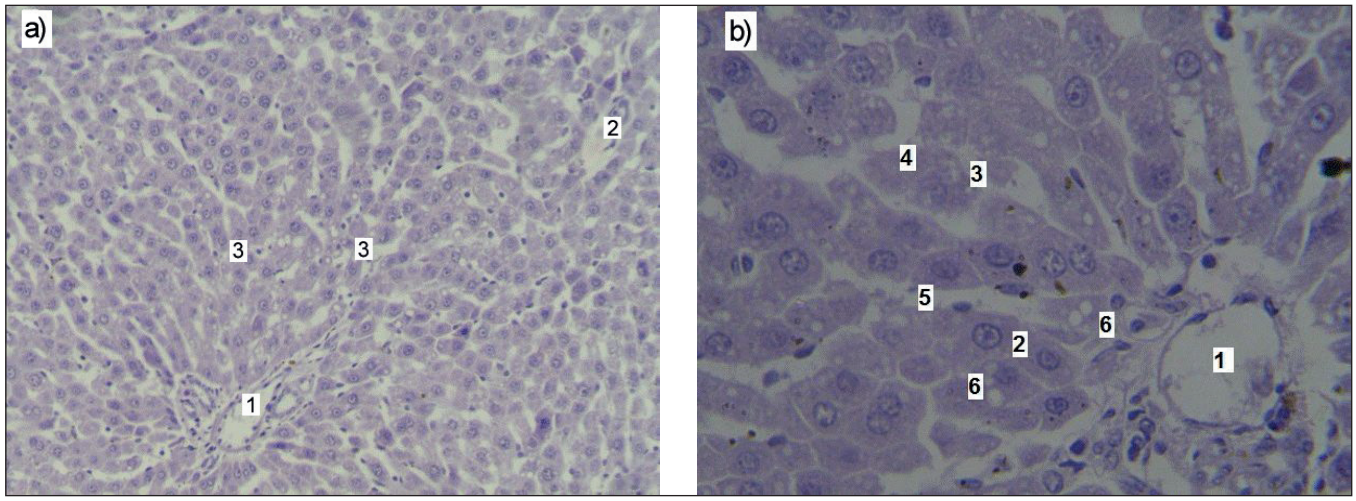

Fig. 1. a) General view of hepatic lobule structure in rats with liver steatosis model. Stained with hematoxylin-eosin, x 200 (1 – portal triad; 2 – central vein; 3 – lipid inclusions);
b) Periportal hepatic lobule part in rats with liver steatosis model. Stained with hematoxylin-eosin, x 600 (1 – portal vein, 2 – hepatocyte with karyolysis and large, optically empty, vacuoles (lipid droplets); 3 – decomposition of hepatic cords due to hepatocytes destruction; 4 – hepatocyte nucleus in the karyolysis phase; 5 – constricted sinusoidal capillaries; 6 – numerous large optically empty vacuoles (lipid droplets).

Changes in periportal and pericentral areas of the acinus, characterized by the presence of hepatocyte karyolysis, decomposition of the hepatic cords due to hepatocytes' destruction, numerous large, optically dense vacuoles (lipid droplets) were revealed during the liver morphological analysis in rats with NAFLD model (Fig. 1a, 1b). In the acinus periportal areas, there were constricted sinusoidal capillaries due to hepatocyte cytoplasm swelling. There is a dramatic sinusoidal dilatation in the acinus pericentral areas (Fig. 1a, 1b).

There was an increased arginine level in the blood of the main group of rats with NAFLD model compared with the control group. In subgroup I-A male rats, arginine level in the blood increased 2-fold compared with subgroup II-A (0.13±0.015 vs 0.064±0.005) mmol/l ($p<0.05$) (Fig. 2c). In subgroup I-B female rats, arginine in the blood increased 2.6-fold times compared to subgroup II-B (0.136±0.014 vs 0.052±0.012) mmol/l ($p<0.05$) (Fig. 2c). There was a tendency to increase the arginine level in rat liver homogenate in the main group compared to the control ($p>0.05$). Thus, in subgroups I-A and I-B the arginine level in liver homogenate was 0,371±0,041 and 0,413±0,021 mmol/g, and in subgroups II-A and II-B – 0,27±0,054 and 0,27±0,023 mmol/g, respectively (Fig. 2a).

Arginase activity in the blood of main group rats in subgroup I-A decreased 2.4-fold compared with subgroup II-A (21.00±3.65 vs 51.40±10.86) μmol/ml ($p<0.05$), in subgroup I-B – 2.1-fold compared to subgroup II-B (14.67±0.66 vs 31.08±4.59) μmol/ml ($p<0.05$) (Fig. 2d).

Arginase activity in liver homogenate decreased 3.4-fold in male rats of subgroup I-A compared to subgroup II-A (0.866±0.041 vs 2.986±0.227) μmol/g ($p<0.05$) (Fig. 2a). In female rats of subgroup I-B arginase activity in liver homogenate decreased 2.9-fold compared with subgroup II-B (0.747±0.183 vs 2.235±0,303) μmol/g ($p<0.05$) (Fig. 2a). A direct correlation was found between serum and liver tissue arginine in male rats of subgroup I-A with NAFLD modeling ($r=+0.8$; $p<0.05$) (Fig. 3a).

Against the background of NAFLD in the blood of subgroup I-A rats, citrulline level decreased 2.4-fold compared to subgroup II-A (241.8±11.61 vs 585.7± 9.53) μmol/ml ($p<0.05$) (Fig. 2e), and in the blood of subgroup I-B rats – 1.7-fold compared to subgroup II-B (397.9±8.64 vs 669.9±69.48) μmol/ml ($p<0.05$) (Fig. 2e). Simultaneously, citrulline content in the liver homogenate of subgroup I-A was 1.8-fold lower than in subgroup II-A (31.64±3.82 vs 57.26±1.92) μmol/g ($p<0.05$) (Fig. 2f) and in rats of subgroup I-B – was 1.6-fold compared subgroup II-B (33.49±2.81 vs 54.07±1.36) μmol/g ($p<0.05$) (Fig. 2f). The NAFLD development was accompanied by a decreased level of citrulline in the blood and liver tissues compared with the control group. The competitive interaction of arginase and NOS is confirmed by the presence of an inverse correlation between blood arginase activity and citrulline content in liver tissues of male rats with NAFLD ($r=-0.98$; $p<0.05$) (Fig. 3b), as well as a direct correlation between levels of arginine in blood and citrulline in liver tissues ($r=+0.98$; $p<0.05$) (Fig. 3c) and inverse correlation between arginase activity and citrulline content in liver tissues of control subgroup II-A male rats ($r=-0.94$; $p<0.05$) (Fig. 3d).

DISCUSSION

Modeling of NAFLD using a 9-week fast food diet resulted in maximum weight gain of male rats, accompanied by significant BMI growth compared to the control group. However, the mass of intra-abdominal fat increased maximally in female rats of the main group with NAFLD model. This fact may indicate a higher risk of diseases development associated with abdominal obesity in females [4].

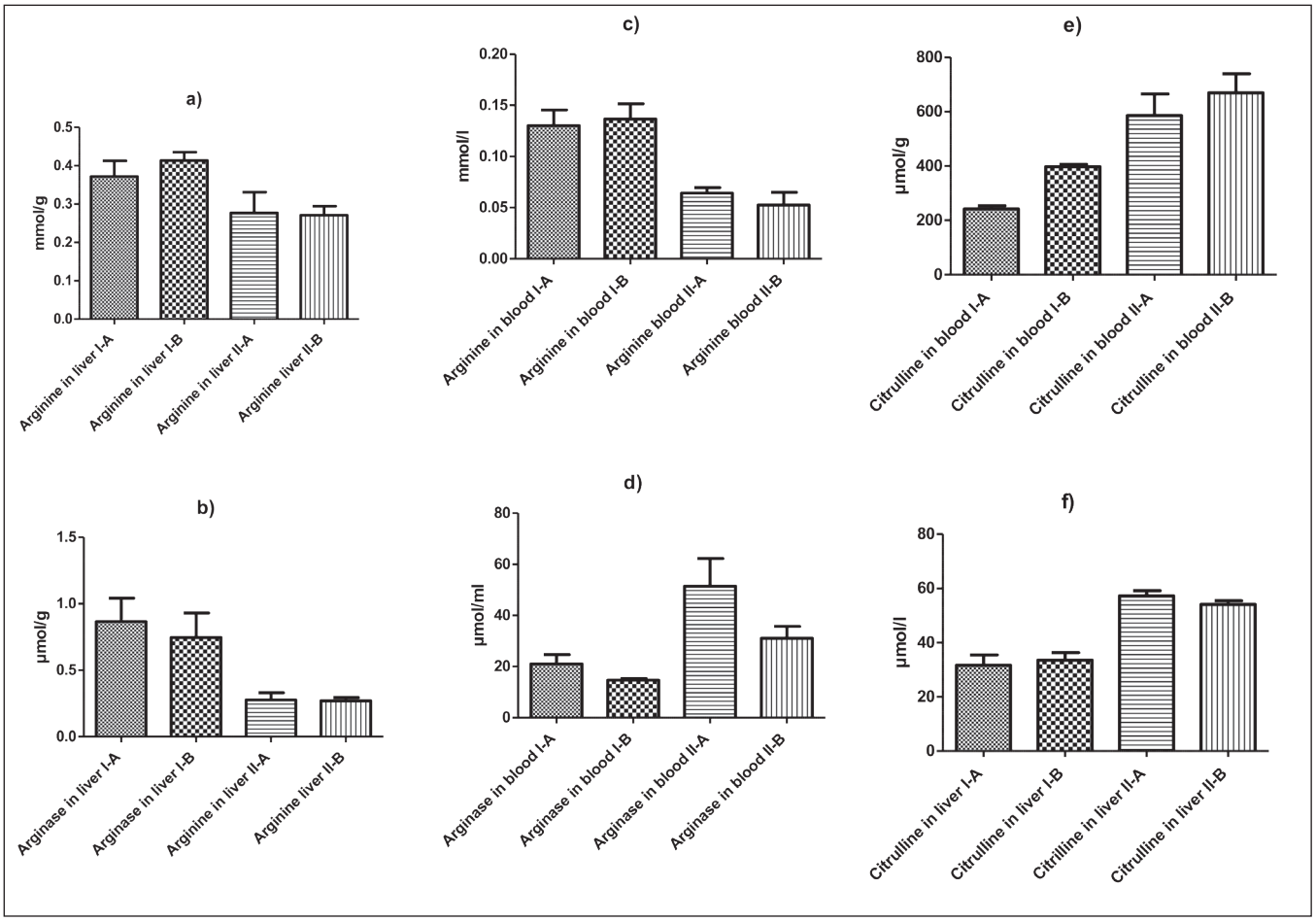

Fig. 2. Arginine/Citrulline-cycle. a) Arginine in liver. b) Arginase in liver. c) Arginine in liver. d) Arginase in blood. e) Citrulline in blood. f) Citrulline in liver. *p<0.05 relative to control (group II).

The use of fast food diet for 9 weeks led to NAFLD histological liver structure transformation, namely, karyolysis of hepatocytes, decomposition of liver cords due to hepatocytes destruction, the presence of numerous large, optically dense vacuoles (lipid droplets), constricted sinusoidal capillaries in the periportal acinus areas due to hepatocyte cytoplasm swelling and sinusoidal dilatation in the pericentral acinus areas. According to the study [1], the use of a high-calorie diet allows to simulate NAFLD in experimental animals with histopathological changes that are similar to humans.

There was an increased arginine content in the blood and liver tissues of experimental animals with NAFLD model. The maximum arginine substrate accumulation in the liver was noted. At the same time, the arginase activity in the blood and liver tissues, which is produced mainly by hepatocytes, was reduced in rats with NAFLD model [5]. Reduced arginase activity in the liver homogenate may indicate impaired liver functionality. The increase in arginine content against the background of NAFLD may be associated with a shift in protein metabolism toward catabolic processes. Reduced arginase activity in liver tissues leads to impaired detoxification of protein catabolism products, as well as reduced ornithine formation. Ornithine is a substrate for polyamines and characterizes the protein-synthesis liver function. That is, NAFLD rat model showed an impaired protein-synthesis and detoxification liver function. It is known that arginine is also a substrate for NO. Reduction of arginine biotransformation with arginase participation leads to metabolic shift to NOS-stimulated citrulline formation. Citrulline may partially replace arginine as a NO substrate donor [5].

Our study results showed the arginase and NO-synthase pathways disruption of arginine metabolism. This fact may be related to the violation of the arginine availability for arginase and NOS [1, 6, 14]. This substrate availability is regulated by transport systems, which provide its recapture. The arginine reuptake is inhibited by L-ornithine, N-aminoethyl-L-ornithine, N-methyl-L-arginine and others [1, 6]. Decreased citrulline is an additional factor that potentiates the progression of liver steatosis [10, 15]. In addition, impaired citrulline formation during the NOS-dependent arginine metabolism reaction can be considered a sign of reduced NO production and endothelial dysfunction.

CONCLUSIONS

The experimental NAFLD model showed an increased arginine level in the blood and liver tissues. The reason for the arginine concentration growth may be a violation of arginine metabolism in the Arginine / Citruline cycle. This opinion is confirmed

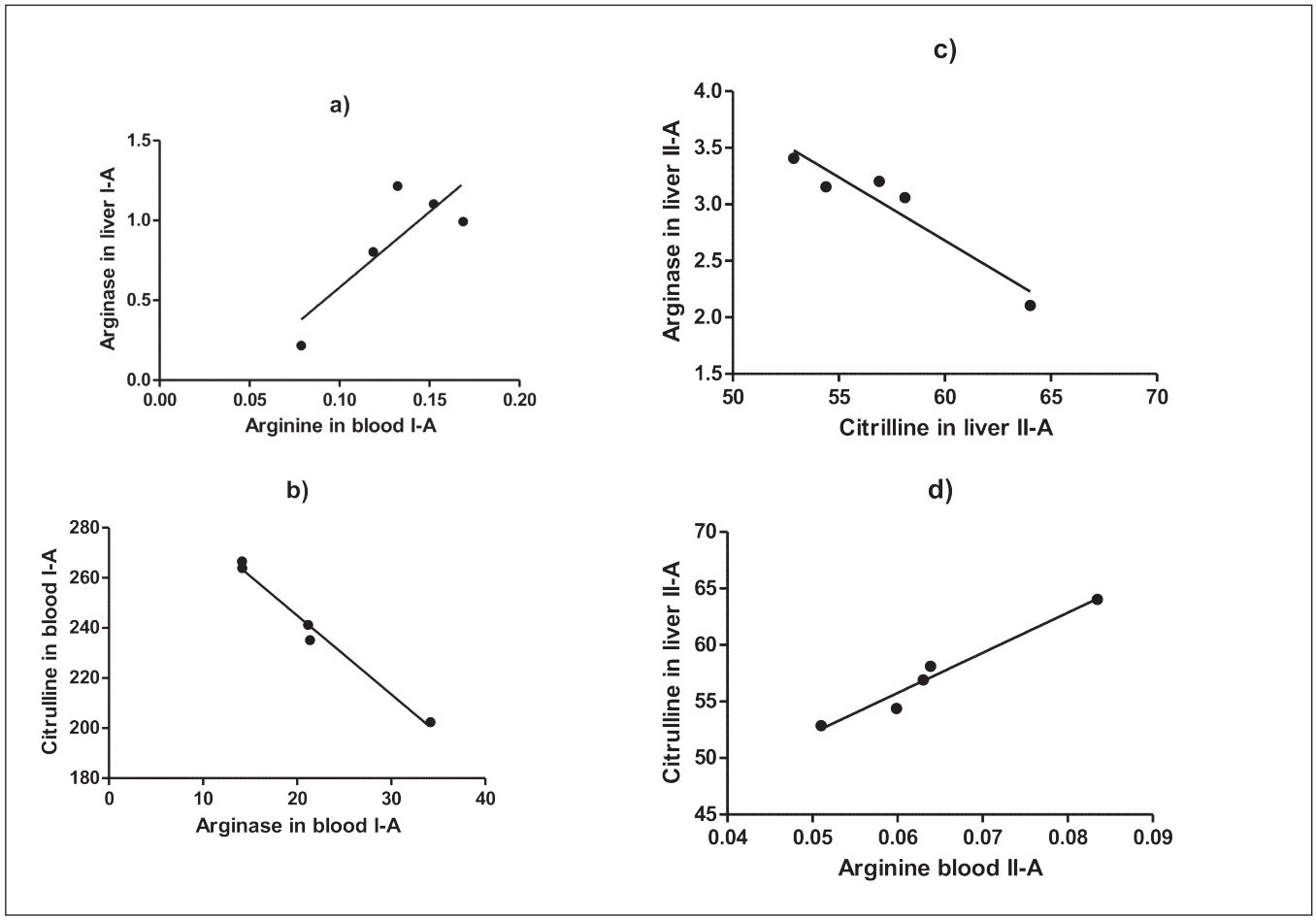

Fig. 3. Arginine/Citrulline-cycle. a) Arginine in liver. b) Arginase in liver. c) Arginine in liver. d) Arginase in blood. e) Citrulline in blood. f) Citrulline in liver. *p<0.05 relative to control (group II).

by decreased arginase activity of in the blood and liver homogenate. At the same time, disorders of arginine metabolism under the influence of NOS system were detected. Citrulline content was decreased in blood and liver tissues in rats with NAFLD model. Thus, it can be considered that Arginine / Citrulline cycle disturbance in rats with NAFLD model was characterized by decreased production of arginase by hepatocytes, as well as low availability of NOS arginine substrate.

REFERENCES

1. Bival`kevich N.V., Denisenko Yu.K., Novgorodczeva T.P. Metodicheskie podkhody` k e`ksperimental`nomu modelirovaniyu nealkogol`noj zhirovoj bolezni pecheni [Methodical approaches in experimental modelling of non-alcoholic fatty liver disease]. RZhGGK. 2015;4:39-45. (In Russian).
2. Hebbard L., George J. Animal models of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol. 2011;8(1):35-44. doi: 10.1038/nrgastro.2010.191.
3. Skrypnyk I.M., Dubrovinska T.V. Optymizatsiia dovhotryvaloho likuvannia rozuvastatynom u khvorykh na infarkt miokarda u poiednanni z nealkoholnym steatohepatytom [Optimization of long-term treatment with rosuvastatin of patients with myocardial infarction in combination with non-alcoholic steatohepatitis]. Likarska sprava 2014; 5-6:113-21. (In Ukrainian).
4. Singh S., Allen A.M., Wang Z. et al. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. Clin Gastroenterol Hepatol. 2015;13(4):643–54. doi: 10.1016/j.cgh.2014.04.014.
5. Granik V.G. Metabolizm L-arginina (obzor) [The metabolism of L-arginine (review)]. Khimiko-farmaczevticheskij zhurnal. 2003;37(3):3-20. (In Russian).
6. Maksymchuk N.O., Konovchuk V.M. Metabolizm arhininu: perspektyvy klinichnoho vykorystannia (ohliad literatury) [Arginine metabolism: prospects for clinical use (review of literature)]. Bukovynskyi medychnyi visnyk. 2017;21(vyp.1(81):205-10. (In Ukrainian).
7. Scheja L., Kluwe J. Arginine and NASH – Do macrophages deliver the first hit?. Journal of Hepatol. 2015;62(2):260-1. doi: 10.1016/j.jhep.2014.11.001
8. Thomas T., Thomas T.J. Polyamine metabolism and cancer. J Cell Mol Med. 2003;7(2):113-126. doi: 10.1111/j.1582-4934.2003.tb00210.x
9. Skrypnyk I., Maslova G., Lymanets T. et al. L-arginine is an effective medication for prevention of endothelial dysfunction, a predictor of anthracycline cardiotoxicity in patients with acute leukemia. Experimental Oncology. 2017;39(4):308-11.
10. Charbonneau A., Marette A. Inducible nitric oxide synthase induction underlies lipid-induced hepatic insulin resistance in mice: potential role of tyrosine nitration of insulin signaling proteins. Diabetes. 2010;59(4):861-71. doi: 10.2337/db09-1238.

11. Dou L., Shi X., He X. et al. Macrophage phenotype and function in liver disorder. Front Immunol. 2020;10:3112. doi: 10.3389/fimmu.2019.03112.
12. Khallou-Laschet J., Varthaman A., Fornasa G. et al. Macrophage plasticity in experimental atherosclerosis. PLoS One. 2010;5(1):e8852. doi: 10.1371/journal.pone.0008852.
13. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009; 158(3): 638–51. doi: 10.1111/j.1476-5381.2009.00291.x.
14. Ming X.F., Rajapakse A.G., Yepuri G. et al. Arginase II promotes macrophage inflammatory responses through mitochondrial reactive oxygen species, contributing to insulin resistance and atherogenesis. J Am Heart Assoc. 2012; 1(4): e000992. doi: 10.1161/JAHA.112.000992.
15. Navarro L.A., Wree A., Povero D. et al. Arginase 2 deficiency results in spontaneous steatohepatitis: a novel link between innate immune activation and hepatic de novo lipogenesis. J Hepatol. 2015; 62(2): 412–20. doi: 10.1016/j.jhep.2014.09.015

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

ORCID and contributorship:
Igor M. Skrypnyk – 0000-0002-3426-3429 [A,E,F]
Ganna S. Maslova – 0000-0002-4729-1736 [A,B,D,E]
Roman I. Skrypnyk – 0000-0003-1828-3371 [B]
Oleksandr F. Gopko – 0000-0003-4116-2869 [C]
Tetiana V. Lymanets – 0000-0001-6021-7066 [B]

Conflict of interest:
The Authors declare no conflict of interest.

CORRESPONDING AUTHOR
Igor M. Skrypnyk
Department of Internal Medicine No 1
Ukrainian Medical Stomatological Academy
23 Shevchenka Str., 36000 Poltava, Ukraine
tel: +380505974908
e-mail: inskrypnyk@gmail.com

Received: 11.03.2020
Accepted: 06.05.2020