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Original scientific paper

The Impact of L-NAME and L-arginine Chronic Toxicity Induced Lesions on Ascites – Pulmonary Hypertension Syndrome Development in Broiler Chickens

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A B S T R A C T

The impact of L-arginine (LA), a precursor for synthesis of nitric oxide (NO), and N-ω -nitro-L-arginine methyl ester (L-NAME, LN), a non-selective inhibitor of the enzyme producing nitric oxide (nitric oxide synthase; NOS) chronic toxicity induced lesions on Ascites – Pulmonary hypertension syndrome (PHS) development was investigated in 140 one-day-old male broiler chickens (ROSS) during the first 5 weeks of life. Every second day the animals were treated intraperitoneally (ip) with L-NAME (10 mg/kg of body weight; BW), L-arginine (100 mg/kg BW), L-arginine and L-NAME in combination (100 mg/kg BW and 10 mg/kg BW, respectively), and with physiological saline (0.90% w/v of NaCl; 0.5 mL/kg BW). Seven birds from each group were euthanized every week. The histopathological examination of the heart, the liver, the lungs, the blood vessels and the lymphoid organs, was performed. Also the organ index values were determined. At the end of the experiment the pre-ascitic condition or ascites – PHS was confirmed in five dead animals in the L-NAME-treated group. In the same group the edema was the most prominent histopathological change confirmed in the heart and in the lungs of the sacrificed chickens. In L-arginine-treated group the congestion and the haemorrhages were the striking changes in the same organs with the highest degree in the last two weeks of trial. While the focal disruption of myocardiofibriole and hepatocytes were predominant lesions in L-NAME-treated chickens ($5^{th}$and 4^{th} weeks, respectively), in L-NAME/L-arginine-treated group only the mild focal myocardial degeneration was seen. According to the most of the results of present investigation, it was concluded that the consecutive treatment with L-NAME provoked ascites – PHS, while L-arginine has protective effect in this animal model of disease.

Key words: *broiler, Ascites – Pulmonary hypertension syndrome (PHS), nitric oxide, L-NAME, L-arginine, histopathology, organ index*

Introduction

Ascites – pulmonary hypertension syndrome (PHS) is a metabolic condition in fast-growing-broilers characterized by an elevated pulmonary vascular resistance (PVR) and pulmonary hypertension (PH), accompained by hypoxemia, and resulting in right ventricular failure (RVF), central venous congestion, and hepatic cirrhosis and ascites[1–9]. As in humans, severe PH leads to severe limitations of functional status and poor survival[10].

Affecting the young broilers (mostly in age of 4–5 weeks, sometimes even earlier in the age of 11–15 days of life[11–12]), and generally causing the mortality from 5–8% in populations worldwide, and even as great as 20 to 30%

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in heavier broiler flocks[13–16], ascites – PHS may cause the significant economic losses in the commercial broiler industry throughout the world, especially if broilers are reared with exposure to cold or at high altitude[12,17–21]. Over the period 1991 to 2009, in broiler chicken production in the Republic of Croatia, the average ascites-PHS mortality rate was 13% of the overall reported infectious and non-infectious disease mortality[22].

Considering these data, one can conclude that the broiler treatment with the pulmonary vasodilatators would reduce the incidence of this cardiovascular disease in broilers. Hypoxia-induced vasoconstriction and subsequent PH was prevented when a dietary L-arginine was used as the main source of a potent endogenous endothelial vasodilatator – nitric oxide in broilers chronically exposed to the cold[23]. The opposite was confirmed in PHS--chickens where the treatment with the L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor, was used[24]. Nitric oxide is a well known physiological and pathophysiological mediator, especially as a potent pulmonary vasodilator in mammals and birds[24–37]. Also the pathogenesis of PHS in broiler chicken is very similar to human idiopathic pulmonary arterial hypertension (IPAH)[38]. In cardiovascular system nitric oxide is produced in blood vessel endothelium, myocardium and endocardium[39], and its impaired endothelial production has been implicated in the pathophysiology of PH in humans[40]. Since the L-NAME was confirmed as a cause of the ascites – PHS[24,29,32,36,37,41–43], and the salutary effect of L-arginine was proved through the measures in prevention and therapy of this disease[40,42,43], we investigated here the effects of the chronic use of these substances in broiler chickens on ascites – PHS development.

Materials and Methods

Chickens and husbandry

One hundred and fourty 1-day-old broiler male ROSS chicks were included in this study and kept in metal wire cages placed in an acclimatised object (temperature of 35°C, relative humidity of 64%). They were allowed 11 days for acclimatization. Food and water were provided *ad libitum*. The animals were fed commercial diet for broilers (»starter« and »finisher«; »Poljoprerada«, Hrvatski Leskovac, Croatia). On the 15th day of life the chickens were vaccinated against Newcastle disease (Pestikal®, LaSota SPF, Pliva, Croatia), using the occulonasal (on) method.

Experimental design and sampling procedures

Chemical substances used in this experiment were L-arginine (ICN Biomedicals, Cleveland, USA), L-NAME (N-ω-nitro-L-arginine methyl ester HCl; ICN Biomedicals, Cleveland, USA) and saline.

Birds were divided into four groups of 35 chickens: 1) control – C; physiological saline – 0.90% w/v of Na Cl (0,5 mL/kg BW); 2) L-arginine – LA; 100 mg/kg BW; 3) L--NAME – LN; 10 mg/kg BW; 4) L-NAME and L-arginine, in combination – L-NAME/Larginine; 10 mg/kg BW and 100 mg/kg BW, respectively. Intraperitoneal (ip) treatment was started on 11th day of life. The animals were treated with the same doses every second day for the next 5 weeks. Seven chicks from each group were killed (a cervical dislocation under chloroform anaesthesia) at the end of every week and necropsied.

Histopathology

For histopathological analysis the tissue samples (the heart, the lungs, the liver, the bursa of Fabricius, the thymus, then spleen, the blood vessels /aorta and pulmonary artery), were fixed in neutral 10%-formalin solution, embedded in paraffin and 4 μm thick sections were prepared (MICROM HM 325, Zeiss, Austria). After deparaffinization, the sections were stained with haematoxylin and eosin (HE). The slices were examined under the light microscope (LEICA DMLB, Germany) and images were captured with digital camera PIXERA Pro 150ES.

Organ index value

Organ index values (the liver-, the heart-, the bursa of Fabricius-, the spleen-, and the thymus – index values) were counted according to the equation:

$$\text{Organ mass / Body mass} \times 1000$$

Statistical analysis

The data were statistically analyzed in accordance with the standard statistical programme (DOS MICROSTAT statistics package Rel. 4.1.06.)[44]. The results obtained by the organ mass-index values data, were statistically analyzed by means of one-way analysis of variance (ANOVA), followed by the Kruskall-Wallis' and the Wilcoxon's test. The Mann-Whitney U-test was used for between two independent groups' comparisons. P values less than 0.05 were considered statistically significant.

Results

Gross lesions

Post-mortem examination did not reveal notable differences between the control and the treated groups of chickens sacrificed at the end of each week. However, five animals from the L-NAME-treated group died after the fifth trial week under the typical signs of ascites – PHS or in pre-ascitic (hypoxic) condition. The gross lesions included: cyanosis of the head and the neck skin and mucous membrane, increased moistness of the thoracic-abdominal cavity or ascites, hydropericardium, dilatation or hypertrophy of the right cardiac ventricle, of the venous sinus and of the *vena cava* in three of five chickens.

Edema and pulmonary hyperemia, an enlarged and hyperemic liver with sporadically pronounced necrosis were noted in all 3 experimental groups of chickens. In the chickens with the so-called preascitic state, cyanosis and thoracic muscular anemia were visible, increased moistness of the thoracic-abdominal cavity, a moderate pulmonary oedema and hepatomegalia.

Histopathology

Heart

While the histopathological examination revealed extremely slight, mostly degenerative changes in the control chicken group (Figure 9), prominent edema and a high degree of parenchymatous degeneration followed by the disruption and necrosis of myofibriole (Figure 1) and myofibrillar disruption and disorganization (Figure 2), as well as focal fibroplasia (Figure 3), were recorded in the L-NAME group at the end of the experiment (5th week). In the trial group treated with L-arginine, the most severe microscopic changes – hyperemia and mostly focal hemorrhages, were seen toward the end of the experiment (from 3rd to 5th trial weeks) (Figure 4). Contrarily, the changes in the animals simultaneously treated with L-arginine and L-NAME were recognized as mild myocardial degeneration and edema.

Lungs

In the lungs, the most striking findings in both L--NAME- and L-arginine-treated groups were perivascular edema and congestion, followed by hemorrhages, especially prominent in the L-arginine-treated chickens at the end of the experiment (Figure 5). Cartilaginous nodules were also recorded from the 3rd to 5th trial week in the L-NAME-treated group (Figure 6), along with the collapsed atria and parabronchial dilatation, and with the hypertrophy of the muscular arteries' wall (Figure 7). Focal mononuclear hyperplasia, the fibrinoid fluid

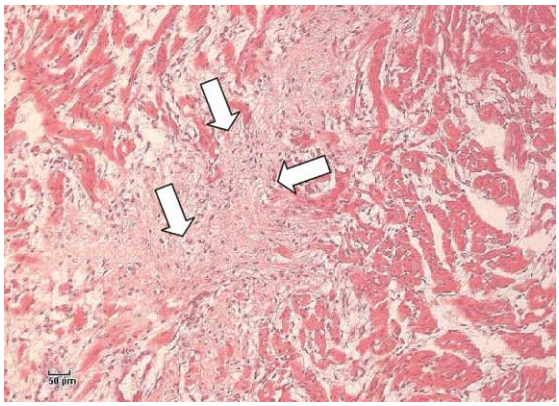

Fig. 3. Focal fibroplasia (arrows) in myocardium (L-NAME--treated group of chickens; 5th wk) (scale bar=50 µm).

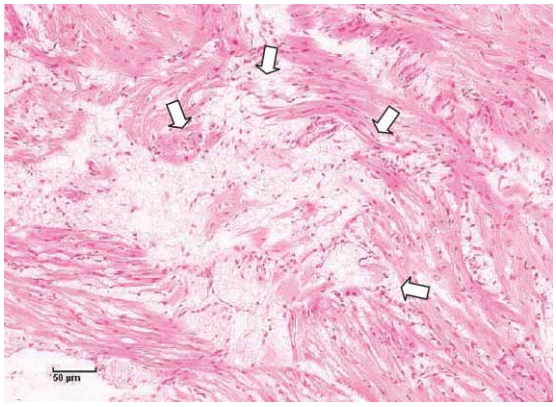

Fig. 1. Diffuse parenchymatous degeneration followed by a prominent interfibrilar edema and focal disruption of myofibriole (arrows) (L-NAME-tretated group of chickens, 5th wk) (scale bar=50 µm).

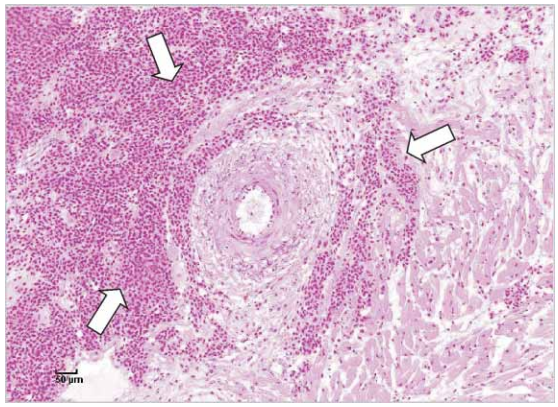

Fig. 4. Hyperemia and mostly focal (perivascular) hemorrhages (arrows) in myocardium (L-arginine-treated group of chickens, 4th wk) (scale bar=50 µm).

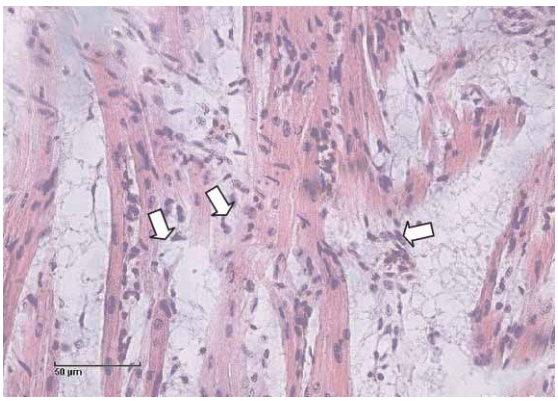

Fig. 2. Disruption and disorganization of the myocardial fibres (arrows) (L-NAME-treated group of chickens, 5th wk) (scale bar=50 µm).

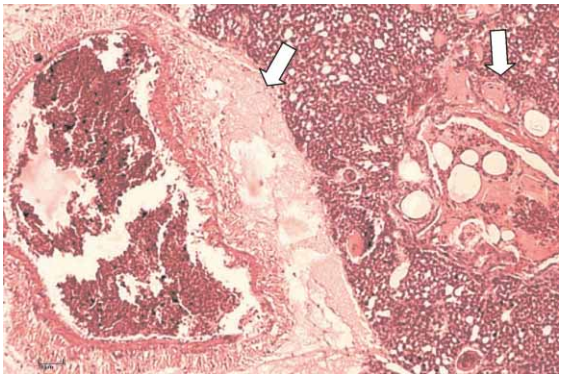

Fig. 5. Lung congestion, perivascular edema and fribrinoid fluid (arrows), followed by hemorrhage (L-arginine-treated group of chickens, 5th wk) (scale bar=50 µm).

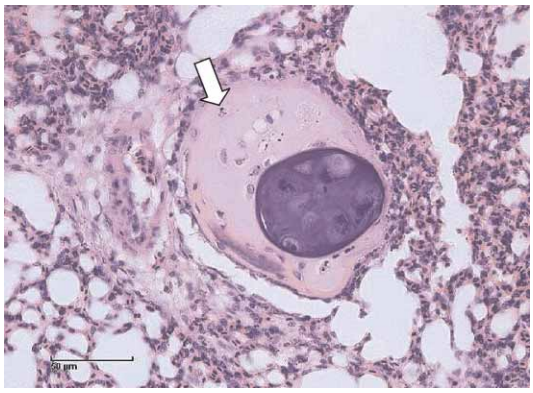

Fig. 6. Cartilaginous nodule in lungs (arrow) (L-NAME-treated group of chickens, 4th wk) (scale bar=50 μm).

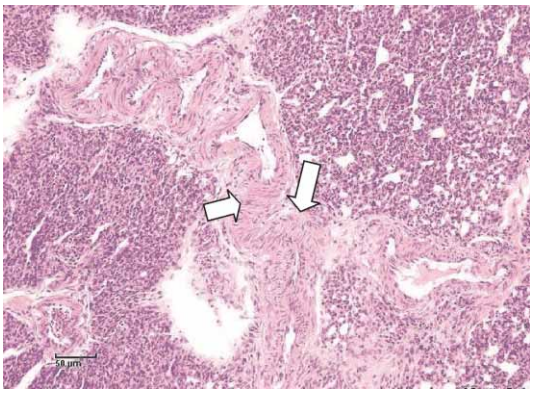

Fig. 7. Hypertrophy of the smooth muscle layer in the small arteries in lungs (arrows) (L-NAME-treated group of chickens, 4th wk) (scale bar=50 μm).

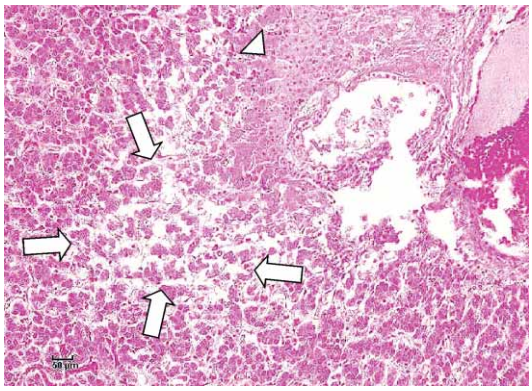

Fig. 8. Perivascular disruption of hepatocytes (arrows) and coagulation necrosis (arrow head) (L-NAME-treated group of chickens, 4th wk) (scale bar=50 μm).

and the parabronchial smooth muscle hypertrophy were seen in the 5th week of the experiment in L-NAME--treated group. Mild to prominent congestion associated with hemorrhages, as well as with the occurrence of cartilaginous nodules in the parenchyma were also seen in the L-arginine-treated group of chickens, these changes being of a higher degree toward the end of the experiment. Just slight to mild edema and congestion were recorded during the experimental period in the L-NAME/ L-arginine-treated group (Figure 10).

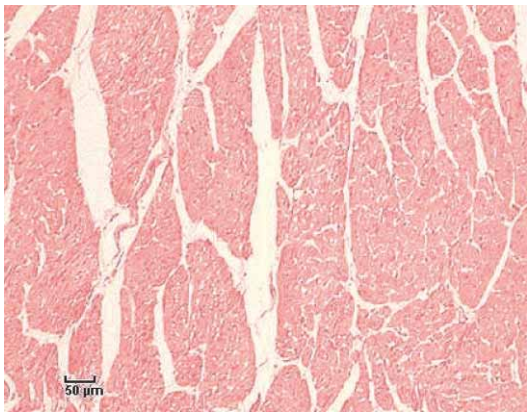

Fig. 9. Heart (control group of chickens, 2nd wk) (scale bar=50 μm).

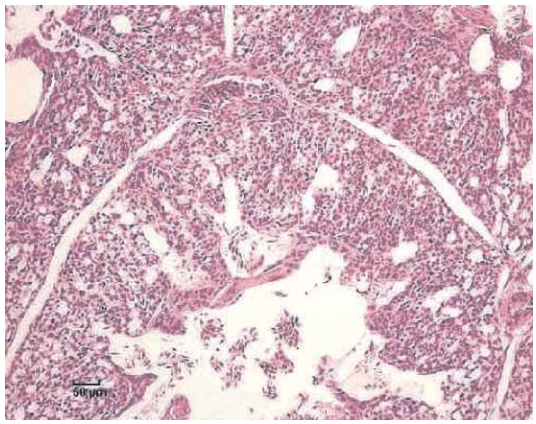

Fig. 10. Lungs no apparent lesions (L-NAME/L-arginine group of chickens, 2nd wk) (scale bar=50 μm).

Liver

The most prominent histopathological changes were seen in the liver in the L-NAME- and in the L-arginine chicken groups. As in the heart and in the lungs, mild to prominent edema (L-NAME-treated group, 5rd wk; Figure 11) and congestion in the L-NAME-treated chickens

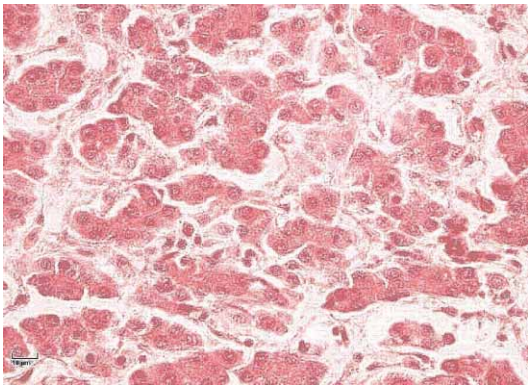

Fig. 11. Prominent interstitial edema in liver (L-NAME group of chickens, 5th wk) (scale bar=10 μm).

along with the hyperemia and hemorrhages of various degree in the L-arginine group, were the most prominent liver changes found in these two trial groups. Also, perivascular disruption of hepatocytes and coagulation necrosis were found in the L-NAME-treated chicken group (4^{th} wk, Figure 8). In the L-NAME/L-arginine-treated chickens mostly mild edema and parenchymatous degeneration were established.

Organ index values

The differences in the mean organ index values as well as the mean body values, the organ index values, standard deviation and the standard error, and the interquartile range in the control and study chicken groups are shown in Tables 1–9.

TABLE 1
STATISTICAL SIGNIFICANCE VALUES OF MEAN LIVER INDEX VALUES IN THE CONTROL AND TREATED GROUPS

Groups	Weeks of trial				
	1	2	3	4	5
C	35.15	29.63	27.45	26.29	24.95
LN	34.79**	29.13	30.36*	24.8#	25.12#
LA	34.09$	28.65	25.96	34.35*	37.08*
LN/LA	29.98*	27.68	24.32**	27.59	34.32*

**, *, #, $– p<0.05; *LN/LA *vs*. C, LN *vs*. C, LA *vs*. C, $LN/LA *vs*. LA; **LN/LA *vs*. LN; #LN *vs*. LA; groups: LN/LA – LN- and L-arginine; LA – L-arginine; LN – L-NAME; C – control

TABLE 2
STATISTICAL SIGNIFICANCE OF MEAN HEART INDEX VALUES IN THE CONTROL AND TREATED GROUPS OF CHICKENS

Groups	Weeks of the experiment				
	1	2	3	4	5
C	6.27	6.70	5.76	5.57	4.67
LN	6.62**	6.32	6.39	5.93	5.21**
LA	6.92	6.97	5.94	5.79	5.65
LN/LA	5.26$	6.88	6.42*	4.74*	6.18$

**, *, $ – p<0.05; *LN/LA *vs*. C; $LN/LA *vs*. LA; **LN/LA *vs*. LN; LN/LA – L-NAME- and L-arginine-treated group; C – control group; LA – L-arginine-treated group; LN – L-NAME-treated group

TABLE 3
STATISTICAL SIGNIFICANCE OF MEAN SPLEEN INDEX VALUES IN THE CONTROL AND TREATED GROUPS OF CHICKENS

Groups	Weeks of trial				
	1	2	3	4	5
C	0.96	0.95	1.77	2.00	1.20
LN	1.07	1.31	1.24	1.73	1.76
LA	1.06	0.81	0.71*	4.64	2.04
LN/LA	0.86	1.05	1.57	2.20	1.76

*p<0.05; *LA *vs*. C; groups: LA – L-arginine, C – control; LN--L-NAME; LN/LA-LN-L-NAME and LA – L-arginine

TABLE 4
STATISTICAL SIGNIFICANCE OF MEAN THYMUS INDEX VALUES IN THE CONTROL AND TREATED GROUPS OF CHICKENS

Groups	Weeks of trial				
	1	2	3	4	5
C	3.75	4.64	3.79	4.9	4.55
LN	3.54	4.24$	3.61	3.75	5.16#
LA	3.02	5.21	3.68	3.18	1.83*
LN/LA	3.47	3.60	4.63	3.59	4.32

$, *, # – p<0.05; $LN/LA *vs*. LA; *LA *vs*. C; #LN *vs*. LA; groups: LN/LA – L-NAME- and L-arginine; LN – L-NAME; LA – L-arginine; C – control

TABLE 5
MEAN BODY WEIGHT AND ORGAN INDEX VALUES, STANDARD DEVIATION (SD), STANDARD ERROR (SE), AND INTERQUARTILE RANGE (M) IN THE CONTROL AND TREATED GROUPS OF CHICKENS (1^{ST} WK OF TRIAL)

Groups		BW (gm)	LI	SI	HI	TI	BFI
C	n	7	7	7	7	7	7
	x	391.21	35.15	0.96	6.27	3.75	3.46
	SD	23.06	5.50	0.33	0.62	1.63	0.82
	SE	8.72	2.08	0.12	0.24	0.62	0.31
	M	390.20	35.38	0.98	6.33	3.33	3.33
LA	n	7	7	7	7	7	7
	x	393.69	34.09	1.06	6.92	3.02	4.00
	SD	55.39	2.70	0.49	0.42	0.47	0.80
	SE	20.94	1.02	0.18	0.16	0.18	0.30
	M	379.30	33.82	1.05	6.95	2.94	4.26
LN	n	7	7	7	7	7	7
	x	409.59	34.79	1.07	6.62	3.54	3.35
	SD	57.02	3.53	1.05	0.53	1.53	0.66
	SE	21.55	1.33	0.40	0.20	0.58	0.25
	M	411.30	35.08	0.75	6.73	3.69	3.33
LN/LA	n	7	7	7	7	7	7
	x	387.00	29.98	0.86	5.26	3.47	3.38
	SD	58.78	2.28	0.44	1.96	1.11	1.02
	SE	22.22	0.86	0.17	0.74	0.42	0.38
	M	362.60	29.47	0.84	5.71	3.14	3.03

Groups: LN/LA – L-NAME- and L-arginine, LN – L-NAME, LA – L-arginine, C – control; Body weight – BW; Liver index – LI, Spleen index – SI, Heart index – HI, Thymus index – TI, Bursa of Fabricius index – BFI; gram – gm; n – number of animals, x – mean values

Discussion and Conclusions

During the whole experimental period there were no notable gross pathomorphological changes in the control and treated groups of chickens, sacrificed at the end of each week. However, at the end of the experiment, five

TABLE 6
MEAN BODY WEIGHT AND ORGAN INDEX VALUES, STANDARD DEVIATION (SD), STANDARD ERROR (SE), AND INTERQUARTILE RANGE (M) IN THE CONTROL AND TREATED GROUPS OF CHICKENS (2nd WK OF TRIAL)

Groups		BW (gm)	LI	SI	HI	TI	BFI
C	n	7	7	7	7	7	7
	x	682.71	29.63	0.95	6.70	4.64	3.75
	SD	44.32	6.41	0.55	0.47	1.34	0.51
	SE	16.75	2.42	0.21	0.18	0.51	0.19
	M	678.00	27.34	1.04	6.72	4.48	4.06
LA	n	7	7	7	7	7	7
	x	649.14	28.65	0.81	6.97	5.21	3.96
	SD	71.29	2.75	0.52	0.92	1.56	0.64
	SE	26.95	1.04	0.20	0.35	0.59	0.24
	M	633.00	28.91	0.95	7.11	4.74	3.96
LN	n	7	7	7	7	7	7
	x	631.29	29.13	1.31	6.32	4.24	3.40
	SD	52.38	5.65	0.36	0.48	0.75	0.49
	SE	19.80	2.14	0.14	0.18	0.28	0.19
	M	632.00	28.16	1.16	6.26	4.47	3.48
LN/LA	n	7	7	7	7	7	7
	x	684.00	27.68	1.05	6.88	3.60	3.63
	SD	56.87	2.47	0.28	0.94	0.84	0.65
	SE	21.50	0.94	0.10	0.35	0.32	0.24
	M	672.00	27.33	0.97	7.06	3.27	3.78

Groups: LN/LA – L-NAME- and L-arginine, LN – L-NAME, LA – L-arginine, C – control; Body weight – BW; Liver index – LI, Spleen index – SI, Heart index – HI, Thymus index – TI, Bursa of Fabricius index – BFI; gram – gm; n – number of animals, x – mean values

TABLE 7
MEAN BODY WEIGHT AND ORGAN INDEX VALUES, STANDARD DEVIATION (SD), STANDARD ERROR (SE), AND INTERQUARTILE RANGE (M) IN THE CONTROL AND TREATED GROUPS OF CHICKENS (3rd WK OF TRIAL)

Groups		BW (gm)	LI	SI	HI	TI	BFI
C	n	7	7	7	7	7	7
	x	1083.14	24.75	1.77	5.76	3.79	2.80
	SD	145.12	1.78	0.53	0.68	0.47	0.60
	SE	54.85	0.67	0.20	0.26	0.18	0.23
	M	1070.00	25.05	1.64	5.78	3.65	2.73
LA	n	7	7	7	7	7	7
	x	1129.86	25.96	0.71	5.94	3.68	8.12
	SD	65.00	2.69	0.63	0.62	1.19	13.33
	SE	24.57	1.02	0.24	0.24	0.45	5.04
	M	1153.00	26.75	0.86	5.62	3.19	3.92
LN	n	7	7	7	7	7	7
	x	1014.29	30.36	1.24	6.39	3.61	2.83
	SD	237.52	5.60	0.72	0.97	1.64	1.09
	SE	89.78	2.12	0.27	0.37	0.62	0.41
	M	1067.00	30.31	0.96	6.48	2.99	2.84
LN/LA	n	7	7	7	7	7	7
	x	1107.57	24.32	1.57	6.42	4.63	2.26
	SD	107.36	1.68	0.97	0.32	1.36	0.33
	SE	40.58	0.64	0.37	0.12	0.51	0.13
	M	1120.00	24.73	1.59	6.45	4.52	2.22

Groups: LN/LA – L-NAME- and L-arginine, LN – L-NAME, LA – L-arginine, C – control; Body weight – BW; Liver index – LI, Spleen index – SI, Heart index – HI, Thymus index – TI, Bursa of Fabricius index – BFI; gram – gm; n – number of animals, x – mean values

chickens were found dead with the signs of pre-ascitic states and of ascites-PHS in the group daily treated with L-NAME over five weeks. The gross lesions seen in the fifth trial week obviously confirmed that the consecutive treatment of the chickens with L-NAME (in a dose of 10 mg/kg BW, ip) has been provoked the pre-ascitic condition or the ascites – PHS[29]. The histopathological findings were typical for pre-ascitic condition as well as for the chickens with ascites – PHS[12,45]. The same findings were confimed in broiler chickens 1, 2 and 4 hours after the start of the intravenous infusion of L-NAME as well as in broilers fed with the diet supplemented with 100 ppm of L-NAME up to 4 week[32]. Perivascular edema of a high degree as well as focal disruption and necrosis of myofibriole and myofibrillar disruption and disorganization (Figures 1 and 2, respectively) were observed in hearts of chickens died in the fifth study week. In hypoxic heart muscle the death of individual cardiac myocytes followed by the lengthening, thinning, and finally disrupting of the cardiac myofibres has been a common phenomenon (dilated cardiomyopathy)[46,47]. So, it seemed to be possible that except of a high degree of interfibrilar edema, this could be also the cause of myofibrilar disruption followed by the disorganization and attempted fagocytosis of the damaged heart muscular tissue (Figure 2). The change of focal myofibriole disruption (Figure 1) could be the consequence of the myocardiotropism of L-NAME, i.e. its cardiotoxic effect[29].

The important fact is that the structure of the bird's heart favors the development of ascites-PHS. The thin-wall right ventricle rapidly responds to the increased action (e.g. in hypoxia) with both dilatation and hypertrophy of the atrioventricular valve itself (that results in valvular insufficiency) and with the development of ascites[48].

The focal fibroplasia (Figure 3) was recorded in LN-treated chickens in 5th wk of trial. According to some investigatiors in case of endocardiosis (the histopathological change recorded in our trial as the mild one in the heart of L-NAME-treated, ascitic bird), the myocardial fibrosis has to be the one of the most common accompanying manifestation[49].

In the fifth study week, numerous, although not extensive, predominantly capillary hemorrhages were another change in the chickens treated with L-NAME. This might be a consequence of increased coronary vascular permeability induced by direct and indirect action of

TABLE 8
MEAN BODY WEIGHT AND ORGAN INDEX VALUES, STANDARD DEVIATION (SD), STANDARD ERROR (SE), AND INTERQUARTILE RANGE (M) IN THE CONTROL AND TREATED GROUPS OF CHICKENS (4th WK OF TRIAL)

Groups		BW (gm)	LI	SI	HI	TI	BFI
C	n	7	7	7		7	7
	x	1556.43	26.29	2.00	5.57	4.90	2.86
	SD	168.03	3.12	0.75	0.48	1.57	0.63
	SE	63.51	1.18	0.28	0.18	0.59	0.24
	M	1586.00	25.65	2.21	5.49	4.87	2.83
LA	n	7	7	7	7	7	7
	x	1366.29	34.35	4.64	5.79	3.18	2.34
	SD	264.76	9.05	8.04	1.55	1.53	0.79
	SE	100.07	3.42	3.04	0.59	0.58	0.30
	M	1442.00	29.30	1.95	5.47	3.08	2.21
LN	n	7	7	7	7	7	7
	x	1568.43	24.80	1.73	5.93	3.75	2.89
	SD	142.21	2.96	0.91	0.61	1.12	0.63
	SE	53.75	1.12	0.34	0.23	0.42	0.24
	M	1520.00	23.29	1.94	6.02	3.46	2.59
LN/LA	n	7	7	7	7	7	7
	x	1591.57	27.59	2.20	4.74	3.59	2.50
	SD	114.01	3.31	2.71	0.45	0.70	0.60
	SE	43.09	1.25	1.02	0.17	0.26	0.23
	M	1565.00	26.47	1.78	4.56	3.69	2.51

Groups: LN/LA – L-NAME- and L-arginine, LN – L-NAME, LA – L-arginine, C – control; Body weight – BW; Liver index – LI, Spleen index – SI, Heart index – HI, Thymus index – TI, Bursa of Fabricius index – BFI; gram – gm; n – number of animals, x – mean values

TABLE 9
MEAN BODY WEIGHT AND ORGAN INDEX VALUES, STANDARD DEVIATION (SD), STANDARD ERROR (SE), AND INTERQUARTILE RANGE (M) IN THE CONTROL AND TREATED GROUPS OF CHICKENS (5th WK OF TRIAL)

Groups		BW (gm)	LI	SI	HI	TI	BFI
C	n	6	6	6	6	6	6
	x	2261.50	24.95	1.20	4.67	4.55	2.62
	SD	281.97	4.05	0.65	0.49	0.67	0.76
	SE	115.11	1.65	0.27	0.20	0.27	0.31
	M	2197.50	24.91	1.25	4.73	4.32	2.46
LA	n	5	5	5	5	5	5
	x	1648.20	37.08	2.04	5.65	3.35	1.83
	SD	205.51	7.87	0.96	1.43	1.19	0.43
	SE	91.91	3.52	0.43	0.64	0.53	0.19
	M	1622.00	36.76	1.67	6.06	3.27	1.73
LN	n	4	4	4	4	4	4
	x	1887.75	25.12	1.76	5.21	5.16	2.77
	SD	126.71	1.13	0.61	1.06	1.03	1.01
	SE	63.36	0.56	0.31	0.53	0.52	0.51
	M	1897.00	25.20	1.76	5.18	5.24	2.59
LN/LA	n	7	7	7	7	7	7 v
	x	1734.57	34.32	1.76	6.18	4.32	2.10
	SD	254.05	9.03	0.48	2.07	2.21	1.18
	SE	96.02	3.41	0.18	0.78	0.84	0.45
	M	1791.00	32.42	1.66	5.21	4.03	1.95

Groups: LN/LA – L-NAME- and L-arginine, LN – L-NAME, LA – L-arginine, C – control; Body weight – BW; Liver index – LI, Spleen index – SI, Heart index – HI, Thymus index – TI, Bursa of Fabricius index – BFI; gram – gm; n – number of animals, x – mean values

L-NAME. However, the highest extent of congestion and hemorrhage, the prevalent changes in the myocardia of L-arginine-treated chickens, was observed over the last two weeks of the trial (Figure 4). These hemorrhages have been presented as scattered intramyocardial, more rarely subepicardial and subendocardial. A role of NO in preventing of platelet accumulation and aggregation in blood vessels wall[50,51] might also contribute to the appearance of the hemorrhages in the hearts of chickens treated with L-arginine over a long period of time.

In the chicken group treated simultaneously with L-arginine and L-NAME, only myocardial degeneration prevailed. It may be presumed that the simultaneous administration of L-arginine with the L-NAME did mitigate, if not prevent, L-NAME's action as a potent vasoconstrictor, at least during the first three trial weeks. In fact, while L-NAME has been acting as a non-selective inhibitor of NOS, supplemental L-arginine has been shown to prevent reduced expression of endothelial nitric oxide synthase (eNOS) in broiler chickens[40]. Since the L-arginine concentrations in birds are correlated with dietary intake (because L-arginine is an essential amino-acid in birds' organism)[31,52], the supplemental L-arginine is essential for the reducing of pulmonary arterial pressure (PAP) and the incidence of pulmonary hypertension syndrome (PHS) in broilers[42]. Obviously, the constitutive NO synthesis contributes modestly to maintaining the basal tone of the pulmonary vasculature[53]. Also the perivascular disruption of hepatocytes and the coagulation necrosis (Figure 8), were found in chickens treated by L-NAME and were considered a result of chronic L--NAME-toxic effect[29]. Sinusoidal congestion, intravascular coagulation, and coagulation necrosis around the central veins were prominent in the L-NAME group of rats but not in the N-ω-nitro-D-arginine (D-NAME) group of rats (both groups were previously treated by dimethylnitrosamine)[54]. Obviously the coagulation necroses, confirmed in livers of animals treated with L-NAME represents the consequences of its vasoconstrictory and/or toxic effect in liver tissue. As in the present experiment, the findings of the interstitial edema, was described by numerous authors as regular histopathological finding in hypoxic or ascitic chickens[55–58].

Development of cartilaginous and/or osseous nodules in the lungs (Figure 6) as a regular concomitant pathological change in ascitic chickens has been well des-

cribed[12,23,59,60], but it has been previously considered as a normal finding in healthy birds[23]. In the fourth week of the study, atrial collapse and parabronchial dilatation was observed in the lungs of the birds treated with L-NAME. The same changes were confirmed in the lungs of ascitic broiler chickens[12,23]. In the L-NAME-treated group of chickens and in the L-arginine-group (Figure 5), a fibrin-like protein matter was visible perivascularly in the fifth week of trial. In the state of PHS, pulmonary capillaries can undergo the so-called »stress-caused failure« due to increased pressure. Then the blood can flow out through pulmonary airways, disturbing gases exchange[61]. Considering the finding of a high degree pulmonary edema, in the same study week (L-NAME group), this finding of perivascular fibrinoid matter could be associated with the increased permeability of pulmonary blood vessel walls. Moreover, during the oxidative lungs damage the reduced pressure in the airways, edema and the finding of protein-like secret in the air spaces after the administration of L-NAME were confirmed[62]. The cause of the microscopic appearance of fibrinoid matter in lungs of L-arginine-treated chickens was not determined. The possibility that the histopathological change of fibrinoid matter could be a plasma would support the accumulating evidence for the involvement of NO in plasma extravasation during the various forms of inflammation[63]. However, the pathomorphological changes typical for the inflammation were not confirmed in L-arginine-treated chickens sacrificed at the end of the present experiment.

In the last weeks of the present trial, the thickening of the medial layer and migration of smooth muscle cells into the subintima of the pulmonary arterioles was observed in the L-NAME-group (Figure 7). Similar pathological changes were described in broilers with chronic PH[64,65]. However, these findings could not be expected in L-arginine group because the proliferation of smooth muscle cells in pulmonary arteriole was inhibited by L-arginine[66]. For the histopathological evaluation it is important to know that some broiler chicken hybrids have an innately thickened arterial wall layer[67], whereas the others attributed this change exclusively to the chickens with PHS[55], as the consequence of elevated pulmonary arteriolar pressure that results in hypertrophy of the smooth muscle layer in the small arterioles of the lungs – the resistance vessels[46].

In the bursa of Fabricius, thymus, and in the spleen of the chickens in the L-NAME group, a low-degree depletion of lymphocyte-type cells was present. The same findings were reported in all the analysed organs of ascitic chickens explaining the findings as immunosuppressive action of a chronic disease[55].

The significance of cardiac index in ascitic chickens is the key to haemodynamic disorders – to hypoxemia and definitive cardiovascular system failure in fast growing broiler chickens. In this production category of the poultry, the value of cardiac index is inheritedly low[17]. In present investigation it was not able to find any regularity concerned the possible elevation of heart index value in L-NAME group toward the end of the experiment, i.e., in a period in which also the other parameters (gross lesions and histopathology) had confirmed the presence of pre-ascitic (hypoxic) and/or ascitic state (Table 2).

It has been interesting that the liver index in L--NAME/L-arginine-group was significantly lower than the index values in both L-NAME-group and in L-arginine-group of chickens in the first trial week (Table 1), and the same relation was recorded during the same week in the heart index values between the same groups of chickens (Table 2). Nevertheless, the relative liver weight followed by the heterophil/lymphocyte ratio and the percentage weights of immunocompetent organs represent the excellent indicators of the stress condition[68]. According to these authors the increasing of the liver weight is one of the signs of the stress.

Relative spleen weight is also a general indicator of stress[68,69]. Although long-term responses to stressors generally result in decreased lymphoid mass[70], the spleen index values shown from the first to the fifth weeks in the present experiment did not confirm this data (Table 3).

In conclusion, the pathomorphology results of investigation of chronic toxicity dynamics of L-NAME confirmed its toxic (vasoconstrictive and hypoxic) effect: pre-ascitic condition and/or ascites – PHS developed in the chickens treated with L-NAME over five weeks. Microscopic changes in the chickens in the L-NAME group were consistent with its systemic action (edema, dysoria) and with topical irreversible damage to individual tissues (tissue tropism /disruption of myofibriole, hepatal cellulolysis/), mainly after a longer period of the compound administration. L-arginine displayed prominent congestions and haemorrhages in the heart, lung and liver tissues.

These effects proved to be directly dependent on the L-arginine and on the L-NAME concentrations, and on the duration of the treatment with these compounds.

In the groups simultaneously administered L-NAME and L-arginine, histopathology analysis established a lower degree to a complete absence of pathological changes otherwise established in the tissues of chickens receiving L-NAME alone. This corroborated the effect of L-arginine, i.e., of NO, confirmed in several literature reports so far, that it prevents and alleviats, or possibly treats vasoconstrictive (ischemic) cardiovascular disorders that in the majority of cases progress from localized to systemic (pulmonary hypertension syndrome and chronic right atrial failure, ascites).

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UČINAK KRONIČNE TOKSIČNOSTI L-NAME I L-ARGININA NA POJAVU ASCITESA – SINDROMA PLUĆNE HIPERTENZIJE U TOVNIH PILIĆA

SAŽETAK

Učinak kronične toksičnosti L-arginina i N-ω-nitro-L-arginin-metil-estera – L-NAME na pojavu ascitesa – sindroma plućne hipertenzije (SPH) istraživan je u 140 tovnih pilića muškoga spola (ROSS) u razdoblju od prvog dana do petog tjedna života. Pilićima podijeljenim u četiri skupine injicirano je intraperitonealno (ip) svaki drugi dan 0,5 mL/kg tj. m. fiziološke otopine (kontrolna skupina, K); 10 mg/kg tj. m. L-NAME (LN-skupina); 100 mg/kg tj. m. (L-arginin; LA--skupina) i 10 mg/kg tj. m. L-NAME i 100 mg/kg tj. m. L-arginina (LN/LA-skupina). Na kraju svakoga tjedna eutanazirano je po sedam pilića iz svake skupine. Uzorci srca, jetre, pluća, krvnih žila i limfoidnih organa podvrgnuti su patolo-

škohistološkoj pretrazi. Određen je također i pokazatelj odnosa mase jetre, slezene, srca i timusa u usporedbi s tjelesnom masom (indeks mase organa). Na kraju pokusa u pet uginulih pilića iz skupine tretirane s L-NAME (LN-skupina) utvrđen je ascites – SPH. U istoj je skupini tijekom pokusa edem utvrđen kao prevladavajuća patološkohistološka promjena u srcu i plućima pilića. U skupini tretiranoj L-argininom najizraženije su bile patološkohistološke promjene punokrvnosti i krvarenja poglavito tijekom posljednja dva tjedna pokusa. Dok su žarišna disrupcija miovlakana srca i disrupcija hepatocita prevladavale u pilića tretiranih s L-NAME (5. tjed. pokusa), u skupini tretiranoj istodobno L-argininom i s L-NAME utvrđena je degeneracija srčana mišića manjeg stupnja jakosti. Razudbom utvrđenim preascitičnim stanjem ili ascitesom-SPH u uginulih tovnih pilića skupine LN krajem petoga tjedna potvrđena je uloga L-NAME u nastanku ove metaboličke bolesti tovnih pilića, dok je nalaz u pilića tretiranih L-argininom ukazao na protektivni učinak glede nastanka ascitesa – SPH u tovnih pilića.