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The effect of L-tryptophan on the heart in rabbits via chronic hypoxia

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Aim: To evaluate the protective effect of tryptophan on an experimentally produced hypoxic myocardial injury via biochemical and pathological parameters.

Materials and methods: A total of 26 rabbits were divided into 3 groups. Group 1 (n = 9) was only exposed to hypoxia. Group 2 (n = 10) was exposed to hypoxia and received L-tryptophan (200 mg/kg per day, orally for 5 days). Group 3 (n = 7) was the control group.

Before the hypoxic injury and after the delivery of the medication, serum samples were taken for troponin-I, creatine kinase myocardial isoenzymes (CK-MB), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), and nitric oxide (NO) analysis, and then the rabbits were sacrificed. Next, the myocardium samples were taken and the myocardial NO, MDA, SOD, and GSH-Px enzyme activity levels were studied histopathologically.

Results: In group 1, Serum GSH-Px and SOD activities were decreased. Conversely, troponin-I, CK-MB, and LDH were elevated. Severe cardiac injury was observed histopathologically. In group 2, serum troponin-I and SOD values were increased. Mild cardiac injury was demonstrated histopathologically.

When groups 1 and 2 were compared, tissue NO and MDA levels in group 1 were higher compared to group 2, but GSH-Px level was found decreased in group 1.

Conclusion: Our findings support that there is a clear effect of the free oxygen radicals and the lipid peroxidation products on hypoxic cardiac injury. In addition, L-tryptophan supplementation has a strong protective effect on hypoxic heart by antioxidant activity.

Key words: Hypoxia, myocardial injury, cardioprotection, tryptophan, oxidative stress

Kronik hipoksili tavşanlarda L-triptofanın kalp üzerine etkisi

Amaç: Bu çalışmanın amacı, deneysel olarak meydana getirilmiş hipoksik miyokardial hasara karşı triptofanın koruyucu etkisini, biyokimyasal ve patolojik parametreler kullanarak değerlendirmektir.

Yöntem ve gereç: Toplam 26 tavşan üç gruba ayrıldı. Grup 1, 9 tavşan yalnızca hipoksiye maruz kaldı. Grup 2, 10 tavşan hem hipoksiye maruz kaldı ve L-triptofan aldı. Grup 3, 7 tavşan kontrol grubu olarak seçildi.

Hipoksik hasar öncesi ve ilaç tedavisi uygulandıktan sonra troponin-I, kreatin kinaz miyokard izoenzimi (CKMB), laktat dehidrogenaz (LDH), glutatyon peroksidaz (GSH-Px), süperoksid dismutaz (SOD), malondialdehid (MDA) ve nitrik oksit (NO) için analizler yapıldı ve tavşanlar sakrifiye edildi. Daha sonra miyokardiyal numuneler alındı ve NO, MDA, SOD, GSH-Px enzim aktivite düzeyleri histopatolojik olarak değerlendirildi.

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Bulgular: Grup 1'de serum GSH-Px ve SOD aktivitesi azaldı. Aksine, troponin-I, CK-MB ve LDH arttı. Histopatolojik olarak şiddetli kardiyak hasar gözlendi. Grup 2'de serum troponin-I ve SOD değerleri arttı. Histopatolojik olarak hafif kardiyomiyopati gösterildi.

Sonuç: Bulgularımız, hipoksik bir kardiyomiyopatide serbest oksijen radikallerinin ve lipit peroksidasyon ürünlerinin belirgin bir etkisi olduğunu desteklemektedir. Aynı zamanda, L-triptofan ilavesi, hipoksik kalp üzerine antioksidan bir etki yaparak güçlü bir koruyucu etkiye sahiptir.

Anahtar sözcükler: Hipoksi, miyokardiyal hasar, kalbin korunması, triptofan, oksidatif stres

Introduction

Chronic hypoxia is associated with increased oxidative stress as evidenced by marked lipid peroxidation and induction of antioxidant enzyme response in various tissue organs (1). Especially, hypoxia and ischemia have an important role on cardiovascular diseases (2). There are studies on etiopathogenesis and prophylaxis of hypoxic and ischemic injuries. By demonstration of the role of free oxygen radicals and antioxidant enzymes in injury, antioxidant treatment modalities are favorable in these days (3).

In the early stages of myocardial ischemia/hypoxia, neutrophils and monocytes infiltrate intima and degenerate the endothelial cells. Free oxygen radicals, cytokine, and nitric oxide are released by the degeneration of the endothelial cells and cause lipid peroxidation. The products of lipid peroxidation, such as malondialdehyde (MDA), result in myocardial damage (4,5).

Creatine kinase (CK-MB), lactate dehydrogenase, and troponin-I (Tn-I) are used to determine the myocardial injury (1). Researchers are still looking for appropriate antioxidant agent to protect myocardium from the hypoxic and ischemic injuries (3).

L-tryptophan is the precursor of melatonin and has a protective effect on the free oxygen radical neutralization (6). Reiter et al. reported that N-acetylserotonin, which is a derivative of melatonin or tryptophan, also has a free oxygen radical neutralization activity. Moosmann et al. reported that membrane lipoproteins, especially in the lipid dense regions, have high concentrations of tryptophan (7,8). High levels of tryptophan protect the lipid layer from peroxidation, which indicates that tryptophan may be used as a pharmacological agent as a cytoprotective antioxidant (9). Today, all the effects of tryptophan are not clear yet, and in the literature there are a limited number of studies on this subject.

In this study, a myocardial hypoxic injury was created experimentally and the protective effect of tryptophan was evaluated by observing troponin-I and CK-MB levels. Because they are involved in tissue injuries, SOD, NO, GPX, MPO, and tryptophan interactions were also evaluated.

Materials and methods

The study was performed on 26 New-Zealand white rabbits weighing between 1300 g and 2600 g. All rabbits were male with an age range of 60-90 days. After a 15-day adaptation period, rabbits were distributed into 3 groups: group 1(n = 9) was only exposed to hypoxia. Group 2 (n = 10) was exposed to hypoxia and received L-tryptophan. Group 3 (n = 7) was the control group and received distillated water.

A hypoxic condition was obtained using a 25 × 25 × 62 cm^3 funnel with input and output ports for air. The funnel was ventilated with 10% oxygen and 90% nitrogen mixture at 5 L/min flow rate for 5 min. Oxygen-nitrogen mixture flow continued for an additional 10 min after the rabbits were placed in the funnel. The oxygen saturation and the heart rate were monitored with a pulse oximeter. The rabbits were let to breathe room air for 10 min (1,10). During this process, when bradycardia or respiratory failure had developed, the experiment was discontinued.

Hypoxia and tryptophan application*:* Same hypoxic condition was applied to the rabbits in group 2 for only 5 min-ventilation. Then, L-tryptophan (Sigma Chemical Company, Sigma, St. Louis. MO) was administered orally in the amount of 200mg/kg per day for 5 days.

After the adaptation period, serum samples were taken from groups 1 and 2 at the beginning of the study (day 0) and day 6 after the hypoxia for troponin-I, CK-MB, LDH, plasma GSH-Px, SOD, MDA, and NO. At the end of day 6, animals were sacrificed and

histopathologic analyses were performed. In addition, cardiac tissue samples were taken to be analyzed.

Frozen serum samples were dissolved and CK-MB and Tn-I levels were analyzed on the same day. Serum CK-MB (with 0.5 cc serum samples) analyses were performed using a Konelab 60i auto analyzer (Thermofisher Scientific, Finland) with the reagent kits produced by Medkim corp. Serum Tn-I levels were studied using an Innotrac Aio Immunoanalyzer (Innotrac diagnostics, Turku, Finland) with Innotrac Aio TM Troponin I Analyte Pen kit (with 0.5 cc serum samples). Serum LDH levels were also studied using a Konelab auto analyzer with LDH reagent kits produced by Medkim in 0.5 cc serum samples.

Serum and myocardial GSH-Px activity determination: The activity was calculated using Paglia and Valentine's combined enzymatic method: measuring the peroxidation rate of H_2O_2 and glutathion (GSH) reaction (11). Tissue GSH-Px activity detection: myocardial homogenate centrifuged at (1/4 w/v) 13200 rpm for 30 min and obtained supernatant tamponated with a phosphate tampon (0.05 M, pH = 7.4) and diluted (1/10). For tissue GSH-Px activity detection, 0.05 mL sample was used (11).

Serum and myocardial SOD activity levels were measured by a method developed by Sun et al. (12). Xanthine oxidase was used for superoxide producer and inhibition of nitroblue tetrazolium (NBT) reduction. Tissue SOD of activity measurement: 0.05 mL supernatant obtained from myocardial homogenate was diluted (1/10) with phosphate tampon (pH: 7.4).

Detection of Serum MDA activity: Jain's method was used: measurement of colorful product of MDA and thiobarbituric acid (TBA) at 532 nm wavelength (12). Tissue MDA detection was performed using a methods developed by Okawa et al. (14).

Tissue NO detection: The 0.05 mL homogenate obtained from the supernatant was diluted (1/4 v/v) by Somogy reactant (10% $ZnSO_4$ and 0.5 N NaOH) and then deproteinized and centrifuged at 1500 rpm for 10 min at + 4°C (15). Using this supernatant, NO (NO_2 + NO_3) levels were measured. The nitrate (NO_3) first reduced to nitrite (NO_2) and the standard scale for nitrite was used to determining the nitrate levels (16).

To histopathological evaluation of the hematoxylin and eosin stained slides were performed using a light microscope. The scoring was achieved by the severity of the histopathological changes (17).

The severity of the cardiac injury degree was graded between zero and 3 as shown below.

0: no evident cardiac injury, 1: mild cardiac injury, 2: moderate cardiac injury, 3: severe cardiac injury.

Statistical Analysis

SPSS 10.0 was used for statistical analysis. Median (minimum-maximum) values were used for comparing the parameters. For comparing the groups, Kruskal-Wallis variance analysis and Mann-Whitney U tests were used; comparisons within the groups were carried out by Wilcoxon signed ranks test. Statistical significance was set at a level of $P < 0.05$.

Results

In group 1, significant increase in troponin-I, CK-MB, LDH, SOD, and a decrease in GSH-Px were detected on day 6 as shown in Table 1 ($P < 0.05$). Severe cardiac injury was observed histopathologically. GSH-Px and SOD activity in this group was also found decreased (Table 1).

In group 2, troponin-I and SOD activity increased significantly as shown in Table 2. ($P < 0.05$). However, increase in troponin-I was less pronounced as compared to group 1. There was no significant change in other parameters as presented in Table 2 ($P > 0.05$).

NO levels in groups 1 and 2 were statistically much higher than the control group ($P < 0.05$). In group 2, NO level was significantly lower than group 1 as shown in Table 3 ($P < 0.05$). When the tissue SOD levels were compared, all groups were very similar ($P > 0.05$). In group 1, the tissue MDA level was statistically much higher compared to groups 2 and 3 ($P < 0.05$), and also group 2 MDA level was statistically much higher compared to group 3 ($P < 0.05$, Table 3). GSH-Px level in group 2 was higher compared to group 1 ($P < 0.05$, Table 3).

When the histopathological findings were compared, the score of group 1 statistically much higher compared to the other groups as shown in Table 4 ($P < 0.05$).

Table 1. Serum CKMB, LDH and Tn-I, plasma MDA, SOD, GSH-Px and NO levels in group 1 median (min-max).

n = 9	Day 0	Day 6	P
Tn-I (ng/mL)	0.21(0.16-0.25)	0.32(0.22-0.41)	<0.05
CK-MB (U/I)	3857(939-8447)	5450(1288-7781)	<0.05
LDH (IU)	660(603-984)	1276(765-8700)	<0.05
MDA (μmol/L)	0.360(0.288-1.232)	0.616(0.384-1.648)	>0.05
SOD (U/L)	1.023(0.929-1.411)	0.882(0.770-0.964)	<0.05
GSH-PX (U/mL)	0.224(0.127-0.488)	0.122(0.096-0.154)	<0.05
NO (μmol/L)	6.04(1.64-8.56)	7.52(0.00-10.50)	>0.05

Table 2. Serum CKMB, LDH and Tn-I, plasma MDA, SOD, GSH-Px and NO levels in group 2 median (min-max).

n = 9	Day 0	Day 6	P
Tn-I (ng/mL)	0.21(0.19-0,32)	0.25(0.20-0.47)	**<0.05**
CK-MB (U/I)	3569(1596-5250)	4285 (2518-6219)	>0.05
LDH (IU)	702(288-2570)	689(388-1498)	>0.05
MDA (μmol/L)	0.392(0.272-1.152)	0.447(0.352-1.088)	>0.05
SOD (U/L)	0.999(0.729-1.294)	1.440(0.858-1.635)	**<0.05**
GSH-PX (U/mL)	0.128(0.104-0.635)	0.130(0.084-0.824)	>0.05
NO (μmol/L)	6.80(3.04-11.72)	7.36(7.10-9.44)	>0.05

Table 3. Comparison of tissue GSH-Px, NO, SOD, and MDA levels.

Group	n	GSH-Px (mU/μg protein) Median (Min-Max)	NO (μmol × 10^{-3}/μg protein) Median (Min-Max)	SOD (U/μg protein) Median (Min-Max)	MDA (nmol/μg protein) Median (Min-Max)
1	9	0.0039(0.0023-0.0043)	0.10 (0.05-0.15)[a]	4.96(2.68-6.96)	0.16(0.10-0.45)[a]
2	10	0.0054(0.0022-0.0083)[b]	0.087 (0.04-0.18)[a,b]	6.09(1.39-11.28)	0.09(0.02-0.27)[a,b]
3	7	0.0046(0.022-0.0055)	0.035 (0.017-0.077)	4.97(1.33-7.26)	0.031(0.007-0.12)

[a] P < 0.05, when compared with group 3
[b] P < 0.05, when compared with group 1

Table 4. Comparison of histopathological cardiac injury scores.

	Group	n	Cardiomyopathy scores		
			Mild (%)	Moderate (%)	Severe (%)
1	Hypoxic	9	0	33	66[a]
2	Hypoxic + L-Tryptophan	10	40	20	40
3	Control	7	0	0	0

[a] P < 0.05, when compared with group 3

Discussion

Cardiovascular diseases, especially in the developed countries, are the main cause of morbidity and mortality in the world. Early detection of hypoxia and ischemia, the main etiology of cardiovascular diseases, and early and appropriate treatment should be prevent mortality (4).

L-tryptophan should have similar effects like melatonin because it is the precursor amino acid of melatonin. There are few studies on the antioxidant activity of L-tryptophan: the way of action is preventing superoxide anion and hydrogen peroxide production. Therefore, it may be used as an antioxidant agent (18,19).

Jaffe et al. reported that Tn-I is more sensitive and specific than CK-MB in the myocardial injury (20). LDH isoenzyme activities are not specific for myocardial injury. CK-MB sensitivity is 92%, and Tn-I sensitivity is over 93% after the first few hours. Vordenwinkler et al. reported that cardiac Tn-I increased in parallel to cardiac Tn-T, CK-MB, and LDH in effluents from an isolated perfused rat hearts after hypoxia-reoxygenation-induced myocardial injury (21).

In this study, Tn-I, CKMB, and LDH levels in the hypoxic group were found significantly elevated, which is in agreement with the literature. Tn-I, CK-MB, and LDH level elevations demonstrate that significant hypoxia caused a myocardial injury. In the hypoxic group that received tryptophan, although mild Tn-I elevation was observed, there was no significant change in CK-MB and LDH. Therefore, this should signify that the tryptophan medication controlled the myocardial injury, and in minor injuries Tn-I is more reliable than CK-MB and LDH because of the minor changes in CK-MB and LDH levels. These results were also supported by the histopathologic findings.

A production of the free oxygen radicals, a lipid peroxidation and a decrease in the antioxidant enzymes are the major factors in the pathogenesis of a hypoxic myocardial injury (3). In the case of the myocardial ischemia reperfusion, Ferrari et al. advocated that the degree of an injury depended on the amount of the free oxygen radicals and the antioxidant defense systems (22). A study by Prasad et al. showed that an increase in MDA was detected (23). Therefore, he concluded that in the ischemic conditions, an elevation in free oxygen radicals results in an inhibition or a decrease of the antioxidant defense system.

In our study, there was a statistically significant decrease in MDA in the tryptophan group than the hypoxic group, and we found the same results in the tissue. These results indicated that the antioxidant activity of tryptophan was from the inhibition of the lipid peroxidation.

Depre et al. concluded that there is an increase in the NO synthase activity after the myocardial ischemia of a rabbit heart (24). This increase causes the NO deposition and myocardial damage.

In our study, NO levels in tissues of the hypoxic group were significantly higher compared to the other groups. High NO levels in the hypoxic group supported that NO had an effect on the hypoxic myocardial injury. The myocardial NO levels in the tryptophan group were lower than the hypoxic group. These results showed that myocardium was protected by tryptophan.

The antioxidant enzymes, which protects the cardiomyocytes from the oxygen radicals, are GSH-P and SOD. Guarnieri et al. reported that there was a decrease in the GSH-Px and SOD activity in the myocardial hypoxemia/ischemia (2,25). Kihlström et al. and Hoshida et al. showed that, after 5 min ischemia, mitochondrial SOD, catalase, and GSH-Px activity increased in a dog heart (26,27).

In our study, the plasma GSH-Px and SOD level decreased significantly in the hypoxic group. In the L-tryptophan group, there was a statistically significant increase in SOD, but the increase in GSH-Px activity was not significant this group. In the tissue we found that GSH-Px activity in L-tryptophan was higher than hypoxemic group and this increase was statistically significant. SOD activity in the tissue did not change significantly. These results showed that the antioxidant activity in hypoxic injury was increased by tryptophan.

In our study, there were severe myocardial damage findings, such as a myocardial fibril swelling, interstitial edema, disorganization, and a necrosis in

the hypoxic group. In the group that received L-tryptophan, we found mild to moderate cardiomyopathy findings, such as a normal myocardium accompanied to a myocardial fibril swelling, interstitial edema, and disorganization. It was observed that L-tryptophan did not protect the heart injury completely, but regressed the hypoxia effects. Our histopathological findings supported that L-tryptophan as an antioxidant could regress the myocardial injury. Our findings were in agreement with Yuan and Llesuy (28,29).

This study showed that L-tryptophan has strong antioxidant activity on hypoxic myocardial injury. Further studies must be carried out on this subject to improve our knowledge.

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