

The effect of benfothiamin and vitamin D in ischemia / Reperfusion model of rat skeletal muscle

Özlem Keskin¹, Mehmet Sipahi¹, Vehbi Yavuz Tokgöz¹, Selçuk Takır²,
Gülname Fındık Güvendi¹

¹ Giresun University, Department of Obstetri & Gynecology, Asst. Prof., MD., Giresun, Turkey.

² Giresun University, Department of Obstetri & Gynecology, Assoc. Prof. MD., Giresun, Turkey.

Abstract

Background: Benfothiamin is a highly potent form of vitamin B1 protecting endothelial function. On the other hand, vitamin D provides restoration of muscular tissue by inhibition of apoptosis and acceleration of cellular proliferation following muscle injury. We assumed that the administration of these vitamins in ischemia/ reperfusion (I/R) injury, could reduce the damage by alteration of the release of various oxidant and antioxidant mediators leading to cellular damage.

Materials and Methods: We assigned 30 Wistar Albino males rats into 5 groups. In the control group (n=6), rats were anaesthetized and total antioxidant capacity (TAS), total oxidant capacity (TOS), malondialdehyde (MDA), superoxide dismutase (SOD) and nitricoxide (NO) level were measured in lower extremity soleus muscle. Benfotiamin and D were given to the groups and the values of these parameters were evaluated in ischemia reperfusion muscle tissue specimens. All tissues were examined histologically.

Results: We detected a significant change in groups 3 and 4 for antioxidant NO level after ischemia and reperfusion. Therefore, we observed that the administration of vitamin D and benfotiamin increased NO levels in muscle especially during reperfusion. The level of other oxidants TOS and MDA and antioxidants TAS and SOD were not significant during I/R at given periods. Overall vitamin D and benfotiamin have acute beneficial effects especially in improving I/R injury of lower extremity, even at non-critical periods.

Conclusion: Acute term effects of benfotiamin and vitamin D can be useful during where changes due to I/R. The effects can be evaluated during long term I/R.

Key Words: Benfotiamin, vitamin D, ischemia / reperfusion injury, skeletal muscle, rats

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Introduction

Lower extremity can be subjected to ischemia due to certain conditions such as injuries, acute peripheral occlusive diseases and surgical intervention with prolonged tourniquet period. Following reperfusion of ischemic extremity by oxygenized blood, reactive oxygen species including free radicals such as hydroxyl ions (OH⁻), superoxide anion (O₂⁻), oxygen (O₂), hydrogen peroxide (H₂O₂) and nitric oxide (NO) are produced. These products within the circulation, lead to ischemic injury of the extremity.^(1,2) However the organism develops an antioxidant defense system in order to prevent cellular damage due to free radicals. This system includes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) antioxidant enzyme systems, which are “free radical scavengers” of indirect pathway.⁽³⁾

Superoxide dismutase (SOD) is an antioxidant enzyme catalyzing the transformation of superoxide free radical (O₂⁻) to hydrogen peroxide (H₂O₂) and molecular oxygen (O₂). It provides the alteration of reactive O₂ metabolites and eliminate them from the cell.

The most important and harmful triggering effect of free radicals within the cell, is lipid peroxidation. Lipid peroxidation is defined as oxidation of polyunsaturated fatty acids by free radicals. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and leads to polymerization of membrane components and their cross linking. In turn, this may affect the status of cellular surface, enzyme activity and ion transport.^(4,5)

I/R injury leads to endothelial cell activation and dysfunction. Endothelial cells are potential target of superoxide radicals and also production site of superoxide radicals. Endothelium produces NO and endothelin (ET) which are responsible from microvascular hemostasis. NO tends to reverse the vasoconstrictor effect of ET in arterial circulation. The opposite is true in veins. In I/R injury, ET/NO ratio is impaired in favor of ET. Therefore arterial vasoconstriction and venous vasodilatation occurs.⁽⁶⁾

Total oxidant capacity TOS and antioxidant capacity TAS measurements show the oxidant and antioxidant status of serum and tissue.⁽⁷⁾ Acute lower extremity

ischemia is a clinical condition leading to significant morbidity and mortality even following elimination of ischemia. In case of delayed intervention, these risks are increased. In addition they may be seen in spite of extremity reperfusion. A clinical event chain leading to acute renal and respiratory insufficiency and dysfunction of heart, intestines and spleen, is initiated.⁽⁸⁾ The initiation and development of these events should be identified in order to determine medical and surgical treatments to prevent this condition.

There are several studies showing the suppressive aspect of benfothiamin on reactive oxygen products.^(9,10) In diabetic rats, benfothiamin normalized many oxygen species.⁽¹¹⁾ In addition, it reduces harmful oxidative effects in mice with streptozocin (STZ) induced diabetes.⁽¹²⁾ Benfothiamin is fat-soluble form of thiamine and can easily pass from cellular membrane which is rich in fatty acid.

There are clinical trials and animal experiment models showing that vitamins A, C, D and E prevent I/R injury. Vitamin D is fat-soluble and is considered to induce endogenous antioxidant pathways and to have neuroprotective properties by reducing inducible nitric oxide synthase. There are especially references on 1,25-dihydroxy vitamin D₃.⁽¹³⁾

In our study, acute effects of administration of benfothiamin and vitamin D were investigated in experimental muscle I/R model. In assessment of this effect, levels of oxidant and antioxidant parameters at pre-determined ischemia and reperfusion periods as well as histopathological tissue samples were examined.

Materials - Methods

In this interventional animal study, 30 male Wistar Albino rats (aged 8-12 weeks and weighing 321±69 g) were included in the experiment, which received ethical approval from the laboratory Animal Unit of Giresun University. The animals were obtained from the same unit, and standard temperature (22±2°C) and humidity (50±5%) controlled rooms were used for preserving the rats until the start of the experiment. In addition, a standard diet and tap water were provided ad libitum for the rats. Following the intervention, all rats were sacrificed by obtaining intra-cardiac blood.

During the interventions, the rats were anesthetized with 50 mg/μl (8.5 cc) of ketamine hydrochloride (HCL) (Ketalar®, Pfizer, Inc., İstanbul, Turkey) and 23.32 mg/ml (1.5 cc) of Xylazine (Rompun®, Bayer Healthcare AG, Leverkusen, Germany) through an 0.3-0.5 cc intra-peritoneal line. Vital parameters during anesthesia were considered observational. Lower extremity was ligated by silk number 0 at proximal level of femoral artery for lower extremity muscle ischemia (**Figure 1**). Maintenance of cyanosis and coldness of the extremity was monitored during ischemia.

Following procedures were applied to the groups consecutively:

***Control group:** Rats were anesthetized and only a biopsy specimen of 1 cm was obtained from right leg soleus muscle and transferred into Eppendorf tube with 10% neutral formalin for histopathological examination. Blood samples were taken from each rat and then they were sacrificed.

***Group 1:** Right lower extremity was ligated from femoral level and ischemia was maintained for 3 hours. Biopsy from soleus muscle was taken by vertical incision of 1 cm on the extremity. Following ischemia, extremity tourniquet was removed and reperfusion was maintained for 3 hours. Biopsy from soleus muscle was taken following reperfusion. Rats were sacrificed by obtaining 3 milliliters of intra-cardiac blood.

***Group 2:** In rats, ischemia was performed at right extremity and concomitantly subcutaneous 332.000 IU/

kg (8.3 mg/kg) vitamin D (Devit 3 IM/Oral ampoule® 300.000IU/ml Deva İlaç) was administered; ischemia was maintained for 3 hours. Biopsy from soleus muscle was taken by vertical incision of 1 cm on the extremity. Following ischemia, extremity tourniquet was removed and reperfusion was maintained for 3 hours. Biopsy from soleus muscle was taken following reperfusion. Rats were sacrificed by obtaining 3 milliliters of intra-cardiac blood.

***Group 3:** Rats received diluted Benfothiamin (S-BenzoyithiaminO-monophosphate B9636-250 MG powder SIGMA-ALDRICH) 70mg/kg via oral route by gastric lavage tube (**Figure2**). Following anesthesia, identical period of I/R was administered to rats and biopsy specimens from soleus muscle were taken. Rats were sacrificed by obtaining 3 milliliters of intra-cardiac blood.

***Group 4:** Rats received same amount of Benfothiamin via same way. Following right leg ischemia, subcutaneous vitamin D ampoule was administered. Same period of I/R was done and biopsy specimens were obtained. Rats were sacrificed by obtaining 3 milliliters of intra-cardiac blood.

Tissue samples taken for TAS, TOS, MDA, SOD and NO were directly transferred into Eppendorf tubes and stored at -80°C. Tissue samples taken for histopathological examination were stored in 10% neutral formalin. SOD, MDA, NO, TAS, TOS determination was as following:



Figure 1



Figure 2

All tissues were homogenized at Rel Assay laboratory. Following homogenization, SOD, MDA and NO tests were done manually according to micro-Elisa method by using Rel Assay Eliza kits on Biotec Elisa device. Other tests were done on Selectra full-automated biochemistry auto-analyzer.

Total Antioxidant Status (TAS)

TAS levels were measured using commercially available kits (Relassay, Turkey). The novel automated method is based on the bleaching of characteristic color of a more stable ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The assay has excellent precision values, which are lower than 3%. The results were expressed as mmol Trolox equivalent/L.⁽¹⁴⁾

Total Oxidant Status (TOS)

TOS levels were measured using commercially available kits (Relassay, Turkey). In the new method, oxidants present in the sample oxidized the ferrous ion-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2$ equivalent/L).⁽¹⁵⁾ Parameters analyzed for ischemia and reperfusion are shown in **Table 1** and **Table 2**, respectively.

Table 1. TAS, TOS, SOD, MDA and NO concentration for ischemia.

Groups	TAS (mmol/l)	TOS ($\mu\text{mol/l}$)	SOD (ng/ml)	MDA ($\mu\text{mol/l}$)	NO ($\mu\text{mol/l}$)	NO concentration
Total n=30	mean \pm SEM	mean \pm SEM	mean \pm SEM	mean \pm SEM	mean \pm SEM	mean \pm SEM
Control (n=6)	0.65 \pm 0.05	10.17 \pm 2.80	8.75 \pm 0.66	1.18 \pm 0.15	1.70 \pm 0.40	133.8 \pm 8.9
G1 (n=6)	0.65 \pm 0.05	14.66 \pm 3.82	5.5 \pm 0.51	1.05 \pm 0.19	1.42 \pm 0.18	189 \pm 41.3
G2 (n=6)	0.38 \pm 0.05	12.03 \pm 0.87	6.11 \pm 0.92	1.008 \pm 0.08	1.33 \pm 0.06	140.5 \pm 6.8
G3 (n=6)	0.53 \pm 0.03	11.14 \pm 1.07	5.41 \pm 0.66	622.36 \pm 281	10327.9\pm2171.8*	125.3 \pm 8.8
G4 (n=6)	0.48 \pm 0.02	11.46 \pm 1.72	7.95 \pm 1.00	825.6 \pm 266.4	15927.5\pm1276.8*	203.3 \pm 34.3

*NO values for ischemia were significant compared to control group ($p < 0.05$).

Table 2. TAS, TOS, SOD, MDA, NO and NO concentration for reperfusion.

Groups	TAS (mmol/l)	TOS ($\mu\text{mol/l}$)	SOD (ng/ml)	MDA ($\mu\text{mol/l}$)	NO ($\mu\text{mol/l}$)	NO concentration
Total n=30	mean \pm SEM	mean \pm SEM	mean \pm SEM	mean \pm SEM	mean \pm SEM	mean \pm SEM
Control (n=6)	0.65 \pm 0.05	10.17 \pm 2.80	8.75 \pm 0.66	1.18 \pm 0.15	1.70 \pm 0.40	133.8 \pm 8.9
G1 (n=6)	0.65 \pm 0.05	14.66 \pm 3.82	5.5 \pm 0.51	1.05 \pm 0.19	1.42 \pm 0.18	189 \pm 41.3
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*NO values for reperfusion were significant compared to control group ($p < 0.05$).

According to the results of histopathological examination of tissue samples:

Muscle tissue samples taken during ischemia and reperfusion for histopathological examination were stained by hematoxylin-eosin (HE). In each group, the samples were examined for hyalinization, necrosis, inflammation and hemorrhage. Significant histopathological properties of groups were shown in (Figure3). Severity score of histopathological examination was rated as 0: none, 1: mild, 2: moderate, and 3: severe. Mean values of groups were shown in Table 3. It was seen in Figure 3: G1: Focal congestion area in the control group, G2: The hyalinisation in muscle tissue. It was seen in figure 4: G4: Active chronic inflammation at the periphery of bleeding areas involved by neutrophils.

Statistics

All numeric data were presented as mean± standard

error of mean(SEM). Two-ways ANOVA multi comparison test was used to compare the parameters between groups. The value P<0.05 was considered as statistically significant.

Results

All experimental subjects completed the study without mortality. In all groups, TAS, TOS, SOD, MDA, NO and NO concentration was statistically non-significant in control group and I/R group of 3 hours(p>0.05). TAS, TOS, SOD, MDA, NO and NO levels of ischemic soleus muscle are shown in Table 1.

There was no statistically significant difference between groups in respect of TAS, TOS, SOD, MDA and NO concentrations for both ischemia and reperfusion at predetermined period. However there was a difference for NO values.

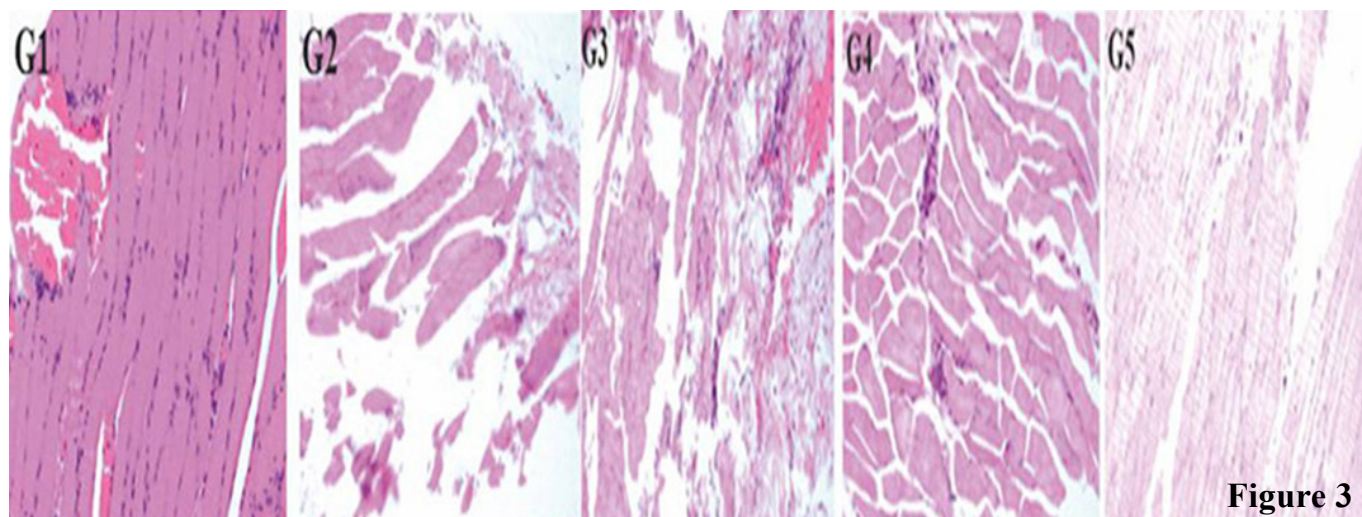


Figure 3

Table 3. Histopathological examination score of muscle.

Groups	Mean Hyalinization	Mean Necrosis	Mean Inflammation	Mean Hemorrhage
Control	1	0	0	0.83
G1	1.33	0	0.33	1.5
G2	1	0	0.16	1.16
G3	1.33	0	0.5	1.33
G4	1	0	1.16	1.33

0: none, 1: mild, 2: moderate, 3: severe.

NO value was significant in group receiving benfothiamin + vitamin D compared to control group during both ischemia and reperfusion. In group receiving only vitamin D, the significance was present only for reperfusion. This showed us benfothiamin considerably increased the acute protective amount of NO during given I/R period and that vitamin D increased remarkably the NO during reperfusion. Concomitant administrations of benfothiamin and vitamin D led also acute increase in NO. Muscle necrosis was not present in any of groups during I/R periods. Hyalinization, inflammation and hemorrhage were mildly present.

Discussion

Acute lower extremity I/R may lead to severe tissue damage and systemic complications. Rate of mortality and amputation due to this condition were reported as 15-52% and 12-22%, respectively.⁽¹⁶⁾ Early diagnosis and early revascularization should be provided as soon as possible. During reperfusion, toxic free oxygen radicals are formed due to re-exposure of tissue to oxygen.⁽¹⁷⁾ In addition to clearance systems developed by the organism to eliminate them, certain substances were also shown to be effective in preventing experimental IR injury. Studies showed that benfothiamin, one of these substances, has suppressive effect on reactive oxygen species.⁽¹⁸⁾ In diabetic rats, benfotihamin normalized many oxygen species.⁽¹⁹⁾ In addition, it reduced harmful oxidative effects in streptozocin (STZ)- induced diabetic rats.⁽²⁰⁾ There are several studies showing that Benfothiamin supplement relieved neuropathic pain, vasodilator effect, demonstrated favorable effects on renal and cardiac systems and contributed to wound healing.⁽²¹⁻²⁴⁾

Although there is evidence for effects of vitamin D on aspects of myogenesis, muscle cell signaling, muscle function, and muscle force^(25,26) the mechanistic action of vitamin D on the injured muscle remains largely unknown. We planned this study by considering that benfotihaminand vitamin D could show protective effects on tissue in IR injury.

Thiamine is not a fat-soluble vitamin. Therefore its oral bioavailability is low. Excess amount of thiamine

is eliminated via urine.⁽²⁷⁾ Benfothiamin is a fat-soluble form of thiamin can easily penetrates cellular membrane rich in fatty acids. According to a study comparing Benfothiamin and Thiamin, overall bioavailability is 420% higher (420% more recovery in cellular plasma).⁽²⁸⁾ By considering these properties, acute effects were evaluated during I/R following oral administration in rats. Each rats received benfothiamin 70 mg/kg by gavage tube and thus individual different dose administration has been prevented.

Biological maximal stimulating dose of vitamin D is 8.3 mg/kg (332.000 IU/kg) and this was subcutaneously administered.

Measurements of tissue oxidants and antioxidants were used to determine the damage caused by I/R in the tissue as well as the improving effect of benfothiamin and vitamin D. Total and individual parameters were analyzed during given I/R periods.

Histological changes were examined in soleus muscle specimens from experimental animals.

During I/R period of 3 hours, the oxidants TOS and MDA and the antioxidants TAS and SOD were not significantly affected by I/R statistically and their concentrations were not significantly changed by administration of both benfothiamin and vitamin D statistically. NO was not changed during given periods of I/R. However it was significantly increased by administration of benfothiamin and vitamin D.

In contrast to vasoconstrictor effect in the muscle due to ischemia, This increase in NO level is protective. The beneficial effect of vitamin D was more significant during reperfusion, while benfothiamin was significantly effective during both ischemia and reperfusion upon its administration alone as well as in combination with vitamin D. The effect of benfothiamin and benfotihamin+ vitamin D may be clinically beneficial in order to induce protective effect against NO in any type of traumatic and degenerative vascular I/R conditions.

Acute and long term effects of benfothiamin and vitamin D can be investigated during more prolonged periods where irreversible changes due to I/R occurs.

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Corresponding author:

Özlem Keskin

Mail: zlemkeskin@hotmail.com

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