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The effect of fullerenol C60 on skeletal muscle after lower limb ischemia reperfusion injury in streptozotocin-induced diabetic rats

Streptozotosin ile diyabet oluşturulan ratlarda alt ekstremite iskemi reperfüzyonuna karşı fullerenol C60'ın etkileri

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Abstract

Aim: Fullerenol, a water-soluble C60-fullerene, has been demonstrated to scavenge free radicals in vitro and in vivo. The aim of the study was to investigate the effects of fullerenol C60 on lower skeletal muscles in a rat model of ischemia/reperfusion (I/R).

Methods: After approval of the ethics committee, 30 Wistar Albino rat were divided into 5 groups with six animals per each as follows: Control (C), diabetes (D), diabetes+fullerenol C60 (DF), diabetes+I/R (group DIR) and diabetes I/R+fullerenol C60 (DIR-F) groups. Streptozotocin was administered to the rats to induce diabetes at a dose of 55 mg/kg. Four weeks after the onset of diabetes, rats were subjected to 2 hours of ischemia and 2 hours of reperfusion. At the end of the reperfusion period, skeletal muscle samples were taken from the lower extremity in all groups for histopathological and immunohistopathological examinations.

Results: Myositis and endothelial caspase 3 enzyme activities were high in all groups, particularly DIR. Compared to C, DF and DIR-F groups, inflammation and myositis were significantly higher in the DIR group (P=0.001, P=0.006, P=0.001, respectively, and P=0.001, P=0.022, P=0.001, respectively). Vascular dilatation and congestion were significantly more prominent in all groups compared to the control group (P=0.001 for all).

Conclusion: Our results confirm that fullerenol C60 has protective effects against skeletal muscle damage resulting from I/R in diabetic rats. Future studies conducted to evaluate these effects may help illuminate the action mechanism of fullerenol C60 and pathophysiology underlying the tissue damage related to I/R injury.

Keywords: Ischemia reperfusion, Fullerenol C60, Caspase 3, Rat

Öz

Amaç: Suda çözünür bir fulleren olan Fullerenol C60'ın, serbest radikalleri in vitro ve in vivo temizleyebildiği gösterilmiştir. Çalışmanın amacı, fullerenol C60'ın iskemi reperfüzyon (I/R) sıçan modelinde alt iskelet kasları üzerindeki etkilerini araştırmaktı.

Yöntemler: Etik kurul onayı alındıktan sonra 30 Wistar Albino sıçan; 5 gruba ayrıldı (n: 6); Kontrol (C), diyabet (grup D), diyabet + fullerenol C60 grubu (DF), diyabet + I/R (grup DIR) ve diyabet I/R + fullerenol C60 (DIR-F). Diyabet için sıçanlara 55 mg / kg streptozotosin uygulandı. Diyabet oluşumundan dört hafta sonra sıçanlara 2 saatlik iskemi ve 2 saatlik reperfüzyon uygulandı. Reperfüzyon döneminin sonunda histopatolojik ve immünohistopatolojik incelemeler için tüm gruplardan alt ekstremite iskelet kası örnekleri alındı.

Bulgular: Miyozit ve endotelyal kaspaz 3 enzim aktiviteleri, özellikle DIR ve C, D, DF ve DIR-F grubunda yüksektir. Enflamasyon DIR grubunda C, DF ve DIR-F grubuna göre anlamlı olarak yüksektir (sırasıyla P<0,001, P=0,006, P<0,001). Myosit hasarı da DIR grubunda kontrol, C, DF ve DIR-F grubuna göre anlamlı derecede yüksektir (sırasıyla P<0,001, P=0,022, P<0,001). Vasküler dilatasyon ve konjesyon D, DF, DIR ve DIR-F grubunda kontrol grubuna göre anlamlı olarak yüksektir (P<0,001, tümü).

Sonuç: Sonuçlarımız, fullerenol C60'ın diyabetik sıçanlarda I/R'den kaynaklanan iskelet kası hasarına karşı koruyucu etkileri olduğunu doğrulamaktadır. Fullerenol C60'ın I/R hasarı üzerindeki etkilerini değerlendirmek için yapılacak gelecekteki çalışmalar, fullerenol C60'ın olası koruyucu etkilerini ve I/R hasarına bağlı doku hasarının altında yatan mekanizmaları anlamaya yardımcı olabilir.

Anahtar kelimeler: İskemi reperfüzyon, Fullerenol C60, Kaspaz 3, Sıçan

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Introduction

Ischemia/reperfusion (I/R) results in serious injuries in tissues and organs. I/R is a complex and biphasic process, which causes cell damage and occurs due to numerous factors [1,2]. Ischemia initiates organ damage and the process of death by reducing the formation of energy required to achieve ionic gradient and hemostasis. Reperfusion causes both local and systemic inflammatory response, which may result in widespread microvascular dysfunction [3]. Infra-renal abdominal aorta clamping results in ischemia of distal body parts. Unclamping after a clamping period causes reperfusion injury of local and distant organs/tissues [4,5].

Some nanoparticles can be used for the treatment of injury due to ischemia [6]. Fullerene, with the chemical formulation of C60, is an allotrope of carbon as a nanoparticle and can react with oxygen free radicals [7-9]. Fullerenol (C60(OH)18-22) is one of the water-soluble derivates of C60 fullerenes which is demonstrated to reduce the severity of oxidative damage during an ischemia period by abolishing reactive oxygen species (ROS). C60 fullerenes function as free radical scavengers [7]. Fullerenol is shown to prevent the catabolic activity of vertebral bone marrow stromal cells by reducing ROS, matrix metalloproteinases (MMPs), and tumor necrosis factor-α (TNF-α) and increasing the activation of antioxidant enzymes [10]. Inflammatory cytokines and apoptotic signals are also reduced by fullerenol [11,12]. Fullerenol C60 has no acute toxicity towards cells/tissues [13-16]. C60 fullerenes can easily accumulate inside the cells/organelles as powerful antioxidants.

The aim of the study was to investigate the potential protective effects of fullerenol C60 on I/R injury in skeletal muscles in a rat model.

Materials and methods

Animals and experimental protocol

This study was conducted upon the consent of Experimental Animals Ethics Committee of Gazi University. All the procedures were performed according to accepted standards of Guide for the Care and Use of Laboratory Animals.

30 Wistar Albino rats (200- 250 g) were used. The rats were kept at 20-21ºC in cycles of 12 hours of daylight and 12 hours of darkness and had free access to food until two hours before the anesthetic procedure. The animals were randomly separated into five groups, each containing six rats. Control group (C), Diabetes group (D), Diabetes+ Fullerenol C60 (DF), Diabetes+ischemia-reperfusion (DIR), Diabetes+ischemia-reperfusion+ fullerenol C60 (DIR-F).

Diabetes was induced by a single intraperitoneal injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA) at a dose of 55 mg/kg. Seventy-two hours after the injection, the blood glucose levels were measured. Rats were classified as diabetic if their fasting blood glucose (FBG) levels exceeded 250 mg/dl, and only animals with FBGs of >250 mg/dl were included in the diabetic groups (D, DF, DIR, DIR-F). The rats were kept alive for four weeks after streptozotocin injection to allow the development of chronic diabetes before they were exposed to I/R.

Control group (Group C): Only midline laparotomy was performed without any additional surgical intervention. After 4 hours of follow-up, they were sacrificed, and skeletal muscle tissue specimens were collected for histopathological and immunohistopathological investigation.

Diabetes group (Group D): Only midline laparotomy was performed without any additional surgical intervention. After 4 hours of follow-up, they were sacrificed, and skeletal muscle tissue specimens were collected for histopathological and immunohistopathological investigation.

Diabetes-Fullerenol C60 group (Group DF): Midline laparotomy was performed without any additional surgical intervention**.** Fullerenol C60 100 $\mu g.kg^{-1}$ was administered intraperitoneally: After 4 hours of follow-up, all rats received ketamine at a dose of 100 mg/kg intraperitoneally and were sacrificed. Skeletal muscle tissue specimens were collected for histopathological and immunohistopathological investigation.

Diabetes-Ischemia-reperfusion group (Group DIR): Midline laparotomy was performed similarly. Infrarenal aorta was left clamped for 2 hours. After removing the clamp, reperfusion was established for another 2 hours. At the end of 4 hours, rats were sacrificed, and skeletal muscle tissue specimens were collected for histopathological and immunohistopathological investigation.

Diabetes-Ischemia-reperfusion group with fullerenol C60 (Group DIRF): After following the same steps in I/R group, fullerenol C60 was administered (100 $\mu g.kg^{-1}$) intraperitoneally 30 minutes before the ischemia period. At the end of 4 hours, rats were sacrificed, and skeletal muscle tissue specimens were collected for histopathological and immunohistopathological investigation.

Histopathological and immunohistopathological evaluation. Tissues were fixed in 10% formaldehyde for 12 hours at room temperature. Sections (3-4 μm thick) were cut from the fixed tissue samples, embedded in paraffin blocks and mounted on poly-L-lysine-coated slides (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), all of which were left overnight at 45˚C. The sections were held for 20 minutes at 75˚C, followed by tap fixation and paraffin extraction. Deparaffinization was performed with a Leica Bond-Max automatic immunohistochemical/*in situ* hybridization stainer (Leica Microsystems GmbH, Wetzlar, Germany). Citrate buffer was applied for antigen retrieval for 30 minutes at 75˚C and washed with bond wash solution (Leica Microsystems GmbH). Sections were blocked with 0.3% hydrogen peroxide for 5 minutes at room temperature, then incubated with primary antibodies against caspase-3 (1:400; p11, C-6; cat. no. sc-271759; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and caspase-8 (1:200; D-8; cat. no. sc-5263; Santa Cruz Biotechnology, Inc.) for 15 minutes. The secondary antibodies (Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK) were incubated with cells for 8 minutes. The Bond™ Polymer Refine Detection system (cat. no. DS9800; Leica Biosystems Newcastle Ltd.) was added as a horseradish peroxidase polymer (a secondary antibody substitute) for 8 minutes. DAB (Leica Microsystems GmbH) was applied to the cells for 6 minutes and the marking became visible. Hematoxylin counterstaining was also performed

at 6 minutes. All steps following blocking of sections with hydrogen peroxide occurred at room temperature.

The stained samples were covered with balsam following washing in water and alcohol and cleared in xylene. The cytoplasmic caspase-3 staining was evaluated in myocyte and endothelia using a light microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan) at a magnification of x400.

For hematoxylin and eosin staining, slides were kept in an oven at 72˚C for 20 minutes, deparaffinized in xylene solution and washed with alcohol three times. Sections were then incubated in hematoxylin for 4 minutes at room temperature, washed and exposed to acid-alcohol and ammonia solutions for a few seconds, then incubated in eosin for 6 minutes at room temperature, and immersed in a descending alcohol series and xylene. Stained slides were covered with slip and evaluated with a light microscope at a magnification of x400.

Statistical analysis

SPSS (IBM Corp., Armonk, NY, USA; version 20.0) was used for all statistical analysis. Descriptive statistics are presented as mean, standard deviation (SD) values. Group averages were compared with one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The myocyte caspase 3 activity in the skeletal muscle tissue was significantly higher in the DIR group as compared to C, D, DF, and DIR-F groups (p=0.001 for all). There was a statistically significant difference between the groups in terms of endothelial caspase 3 enzyme activity in skeletal muscle tissue ($P=0.001$). Endothelial caspase 3 in DIR group was significantly higher than that in C, D, DF, and DIR-F groups ($P=0.001$ for all). Additionally, that of the D group was significantly higher than that of the DIR-F group ($P=0.005$) (Table 1, Figure 1-5).

Inflammation in skeletal muscle tissue in the DIR group was significantly higher than that in C, DF, and DIR-F groups (P=0.001, P=0.006, P=0.001, respectively). In addition, inflammation in D group was significantly higher than that of C, DF and DIR-F groups ($P=0.001$, $P=0.006$, $P=0.001$, respectively) (Table 2, Figures 6–10).

The groups were compared in terms of skeletal myocyte damage ($P=0.001$), which was significantly higher in the D, DF, DIR, and DIR-F groups than that in the C group ($P=0.001$ for all). It was also significantly higher in DIR group compared to C, DF, and DIR-F groups ($P=0.001$, $P=0.022$, $P=0.001$, respectively) (Table 2, Figures 6–10).

Vascular dilatation and congestion were higher in D, DF, DIR, and DIR-F groups compared to the control group ($P=0.001$ for all) (Table 2, Figures 6–10)).

Table 1: Muscle tissue caspase 3 values [Mean (SD)]

	Group C (n=6)	Group D (n=6)	Group DF (n=6)	Group DIR (n=6)	Group DIR-F (n=6)	*P*-value **
Myocyte	0.00 (0.00)*	0.00 (0.00)*	0.00 (0.00)*	2.00 (0.26)	0.00 (0.00)*	0.001
Endothelial	1.17 (0.17)*	2.00 (0.00)*	1.67 (0.33)*	3.33 (0.21)	0.83 (0.17)*,&	0.001

*P***: Significance level with One Way ANOVA test $P<0.05$, *$P<0.05$: Compared with group DIR, &$P<0.05$: Compared with group D, Note: zero value indicates no damage

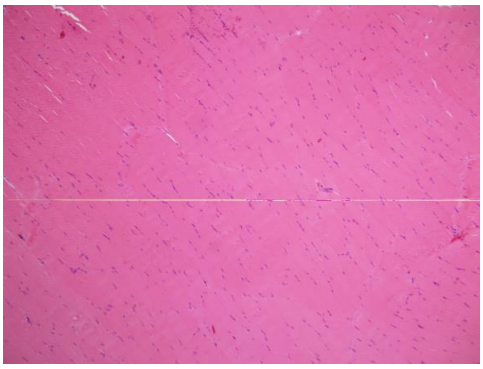

Figure 1: No inflammation, fibrosis or vascular dilation were observed in the muscle tissue taken from the control group (H/E x 100)

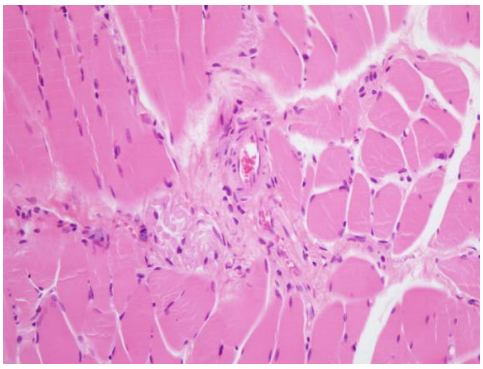

Figure 2: In the muscle sample from the diabetic group, there was minimal inflammation, vascular dilation, congestion between muscle fibers, and damage to myocytes (H/E x 400)

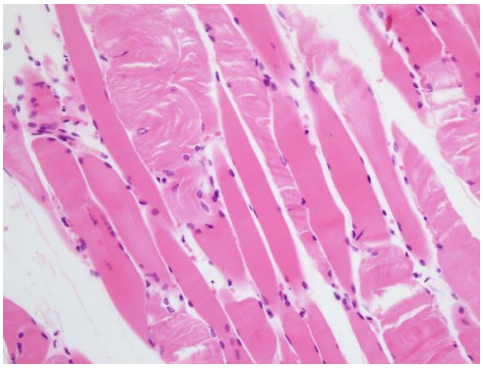

Figure 3: In the muscle sample from the diabetic-ischemia group, there was considerable damage, inflammation, vascular dilation, and congestion in myocytes (H/E x 400)

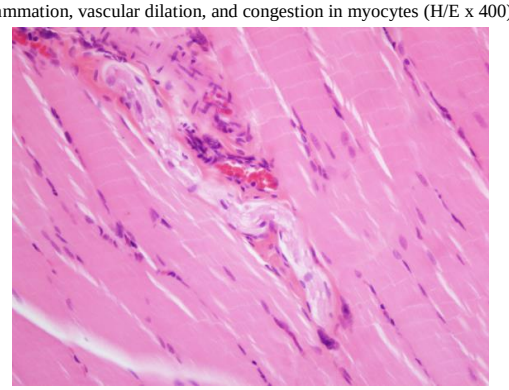

Figure 4: In the muscle sample from the diabetic-fullerenol group, damage, vascular dilation, and congestion were observed in myocytes (H/E x 400)

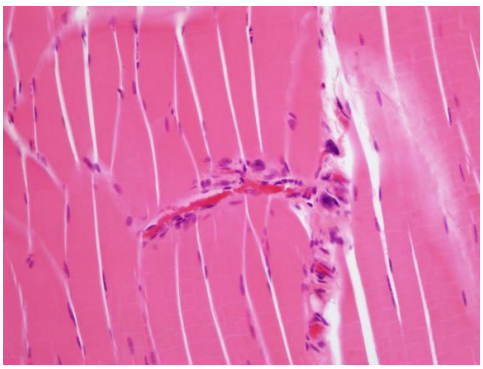

Figure 5: In Diabetic-ischemia-fullerenol group, various myocytes were damaged, vascular dilation, and congestion were observed (H/E x 400)

Table 2: Histopathological examination of muscle tissue [Mean (SD)]

	Group C (n=6)	Group D (n=6)	Group DF (n=6)	Group DIR (n=6)	Group DIR-F (n=6)	*P*-value **
Inflammation	0.00 (0.00)*,&	1.00 (0.00)	0.33 (0.21)*,&	1.00 (0.00)	0.17 (0.17)*,&	0.001
Fibrosis	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	-
Vascular dilation	0.00 (0.00)	1.00 (0.00)+	1.00 (0.00)+	1.00 (0.00)+	1.00 (0.00)+	0.001
Congestion	0.33 (0.21)	1.00 (0.00)+	1.00 (0.00)+	1.00 (0.00)+	1.00 (0.00)+	0.001
Steatosis	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	-
Myocyte injury	0.00 (0.00)	1.50 (0.22)+,*	1.33 (0.21)+,*	2.67 (0.21)+	1.67 (0.17)+	0.001

*P***: Significance level with One Way ANOVA test *P*<0.05, **P*<0.05: Compared with group DIR, +*P*<0.05: Compared with group C, Note: zero value indicates no damage

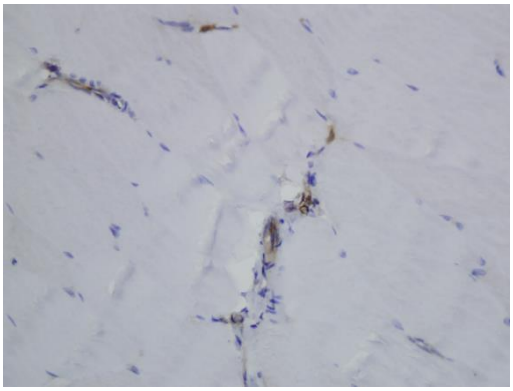

Figure 6: In Caspas-3 immunohistochemical study on muscle tissue in the control group, mild-moderate staining was detected only in endothelial cells(x400)

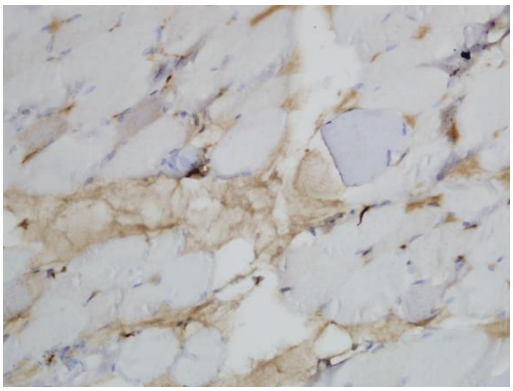

Figure 7: According to Caspase-3 immunohistochemical study of the muscle sample in the diabetic group, there was moderately positive staining in damaged myocytes and endothelial cells (x400)

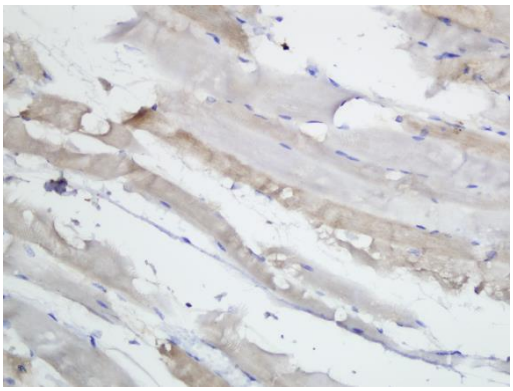

Figure 8: In Caspase-3 immunohistochemical study of the muscle sample in the diabetic-ischemia group, moderate positive staining was observed in the damaged myocytes (x400)

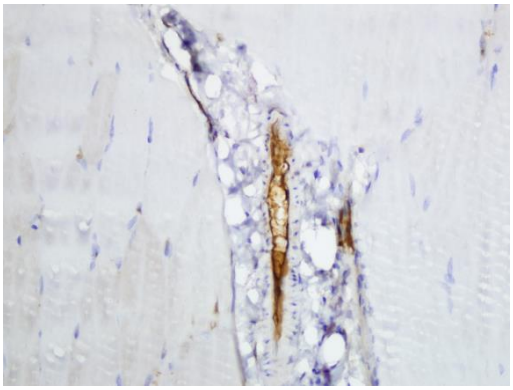

Figure 9: In the muscle sample from the diabetic-fullerenol group, only positive staining detected in the endothelial cells, but no staining in myocytes (x400)

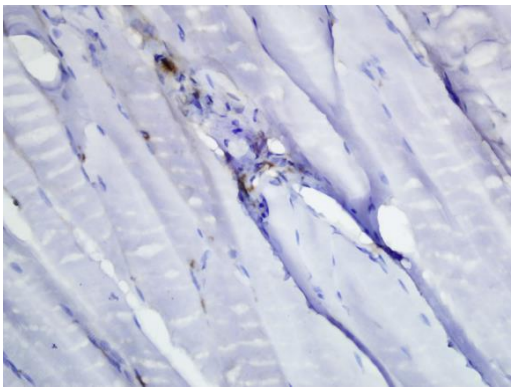

Figure 10: In the muscle sample from diabetic- ischemia-fullerenol group, only positive staining was detected in endothelial cells, but no staining in myocytes (x400)

Discussion

Reperfusion injury is still being studied today [17]. Ischemia reperfusion injury is especially important for not only adjacent organs but distant organs as well, such as the lungs, kidney, and heart [2,18,19]. After ischemia, reperfusion increases the rate of injury caused by the ischemic period and aggravates the damage [2,18]. Reperfusion generates ROS. Then, ROS causes lipid peroxidation in cell membranes [20]. Various I/R injury models have been used [21-24] and many studies have been conducted about I/R [25-27]. It is known microcirculation may be affected by diabetes, along with I/R, which is the reason we used STZ-induced diabetic rats to determine the effects of fullerenol.

Reperfusion injury has some features such as vasoconstriction, thrombosis, edema, leukocyte infiltration, and increased free radicals [28]. Various methods have been described to evaluate muscle injury due to I/R such as mitochondrial enzyme activity, the permeability of vessels, lactate dehydrogenase levels, neutrophil infiltration [28-30]. Histologic examination may also be used in the evaluation of I/R injury [31]. A histologic examination can show myocyte injury directly, including the integrity of the cell membrane, gaps within the cell, and staining intensity [30,32]. In previous studies, morphological changes due to I/R was shown [33-35]. In this study, we used histopathological examination to show I/R injury and the effect of fullerenol on I/R injury of skeletal muscle.

Previous studies found that fullerene derivatives are potent antioxidants [36,37] and have tissue-protective effects against oxidative damage [38,39]. In addition, it is known that C60 can react with up to 34 methyl radicals and release nitric oxide (NO). Therefore, we used fullerene derivates in our study. A previous study demonstrated that fullerenol attenuated ischemia-induced lung injury [36]. Foroshani et al. [40] stated that fullerenol decreased ischemia-induced brain edema. Zavodovsky et al. [41] conducted a study about the influence of C 60 fullerene on I/R injury in the skeletal muscle and found that fullerene reduced ischemic muscle trauma. Erer et al. [42] used iloprost to find that lung injury induced by skeletal muscle I/R was alleviated. Another study showed that fullerene derivates decreased neurological dysfunction and brain edema and had protective effects against ischemia-induced damage [43]. Our findings also revealed that fullerenol C60 has protective effects against lower skeletal muscle damage due to ischemia-reperfusion injury.

Limitations

We have some limitations to our study. First, we investigated the effect of fullerenol C60 on I/R injury in skeletal muscle by using immunohistological examination only. We did not use any grading and scoring methods to show skeletal muscle injury. In addition, we did not examine any biochemical parameters and ROS levels.

Conclusion

We showed that fullerenol C60 has protective effects against skeletal muscle damage after I/R in diabetic rats. We believe that researching nanoparticles, the most important raw material of the future, particularly fullerenol C60, can help understand the possible protective effects and mechanisms underlying I/R damage.

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