Effect of Fullerenol C60 on Erythrocyte Deformability During Ischaemia-Reperfusion Injury of Lower Extremity in Diabetic Rats

Fullerenol C60'ın Diyabetik Ratlardaki Alt Ekstremite İskemi Reperfüzyon Hasarı Sırasındaki Eritrosit Deformabilitesine Etkisi

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ABSTRACT

Background: Fullerenol, a water-soluble C60-fullerene derivative synthesized by Chiang et al, has been demonstrated to be able to scavenge free radicals in vitro and in vivo. Although its protective effects have been already studied and shown in ischemia reperfusion (IR) injury, additional investigation is necessary for its effect on erythrocyte deformability. The purpose of our study was to look into the effects of fullerenol C60 on erythrocyte deformability in rat lower extremity ischemia reperfusion injury model.
Materials and Methods: After approval of the Ethics Committee, 30 Wistar Albino rat were divided into 5 groups (n:6) as; Control (C), Diabetes (group D), diabetes+ fullerenol C60 group (DF), diabetes+ IR (group DIR) and diabetes IR+ fullerenol C60 (DIRF). 55 mg/kg streptozotocin was administered to the rats for diabetes. After the period of 72 hour, blood glucose concentration was mesured, 250 mg/dl and above were considered as diabetic rat. Four week after the formation of diabetes, rats were subjected to 2 hour ischemia and 2 hour reperfusion. Erythrocyte packs were prepared from heparinized blood samples and deformability measurements were performed.
Results: The deformability index was significantly increased in diabetic rats; however, it was similar in group D, DF and DIRF. It was significantly increased in group DIR when compared to group C, D, DF and DIRF. The relative resistance was increased in I/R models.
Conclusion: This study aimed to investigate the effects of IR on erythrocyte deformability which may lead to disturbance in blood flow and hence tissue perfusion in infrarenal rat aorta. We found that fullerenol C60 had beneficial effects by reversing undesirable effects of IR. In our opinion, further studies with larger volume are required to support our promising results.

Key Words: Erythrocyte deformability, ischemia reperfusion, fullerenol C60, rat

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ÖZET

Amaç: Fullerenol Chiang ve arkadaşları tarafından sentezlenen C60-fullerenin suda çözünebilen bir bileşenidir. Bu bileşiğin in vitro ve in vivo olarak serbest radikalleri tuttuğu gösterilmiştir. Bu bileşiğin iskemi reperfüzyon (İR) hasarındaki koruyucu etkileri gösterilmiş olmakla birlikte eritrosit deformabilitesi üzerindeki etkilerinin yapılacak çalışmalarla gösterilmesi gereklidir. Bu çalışmanın amacı ratlardaki alt eksremite iskemi reperfüzyon hasarı modelinde fullenerol C60'ın etkilerini araştırmaktır.
Yöntem: Etik kurul onayı alındıktan sonra 30 adet Wistar Albino rat eşit olarak 5 gruba ayrılmıştır (n:6); Kontrol (K), Diyabet (grup D), diyabet+ fullerenol C60 grup (DF), diyabet+ İR (grup DİR) ve diyabet İR+ fullerenol C60 (DİRF). Diyabet oluşturmak için ratlara 55 mg/kg streptozotosin uygulanmıştır. 72 saat sonra kan glukoz düzeyi 250mg/dl üzerindeki ratlar diyabetik ratlar olarak değerlendirilmiştir. Diyabet oluşturulduktan dört hafta sonra ratlar 2 saat süre ile iskemi ve ardından 2 saat süre ile reperfüzyona maruz bırakılmıştır. Heparinli kan örneklerinden eritrosit paketleri hazırlanmış ve deformabilite ölçümleri yapılmıştır.
Bulgular: Diyabetik ratlarda deformabilite indeksi anlamlı olarak yükselmiştir; bununla birlikte grup D, DF ve DİRF'deki deformabilite indeksi değerleri aynı bulunmuştur. Grup K, D, DF ve DİRF ile karşılaştırıldığında ise Grup DİR'de elde edilen deformabilite indeksi değerleri anlamlı olarak yüksek bulunmuştur.
Sonuç: Bu çalışmanın amacı kan akımında düzensizliğe ve ratların infrarenal aortasındaki doku perfüzyonununda bozukluğa neden olan iskemi reperfüzyonun eritrosit deformabilitesi üzerindeki etkilerini araştırmak idi. Fullerenol C60'ın iskemi reperfüzyonun istenmeyen etkilerini geri çevirmede yararlı etkileri olduğunu tespit ettik. Çalışmamızın umut verici bulgularını destekleyecek geniş ölçekli yeni çalışmalara ihtiyaç olduğunu düşünmekteyiz.

Anahtar Sözcükler: Eritrosit deformabilitesi, iskemi reperfüzyon, fullerenol C60, rat

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INTRODUCTION

Ischemia reperfusion injury (IR) in lower extremity is a frequent and important clinical phenomenon. Reperfusion period following an ischemic insult may paradoxically cause increased rates of mortality and morbidity due to systemic complications. Local edema and muscle tissue necrosis are likely to be followed by systemic inflamatory response syndrome and multiple organ failure (kidney, respiratory and circulatory system etc.) as reperfusion advances (1-3).

It is known that free radicals are a major pathogenic factor in the development of ischemic damage in the muscle tissue (4). Preliminary biological studies of water-soluble pristine C_{60} fullerenes (5–9) have shown that at low (physiological) concentrations, they do not exhibit acute toxic effects on the normal cells (10–12), they are not allergenic and immunogenic and they able to regulate free-radical processes in the cells and tissues, in particular, neutralize excess free radicals (13, 14). Consequently, the use of biocompatible and bioavailable C_{60} fullerenes as powerful antioxidants (15) opening up new potential opportunities for the prevention and correction of ischemic-reperfusion pathological processes in the muscle tissue.

Beneficial effect of fullerenol C60 (OH) 24 on the activity of antioxidant enzymes was confirmed in erythrocytes after a single dose administration of doxorubicin in rats pretreated with C60 (OH) 24 (16) as well as in hepatocytes from rats with colorectal cancer (17) and mammary carcinomas (18), muscle IR (19), and cardiac IR (20) were investigated in several studies. However, it is still unknown if fullerenol C60 nanoparticles can be used to attenuate erythrocyte deformability caused by IR. Our study aims to look into the potential effect of fullerenol C60 on lower extremity muscle ischemia and subsequent IR injury which is provoked with the tourniquet method.

MATERIALS and METHODS

Animals and Experimental Protocol

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

Thirty 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 (DIRF).

Diabetes was induced by a single injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA), at a dose of 55 mg/kg (i.p) body weight. 72 hours afeter 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 (diabetes, diabetes+Fullerenol C60, diabetes+ischemia-reperfusion and diabetes+ Fullerenol C60 -ischemia-reperfusion). 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): Midline laparotomy was done alone without any additional surgical intervention. After 4 hours of follow-up, blood sample was collected and subjects were sacrificed.

Diabetes group (Group D): Midline laparotomy was done alone without any additional surgical intervention. After 4 hours of follow-up, blood sample was collected and subjects were sacrificed.

Diabetes-Fullerenol C60 group (Group DF): Similarly Midline laparotomy was done alone without any additional surgical intervention**.** Fullerenol C60 100 $\mu g.kg^{-1}$ was administered intraperitoneally and again after 4 hours of follow-up, blood sample was collected and subjects were sacrificed.

Diabetes-Ischemia-reperfusion group (Group DIR): Midline laparotomy was done similarly. Infrarenal aorta was left clamped for 2 hours. After removing the clamp, reperfusion was established for another additonal 2 hours. At the end of 4 hours, blood samples were collected from the abdominal aorta and subjects were sacrificed

Diabetes-Ischemia-reperfusion group with fullerenol C60 (Group DIRF): After following the same steps in I/R group, fullerenol C60 was given (100 $\mu g.kg^{-1}$) intraperitoneally 30 minutes before the ischemia period. At the end of 4 hours, blood samples were collected from the abdominal aorta and subjects were sacrificed.

After ketamine (100 $mg.kg^{-1}$,ip) injection intraabdominal blood samples were collected. Erythrocyte packs were prepared with heparinized total blood samples. Erythrocyte suspensions of 5% hematocrit with phosphate buffered saline (PBS) were used for deformability measurements.

Deformability Measurements:

First the samples were centrifuged for ten minutes at 1000 rpm and then serum and the buffy coat on erythrocytes were removed. Then, isotonic PBS buffer was added to the collapsing erythrocytes. This mixture of PBS and erythrocytes was centrifuged for another ten minutes at the same speed of 1000 rpm. Subsequently, liquid was removed from the upper surface. Finally pure red cell packs were obtained from three consequent washing process. PBS buffer was mixed with erythrocyte packs in order to obtain a value. And those mixed suspensions with 5% hematocrit were used for deformability measurement. These procedures were done at 22 ºC.

Deformability parameters were analyzed with the constant-current filtrometer system. Samples of 10 ml erytrocytes suspension - PBS buffer were prepared for measurement. There was a constant flow rate of 1.5 ml/min through an infusion pump. We used a 28 mm nucleoporin polycabonate filter which has a pore diameter of 5 µm. A transducer detected the pressure changes during the erythroctes passage through the filter and the collected data was transferred to computer with MP 30 data equation system (Biopac Systems Inc, Commat, USA). The pressure of the system was calibrated before each measurement. Buffer (P_T) and then erythrocytes (P_E) were passed subsequently through from the filtration system and pressure changes were measured. The relative refractory period value (Rrel) was calculated by relating the pressure value of erythrocyte suspension to pressure value of buffer. Increase in Rrel as the deformability index was interpreted as adverse effect on erythrocyte deformability.

Statistical Analysis

SPSS 17.0 software program was used for statistical analyses and $p<0.05$ was considered statistically significant. The findings were expressed as mean ± standard deviation. Kruskal-Wallis variance analysis was preferred for data evaluation. The variables with significance were evaluated with Bonferroni corrected Mann-Whitney U test.

RESULTS

The deformability index was significantly increased in diabetic rats ($p<0.0001$); however, it was similar in group D, DF and DIRF.

It was significantly increased in group DIR when compared to group C, D, DF and DIRF ($p<0.0001$, $p=0.001$, $p=0.003$, $p=0.033$, respectively). The relative resistance was increased in IR models (Figure 1).

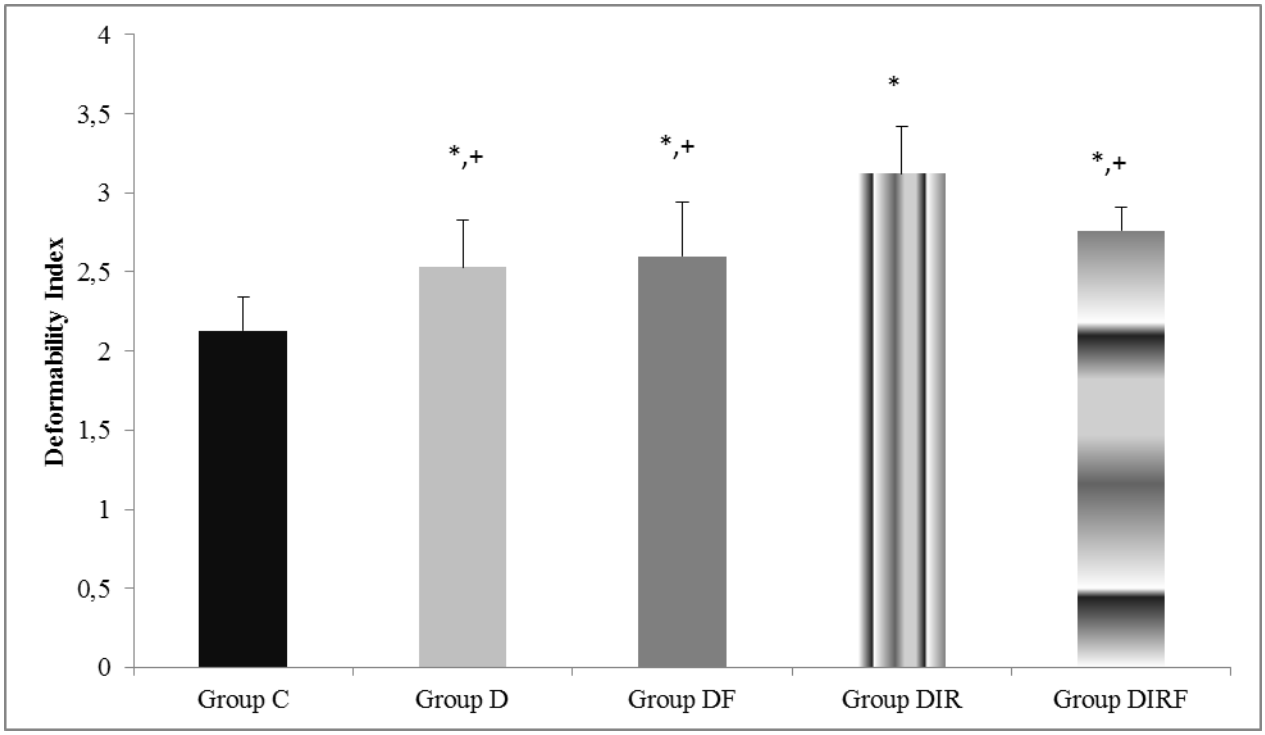


Figure 1: Erythrocyte deformability index values of the groups. Each bar represents the mean ± sd.
* p<0.05 compared to the Group C
+ p<0.05 compared to the Group DIR

DISCUSSION

It's a well-known fact that the oxidative nature of the IR process may lead to numerous unsolicited outcomes and insults in living organisms and tissues (21,22).

Previous results have shown that fullerenol nanoparticles can bind to the erythrocyte cytoskeleton and membrane proteins (23,24). Moreover, Grebowski and Krokosz showed that proved that fullerenol C60(OH)36 preferentially binds either to band 3 protein (a transmembrane protein responsible for anion Exchange which also acts as an anchor for many cytoskeletal proteins, including spectrin) or to membrane ATPases. Grebowski and Krokosz showed that interaction between fullerenol and membrane proteins is based on the available – SH groups of membrane proteins (25).

Several studies have tested the efficacy of C60(OH)36 nanoparticles in animal models of tissue damage. One of the first reports on the potential antioxidative properties of fullerenol is from the 1995 study, where Chiang et al. (26) showed that fullerenol acted as an effective scavenger of superoxide anions ($O_2^{\cdot-}$) generated by the xanthine-xanthine oxidase system. The antioxidant properties of fullerenols have also been extensively described by Nielsen et al. (27) and Markovic and Trajkovic (28) in their comprehensive review papers. In addition to the ability for scavenging reactive oxygen species (ROS), fullerenols also inhibit the reaction of reactive nitrogen species by reacting directly with nitric oxide (NO) (29).

Milic et al (16) showed that the results obtained by a concomitant administration of fullerenol and doxorubicin show a potential role for fullerenol in protecting hemoglobin and the erythrocytes. However, the results of the antioxidative enzymes show the existence of oxidative stress in red blood cells, but without a significant alteration in activity, compared to the control group in both experimental series.

Erythrocytes are responsible for the delivery of oxygen and vital molecules to the final organ capillaries as well as of metabolic wastes. They must be able to extend and curve to move in the capillary level hence this capacity, termed as "deformability" is much more important in microcirculation. Decreased erythrocyte deformability alters the oxygen delivery capacity and also has a negative impact on the survival of the circulating erythrocytes (30-32).

Grebowski et all (34) investigated research the radioprotecting potential of fullerenol C60(OH)36 on human erythrocytes irradiated by high-energy electrons of 6 MeV. The results demonstrate that C60(OH)36 at concentration of 150 µg/mL protects the erythrocytes against the radiation-induced hemolysis (comparing to non-protected cells, observed 30% and 39% protection for 0.65 and 1.3 kGy irradiation doses, respectively). Fullerenol C60(OH)36 protects human erythrocytes against high-energy electrons induced damage, however, can enhance radiation-induced functional perturbations of membrane proteins.

We think that measurement of erythrocyte deformability can be used as a parameter in cases of IR because its impairment leads to disturbance of microvascular perfusion and related problems. We were able to document the potential beneficial effect of fullerenol C60 on maintaining erythrocyte deformability after IR but we still think these promising results should further be supported by more detailed studies with larger volumes.

Conflict of interest
No conflict of interest was declared by the authors.

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