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

Fullerenol C60’in Diyabetik Ratlardaki Alt Ekstremite İskemi Reperfüzyon Hasarı Sırasında Eritrosit Deformabilitesine Etkisi

Gülay Kip1, Hakan Kartal1, Faruk Metin Çomu2, Yücel Polat3, Mustafa Arslan4, Ayşegül Küçük5

1Department of Paediatric Dentistry (Anaesthesiology and Reanimation Specialist), Gazi University Faculty of Dentistry, Ankara, Turkey
2Department of Cardiovascular Surgery, Gulhane Medical Faculty, Gulhane Education and Research Hospital, Ankara-Turkey
3Department of Physiology, Kırıkkale University Medical Faculty, Kırıkkale- Turkey.
4Department of Cardiovascular Surgery, Mehmet Akif Ersoy Thoracic and Cardiovascular Surgery Training and Research Hospital, Ankara-Turkey
5Department of Anaesthesiology and Reanimation, Gazi University Medical Faculty, Ankara-Turkey.

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 measured, 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 IR 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

Received: 03.13.2019  Accepted:05.08.2019

ÖZET

Amaç: Fullerenol Chiang ve arkadaşları tarafından sentezlenen C60-fullererenin suda çözünebilen bir bileşenidir. Bu bileşikin in vitro ve in vivo olarak serbest radikalleri tuttuğu gösterilmiştir. Bu bileşikin iskemi reperfüzyon (IR) hasarındaki koruyucu etkileri gösterilmiştir olmakla birlikte eritrosit deformabilitesi üzerindeki etkilerinin çalışılarak çalışılmasına gösterildi. Bu çalışmanın amacı ratlardaki alt ekstremitenin iskemi reperfüzyon hasarı modelinde fullerenol C60’ın etkilerini araştırmaktır.

Yöntem: Etki kurul onayı alınanızdan sonra 30 adet Wistar Albino rat eşit olarak 5 gruba ayrılmıştır (n:6); Kontrol (K), Diyabet (grup D), diyabet+ fullerenol C60 grup (DF), diyabet+ IR (grup DIR) ve diyabet IR+ fullerenol C60 (DIRF). Diyabet oluşturmak için ratlara 55 mg/kg streptozotocin uygulanmıştır. 72 saat sonra kan glukoz düzeyi 250mg/dl üzerindeki ratlar diyabetik ratlar olarak değerlendirilmiştir. Diyabet oluşturulan ratlar 2 saat süre ile iskemi ve ardından 2 saat süre ile reperfüzyona maruz bırakılmıştır. Hiperpansitik ve örneklendikten eritrosit paketleri hazırlanmış ve deformabilite ölçümleri yapılmıştır.

Bulgular: Diyabetik ratlarda deformabilite indeksi anlamlı olarak yükselmiştir; bununla birlikte grup D, DF ve DIRF’deki deformabilite indeksi değerleri aynı bulunmuştur. Grup K, D, DF ve DIRF ile karşılaştırıldığında ise Grup DIRF’de elde edilen deformabilite indeksi değerleri anlamlı olarak yüksek bulunmuştur.

Sonuç: Bu çalışmanın amacı akımda düzensizliği ve ratların infrarenal aortasındaki doku perfüzyonunun bozulacağı neden olan iskemi reperfüzyonun eritrosit deformabilitesini üzerindeki etkilerini araştırmaktır. Fullerenol C60’ın iskemi reperfüzyonunun istenmeyen etkilerini geri çevirmeye yardımcı etkisini olduğunu tespit etmek. Çalışmanının umut verici bulgularını destekleyecek geniş ölçekli yeni çalışmalara ihtiyaç duyulmaktadır.

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

Geliş Tarihi:13.03.2019  Kabul Tarihi:28.05.2019

Address for Correspondence / Yazışma Adresi: Mustafa Arslan, MD Gazi University Medical Faculty Department of Anaesthesiology and Reanimation 06510 Ankara- Türkiye. E-mail: mustarslan@gmail.com
©Telif Hakki 2019 Gazi Universitesi Tip Fakültesi - Makale metnine http://medicaljournal.gazi.edu.tr/ web adresinden ulaşılabilir.
©Copyright 2019 by Gazi University Medical Faculty - Available on-line at web site http://medicaljournal.gazi.edu.tr/ doi:http://dx.doi.org/10.12996/gmj.2019.96
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 inflammatory 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), and 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 fullerene C_{60} (OH) 24 on the activity of antioxidant enzymes was confirmed in erythrocytes after a single dose administration of doxorubicin in rats pretreated with C_{60} (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 fullerene C_{60} nanoparticles can be used to attenuate erythrocyte deformability caused by IR. Our study aims to look into the potential effect of fullerene C_{60} 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 20-21°C 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 C_{60} (DF), Diabetes+ischemia-reperfusion (DIR), Diabetes+ischemia-reperfusion+Fullerenol C_{60} (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 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 (diabetes, diabetes+Fullerenol C_{60}, diabetes+ischemia-reperfusion and diabetes+ Fullerenol C_{60} -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 IR.

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 C_{60} group (Group DF): Similarly Midline laparotomy was done alone without any additional surgical intervention. Fullerenol C_{60} 100 μg.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 additional 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 C_{60} (Group DIRF): After following the same steps in IR group, fullerenol C_{60} was given (100 μg.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°C.

Deformability parameters were analyzed with the constant-current filtrometer system. Samples of 10 ml erythrocytes 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 nikeloporfir polycarbonate filter which has a pore diameter of 5 μm. A transducer detected the pressure changes during the erythrocytes 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_b) 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).
induced hemolysis (comparing to non-protected cells, observed 30% and concentration of 150 μg/mL protects the erythrocytes against the radiation-electrons of 6 MeV. The results demonstrate that C60(OH)36 at fullerenol C60(OH)36 on human erythrocytes irradiated by high-energy 32).

Decreased erythrocyte deformability also has a negative impact on the survival of the circulating erythrocytes (30-32). The ability to extend and curve to move in the capillary level hence this capacity, is much more important in microcirculation. 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).

Grebowksi et al (34) investigated research the radioprotecting potential of fullerene 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.

**REFERENCES**

1. Duru S, Koca U, Oztekin S, Olguner C, Kar A, Coker C, et al. Antithrombin III pretreatment reduces neutrophil recruitment into the lung and skeletal muscle tissues in the rat model of bilateral lower limb and reperfusion: A pilot study. Acta Anaesthesiol Scand 2005;49:1142-8.

2. Turchániny B, Tóth B, Rácz I, Vendégh Z, Furész J, Hamar J. Ischemia reperfusion injury of skeletal muscle after selective deafferentation. Physiol Res 2005;54:25-32.

3. Lin B, Ginsberg M, Busto R, Li L. Hyperglycemia triggers massive neutrophil deposition in brain following transient ischemia in rats. Neurosci Lett 2000;278:1-4.

4. Walters TJ, Garg K, Corona BT. Activity attenuates skeletal muscle fiber damage after ischemia and reperfusion. Muscle Nerve 2015;52:640-8.

5. Prylutska SV, Matyshevskaya OP, Grynyuk II, Prylutsky YU, Ritter U, Scharff P. Biological effects of C60 fullerenes in vitro and in a model system. Mol Cryst Liq Cryst 2007;468:265-74.

6. Scharff P, Prylutska SV, Prylutsky YI, Grynyuk II, Ritter U, Scharff P. Pristine C60 fullerenes inhibit the rate of tumor growth and metastasis. Exp Oncol 2011;33:162-4.
9. Zay SY, Zavodovsky DA, Bogutska KI, Nozdrenko DN, Prylutskyy YI. Prospects of C60 fullerene application as a mean of prevention and correction of ischemic-reperfusion injury in the skeletal muscle tissue. Fiziol Zh 2016;62:66-77.

10. Prylutskyy SV, Matyshevska OP, Golub AA, Prylutskyy YuI, Potebnya GP, Ritter U, et al. Study of C60 fullerenes and C60-containing composites cytoxicity in vitro. Mater Sci Engineer C 2007;27:1121-24.

11. Prylutskyy SV, Grynyuk II, Grebinyk SM, Matyshevska OP, Prylutskyy YuI, Ritter U, et al. Comparative study of biological action of fullerenes C60 and carbon nanotubes in thymus cells. Mat-wiss u Werkstofftech 2009;40:238-41.

12. Tolkachov M, Sokolova V, Korolovych V, Prylutskyy YuI, Eyple M, Ritter U, et al. Study of biocompatibility effect of nanocarbon particles on various cell types in vitro. Mat-wiss u Werkstofftech 2016;47:216-21.

13. Burala AP, Sidorik YP, Prylutskyy SV, Matyshevska OP, Golub OA, Prylutskyy YI, et al. Catalytic system of the reactive oxygen species on the C60 fullerene basis. Exp Oncol 2004;6:326-7.

14. Burala AP, Sidorik YP, Prylutskyy SV, Matyshevska OP, Golub OA, Prylutskyy YI, et al. Comparative study of biological action of fullerenes C60 and carbon nanotubes in thymus cells. Mat-wiss u Werkstofftech 2008;16:698-705.

15. Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR, Moussa F. C60 fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. Nano Lett 2005;5:2578-85.

16. Milic VD, Stankov K, Injac R, Djordjevic A, Srdjenovic B, Govedarica B, et al. Activity of Antioxidative Enzymes in Erythrocytes after a Single Dose Administration of Doxorubicin in Rats Pretreated with Fullerolen C60(OH)24. Toxicol Mech Methods 2009;19:24-8.

17. Injac R, Perse M, Cerne M, Potocnik N, Radic N, Govedarica B, et al. Protective effects of fullerolen C60(OH)24 against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats with colorectal cancer. Biomaterials 2008;30:1184-96.

18. Shen C, Xing G, Wang J, Zhao Y, Li B, Tang J, Jia G, et al. Multihydroxylated (Gd@C82(OH)22)n nanoparticles: antineoplastic activity of high efficiency and low toxicity. Nano Lett 2005;5:2050-7.

19. Nozdrenko DM, Zavodovskiy DD, Matvienko TY, Zay SY, Bogutska KI, Prylutskyy YI et al. C60 Fullerene as Promising Therapeutic Agent for the Prevention and Correction of Skeletal Muscle Functioning at Ischemic Injury. Nanoscale Res Lett 2017;12:115. doi: 10.1186/s11671-017-1876-4.

20. Thompson LC, Urankar RN, Holland NA, Vidanapathiraka AK, Pitzer JE, Han L, et al. C60 exposure augments cardiac ischemia/reperfusion injury and coronary artery contraction in Sprague Dawley rats. Toxicol Sci 2014;138:365-78.

21. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. J Surg Res 2008;147:153-9.

22. Xu Z, Yu J, Wu J, Qi F, Wang H, Wang Z, et al. The effects of two anesthetics, propofol and sevoflurane, on liver ischemia/reperfusion injury. Cell Physiol Biochem 2016;38:1631-42.

23. Grebowski J, Krokosz A, Puchala M. Membrane fluidity and activity of membrane ATPases in human erythrocytes under the influence of polyhydroxylated fullerene. Biochim Biophys Acta 2013;1828:241-8.

24. Grebowski J, Krokosz A, Puchala M. Fullerolen C60(OH)36 could associate to bond 3 protein of human erythrocyte membranes. Biochim Biophys Acta. 2013;1828:2007-14.

25. Grebowski J, Krokosz A. The Effect of Highly Hydroxylated Fullerolen C60(OH)36 on Human Erythrocyte Membrane Organization. Journal of Spectroscopy 2015;10.1155/2015/825914.

26. Chiang LY, Lu FJ, Lin JT. Free radical scavenging activity of water-soluble fullerols. J Chem Soc Chem Commun 1995;12:1283-4.

27. Nielsen GD, Roursgaard M, Jensen KA, Poulsen SS, Larsen ST. In vivo biology and toxicology of fullerenes and their derivatives. Basic Clin Pharmacol Toxicol 2008;103:197-208.

28. Markovic Z, Trajkovic V. Biomedical potential of the reactive oxygen species generation and quenching by fullerenes (C60). Biomaterials 2008;29:3561-73.

29. Mirkov SM, Djordjevic AN, Andric NL, Andric SA, Kostic TS, Bogdanovic GM, et al. Nitric oxide-scavenging activity of polyhydroxylated fullerolen, C60(OH)24. Nitric Oxide 2004;11:201-7.

30. Zinchuk VV. Erythrocyte deformability: physiological aspects. Usp Fiziol Nauk 2001;32:66-78.

31. Kip et al. Red cell membrane damage. J Heart Valve Dis 1998;7:387-95.

32. Sivilotti ML. Oxidant stress and haemolysis of the human erythrocyte. Toxicol Rev 2004;23:169-88.

33. Grebowski J, Kazmierska P, Litwinienko G, Lankoff A, Wolszczak M, Krokosz A. Fullerolen C60(OH)36 protects human erythrocyte membrane against high-energy electrons. Biochim Biophys Acta Biomembr 2018;1860:1528-36.