Protective effects of vitamin E against myocardial ischemia/reperfusion injury in rats

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ABSTRACT

الأهداف: تقييم التأثير الوقائي لفيتامين هـ علي عضلة القلب ضد الضرر الذي ينتج عن إعادة التروية بعد الإقفار بالمقارنة مع عقار النيتروجلسرين.

الطريقة: أجريت هذه الدراسة العشوائية في قسم وظائف الأعضاء – كلية الطب – جامعة عين شمس خلال الفترة ما بين 1 يونيو 2009 حتى 31 أغسطس 2009م القاهرة – مصر. تم إجراء العملية على 28 فأر أنثى من نوع ويستر تتراوح أوزانهم من 150-200gm. تم تقسيم الفئران إلى 4 مجموعات، المجموعة الضابطة (غير معالجة)، المجموعة المعالجة بالنيتروجلسرين GTN تم حقنها بعقارالنيتروجلسرين GTN عن طريق الحقن البريتونى 25 دقيقة قبل التضحية بجرعة مقدارها 120µg/kg bw . المجموعة المعالجة بفيتامين هـ تلقى الفئران فيتامين هـ عن طريق الفم 16-20 ساعة قبل التضحية بجرعة مقدارها 250mg لكل فأر، وكذلك المجموعة المعالجة بفيتامين هـ و النيتروجلسرين GTN تلقى الفئران فيتامين هـ و النيتروجلسرين GTN كما في كلا المجموعتين التي تم علاجها بفيتامين هـ و عقار النيتروجلسرين GTN. بعد التضحية، تم استئصال القلب و ترويته بمحضر لانجندروف و تعريضه للاقفار لمدة 30 دقيقة، ثم إعادة التروية لمدة 30 دقيقة. كما تم قياس أنسجة القلب لتقدير MDA و NAD^+، و الفحص النسيجي بعد عملية التروية.

النتائج: أظهرت نتائج المعالجة بفيتامين هـ في تقوية الشفاء بعد الإقفار وذلك من العامل الانقباضي في المجموعة المعالجة بفيتامين هـ (المجموعة المعالجة بفيتامين هـ، و المجموعة المعالجة بفيتامين هـ و عقار النيتروجلسرين GTN) مقارنة بمجموعة التحكم. كما تم تقوية جريان الشريان بعد الشفاء من التروية مقارنة للمجموعة المعالجة بعقار النيتروجلسرين GTN. أظهرت مؤشرات الانتكاسة للنسيج الإقفاري، و MDA، و NAD^+ التأثير الوقائي لفيتامين هـ. كما أظهرت دراسة الأنسجة للنسيج القلبي لهذه الفئران تحسن الميتوكوندريا و الخلايا القلبية.

خاتمة: إعطاء فيتامين هـ يوفر حماية للقلب من الضرر الذي ينتج عن إعادة التروية بعد الإقفار الدموي مقارنة بالمعالجة بعقار النيتروجلسرين GTN، تشير النتائج إلى دور فيتامين هـ في تحسن وظائف الميتوكوندريا خلال إعادة التروية بعد الإقفار.

Objectives: To clarify the cardioprotective effects of a short course of vitamin E treatment (vit E) as compared with a nitric oxide donor, nitroglycerin (GTN) against ischemia-reperfusion induced heart injury in rats.

Methods: This randomized control study was conducted in the Physiology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt from 1st June to 31 August 2009. This work was undertaken on 28 female Wistar rats weighing 150-200 gm. Rats were allocated into 4 groups; control group (non-treated), GTN-treated group (rats received GTN intraperitoneally 25 minutes before sacrifice, in a dose of 120 µg/kg body weight), vit E-treated group (rat received vit E by oral tubal feeding 16-20 hours before sacrifice, in a dose of 250 mg/rat), and vit E and GTN-treated group (rats received vit E and GTN as in both GTN-treated group and vit E -treated group). After sacrifice, the hearts were excised and perfused in a Langendorff preparation and subjected to 30 minutes global ischemia and reperfused for 30 minutes. Following reperfusion, heart tissues were used for assessment of malondialdehyde (MDA) and nicotinamide adenine dinucleotide $(NAD)^+$, and for histological examination.

Results: Vitamin E treatment resulted in an enhanced post-ischemic recovery of systolic function in vit E-treated groups (vit E-treated group, and vit E and GTN-treated group) compared to the control group. Post-ischemic recovery of coronary flow was enhanced in the vit E-treated group compared to the GTN-treated group. Post ischemic tissue degeneration indicators: MDA, and NAD^+ indicated a cardioprotective effect of vit E. Histological study revealed marked improvement of myocytes and mitochondrial structure in the vit E-treated group as compared with the control group.

Conclusion: Preconditioning with vit E treatment afforded substantial recovery of post-ischemic contractile, and vascular functions compared to GTN treatment, the mechanism might involve less opening of mitochondrial permeability transition during postischemic reperfusion.

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Prevention of ischemia-reperfusion (I/R) injury is crucial for successful cardiac surgery. In cardiac surgery, it is reported that pharmacological agents can be administered prior to ischemia, enabling them to exert their protective effects on mitochondria prior to ischemia and reperfusion. The role of α-tocopherol as a chain-breaking antioxidant is well characterized in vitro; it is considered the major lipophilic antioxidant in the human body, specifically by its reaction with peroxyl free radicals.[1] It has been demonstrated that vitamin E (vit E) deficiency is responsible for increased myocardial injury caused by oxidative stress and that I/R of the heart is associated with a blunting in cardiac α-tocopherol levels.[2] Vitamin E has been extensively assayed in experimental animal diseases, and in the protection and treatment of human diseases. In the CHAOS study,[3] it has been reported that more than 2,000 patients with angiographic evidence of coronary disease were randomized to receive 400 or 800 intrauterine (IU)/day alpha-tocopherol for one year. This study led to a reduction in relative risk of cardiovascular mortality, and nonfatal myocardial infarction compared to placebo.[3] Research provided evidence that vit E intakes much higher than the current recommended dietary allowance could contribute to or improve human health. It has been reported that dietary requirements to prevent deficiency and maintain apparent health is substantially less than optimal amounts necessary to provide protection against degenerative conditions and chronic diseases. Results of a number of studies suggested that increased vit E intake is associated with decreased risk of coronary heart disease, and certain types of cancer as well as enhancement of immune function.[3,4] A review of the literature concerning safety, and tolerance of oral vit E demonstrated that vit E is relatively nontoxic.[4,5] In a 91-day study of rats receiving up to 316-443 mg vit E/animal/day, vit E had no adverse effects on weight gain, food intake, organ weights, hematology or serum chemistry values.[6] In the heart and cardiovascular system, nitric oxide (NO) plays a significant role. The specific roles of NO in the heart in general and on cardiac mitochondria in particular remain controversial. It has been reported that both endogenous and exogenous sources of NO exert important modulatory effects on mitochondrial function.[7] Nitric oxide donors have been shown to induce a powerful cardioprotection against (I/R) injury in mice.[8] However, literature reporting varying results of NO therapy, with some investigators reporting cardioprotective effects, whereas others report cardiotoxic effects.[9] Mitochondrial permeability transition (MPT) is a nonspecific pore in the inner mitochondrial membrane. It has been reported that the opening of the MPT converts the mitochondria from an organelle that provides adenosine triphosphate to sustain heart function into an instrument of cell death by apoptosis if the insult is mild, and to necrosis if the insult is profound.[10] It is hypothesized that a major component of I/R injury is necrotic cell death, which is widely thought to be the consequence of opening the MPT as reported by Costa et al.[11] Functional recovery of the Langendorff-perfused heart from ischemia inversely correlates with the extent of the opening, and inhibition of the MPT provides protection against reperfusion injury.[10] Kim et al[12] reported that radical oxygen species (ROS) generated during early reperfusion is the primary activator of the MPT, and cardiomyocyte death. Some recently developed, intracellularly targeted scavengers have been reported to provide some reduction in infarct size.[13] Antioxidants such as vitamins C, and E have also been suggested to scavenge ROS and reduce ischemic injury.[14] The present study, therefore, was performed with the following objectives, first, to determine whether a short course of oral administration of vit E in a megadose as compared to a NO donor nitroglycerin (GTN) can provide sufficient protection of the heart against reperfusion induced injury, and second, to determine whether a combined regimen of vit E and a NO donor confound superior protection to the hearts against this insult, and to investigate the effect of each of these pharmacological preconditioning agents on mitochondria and MPT.

Methods. This randomized control study was conducted in the Physiology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt from 1st June to 31 August 2009, and was approved by the ethics committee of FMSU REC, Cairo, Egypt. This work was undertaken on 28 female Wistar rats weighing 150-200 gm. The rats were maintained under standard conditions of boarding. The investigation conforms to the guide for the care and use of laboratory animals published by the United States National Institutes of Health. Rats were allocated into 4 groups: a) control group (non-treated), b) (GTN-treated) group (rats received GTN intraperitoneal 25 minutes before sacrifice, in a dose of 120 μg/kg bw),[15] c) vit E-treated group (rat received vitamin E by oral tubal feeding 16-20 hours before sacrifice, in a dose of 250mg/rat) d) vit E and GTN-treated group (rats received vit E and GTN as in both GTN-treated group and vit E -treated group).

Experimental procedures. On the day of the experiments, rats were weighed and injected intraperitoneally with heparin sodium, 1000 IU (B. Braun Melsungen AG.D-34209 Melsungen, Germany). One hour later, the rats were anesthetized with thiopental sodium 40 mg/kg intraperitoneally (Sandoz GmbH, Kundl, Austria). The hearts were removed using standard surgical techniques.

Perfusion of isolated hearts. The perfusion of isolated hearts was performed according to the method described by Ayobe and Tarazi,[16] and El-Bahai et al.[17] The hearts were perfused in a Langendorff preparation, with retrograde perfusion under constant pressure (55 mm Hg) without recirculation. The perfusion medium used was the modified Krebs-Henseleit bicarbonate buffer of pH 7.4, equilibrated with O_2 and CO_2 (95:5) at 37°C. Tension developed by the heart was measured by light weight (1-30 g range) K-30 HSE isometric force transducer, which is connected through a strain gauge (half-bridged Bioscience FC 117 coupler) to a two-channel oscillograph (MD2 Bioscience, Washington, DC., USA). A one-gram weight was attached to the heart apex and left to hang freely, thus exerting resting tension of one gram. The heart was left to stabilize for 10 minutes. A baseline recording was then obtained at a paper speed of 50 mm/second, to determine the baseline heart beating rate, developed peak tension, time to peak tension, rate of tension development (dT/dt) and half relaxation time (1\2 RT). Myocardial flow rate (MFR) was determined by volumetric collection of the fluid passing out of the heart for 3 minutes, and expressed in ml/minute. Ischemia was induced by stopping of the perfusion fluid for 30 minutes. Afterwards, the hearts were reperfused for an additional 30 minutes. At the end of 30 minutes of perfusion, recordings were obtained at paper speed (50 mm/second) for one minute. In the meantime, the myocardial flow rate was measured at the same intervals by timed volumetric collection. Following the heart perfusion, hearts were washed with normal saline, blotted dry by filter paper, and were further cleaned from fat and fibrous tissue. Results were expressed as the percentage change of the measured parameters relative to baseline values to normalize individual differences between basal values among each group. The ventricles were used to isolate mitochondria. Mitochondria were isolated by conventional procedures of differential centrifugation.[18] Hydrolysis of mitochondrial nicotinamide adenine dinucleotide $(NAD)^+$ directly reflects MPT opening. The NAD^+ was measured after perchloric acid extraction. To achieve this, in the case of isolated mitochondria, 0.1 ml of 21% (v/v) perchloric acid was added to 1 mg of protein/ml suspensions.[18] The concentration of NAD^+ in the perchloric acid extract of the cardiac mitochondria was measured using an alcohol dehydrogenase reaction. The reaction mixture contained 1000 µl of buffer-substrate (0.1 M Tris acetate [pH 8.80] and 0.5 methanol), 100 µl of the tissue extract neutralized and 20 µl of alcohol dehydrogenase. The reaction was initiated by the addition of enzyme, and change in absorbance at 340 nm was recorded by a spectrophotometer.[19] The MDA was estimated in cardiac homogenates by the double heating method of Draper and Hadley.[20]

Electron microscopic study. Parts of the lower half of the left ventricle were fixed in 4% glutaraldehyde, dehydrated and embedded in resin. Sections of 60 nm thickness were cut on copper grids and stained with uranyl acetate followed by lead citrate for examination by the electron microscope.[21]

Statistical analysis. Armitage et al,[22] Statistical significance for perfusion study datum was determined using a one-way analysis of variance (ANOVA) with post-hoc test, significance calculated by least significant difference multiple range-test to find inter-group significance. A confidence level of 95% was considered statistically significant. Statistical significance for data of NAD^+ as well as MDA was determined using non-parametric Mann-Whitney test. The level of significance was accepted as $p<0.05$.

Results. Post I/R mechanical performance of the isolated hearts: There was enhancement of the recovery of systolic function of hearts isolated from rats treated with vit E (vit E-treated group, and vit E and GTN-treated group). The percentage recovery of the rate of (dT/dt) was significantly increased in I/R hearts treated with vit E (vit E -treated group, and vit E and GTN-treated group) compared to controls. Myocardial flow rate in the I\R hearts treated with vit E (vit E-treated group) showed less reduction at 30 minutes reperfusion as compared to GTN-treated rats. There was no significant difference in the recovery of diastolic function (%1\2 RT), or the chronotropic activity (%BR) among the studied groups (**Table 1**).

MDA assay. The MDA level (lipid peroxidation marker) was significantly lower in the ischemic-reperfused hearts treated with vit E as compared to controls (**Figure 1**). Changes of intracellular NAD^+ content: The level of NAD^+ in the ischemic-reperfused hearts of vit E treated- rats was significantly higher than their matching controls. The enhancement of NAD^+ content induced by vit E treatment was attenuated when GTN treatment was added (vit E and GTN-treated group) (**Figure 2**).

Histological examination. Control group: Electronic microscope examination of the control group revealed marked distortion of the cardiac myocytes. The myofilaments were irregular, distorted, loose and discontinuous in many areas resulting in disruption of the regular striated appearance. The mitochondriae appeared disorganized, pleomorphic in shape, and widely spaced. Some appeared electron dense with hardly visible cristae. Others showed irregular outline and loss of cristae. (**Figure 3**).

The GTN-treated group. Examination revealed mild improvement as regards the structure. Myofilaments still appeared irregular and discontinuous in some areas yet they were more dense than the control group. The

Table 1 - Percentage changes from baseline values of cardiac activity at 30 minutes reperfusion after 30 minutes of ischemia (I/R) of perfused hearts isolated from; control rats, GTN-treated rats (GTN-tr.), vitamin E-treated rats (vitE-tr.), and vitamin E & nitroglycerin-treated rats (vit E & GTN-tr.).

Group (n=7)	%BR	%dT/dt	%½RT	%MFR
Control	-16.21±4.93	-23.75±6.88	22.85±9.84	-21.78±13.07
GTN-tr.	-21.16±11.04	22.94±20.64	46.43±19.57	-53.31±9.74
Vit E-tr.	15.37±26.21	39.66±24.43[a]	10.71±12.02	-17.57±17.68[b]
Vit E and GTN-tr.	-15.10±7.11	38.48±23.67[a]	14.29±5.04	-25.44±4.24

Data are presented as mean ± SEM. [a]Significance calculated by least significant difference (LSD) at p=0.03 from control group, [b]Significance calculated by LSD at p=0.04 between vit E-tr., and GTN-tr groups. BR - beating rate, dT/dt - rate of tension development, RT - relaxation time, MFR - myocardial flow rate. GTN-tr - rats received nitroglycerin intraperitoneal 25 minutes before sacrifice, in a dose of 120 µg/kg body weight, vitE-tr - rats received vitamin E by oral tubal feeding 16-20 hours before sacrifice, in a dose of 250mg/rat, and vit E and GTN-treated group - rats received vitamin E and GTN as in both GTN-treated group and vit E -treated group.

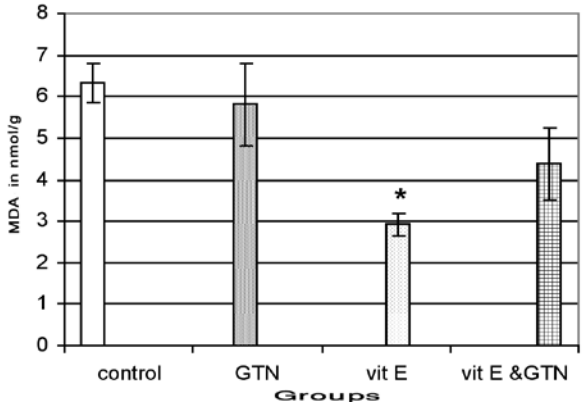

Figure 1 - The malondialdehyde (MDA) levels (nmol/g weight hearts) after 30 minutes reperfusion in control rats, nitroglycerin-treated rats (GTN), vit E, and vit E and GTN. Data are presented as mean ± SD *p=0.004 as compared to control rats.

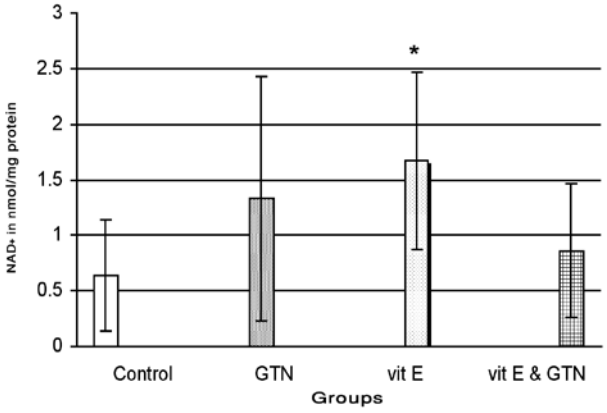

Figure 2 - The NAD^+ levels (nmol/mg protein) in isolated mitochondria after 30 minutes reperfusion in control rats, nitroglycerin-treated rats (GTN), Vitamin E-treated rats (vit. E), and Vitamin E and GTN. Data are presented as mean ± SD,*p=0.03 as compared to control rats.

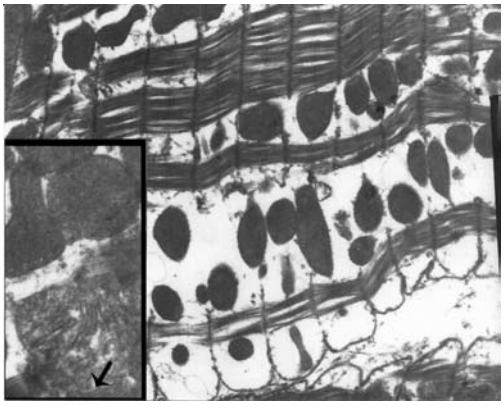

Figure 3 - Showing irregular, distorted myofilaments. They appear loose and discontinuous. Mitochondriae are pleomorphic and widely spaced (control group x6000). Arrow shows disruption in the mitochondrial membrane (x15000).

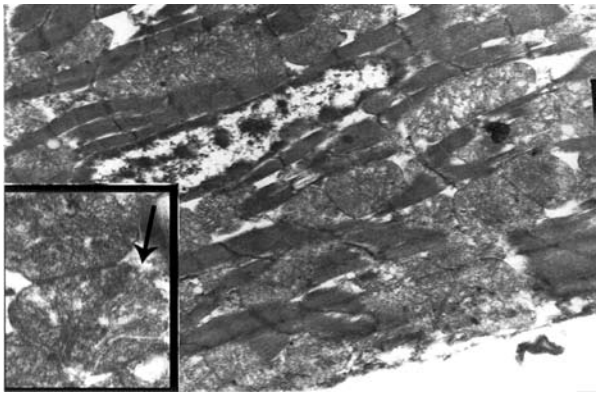

Figure 4 - Myofilaments appear irregular but more dense. (NTG group X6000). Inset - mitochondriae show disrupted cristae, and arrow shows discontinuous mitochondrial membrane (x15000). NTG group - group received nitroglycerin.

mitochondriae were more uniform in shape. Many of them showed disrupted cristae and discontinuous mitochondrial membrane (**Figure 4**).

The vit E-treated group. Examination showed regular, dense and continuous myofilaments giving the striated appearance. The mitochondriae were more or less regular in shape and size. They revealed transverse, parallel and regular cristae as well as continuous mitochondrial membrane. The structure showed marked improvement as compared to the control group (**Figure 5**).

The vit E & GTN-treated group. Examination of this group revealed moderate improvement. The myofilaments appeared regular and dense resulting in striated appearance. The mitochondriae appeared electron dense with unclear cristae (**Figure 6**).

Discussion. This in vitro study of cardiac performance reflects the prevailing conditions at the time of the experiment. Our results demonstrated that a short course of vit E treatment induced preconditioning in the hearts. The vit E therapy, enhanced contractile, and vascular recovery, and attenuated oxidative stress in cardiac tissue, as demonstrated by the decrease of MDA in cardiac tissue. Moreover, this therapy protected the hearts against MPT opening as indicated by significant increase of NAD^+ in cardiac tissue. Histological examination showed less mitochondrial injury induced by reperfusion in the hearts of this group, with preservation of the myocytes structure. Moreover, the present study clearly demonstrated that preischemic treatment with GTN; a NO donor did not provide significant protection of hearts against I/R-induced contractile dysfunction and tissue injury. Peroxynitrite ($ONOO^-$), the reacting product of NO and O_2^-, is a potent cytotoxic agent. It is highly reactive with a wide variety of molecules, including deoxyribose, cellular lipids, and protein sulfhydryls, and results in oxidative tissue damage.[23] In this study, GTN treatment did not attenuate the reperfusion-mediated increase of MDA in cardiac tissues of GTN-treated rats. This finding suggested that $ONOO^-$ might be formed excessively in post-ischemic myocardial tissue. In the current study, supplementation of NO- donor could have raised cardiac tissue NO concentration, with the coincident increase in free radical generation at reperfusion established the conditions of $ONOO^-$ formation. Therefore, we suggest that exogenous NO failed to provide cardioprotection, due to concomitant increase of $ONOO^-$. In this study, the non significant increase of NAD^+ content in isolated hearts from GTN-supplemented rats indicated failure of GTN to attenuate MPT opening. The opening of that channel lead to mitochondrial swelling, release of apoptotic molecules and eventually cell death. This suggestion was further confirmed by histological examination of isolated hearts from GTN-supplemented rats. Dhalla et al[24] reported a depletion of endogenous antioxidants in the ischemic hearts upon reperfusion. Various studies have reported the beneficial effects of antioxidants as these agents render resistance to the hearts against I/R injury. However, other investigators have failed to observe such results. The slow incorporation of vit E into tissues, due to its marked lipophilicity, is probably responsible for its failure as a cardioprotective compound as shown during the acute administration of α-tocopherol after I/R injury induced in the pig.[2]

Several studies reinforce the importance of localization and timing in cardioprotection. Delivery of the antioxidants to the right compartment in the right time period is very difficult to achieve in controlled

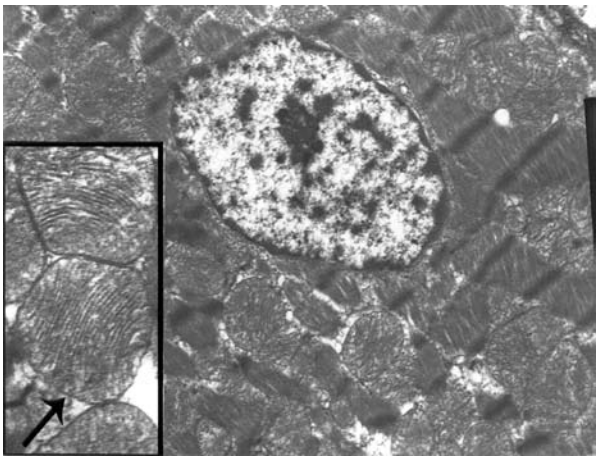

Figure 5 - Showing regular, dense and continuous myofilaments. (Vit E group x6000). Inset - mitochondriae are regular in shape. They reveal transverse, parallel cristae. Arrow shows continuous mitochondrial membrane. (x15000). Vit E group - received vitamin E.

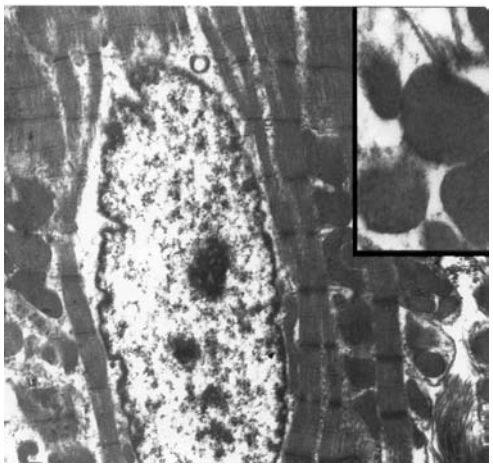

Figure 6 - Showing regular and dense myofilaments (NTG and Vit E group x6000. Inset - mitochondriae are electron dense with unclear cristae. (x15000). NTG and Vit E group - received nitroglycerin and vitamin E.

animal studies, and even more difficult in patients. As a result of the controversy in the animal studies, and the failed clinical trials, it is often concluded that inhibition of ROS will not influence infarct size. A more realistic assessment is that to have a significant benefit in reducing infarct size requires the correct delivery of mitochondrial targeted antioxidants perhaps in conjunction with other therapies. So in this study, we tried to give the animals vit E in a large dose, with a sufficient time to provide its effects. The demonstration that prior exposure to a low concentration of H_2O_2 protects against MPT opening may be of pathophysiological importance for cardioprotection.[11] By the same reasoning, in preconditioning, we speculate the need for an antioxidant that attenuates the large burst of oxidative stress at the start of reperfusion without completely neutralizing free radicals.

We suggest that vit E as a physiological antioxidant acted to scavenge free radicals without completely neutralizing them, thereby affording a significant preconditioning effect. Reduction of free radical formation inhibits MPT opening, thereby affording preconditioning. This is clearly demonstrated in the current study, since there was a significant increase of NAD^+ content of reperfused hearts in the vit E-treated groups. Histological examination confirmed this result, as it revealed marked protection of the normal structure of myocytes and mitochondria. Addition of GTN treatment to vit E attenuated its cardioprotective effect.

In summary, the findings of the present study provide evidence that a short course of vit E treatment protected the heart against reperfusion-injury compared to a NO donor. The MPT is an important target of this protection. Further studies should be conducted to test the possibility of using vit E in cardiac surgery.

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