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Myocardial Ischemia-Reperfusion/Injury

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1. Introduction

Refers to myocardial, vascular, or electrophysiological dysfunction that is induced by the restoration of blood flow to previously ischemic tissue. Tissue damage caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function.

Early reperfusion of ischemic myocardium is an accepted approach for the management of patients with acute coronary syndromes. In addition, surgical interventions requiring interruption of blood flow to the heart, out of necessity, must be followed by restoration of perfusion. Reperfusion, although essential for tissue and/or organ survival, is not without risk due to the extension of cell damage as a result of reperfusion itself.

Since rupture of the plasma membrane is a prominent feature of necrosis and ischemia-reperfusion injury and is a lethal event, it is worth considering what might lead to rupture of the plasma membrane. Rupture of the plasma membrane could be facilitated by calpain or some other protease cleavage of the cytoskeleton. Complete loss of ATP would also inhibit ion pumps, which would result in swelling perhaps rupturing the plasma membrane, particularly if the cytoskeleton has been weakened (Murphy & Steenbergen, 2008).

It is possible that a combination of protease activation with loss of ATP, ion dysregulation and cell swelling all conspire to rupture the plasma membrane. A rise in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ has been consistently observed in ischemia and early reperfusion. A rise in $[Ca^{2+}]_i$ will lead to activation of calpains, which could be involved in cleaving proteins leading to plasma membrane rupture. Calpain activates the proapoptotic BID, and also cleaves Atg5, shifting the balance from autophagy to apoptosis. An increase in $[Ca^{2+}]_i$ and ROS can lead to activation of an inner mitochondrial large-conductance channel known as the Mitochondrial permeability transition (MPT). Opening of this channel would lead to loss of ATP and mitochondrial function, which would quickly lead to mitochondrial swelling and release of cytochrome *c*, which could activate apoptosis. If a large number of mitochondria in a cell undergo opening of the MPT, the cell will lose the capacity to make ATP, and the cell will lose ion homeostasis, resulting in cell swelling, membrane rupture, and cell death (Murphy & Steenbergen, 2008).

Platelet-dependent thrombus formation is a key event in the pathogenesis of acute myocardial infarction (AMI). Platelets mediate both thrombotic occlusion of the entire epicardial coronary artery and also accumulate in the microcirculation resulting in impairment of microcirculation and provoking myocardial ischemia during reperfusion (Gawaz, 2004)..

Despite an improved understanding of the pathophysiology of this process and encouraging preclinical trials of multiple agents, most of the clinical trials to prevent reperfusion injury have been disappointing. Despite these problems, adjunctive therapies to limit reperfusion injury remain an active area of investigation. In these studies vitamin E has been tried to ameliorate lethal reperfusion injury.

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

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 (vitamin E) 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 (Navarro et al., 2005). It has been demonstrated that vitamin 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 (Altavilla et al., 2000). Vitamin E has been extensively assayed in experimental animal diseases, and in the protection and treatment of human diseases.

Research provided evidence that vitamin E intake 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 vitamin E intake is associated with decreased risk of coronary heart disease, and certain types of cancer as well as enhancement of immune function (Ricciarelli et al., 2002 ; Dong et al., 2009). Literatures concerning safety, and tolerance of oral vitamin E demonstrated that vitamin E is relatively nontoxic (Dong et al., 2009; Hanson et al., 2007). In a 91-day study of rats receiving up to 316-443 mg vitamin E/animal/day, vitamin E had no adverse effects on weight gain, food intake, organ weights, hematology or serum chemistry values (Krasavage et al., 1977). 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 (Davidson & Duchen, 2006). Nitric oxide donors have been shown to induce a powerful cardioprotection against I/R injury in mice (Wang et al., 2005). However, literature reporting varying results of NO therapy, with some investigators reporting cardioprotective effects, whereas others report cardiotoxic effects (Bell et al., 2003). 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 (Halestrap et al., 2004). 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 previous literature (Costa et al., 2006). 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 (Halestrap et al., 2004). Previous literature (Kim et al., 2006) 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 (Sheu et al., 2006). Antioxidants such as vitamins C, and E have also been suggested to scavenge ROS and reduce ischemic injury (Qin et al., 2006). A study, therefore, was performed with the following objectives, first, to determine whether a short course of oral administration of vitamin 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 vitamin 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.

2.1 Methods

This study was undertaken on female Wistar rats weighing 150-200 gm. 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 (Zhou et al., 2002) ,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 vitamin 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. One hour later, the rats were anesthetized with thiopental sodium 40 mg/kg intraperitoneally.

In vitro studying of isolated hearts: Hearts were excised and perfused in a Langendorff preparation with the standard Krebs-Henselite Bicarbonate (KHB) buffer, pH 7.4 equilibrated with O2:CO2 (95%:5%) at 37 ^{o} C (Ayobe &Tarazi, 1983). After 20 minutes stabilization period, baseline cardiac activities were recorded using isometric force transducer connected to a two-channel oscillograph.

Ischemia Reperfusion Technique:: After recording baseline cardiac activity, 30 minutes of ischemia was induced by stopping the perfusing fluid, and at 30-minute reperfusion, the cardiac activity was recorded again.

Cardiac activity was assessed by the following parameters: heart beating rate (BR), rate of tension development (dT/dt) and half relaxation time (1\2 RT). Myocardial flow rate (MFR) was measured at the same intervals by timed volumetric collection. 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 cardiac chambers were weighed. The ventricles were used to isolate mitochondria. Mitochondria were isolated by conventional procedures of differential centrifugation. Hydrolysis of mitochondrial nicotinamide adenine dinucleotide (NAD $^{+}$) directly reflects MPT opening. The NAD^{+} was measured after perchloric acid extraction (Di Lisa et al., 2001; Yamazaki et al., 2004). The malondialdehyde (MDA) was estimated in cardiac homogenates by the double heating method of Draper and Hadley (Draper & Hadley, 1990). Data for MDA and NAD^{+} was calculated by non-parametric Mann –Witney test.

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 (Hunter, 1984).

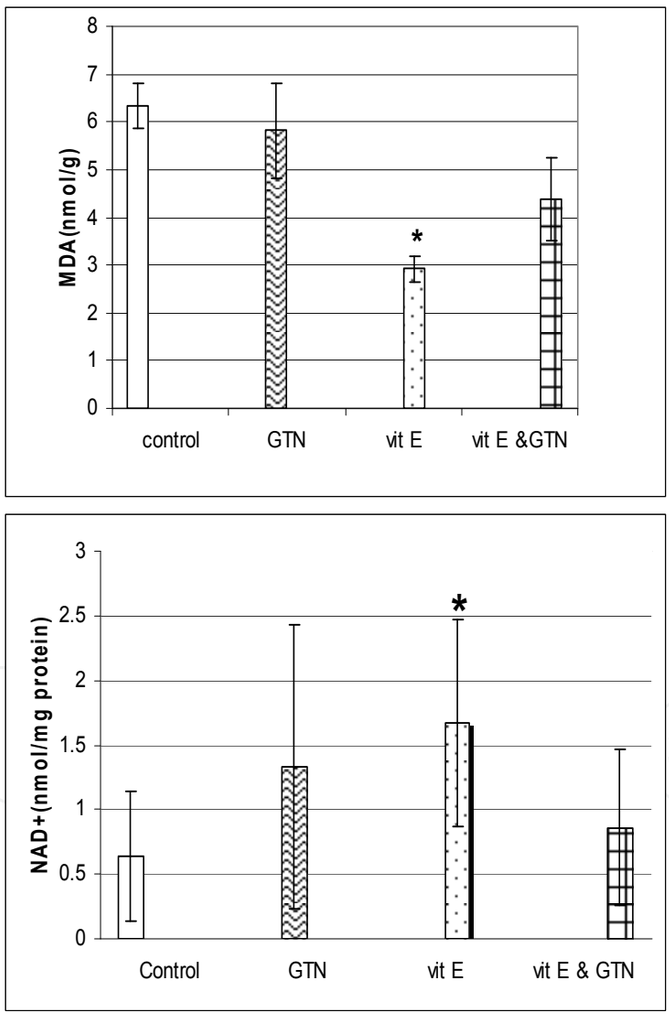


Fig. 1. The malondialdehyde (MDA) and mitochondrial nicotinamide adenine dinucleotide (NAD $^{+}$) levels after 30 minutes reperfusion in control rats, nitroglycerine-treated rats (GTN), vit E-treated rats and vit E & GTN-treated rats. Data are presented as means ± SD * P = 0.004 as compared to control rats.

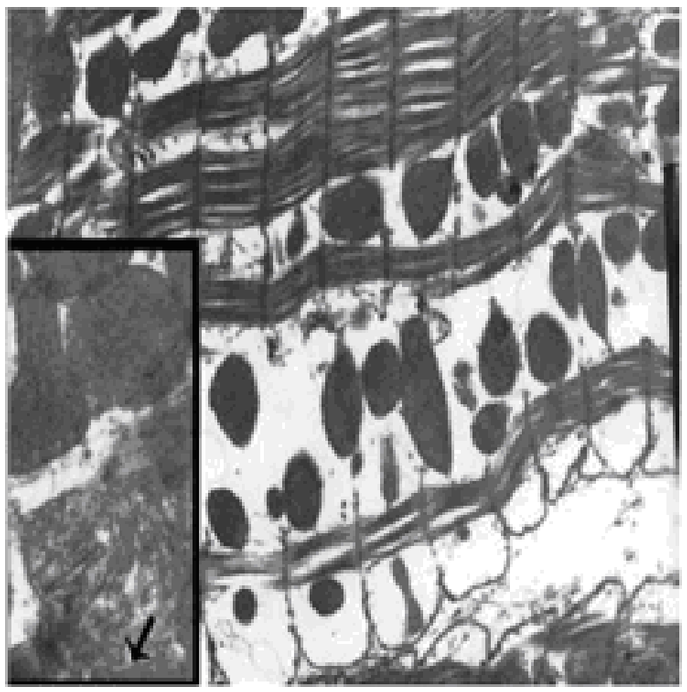


Fig. 2. Showing irregular, disorted myofilaments. They appear loose and discontinuous.Mitochondiae are pleomorphic and idely spaced (controll group x60000). Arrow shows disruption in the mitochondrial membrate (x15000).

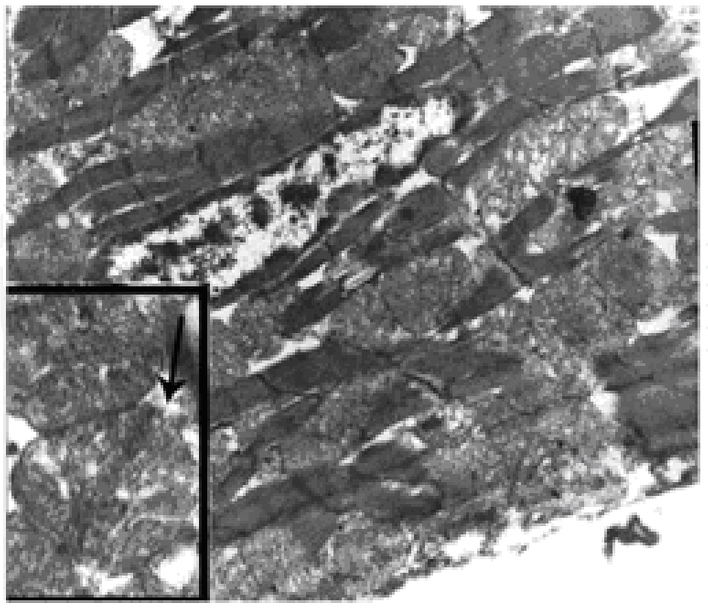


Fig. 3. Myofilaments appear irregular but more dense. (NTG group x6000). Inset – mitochondriae show disrupted cristae and arrow shows discontinuous mitochondrial membrane (x15000). GTN group – group received nitroglycerin

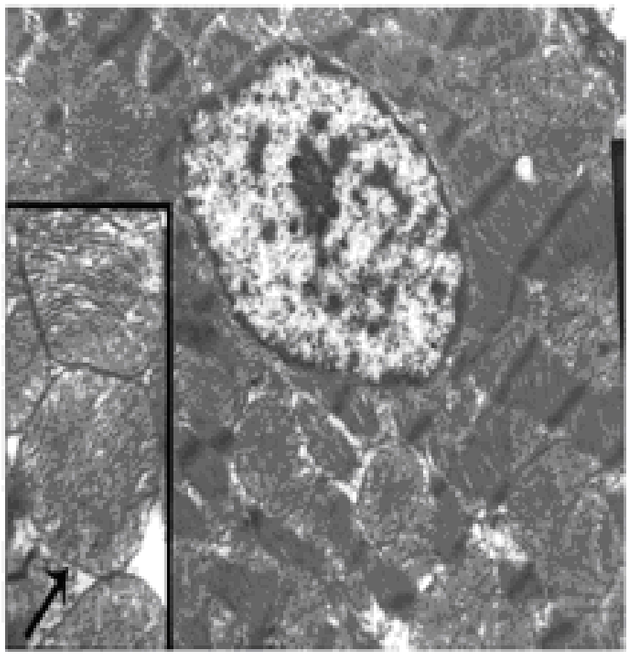


Fig. 4. Showing regular dense and continuous myofilaments. (Vit E group x6000). Inset – mitochondriae are regular in shape. They reveal transverse, parallel cristae.Arrow shows continuous mitochondrial membrane. (x15000). Vit E group – received vitamin E.

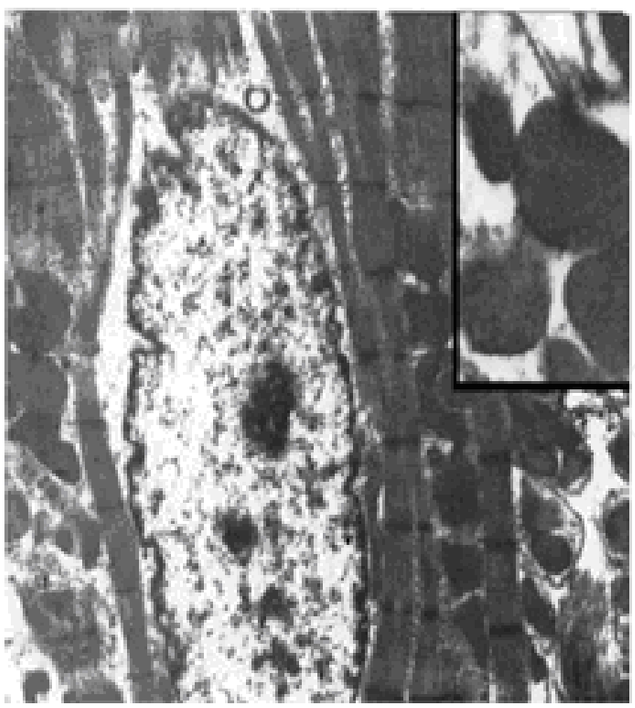


Fig. 5. Showing regular and dense myofilaments (GTN and Vit E group x6000.). Inset – mitochondriae are electron dense with unclear cristae. (x15000). GTN and Vit E group – received nitroglycerin and vitamin E.

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. n: is the number of rats. 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, ½ RT -half relaxation time, MFR - myocardial flow rate.

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 and GTN-tr.).

2.2 Effects of vitamin E versus nitroglycerine on I/R injury

Our results demonstrated that a short course of vitamin E treatment induced preconditioning in the hearts. The vitamin 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 apotent 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 (Farshid et al., 2002). 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. Previous literature (Dhalla et al., 2000) 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 vitamin 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 (Altavilla et al., 2000). 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 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 vitamin 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 (Costa et al., 2006). 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 vitamin 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 vitamin 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 vitamin E attenuated its cardioprotective effect. In summary, the findings of the present study provide evidence that a short course of vitamin E treatment protected the heart against reperfusion-injury compared to a NO donor. The MPT is an important target of this protection.

3. Effects of vitamin E treatment on age-associated changes in cardiac responses to the injury of post I/R

Aging is one of the most important risk factors for the development of cardiovascular disease. Aging is characterized by loss of myocytes, remodeling and impaired contractile function in the heart. The rate of programmed cell death in the left ventricle increases with age (Kwak et al., 2006). Meanwhile the aged heart faces a high risk of free radical injury owing to slow generation of antioxidant enzymes by its cells, and a general decline in this system may be another reason for the development of myocardial dysfunction (Asha Devi et al., 2003 a).

It is well established that nitric oxide (NO), is constitutively generated within the heart, not only by the endothelium but also by the myocytes (Balligand et al., 1995). Numerous previous animal studies have reported that there is a loss of NO biological activity and /or biosynthesis during aging (Matz et al., 2000).

Oxygen free radicals increase in concentration upon reperfusion of ischemic cardiac tissue (Xia et al., 1996). It is well established that these reactive species can interact with and damage various cellular components (Berlett & Stadtman, 1997). Free radical production in the heart has also been reported to increase with age (Sohal et al., 1990). Thus, although restoration of blood flow is the sole method for salvaging ischemic tissue, oxidative damage may occur during reoxygenation and contribute to ischemia–reperfusion injury.

Regular exercise is a key component of cardiovascular risk prevention strategies. Physical activity is known to cause generation of free radicals. Exercise training in old animals failed

to enhance antioxidant activity (Hatao et al., 2006). However swim training especially at low-intensity, was found to be beneficial as a major protective adaptation against oxidative stress particularly in the older myocardium (Kiran et al., 2004). The tocopherols (major vitamers of vitamin E) are believed to play a role in the prevention of human aging-related changes, and are of particular interest, mainly because of their antioxidative properties (Asha Devi et al., 2003 b).

From the aforementioned data, it seems possible that free radical - mediated injury is a major issue in the increase of aging related reperfusion-induced myocardial dysfunction. Exercise training program could not always protect the aging heart satisfactorily. We tried to test the hypothesis that using an antioxidant as vitamin E could lead to better protection of the aging hearts as compared with exercise training.

Therefore, a study was conducted to compare the effects of vitamin E treatment versus swim- exercise training on age- associated changes in cardiac responses to the injury of post I/R.

3.1 Methods

This work was undertaken on female Wistar rats aged 16-18 months. Rats were allocated into 3 groups: a) Control group of aged rats, b) Exercise -trained aged rats subjected to daily physical exercise by swimming in the water tank, for 2 hours daily, 6 days aweek for 2 weeks, and c) Vitamin E-treated aged rats subjected to daily injection of vitamin E (300 mg/kg) intraperitoneally, 6 days aweek for 2 weeks.

The swim-training program adopted was according to Refaat et al., (1989), in a swimming tank, filled with water and maintained at thermoneutral temperature of 30°c. A motor fan in the tank stirs strong water currents. This ensures uniformity of temperature and forces the rats to swim actively all the time.

3.1.1 Experimental procedures

They were done as previously described (Ayobe &Tarazi, 1983). Peak developed tension per left ventricular weight as well as coronary flow rate per left ventricular weight were calculated relative to left ventricular weight (PT/LV, g /100mg)& (CF/LV, ml /100mg/min).

Nitrate concentration in coronary effluents, as a stable product of nitric oxide (NO), was measured by an endpoint one-step enzymatic assay using nitrate reductase, as described by Bories and Bories, (1995), and modified in tissues by Kassim, (1997).

3.1.2 Effects of exercise on cardiac weights and post Ischemia-reperfusion (I/R) responses

In this study, exercise was found to exert a trophic effect on cardiac chambers manifested by significant increase of the absolute & relative weights of atria, right ventricle, and left ventricle. Swimming is well recognized for its effectiveness in inducing myocardial hypertrophy (Evangelista et al., 2003). Exercise can protect cardiac function of the aging heart by protection against; loss of cardiac myocytes, reduction in number of myonuclei, reactive hypertrophy of remaining myocytes, and increased connective tissue in left ventricle (LV) of the aging rat heart. This protection is achieved through attenuation of age-

induced elevation in Bax/Bcl-2 ratio, thereby inhibits apoptosis (Asha Devi et al., 2003a), and exerts its trophic effect.

Although exercise-trained rats had significant increase in LV and LV/BW that indicates LV hypertrophy, they showed Peak developed tension similar to control which could be attributed to the increase of free radical in aged rats induced by exercise. Oxygen free radicals are known to have deleterious effects on contractile force of heart (Gao et al., 1996).

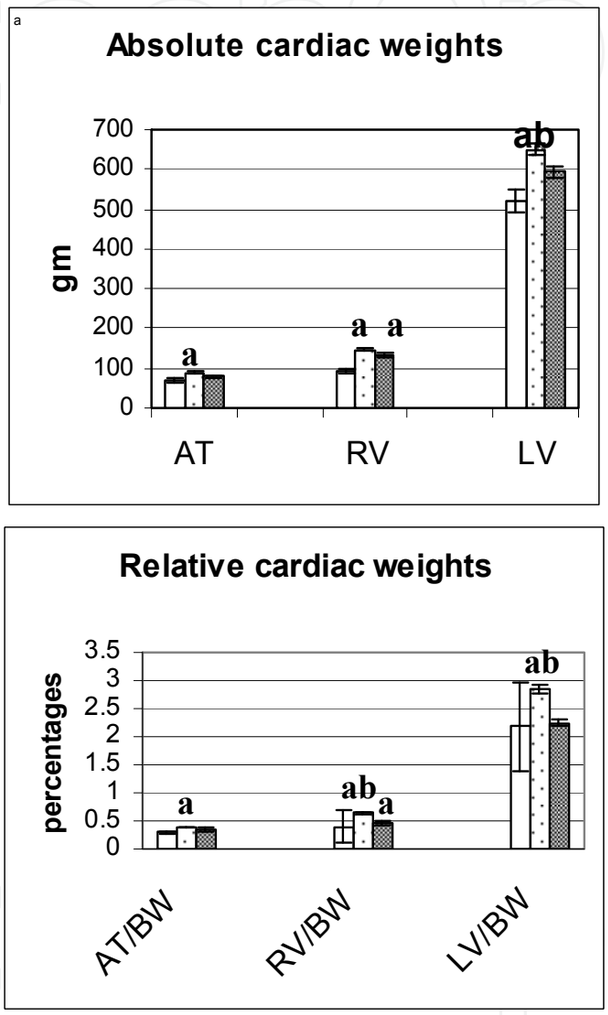


Fig. 6. Mean values± SEM of: absolute weights (mg) of atria (AT), right ventricle (RV), left ventricle (LV), and their relative weights (mg/g) (AT/BW), (RV/BW), (LV/BW) in control rats □ , exercise-trained rats ⬚, and vit. E-treated rats ▩.

a. Significance calculated by least significant difference (LSD) at $P < 0.05$ from control group.
b. Significance calculated by LSD at $P < 0.05$ between vit E-treated, and exercise-trained groups

The results of ischemia-reperfusion of isolated hearts from both control and exercise-trained aged rats have shown the lack of tolerance to ischemia-reperfusion injury as regard

inotropic activities as well as myocardial flow rate. This could be explained in view of the increase in free radicals generation induced by exercise, even if small, was added to the increased free radicals generation induced by aging exceeding the antioxidant defense capacity of the heart leading to loss of beneficial cardio-protective effect of exercise on ischemia-reperfusion injury (Hatao et al., 2006).

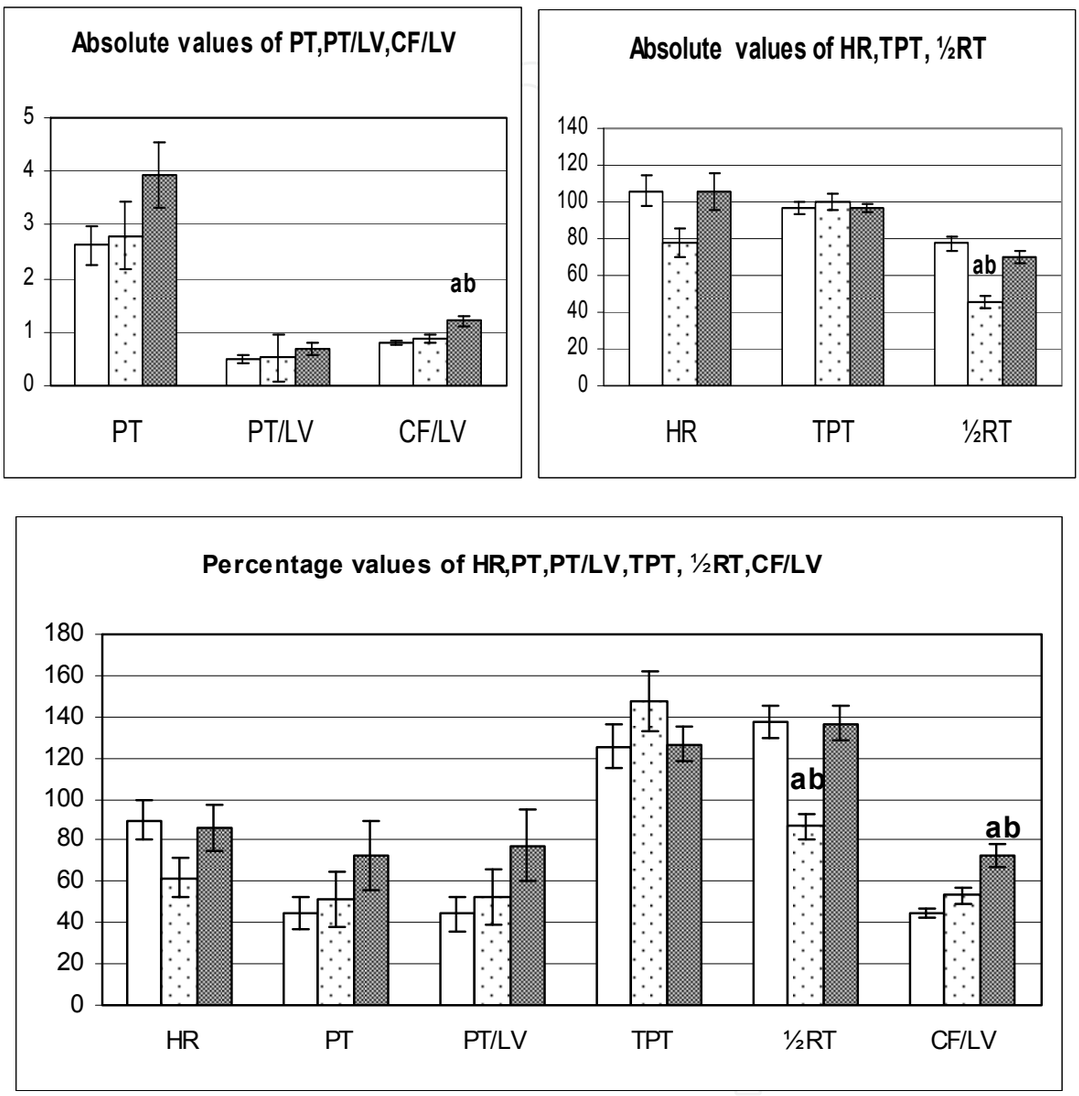


Fig. 7. Mean absolute and percent values ± SEM of: heart beating rate(HR), Peak developed tension(PT), Peak developed tension per left ventricular weight(PT/LV), Time to peak tension (TPT), Half relaxation time(½ RT), and Coronary flow rate per left ventricular weight (CF/LV), at 30 minutes reperfusion of hearts isolated from control rats ☐ , exercise-trained rats ⬚ , and vit. E- treated rats ▩ .

a. Significance calculated by least significant difference (LSD) at $P < 0.05$ from control group.
b. Significance calculated by LSD at $P < 0.05$ between vit E-treated, and exercise-trained groups

The increased free radicals generation such as superoxide anion could react with NO to form peroxynitrate, decreasing NO biological activity. Peroxynitrate molecule by itself might play a significant role in oxidative tissue damage. This is supported in this study by the absence of increase of nitrates in the coronary effluent from exercise- trained aged rats at 30 min reperfusion.

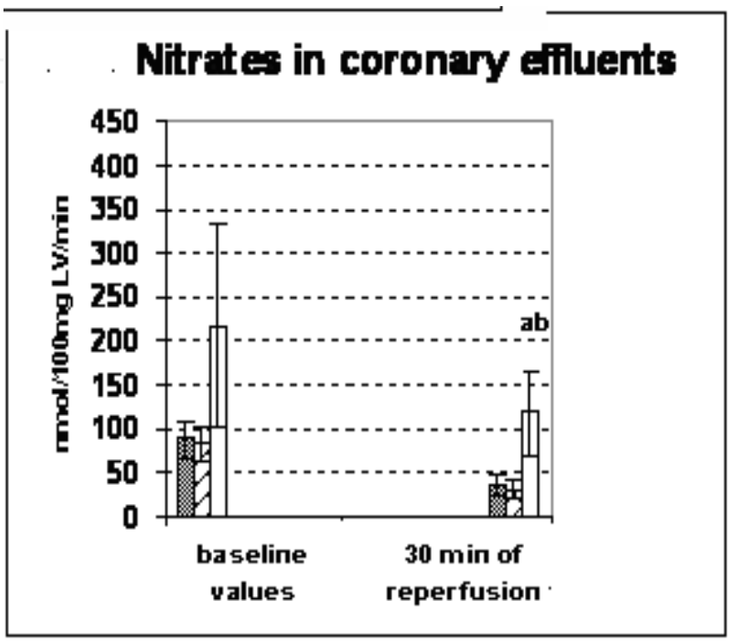


Fig. 8. Mean values ±SEM of nitrates in coronary effluents (nmol/100mg LV/min) at baseline values, and 30 minutes of reperfusion in control rats ▩, exercise-trained rats ▨, and vit. E-treated rats □.

a. Significance calculated by least significant difference (LSD) at $P < 0.05$ from control group.
b. Significance calculated by LSD at $P < 0.05$ between vit E-treated, and exercise-trained groups

Also, the heart beating rate of the exercise-trained rats could not be preserved after 30 min of reperfusion. This could be attributed to the increased free oxygen radicals in these rats, since oxyradicals have been implicated as a possible cause of reperfusion arrhythmias (Tanguy et al., 1998). However, the rate of relaxation of hearts excised from exercise-trained rats showed better tolerance to I/R after initial intolerance, and such improvement is in accordance to a recent report by Libonati et al., (2005), who found that sprint training in rats improves post-ischemic left ventricular diastolic stiffness due to up-regulation of myocardial glycolysis, with ATP production that protects the heart from ischemia-reperfusion as it plays an important role in actin-myosin rigor bond dissociation, and regulating myocardial diastolic function.

Effects of vitamin E on cardiac weights and Post Ischemia-Reperfusion (I/R) responses:

Vitamin E has been reported to stimulate upregulation at the expression level of B cl-2 gene, B cl-2 molecule is involved in the inhibition of apoptosis (Azzi et al., 2004).

Therefore, vitamin E by preventing apoptosis of myocardial cells could be expected to induce hypertrophy of the heart (trophic effect). However in case of vitamin E–treated rats, results have shown that the effect of vitamin E on the rate of growth of right and left ventricles was not similar. While the rate of growth of right ventricle was increased by the anti-apoptosis effects of vitamin E. The increase in left ventricle & left ventricle/ body weight was insignificant. This could be attributed to the favorable effects of vitamin E on reduction of blood pressure and blood viscosity (Costa et al., 2005). These two factors have been claimed to induce growth of left ventricle during aging (Ghali et al., 1997). Thereby, by eliminating these two factors, the rate of growth of left ventricle was decreased under the influence of vitamin E supplementation counteracting its trophic effect in aged rats.

In contrast to aged control rats and exercise-trained aged rats, the isolated hearts from vitamin E-treated rats showed better tolerance as regard inotropic activity, especially force generation measured as peak developed tension as well as coronary flow rate. In accordance, Venditti et al., (1999) showed a protective action of vitamin E treatment against lipid peroxidation and cardiac dysfunction associated with ischemia- reperfusion in rats. Vitamin E decreases free radical deleterious effect on cell membrane, proteins, DNA, and decreases both ROS mediated direct ischemia-reperfusion mediated injury, as well as peroxynitrite-mediated injury and thereby preserving NO through preventing its inactivation by its transformation to peroxynitrite (Kamat, 2006). In support, this study showed significant increase of nitrates in coronary effluent from vitamin E- treated aged rats measured at 30 min of reperfusion.

In view of the aforementioned data, vitamin E proved to be a cardio-protective agent as it attenuated the injury of post ischemia-reperfusion on the aged myocardium. This beneficial effect of vitamin E could be partially attributable to preservation of NO biological activity by preventing its transformation to the toxic compound peroxynitrite.

On the other hand, the swim-training program adopted in this study, as regard tolerance to ischemia-reperfusion apart from improvement of half relaxation time, the swim-training program did not show other promising cardio-protective effects against cardiac changes associated with the aging process.

Therefore, vitamin E supplementation could be recommended to aged people especially patients suffering from ischemic heart disease. Vitamin E could be used either alone or in combination with exercise training taking into consideration the duration and intensity of exercise program.

Vitamin E and platelet function

Platelets play a critical role in the pathophysiology of reperfusion (Gawaz, 2004). Platelet function is not static during ischemia-reperfusion. Instead, during ischemia regional platelet aggregability is increased. Systemic and regional platelet aggregability also increases during myocardial reperfusion. The mechanism of these responses is unknown but may be related to regional endothelial dysfunction created by ischemia. The response observed could also be explained by the release of proaggregatory mediators in the coronary and/or systemic circulation during ischemia-reperfusion (Gurbel et al., 1995).

Reperfusion induces an important inflammatory response, characterized by a massive production of free radicals and by the activation of the complement and leucocyte neutrophils (Gourdin et al., 2009). Platelets and neutrophils act synergistically in provoking postreperfusion cardiac dysfunction (Lefer et al., 1998). Activated platelets play an important role in the process of myocardial ischemia-reperfusion injury, and platelet-derived P-selectin is a critical mediator (Xu et al., 2006), whereas platelet P-selectin promotes platelet interactions with leukocytes. Because platelets release potent proinflammatory chemokines and modulate leukocyte function, platelet accumulation in the postischemic microvasculature might significantly contribute to the manifestation of I/R injury (Massberg et al., 1998).

Reperfusion of the tissue, subsequent to ischemia, results in burst of oxygen consumption with consequent generation of oxygen derived free radicals; the oxidant-anti oxidant status of the tissue is thrown out of balance and multi dimensional free radical mediated damage ensues. Since vitamin E is a potent natural anti-oxidant, its administration is expected to restore the imbalance.

Effect of administration of 600 mg vitamin E each day, for six days, was observed on activity of some of the anti-oxidant enzymes and levels of malondialdehyde (as an index of free radical mediated damage) in the platelets of patients reperfused after myocardial infarction. It has been found that vitamin E administration significantly lowers the level of malondialdehyde in the patients. Vitamin E administration increases the activities of anti oxidant enzymes (superoxide dismutase, glutathione reductase and catalase) tested both in the patients and healthy controls. However, lowering of lipid peroxidation upon administration of vitamin E is specific for patients. These findings exhibit beneficial role of vitamin E administration in the management of the patients reperfused after myocardial infarction (Dwivedi et al., 2005).

The results of Chen et al, (2002) suggested that the reduction of myocardial I/R injury with vitamin E supplementation may be the result of the inhibition of polymorphonuclear neutrophil (PMN) CD11b expression.

Vitamin E supplementation in healthy subjects or patients with hypercholesterolemia was shown to diminish platelet function as assessed by ex vivo platelet aggregation of 11-dehydrothromboxane B_2, a marker of in vivo platelet activation (Calzada et al., 1997; Davi et al., 1997). Celestini et al., (2002) demonstrated that vitamin E can potentiate the antiplatelet activity of aspirin by inhibiting the early events of platelet activation pathway induced by collagen.

A study from our laboratory was to examine the possibility that vitamin E administration could exert an effect on blood elements and platelet aggregation.

Materials and Methods

Albino rats of both sexes weighing 180- 220 gm fed on a standard rat diet and fasted for 18-24 hours before sacrifice were used in this study.

In vivo study: A total of 30 rats were used in this study. They were divided in two groups. Group I: Saline control group; rats in this group were injected with saline instead of vitamin E, daily for 5 consecutive days. Group II: vitamin E treated group; rats were injected with

vitamin E (300 mg/kg b.w.) intraperitoneally for five consecutive days. After 5 days all injections were stopped for 2 days. By the seventh day rats were anesthetized with pentobarbitone in a dose of 40 mg/kg b.w.

Collection of blood samples: blood samples were obtained by arterial puncture from the abdominal aorta. One ml samples were collected into tubes containing EDTA for examination of RBCs and platelet counts, hemoglobin content and hematocrite values.

Another blood samples were collected in chilled plastic tubes containing sodium citrate 3.8gm/100 ml (9 volumes of blood to 1 volume of sodium citrate) and gently shaken. These blood samples were used for study of platelet aggregation. The citrated blood was centrifuged at 1500 r.p.m. for 5 min. The supernatant platelet rich plasma (PRP) was pipetted into clean plastic tubes. The remaining blood sample was centrifuged at 10,000 r.p.m. for 10 min. to prepare platelet poor plasma (PPP). Standard PRP: the number of platelets in PRP was counted using coulter T-660 counter. The platelet number was adjusted to a standardized number of 3×10^5 platelet per µl by dilution with autologus platelet poor plasma.

Aggregation study: platelet aggregation was performed using Chrono-Log automatic aggregometer (model 540-VC, Chrono-Log Corp, Harvertown, USA) coupled with computer and printer. ADP as an aggregating agent was used at a final concentration of 10 uM. The maximum aggregation was recorded after 3 min.

In vitro of vitamin E on platelet aggregation

Collection of blood samples: blood was collected from normal rats, anaesthetized by pentobarbitone, by arterial puncture from abdominal aorta into chilled tubes containing sodium citrate 3.8 gm%. Preparation of standard PRP was carried out as described in the in vivo experiments.

The in vitro effect of vitamin E on ADP- induced platelet aggregation was studied by exposing PRP to rising concentrations of the vitamin 1,2,3,4 and 5 mg/ml. Equal volumes of saline were added to control samples.

Aggregation study: was carried as described above in vivo experiments.

Statistical analysis of the data was done using Student's "t" test for unpaired data according to Fisher and Yates (1957) $P < 0.05$ was considered significant.

Regression study: linear regression analysis was used to relate different parameters to a certain outcome (platelet aggregation) to find out the highest beta coefficient and the most important factor affecting this outcome. This analysis was performed on SPSS windows version eight.

Results

Table 1 portrays the results of in vivo effects of vitamin E on hematological parameters. RBCs count, Hb content and PCV showed slight and insignificant changes in vitamin E treated rats compared to their saline controls. The number of platelets was insignificantly decreased in vitamin E treated animals. However platelet aggregation induced by ADP showed a significant decrease ($P < 0.05$) in this group (table 2 and figure 9).

Regression analysis: as seen in fig. 10, multiple regression analysis of platelet aggregation against other parameters. Only a significant negative correlation between platelet number and platelet aggregation was seen in vitamin E treated group ($P < 0.03$).

In vitro effect of vitamin E on platelet aggregation: the platelet aggregation effect of ADP in presence of rising concentration of vitamin E showed significant inhibition ($P < 0.01$) only when vitamin E was added at a final concentration of 5 mg/ml. Addition of vitamin E in smaller concentrations of 2- 4 mg/ml final concentration produced insignificant inhibition of platelet aggregation. Almost no effect was seen when vitamin E was added at a final concentration of 1 mg/ml (table 3 and figure 11).

Parameters / Group treatment	RBCs x 10^6/ul	Hb gm/dl	PCV %	Platelet count x 10^3/ul	Platelet aggregation %
Saline control (15)	6.54±0.3	12.24±0.5	36.15±1.2	1146±71.12	68.1±1.9
Vitamin E treated rats (15)	6.33±0.2	11.93±0.3	35.3±0.9	1068±53.1	58.9±4
P	NS	NS	NS	NS	< 0.05

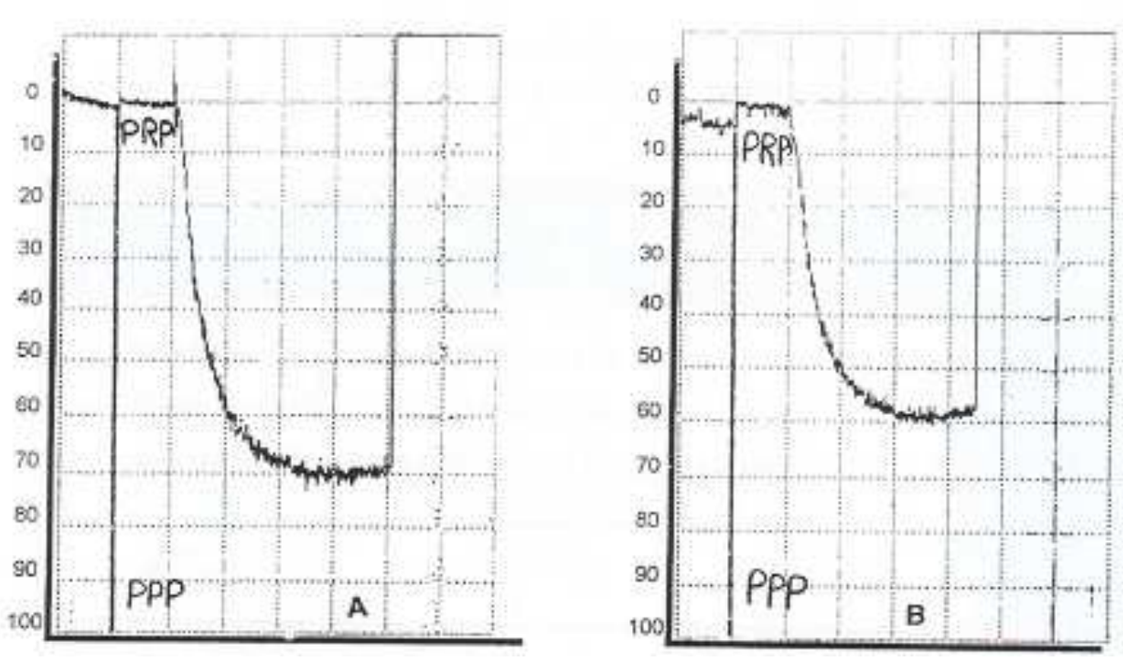
Data are mean± SEM In parenthesis is the number of observations NS: non significant

Table 2. Red blood cell count (RBCs), hemoglobin level (Hb), packed cell volume (PCV), platelet count and platelet aggregation in saline control and vitamin E treated rats.

Additions to normal PRP					
	Final concentration of vitamin E				
Saline control (12)	1mg/ml (8)	2mg/ml (9)	3mg/ml (11)	4mg/ml (11)	5mg/ml (13)
50.08±2.6	50.75±2	47.1±2.32	44.36±2.85	44.7±3.4	37.23±3
P	NS	NS	NS	NS	< 0.01

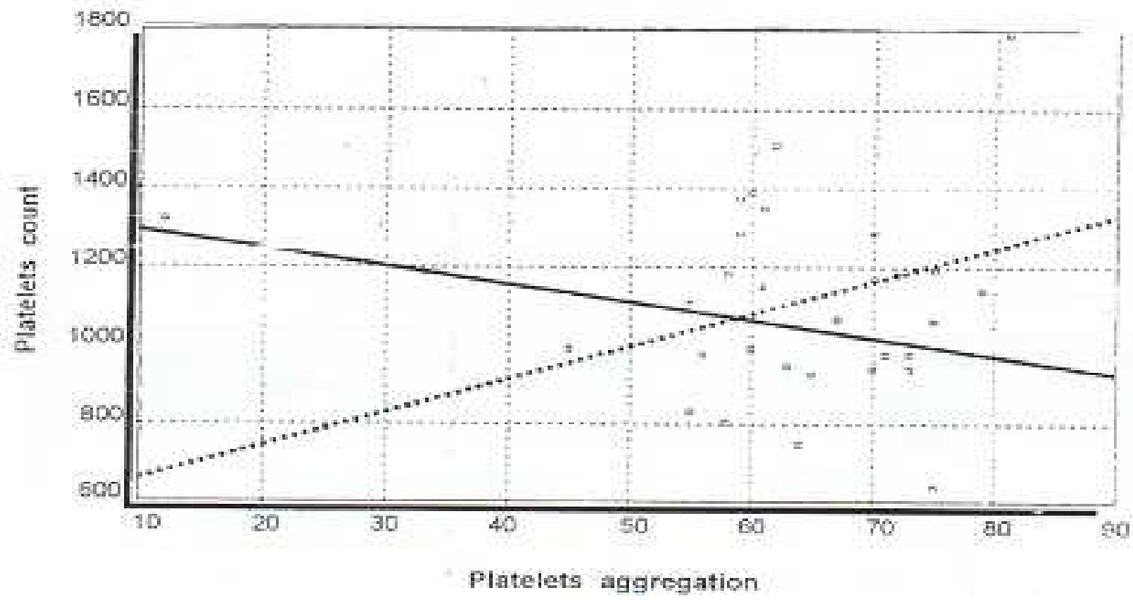
Data are mean± SEM In parenthesis is the number of observations NS: non significant

Table 3. In vitro effect of vitamin E on platelet aggregation of normal rat PRP in presence of different concentrations of vitamin E compared to saline control



ADP-induced platelet aggregation%

Fig. 9. Tracing of ADP- induced platelet aggregation of Vitamin E-treated rats (B) compared to saline treated rats (A).



Vitamin E- treated rats ——————

Saline- treated rats ••••••••••••

Fig. 10. Correlation between platelet aggregation and platelet count among vitamin E-treated rats and saline treated rats (by multiple regression analysis)

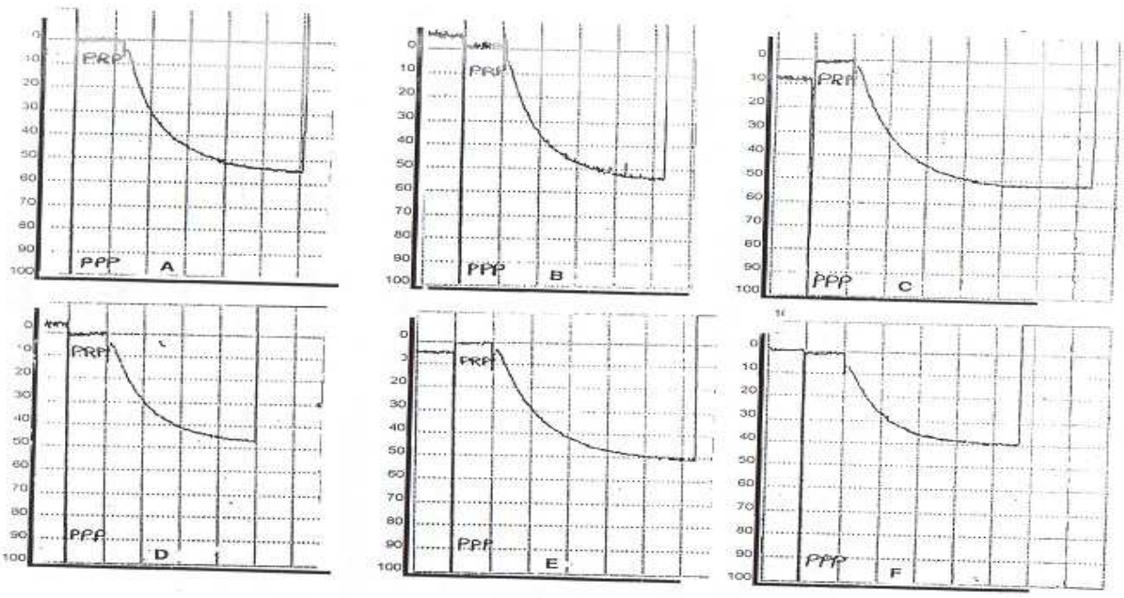


Fig. 11. Tracing of ADP- induced platelet aggregation of normal rats PRP in presence of vitamin E in concentrations of 1, 2, 3, 4 and 5 mg/ml respectively (B- F) compared to saline control (A)

The data reported here demonstrated that administration of megadose of vitamin E (300 mg/kg for one week) to rats, produced slight and nonsignificant changes in red blood cell counts, hemoglobin content, packed cell volume. Platelet counts showed an insignificant decrease. However, the platelet aggregation responses to ADP of PRP from treated rats were significantly inhibited. This finding shows the safety of vitamin E in this supra-physiological dose of 300 mg/kg on blood parameters tested.

On addition of vitamin E to normal PRP in vitro, the platelet aggregating effect of ADP showed significant inhibition only when vitamin E was added at a final concentration of 5 mg/ml. Addition of vitamin E in smaller concentrations of 2- 4 mg/ml produced insignificant inhibition of platelet aggregation. Almost no effect was seen when vitamin E was added at a final concentration of 1 mg/ml.

From these data, it can be concluded that this supra physiological dose of vitamin E is safe concerning the blood parameters tested.

The observation in the present study that vitamin E when added in vitro to normal rat PRP caused significant inhibition of platelet aggregation in response to ADP; illustrate that vitamin E by itself exerts a direct antiplatelet effect. Higashi & Kikuchi (1974) were the first to demonstrate that vitamin E inhibits the aggregation of platelets using hydrogen peroxide as the aggregating stimulus. Subsequent studies by Steiner & Anastasi, (1976) demonstrated that vitamin E also inhibited platelet aggregation response to epinephrine, collagen and ATP. Moreover, Freedman and Keaney, (2001) found that platelet incorporation of vitamin E both in vitro and in vivo leads to dose-dependent inhibition of platelet aggregation in response to agonists such as arachidonic acid and phorbol ester.

Although it is best known for its antioxidant activity the exact mechanism of the antiplatelet effect of vitamin E is not exactly known and one of the following mechanisms may operate. First, it can be attributed to altered metabolism of prostaglandins. The vascular generation of prostacyclin (PGI_2) is higher and the platelet thromboxane A_2 generation is lower than normal. This view is supported by the findings of Steiner and Anastasi (1976) and others (Karpen et al., 1981; Pritchard et al., 1982; Pignatelli et al., 1999) that vitamin E inhibits platelet thromboxane A_2 synthesis. On the other hand, PGI_2 synthesis is stimulated possibly by reduction of cellular peroxide level (Gilbert et al., 1983). Second, the inhibition of platelet aggregation can be explained by the ability of vitamin E to inhibit intracellular mobilization of sequestrated calcium from the dense tubular system of the cytoplasm (Srivastava, 1986). Third, by its membrane stabilizing action, vitamin E would impair platelet release reaction. This view is supported observations of Feki et al., (2001) that vitamin E by its antioxidant effect, protects molecules and tissue against the deleterious effect of free radicals and also contributes to the stabilization of biological membranes. Fourth, unrelated to its antioxidant action, vitamin E was shown to inhibit protein kinase C (PKC) in various cell types with consequent inhibition of platelet aggregation (Azzi et al., 2002; Freedman et al., 1996; Freedman and Keaney, 2001). Further, vitamin E includes inhibitory effects are the result of specific interactions with component of the cell e.g. proteins, enzymes and membranes (Ricciarelli et al., 2002).

Vitamin E attenuated P-selectin expression on activated human platelets and thus inhibited the P-selectin–dependent function, platelet–mononuclear cell (MNC) interaction. The mechanism probably was related to the inhibition of PKC activity in platelets. Since P-selectin is an important atherothrombogenic adhesion molecule, this finding will provide us new insights into the mechanism by which dietary vitamin E inhibits thrombosis and atherogenesis and thereby reduces the risk of coronary artery diseases (Murohara et al, 2004).

Although these studies from our laboratory have shown that vitamin E administered in megadose, could provide a protective effect against the cardiac responses to the injury of post I/R. Further studies should be conducted to test the possibility of using vitamin E in cardiac surgery.

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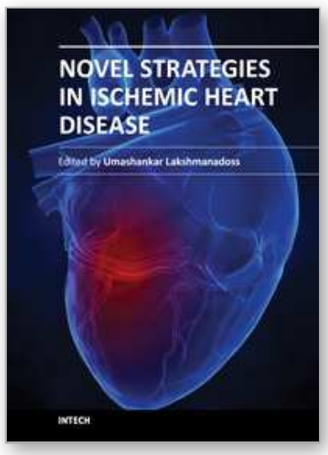

Novel Strategies in Ischemic Heart Disease

Edited by Dr. Umashankar Lakshmanadoss

ISBN 978-953-51-0184-0
Hard cover, 450 pages
Publisher InTech
Published online 29, February, 2012
Published in print edition February, 2012

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