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Research Article

Grape Seed Extract and Zinc Containing Multivitamin-mineral Nutritional Food Supplement Protects Heart against Myocardial Ischemia-reperfusion Injury in Wistar Rats

Satyam SM[1], Bairy KL[2]*, Pirasanthan R[3] and Vaishnav RL[2]

[1]Department of Pharmacology, Manipal University, India
[2]Department of Pharmacology, Manipal University, India
[3]Department of Pharmacology, Nepalgunj Medical College, Nepal

***Corresponding author:** Bairy KL, Department of Pharmacology, Kasturba Medical College, Manipal University, Manipal-576104, Karnataka, India

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Abstract

Zincovit tablets have been used as nutritional food supplement over a prolonged period of time. In previous studies, we had reported combined formulation of grape seed extract and Zincovit tablets for its strong *in-vitro*, *in-vivo* antioxidant, anti-hyperglycemic and anti-cataractogenic potential. Consequently, the aim of the present study was to investigate the cardio-protective effect of single combined formulation of grape seed extract and Zincovit tablets using a Langendorff model of ischemia-reperfusion in Wistar rats. Combined formulation of grape seed extract and Zincovit tablets significantly attenuated ischemia-reperfusion induced cardiac injury in terms of increased coronary flow rate, decreased creatine kinase activity in coronary effluent, decreased MDA, 4-HNE and increased protein thiol content in heart tissue homogenate. The present study revealed that the combined formulation of grape seed extract and Zincovit tablet is the potential functional nutritional food supplements that could offer a novel therapeutic opportunity against myocardial ischemia-reperfusion injury in Wistar rats.

Keywords: Grape seed extract; Zincovit tablets; Cardiovascular disease; Langendorff ischemia-reperfusion model; 4-hydroxynonenal; Creatine kinase

Introduction

According to the WHO in 2011, ischemic heart disease accounted for 7 million deaths worldwide. Acute myocardial infarction (AMI) is the leading cause of mortality worldwide [1]. It is necessary to consider not only its impact in mortality, but also the impairment in the life quality of patients surviving this vascular accident. Following acute myocardial ischemia, restoring coronary blood flow with the rapid use of pharmacological or mechanical interventions, such as thrombolytic treatment, angioplasty or coronary bypass surgery have been used to recover the myocardial perfusion, as it allows to reestablish the blood flow in the cardiac zones affected by the occlusion of a branch of the coronary artery. Nevertheless, as a consequence of this procedure, the ischemic zone is reperfused, giving rise to an ischemia-reperfusion event that generates increased production of reactive oxygen species (oxidative stress) [2], thus enhancing the previously established tissue damage (lethal reperfusion), as these reactive species attack biomolecules such as lipids, DNA, and proteins and trigger cell death pathways [3]. One of the studies on animal models of acute myocardial ischemia suggests that lethal reperfusion accounts for up to 50% of the final size of a myocardial infarct, a part of the damage likely to be prevented [4]. Oxidative stress is considered as one of the key factors that contribute to ischemia-reperfusion injury [5]. A large number of strategies have been aimed at to ameliorate lethal reperfusion injury, but the beneficial effects in clinical settings have been disappointing till date. Therapeutic strategies are designed to reduce free radical induced damage, either by intervening in the process by which free radicals are formed or by scavenging the free radicals that have already been formed [6].

Zincovit tablet is an advanced combined formulation of vitamins, minerals and grape seed extract (Table 1). Long-term daily administration of grape seed extract offers enhanced antioxidant potential and protection against tissue lipid peroxidation and protein oxidation [7]. The biologically active constituents of grape seed extracts are proanthocyanidins, which represent a variety of polymers of flavan-3-ol, such as catechin and epicatechin and have a strong antioxidative effect in aqueous systems [8]. In previous studies, we have reported combined formulation of grape seed extract and Zincovit tablets for its strong *in vitro, in vivo* antioxidant, anti-hyperglycemic and anti-cataractogenic potential [9-13]. Consequently, the aim of the present study was to investigate the cardio-protective effect of single combined formulation of grape seed extract and Zincovit tablets (Nutritional food supplement) using a Langendorff model of ischemia-reperfusion in Wistar rats.

Materials and Methods

Drugs and reagents

Single combined formulation of grape seed extract and zinc containing nutritional food supplement (Zincovit tablet) was obtained as kind gift from Apex Laboratories Private Ltd., Chennai (India). Thiobarbituric acid (TBA), Trichloroacetic acid (TCA) and 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB) were procured from Sigma Aldrich, Mumbai (India).Creatine kinase and 4-hydroxynonenal (4-HNE) assay kits were purchased from Bioassay Systems (USA) and MyBioSource Inc. (USA) respectively. Di-sodium hydrogen

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Table 1: Composition of Zincovit tablet.

Ingredients	Per tablet contains
Vitamin C	75 mg
Vitamin B_3	50 mg
Vitamin E	15 mg
Vitamin B_1	10 mg
Vitamin B_2	10 mg
Vitamin B_5	10 mg
Vitamin B_6	2 mg
Folic acid	1 mg
Vitamin A	5000 IU
Vitamin D_3	400 IU
Biotin	150 mcg
Vitamin B_{12}	7.5 mcg
Zinc	22 mg
Magnesium	18 mg
Silica	1 mg
Manganese	0.9 mg
Copper	0.5 mg
Iodine	150 mcg
Boron	150 mcg
Selenium	50 mcg
Chromium	25 mcg
Molybdenum	25 mcg
Grape Seed Extract	50 mg

phosphate, Sodium-di-hydrogen phosphate, Di-potassium hydrogen phosphate, Potassium-di-hydrogen phosphate, Potassium chloride, Sodium chloride, Sodium hydroxide, Ethylene-di-amine-tetra-acetic acid (EDTA) and all other chemicals were obtained from Merck Chemicals, Mumbai (India). All reagents were analytical grade. All reagents except for the phosphate buffers were prepared every day and stored in a refrigerator at +4°C. The reagents were equilibrated at room temperature for 30 minutes before use, either at the start of analysis or when reagent containers were refilled.

Preparation of aqueous solution of Zincovit tablets for oral administration

Zincovit tablet is a single combined formulation of vitamins, minerals and grape seed extract. Each tablet of Zincovit weighs 850 mg. 10 tablets of Zincovit were crushed and fine powder form was dissolved in 100 ml of distilled water containing 2 g gum acacia (2% gum acacia). The aqueous solution of Zincovit tablets was stored in an amber colored bottle at 4°C in refrigerator.

Animals

Inbred healthy male albino Wistar rats (6-8 weeks old, weighing 150-250 g) were used in this experiment. They were obtained from Central Animal Research Facility, Manipal University, Manipal. The rats were housed in separate polypropylene cages, maintained under standard conditions with temperature (22–24°C), 12-h light/12-h dark cycle and relative air humidity 40–60%. The animals were acclimatized to the laboratory conditions for one week before the start of the experiment. The animals were provided with a normal pellet diet (Amrit Feeds Ltd., Pune, India) and water ad libitum. Animals described as fasted were deprived of food for 16-h but had allowed free access to water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/KMC/86/2012) and experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines.

Experimental design

In the experiment, 24 adult male Wistar rats were divided into four groups (n= 6). The dose of Zincovit tablet for a 70 kg human is 850 mg (one tablet per day). This dose was converted into 200 g rat dose by multiplying with 0.018 according to the Paget and Barnes [14] and further multiplied by 5 to get the dose for per kg rat. Following the calculation, the dose of Zincovit tablets- 850 mg/day for 70 kg human becomes equivalent to 80 mg/kg/day of rat. Then, double (160 mg/kg/day) and half (40 mg/kg/day) of this equivalent dose was selected to investigate the therapeutic role of the test drug in mytocardial ischemic-reperfusion injury model. The corresponding doses of Zincovit tablets with grape seed extract were administered orally till 21 days as follow-

Group I: Control rats received 2% gum acacia (1ml/kg/day) **Group II:** Rats received Zincovit tablets with grape seed extract (40 mg/kg/day) **Group III:** Rats received Zincovit tablets with grape seed extract (80 mg/kg/day) **Group IV:** Rats received Zincovit tablets with grape seed extract (160 mg/kg/day).

Heart isolation

On 22nd day, all the rats were sacrificed by cervical dislocation, according to the annexure-6 of euthanasia of laboratory animals in the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility. The heart was rapidly excised and placed in ice-cold Krebs-Henseleit buffer solution containing NaCl, 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.0mM. The heart was attached to a Langendorff apparatus via the aorta for retrograde perfusion with Krebs-Henseleit buffer solution for a 10-min washout period at a constant perfusion pressure equivalent to 100 cm water (10 kPa) [15,16]. The perfusate was equilibrated with medical oxygen (O_2) 100% inhalational gas, maintained at 37°C and pH 7.4. During the washout period, the pulmonary vein was cannulated and the Langendorff preparation was switched to the working mode.

Ischemia and reperfusion

Isolated hearts (n = 6 in each group) were subjected to 30 min of global ischemia followed by 60 min of reperfusion [17,18]. The left atrial inflow and aortic outflow lines were clamped during ischemia at a point close to their origin, and reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. During the whole experiment, to prevent the myocardium from drying out, the Krebs-Henseleit buffer solution was sprayed above the isolated heart attached to Langendorff apparatus.

Measurement of coronary flow rate

Before ischemia and during reperfusion, coronary flow rate was measured with a timed collection of the coronary effluent that dripped from the heart in a beaker. The collected coronary effluent per minute was measured with calibrated pipette to determine the coronary flow rate (ml/min).

Measurement of creatine kinase (CK) activity

Creatine kinase activity in the coronary effluent at 5th minute of reperfusion was measured according to the standard protocol given along with the Creatine kinase assay kit of Bioassay Systems (USA).

Preparation of heart tissue homogenate

After the 60 minute of reperfusion, heart was taken off from Langendorff apparatus and further heart homogenates (10% w/v) were prepared in cold 50mM potassium phosphate buffer (pH 7.4) using a homogenizer (Model RQ-127A/D, REMI Laboratory Instruments, Mumbai, MH, India). The unbroken cells and cell debris were removed by centrifugation at 10000 rpm for 30 minutes using a centrifuged at 1000 rpm for 10 min using a refrigerated centrifuge (MIKRO 22R, Andreas Hettich GmbH & Co. KG, Germany). The resulting supernatant was stored at -20°C. The supernatant was used for the estimation of malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and protein thiol content. All the biochemical parameters were estimated in triplicate manner and optical density was also read for reagent and sample blank by using an ELISA reader Bio Tek Instruments ELx800- MS, (USA).

Measurement of malondialdehyde (MDA) content

To 20µl heart homogenate samples, 200µl 0.67% thiobarbituric acid and 100µl 20% trichloroacetic acid were added and incubated at 100˚C for 20 minutes. Then, it was centrifuged at 12000 rpm for 5 minutes and 100µl of supernatant was transferred to 96- wells of micro test plate and optical density of supernatant was read at 540nm [10,19].

Measurement of 4-hydroxynonenal (4-HNE) content

4-HNE is able to bind to proteins and form stable adducts, which is generally used to represent the content of 4-HNE. A commercially available ELISA kit (MyBioSource Inc. USA) was used for the measurement of HNE-protein adducts content. Briefly, 100µl of tissue homogenate was added to a 96-well protein binding plate and incubated at 37°C for 2 h. Then the 4-HNE protein adducts were probed with an anti-HNE-His antibody, followed by an HRP conjugated secondary antibody. After adding stop solution, the absorbance of each well on a micro plate was read at 450 nm immediately. The HNE-protein adducts content was determined by comparing with a standard curve that was prepared from predetermined HNE-BSA standards. The 4-HNE content was expressed as µg/ml in heart tissue homogenate.

Measurement of protein thiol (PT) content

20µl of heart tissue homogenate sample was added in the mixture of 180µl disodium edetate (2mM disodium edetate in 0.2 M disodium hydrogen phosphate) buffer solution and 4µl DTNB solutions (10mM DTNB in 0.2 M disodium hydrogen phosphate) in 96-wells of micro test plate and after 5 minutes of incubation under room temperature optical density was read at 412nm [10].

Statistical analysis

Using Statistical Package for the Social Sciences (SPSS version 16.0; SPSS Inc., Chicago, USA), data were expressed as mean ± standard error of mean and analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey test. A level for $P \leq 0{\cdot}05$ was considered to be statistically significant.

Results

Effect on coronary flow rate

In the group of animals treated with combined formulation of

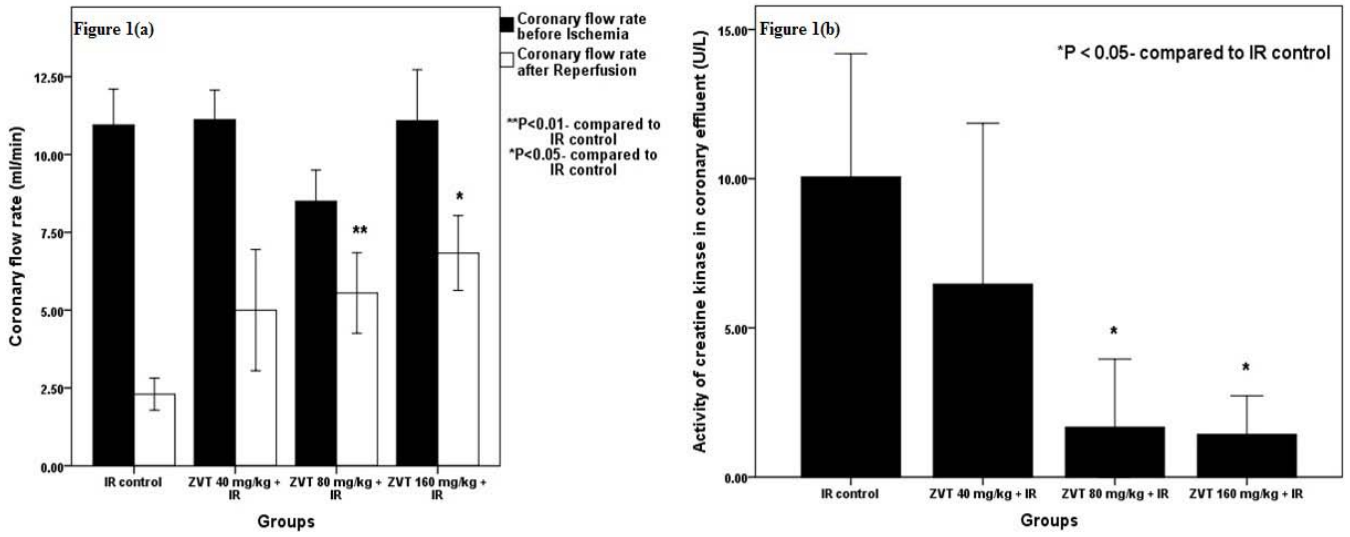

Figure 1: Effect of pre-treatment with different doses of combined formulation of grape seed extract and Zincovit tablets among experimental animal groups on: (a) Coronary flow rate (ml/min) before ischemia and after reperfusion (b) Activity of creatine kinase in the coronary effluent collected at 5th minute of reperfusion n = 6, number of rats in each group; IR, ischemia-reperfusion; ZVT, zincovit tablets with grape seed extract. Values are mentioned as mean. Error bars, +/- 2 standard error of the mean. **indicates statistically significant difference compared with ischemia-reperfusion control (p< 0.01), *indicates statistically significant difference compared with ischemia-reperfusion control (p< 0.05).

grape seed extract and Zincovit tablets at the dose of 80 mg/kg (p = 0.003) and 160 mg/kg (p = 0.021), coronary flow rate (ml/min) was significantly increased after reperfusion in comparison with untreated animals (Figure 1a).

Effect on creatine kinase activity (CK)

Combined formulation of grape seed extract and Zincovit tablets at the dose of 80 mg/kg (p = 0.019) and 160 mg/kg (p = 0.015) significantly decreased creatine kinase activity caused by ischemia-reperfusion injury (Figure 1b).

Effect on malondialdehyde content (MDA)

There was significant decrease for malondialdehyde amount in heart tissue homogenate of all the animals treated with combined formulation of grape seed extract and Zincovit tablets at the dose of 40 mg/kg (p< 0.001), 80 mg/kg (p< 0.001) and 160 mg/kg(p< 0.001) as compared to ischemia-reperfusion control rats (Table 2).

Effect on 4-hydroxynonenal content (4-HNE)

In comparison with ischemia-reperfusion control rats, 4-hydroxynonenal (4-HNE) content was decreased significantly (Table 2) for all the animal groups that were treated with combined formulation of grape seed extract and Zincovit tablets at the dose of 40 mg/kg (p< 0.001), 80 mg/kg (p< 0.001) and 160 mg/kg (p< 0.001).

Effect on protein thiol content (PT)

There was significant increase of protein thiol content in heart tissue homogenate for the group of animals treated with combined formulation of grape seed extract and Zincovit tablets at the dose of 160 mg/kg (p = 0.004) when compared to ischemia-reperfusion control rats (Table 2).

Discussion

In this study, by using a Langendorff model of ischemia-reperfusion, we evaluated the beneficial effects of combined formulation of grape seed extract and Zincovit tablets (nutritional food supplement) on ischemia-reperfusion induced injury. A series of biochemical and metabolic changes in myocardial tissue occur due to deprivation of oxygen and nutrient supply during ischemia. Consequently mitochondrial damage and ATP depletion impair myocardial contractile function [20]. Anaerobic glycolysis due to the absence of oxygen results in the accumulation of lactate and intracellular pH reduction (to <7.0) which leads to activation of the Na^+/H^+ ion exchange, thus extruding protons from the cell in exchange for Na^+ entry. Furthermore the impaired function of (Na^+/K^+) ATPase contributes to exacerbate the intracellular Na^+ and Ca^{2+} overload [21]. During reperfusion, the level of tissue oxygenation increases following restoration of blood flow, which is followed by a burst of reactive oxygen species generation (ROS) that leads to the syndrome of reperfusion injury [2].

Table 2: Effect of combined formulation of grape seed extract and Zincovit tablets on malondialdehyde (µmoles/ml), 4-hydroxynonenal (µg/ml) and protein thiol (µmoles/ml) in heart tissue homogenate Groups.

(n=6)	MDA	4-HNE	Protein thiol
I- IR control (2% gum acacia)	2.22±0.45	3.97±0.29	186.30±27.58
II- ZVT +IR (40 mg/kg/day)	0.28±0.06***	1.31±0.36***	298.85±21.37
III- ZVT +IR (80 mg/kg/day)	0.18±0.07***	0.50±0.16***	306.95±76.49
IV- ZVT +IR (160 mg/kg/day)	0.23±0.06***	2.02±0.27***	468.28±56.95**

n: number of rats in each group; IR, ischemia-reperfusion; ZVT, zincovit tablets with grape seed extract; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal. Values are mentioned as mean ± standard error of the mean. Table 2 shows the reactive aldehydes- MDA, 4-HNE and non-enzymatic antioxidant- protein thiol contents in heart of different experimental groups after myocardial ischemia-reperfusion injury. ***indicates statistically significant difference compared with ischemia-reperfusion control (p< 0.001), **indicates statistically significant difference compared with ischemia-reperfusion control (p< 0.01).

The results clearly showed that ischemia-reperfusion treatment led to cardiac dysfunction (decreased coronary flow rate) accompanied by the increased 4-HNE and MDA contents and creatine kinase activity. Oxidative stress is an important key factor that contributes to ischemia-reperfusion injury. There are reports that reactive aldehydes are significantly accumulated during ischemia-reperfusion due to the increased oxidative stress [22,23]. These reactive aldehydes, such as 4-HNE, are highly toxic and can form protein adducts with the amino acid residues of cysteine, histidine or lysine, which lead to myocardial tissue damage and cardiac dysfunction during ischemia-reperfusion [24]. Antioxidant treatment is considered as a potential strategy to prevent myocardial ischemia-reperfusion injury [25,26]. In comparison with ischemic-reperfusion control (untreated) group, pre-treatment with combined formulation of grape seed extract and Zincovit tablets (nutritional food supplement) especially at the dose of 80 and 160 mg/kg significantly attenuated ischemia-reperfusion induced cardiac injury. It increased coronary flow rate after reperfusion, decreased creatine kinase activity in coronary effluent collected at 5th minute of reperfusion, decreased MDA, 4-HNE and increased protein thiol content in heart tissue homogenate. This effect was not observed in a dose dependent manner. The reason behind this could be excess of antioxidants itself can promote the lipid peroxidation and further generation of reactive aldehydes like MDA, 4-HNE etc.

Earlier we had reported combined formulation of grape seed extract and Zincovit tablets for its strong *in vitro, in vivo* antioxidant, anti-hyperglycemic and anti-cataractogenic potential [9-13]. In one of the study, it has been reported that myocardium of rats fed proanthocyanidins was more resistant to injury caused by ischemia and reperfusion than was the myocardium of untreated control rats. They suggest that proanthocyanidins may not bind to the myocardium, but may instead remain active for several days or weeks and act as a sink for hydroxyl radicals [6]. Proanthocyanidins present in the grape seed extract may interact with intracellular calcium ions, leading to a reduction in the ionized calcium content. One of the studies suggests that flavonoids may increase the binding affinity of a substrate or improve the electron transfer efficacy between NADPH-ferrihemoprotein reductase and the P-450 enzyme [27] thereby providing further protection against reperfusion-induced calcium overload. Furthermore, proanthocyanidins may act as a regenerator of other antioxidants, keeping the concentrations of other antioxidants high enough to affect the formation of hydroxyl radicals. One of the studies suggests that vitamin E supplement prevents the depression of left ventricular function, as well as the elevation of malondialdehyde content and conjugated diene formation in the infarcted rat heart [20]. A synergistic effect of vitamins C and E along with zinc could be expected based on the different environments in which they act-

vitamin C acts in the hydrophilic milieu, scavenging reactive oxygen species, zinc located in the interphase of the bilayer prevents iron or copper binding to the membrane and alpha-tocopherol in the hydrophobic domains of the bilayer inhibits the lipid oxidation free-radical chain reaction [28]. Magnesium inhibits Malondialdehyde (MDA) formation in endothelial cells and low Magnesium oxide induced lipid peroxidation [28]. Taken together, the decreased MDA, 4-HNE, Creatine kinase and increased both protein thiol and coronary flow rate after ischemia-reperfusion injury in the current study might be attributed to the synergistic interplay of constituents of Zincovit tablets, such as-grape seed extract proanthocyanidins which comprise only procyanidins [subunits constituted of (+) catechin (C) and (−)-epicatechin (EC)], Vitamins A, B, C, D, E, folic acid, biotin and minerals like zinc, copper, selenium, magnesium, manganese, chromium and molybdenum mainly, which are promoters of antioxidant activity and act against oxidative stress (Table 1).

Conclusion

Thus, the present study demonstrates that the single combined formulation of grape seed extract and Zincovit tablet is the potential functional nutritional food supplements that could offer a novel therapeutic opportunity against myocardial ischemia-reperfusion injury in Wistar rats. The therapeutic effect seen in animal studies cannot always be entirely extrapolated to humans. Hence, clinical evaluation should be performed to precisely define the cardio-protective role of Zincovit tablets with grape seed extract in humans. Our study opens the perspective to clinical studies could improve the clinical outcome of patients subjected to percutaneous angioplasty, a novel view likely to give rise to the performance of clinical trials devised to demonstrate the validity of this paradigm as nutritional food supplement. "This information would eventually complement our findings, opening the way to sustain ischemic heart disease development in human population".

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