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Effect of Natural Polyphenols on Reactive Oxygen Species in Patients with Myocardial Infarction: A Double Blind Placebo Controlled Trial

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Abstract
Introduction: *Reactive Oxygen Species (ROS) and Reactive Nitogen Speices are lethal in nature. These leads to Oxidative stress. Oxidative stress stimulates the secretion of various cytokines and leukocyte adhesions to smooth vascular cells. This promotes the entry of Monocytes to sub-intimal space of coronary arteries. These Monocytes consumes Oxidized LDL particles and converts in to foam cells and leads to MI.*
Material and Methods: *A Pomegranate Extract of Whole Fruit (PEWF) was prepared as tablet of 300 mg to investigate its effects in synthesis of antioxidants and Antioxidative enzymes in patients with MI. Total 100 participants of either gender with nested cases of MI were included in present study. All participants were assigned in two groups (50 each). One group was under "Add On" therapy of PEWF and matching placebos of same colour, shape and size were used as comparator agent for second group (300 mg BD for 1 month).*
Results: *Results were compared by Z test, Chi square test and coefficient of variations. Statistical analysis proves the prognostic effect after active medication (p<0.05). Study results indicate the rejection of Null Hypothesis (H$_0$) and acceptance of Alternative Hypothesis (H1).*
Conclusion: *Results highlighted that those participants who were under "add-on" therapy of PEWF showed high levels of Total Antioxidants and Antioxidative Enzymes in blood. This is a good sign of prognosis. Thus PEWF should be consumed in diet as food supplementation.*
Keywords: *Myocardial infarction, Coronary Artery Disease, Pomegranate Extract of Whole Fruit, Total Antioxidants Activity, Glutathione Reductase.*

Introduction

In 1954, Garshman and Gilbert speculated that lethal effects of ionizing radiations might be ascribed to formation of Reactive Oxygen Species (ROS)[1]. Since then ROS and Reactive Nitrogen Species (RNS) have gained notoriety. In terms of

scientific literature, ROS are group of free radicals which leads to oxidative stress and free radical damages in body. These are major risk factors for progression of chronic and degenerative diseases such as Cancer, Aging, Arthritis, Coronary Artery Diseases (CAD), Autoimmune diseases and Neurodegenerative disorders[2]. Among these, incidences of CAD are increasing globally[3]. Literatures reviews suggest that ROS stimulate leukocyte adhesions to smooth vascular cell by activation of cytokine like vascular cell adhesion molecules (VCAM). This action promotes the Monocytes adhesion to vascular endothelial cell and entry of Monocytes to sub-intimal space of coronary arteries[4]. This mechanism is further stimulated by signaling molecules like Selectin, Integrins and Monocyte chemoattractent protein1 (MCP1)[5]. ROS and RNS also stimulate generation of Oxidized Low Density Lipoproteins cholesterol (ox-LDL-C) and also prevent nitric oxide mediated arterial relaxation[6]. These overall processes lead to Myocardial Infarction (MI).

In recent years there is upsurge in quest to elucidate the role of antioxidants in prevention of diseases. An antioxidant referred to as any substance that, when present at low concentration compared with the oxidizable substance; can delay or prevent oxidation of that substrate[7]. Antioxidants act as "free radical scavengers". These Antioxidants will prevent and repair all damages caused by ROS and RNS. There for Polyphenols and Antioxidants can enhance the immune defense, lower the risk of cancers and degenerative diseases. These antioxidants are either may be naturally produced in situ (endogenous antioxidants), or externally supplied through foods and by supplements. Some externally supplied, artificial and synthetic antioxidants such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxyltouline (BHT) are now restricted to use in foods as these are suspected to be carcinogeni[8]. Therefore natural antioxidants especially of plant origin are under considerations for these days.

Plants are rich source of compounds that do not appear essential for primary metabolism; these non nutritive compounds are known as phytochemicals. These phytochemicals are known as polyphenols. Polyphenols are synthesized by plants to protect them self against ROS, harmful ultra violet radiations and from active pathogens. These polyphenols are known to have potential health benefits[9]. Recently, the interest of using these plant based polyphenols as therapeutic agents are increasing because these herbs are effective and not having any side effects[10].

Polyphenols are widely present as constituents of plants based food items (such as fruits, vegetables, cereals, legumes and chocolates) and beverages (such as tea, coffee, beers and wines). The level of polyphenols and antioxidants are diverse in different food items. Antioxidative capacities of different beverage such as pomegranate juice, black cherry juice, cranberry juice, grape juice, orange juice and black tea were tested in USA in year 2008. It is found that Antioxidative potencies of different foods are in following order: pomegranate juice> Red wine> Grape juice> Blue berry Juice > Black berry juice> orange juice> Apple juice[11]. Studies reported that Pomegranate peels (skin and pericarp) are having higher polyphenols content than seeds and pulp. Total polyphenols content from peel was found 85.20 ±4.87 mg gallic acid equivalents per gram of dry weight and total polyphenols content from seeds were 7.94 ±1.25 mg gallic acid equivalents per gram of dry weight[12]. This is now well established that pomegranate is a richer source of Polyphenols and Antioxidants.

Literatures reviews suggest that all studies till now have been conducted to assess the effects of polyphenols in human and as well as in experimental animals are by administration of pomegranate juice of seeds and pulp. However, no study has investigated the effect of Pomegranate Extract of Whole Fruit (PEWF) containing the combination of total polyphenols from peels (Skin and Pericarp), Pulps and Seeds all together. Hence we prepared Pomegranate Extract of Whole Fruit

(PEWF) in the form of a tablet of 300 mg, which is a powerful source of polyphenols and antioxidants to investigate; whether the administration of PEWF for one month has any prognostic effects on serum Total Antioxidant Status in patients with MI?

Hypothesis

As PEWF is rich in natural antioxidants and polyphenols, the consumption of this may improve Total Antioxidant Status in blood. Null Hypothesis (H_0) will be implemented during the trial.

Material & Methods

1. Trial Design

A randomized, double-blind, placebo controlled, parallel trial was conducted in Base Hospital, Srikot, Pauri-Grahwal, Uttarakhand, India attached to Veer Chandra Singh Garhwali Government Institute of Medical Sciences & Research, Srikot, Pauri Garhwal, Uttarakhand ,India (VCSGGIMSR) and Netaji Subash Chander Bose Subharti Medical College (SMC) and C.S. Subharti Hospital, Meerut (U.P.) India, in collaboration with Department of Biochemistry, Pharmacology and Medicine.

2. Inclusion and exclusion criteria

A total 100 patients of both men and women with MI and satisfying inclusion and exclusion criteria were enrolled in this trial by applying the formula.

$$n = [(z\sigma)/E]^2$$

Where 'Z' is constant value 1.96 for the Confidence level 95%

'σ' is standard deviation for the sample size , which is ± 3.

'E' is error which is 0.9

'n' is total number of participants in each group.

Inclusion Criteria

1. Men / Women: aged 20-60 years
2. MI as per signs and symptoms: Chest pain (radiating to left arm or left side of the neck), shortness of breath, nausea, sweating, anxiety, palpitation.
3. Permanent residents of the trail area.

Confirmatory tests

1. E.C.G. with changes - Q and ST segment elevation of 1mm or more in two neighboring leads.
2. Creatine Phospho Kinase MB (CPK-MB): Levels more than the reference ranges (0-25 U/L at 37^0c).
3. Troponin -T: Levels more than the reference range. (>0.1 ng/ml)

Exclusion Criteria

1. Patients with acute illness, pregnant, lactating, and postoperative patients.
2. Patients with CNS disorders, systemic chronic diseases e.g. renal failure and chronic hepatic disease.
3. Postoperative conditions like angiography, angioplasty or any other surgical intervention.

3. Method of Randomization

Selected participants were randomized as per criteria given in Table 1 by generating a list of sequential assignments to the treatment group, using the "random seed" function in the Statistical Package for the Social Sciences (SPSS) software program, version 16.0.

Table1: Assignment of participants

Dose	PEWF(Active) 1 BD x 1 month	Placebo 1 BD x 1 month
Myocardial Infarction	n=50	n= 50

4. Assessment of treatment effect

Fasting 4 ml venous blood samples were collected in vacutainer. After coagulation, samples were centrifuged at 8000 RPM for 15 min and serum was collected in separate test tube. Samples were processed on the same day to assess Antioxidants status in Blood by Total Antioxidants Activity (TAA), Glutathion Peroxidase (GPX), Glutathion Reductase (GSH) and Superoxide dismutase (SOD) by spectrometric methods on semi-automatic analyzer.

5. **Trial medicines**

Trial medicines were given on **"add-on basis"** in addition to other prescribed medicines.

5.1 Description of the medicine: The active medicine had PEWF. Matching placebo of same color, shape, size and weight was used. The PEWF/placebo were given orally, as tablets of 300 mg twice daily (BD) for one month.

5.2 Trial procedure

1. Duration of treatment

Participants were treated daily with either active medicine or placebo for one month. Regular follow-up of patients were done by frequents visits and personal communications.

Visit I (Week 0), screening visit (Pre Dug Analysis):

After obtaining an informed consent, nested cases of MI were included. Venous blood sample was collected for assessment of Total Antioxidants status in blood. Baseline titer was obtained and recorded.

Visit II (Week 1)

The participants were under the **"add-on"** therapy of PEWF / placebo and the treatment doses were issued for 15 days initially and participants were recalled for next visit.

Visit III (Week 3)

Follow-up information was obtained regarding any adverse effects of intervention. The participants were questioned regarding any missed doses of trial medicine and next dose of medicines were issued for next 15 days.

Visit IV (Week 5), Final Visit (Post Drug Analysis):

The participants were questioned regarding any missed doses of the trial medicine. All biochemical parameters related to Total Antioxidants status in blood were repeated and recorded.

5.3 Assessment of Compliance:

The participants; who had 80% consumption of PEWFs / placebos, were considered to be compliant.

Results

Table: 2 Age wise distribution of participants with MI in groups

Age (Years)	PEWF (Active)	Placebo	Total
40-50	11	09	20
50-55	15	18	33
55-60	24	23	47
Total participants	**n=50** **Men:44** **Women:06**	**n=50** **Men:47** **Women:03**	**100**

n= Total number of participants.

Table: 3- Descriptive statistics for PEWF (active) medication in Pre and Post Drug Analysis

Sr.no	Parameters	Pre Dug analysis n=50		Post Drug Analysis n=50	
		Mean (X̄)	Std. Deviation (±SD)	Mean (X̄)	Std. Deviation (±SD)
1	TAA(mMol/l)	0.68	0.18	1.31	0.24
2	GPX(U/l)	2729.8	179.82	4673.4	804.06
3	GSH(U/L)	24.79	4.98	65.30	8.05
4	SOD(U/L)	118.63	18.14	295.95	37.11

n= Total number of participants.

Table: 4- Descriptive statistics for Placebo in Pre and Post Drug Analysis

Sr.no	Parameters	Pre Dug analysis n=50		Post Drug Analysis n=50	
		Mean ($\overline{X}$)	Std. Deviation ($\pm SD$)	Mean ($\overline{X}$)	Std. Deviation ($\pm SD$)
1	TAA(mMol/l)	0.72	0.43	0.96	0.32
2	GPX(U/l)	2731.2	208.88	3123.4	256.07
3	GSH(U/L)	27.50	4.81	34.34	7.40
4	SOD(U/L)	111.33	23.97	130.52	17.55

n= Total number of participants.

Table: 5- Z Statistics of PEWF (active) for post drug in comparison to pre drug analysis

Paired Samples Test				
Pairs	Parameters	Z Test	Degree of Freedom	Sign(2 Tailed)
Pair 1	TAA for Pre and Post Drug Analysis	228.47	7254	0.00
Pair 2	GPX for Pre and Post Drug Analysis	285.95	7254	0.00
Pair 3	GSH for Pre and Post Drug Analysis	271.14	7254	0.00
Pair 4	SOD for Pre and Post Drug Analysis	353.25	7254	0.00

Table: 6- Z Statistics of Placebo for post drug in comparison to pre drug analysis

Paired Samples Test				
Pairs	Parameters	Z Test	Degree of Freedom	Sign(2 Tailed)
Pair 1	TAA for Pre and Post Drug Analysis	158.423	4528	0.00
Pair 2	GPX for Pre and Post Drug Analysis	12.954	4528	0.00
Pair 3	GSH for Pre and Post Drug Analysis	33.914	4528	0.00
Pair 4	SOD for Pre and Post Drug Analysis	86.435	4528	0.00

Table: 7 Chi square test of PEWF (active) for post drug in comparison to pre drug analysis

Paired Samples Test				
Pairs	Parameters	Chi square	Degree of Freedom	Sign(2 Tailed)
Pair 1	TAA for Pre and Post Drug Analysis	132.1	1	0.00
Pair 2	GPX for Pre and PostDrug Analysis	8.510	2	0.00
Pair 3	GSH for Pre and Post Drug Analysis	117.01	1	0.00
Pair 4	SOD for Pre and Post Drug Analysis	14.791	3	0.00

Table: 8 Chi square test of Placebo for post drug in comparison to pre drug analysis

Paired Samples Test				
Pairs	Parameters	Chi square	Degree of Freedom	Sign(2 Tailed)
Pair01	TAA for Pre and Post Drug Analysis	100.004	4	0.00
Pair02	GPX for Pre and PostDrug Analysis	65.693	1	0.00
Pair03	GSH for Pre and Post Drug Analysis	14.748	1	0.00
Pair04	SOD for Pre and Post Drug Analysis	21.874	4	0.00

Table: 9 Coefficient of Variations for PEWF (active) & placebo medicine after post drug analysis

Descriptive Statistics	PEWF (ACTIVE)			PLACEBO		
Post Drug Analysis	Mean ($\overline{X}$)	Std. Deviation ($\pm SD$)	C.V.	Mean ($\overline{X}$)	Std. Deviation ($\pm SD$)	C.V.
TAA(mMol/l)	1.31	0.24	0.41	0.96	0.32	0.22
GPX(U/l)	2729.3	179.8	0.47	3123.4	256.07	0.27
GSH(U/L)	65.30	8.05	0.27	34.34	7.40	0.13
SOD(U/L)	295.95	37.11	0.19	130.53	17.55	0.12

Figure 1: Line graph presentation for Coefficient of Variations for PEWF (active) & placebo medicine after post drug analysis

	TAA	GSH	GPX	SOD
C.V. of PEWF	0.41	0.27	0.47	0.19
C.V. of Placebo	0.22	0.13	0.27	0.12
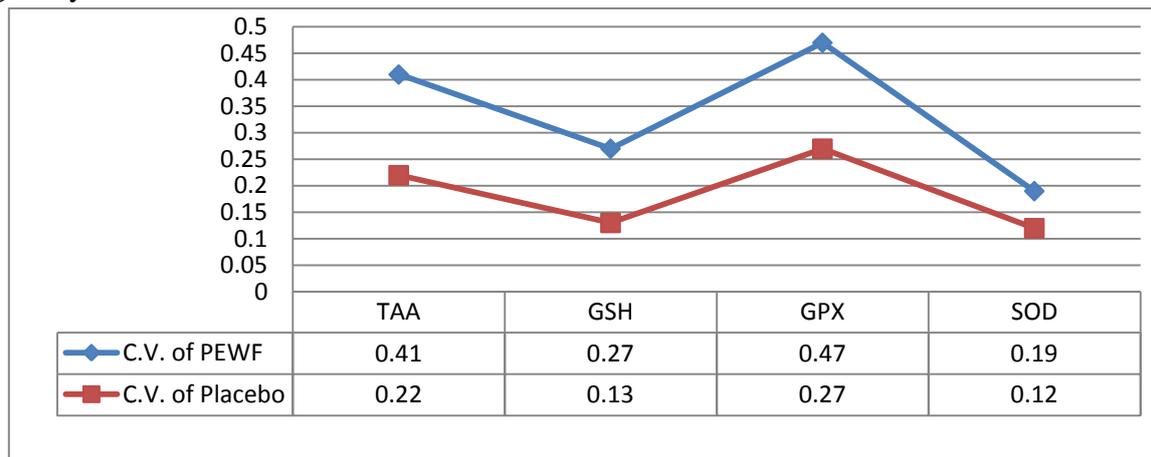
Interpretation

Total 100 participants, of either gender between 40-60 years participated in this trial (Table: 2). Amongst these, 20 were from 40-50 years age group, 33 from 50-55 years and 47 from 55-60 years age group. We did not get participants of 20-40 years. The participants were distributed randomly to either of two groups of 50 each. One group of 50 participants (44 men and 6 women) received PEWF (active) and second group of 50 participants (47 men and 3 women) received placebo. All participants were instructed to continue with prescribed medicines by the clinicians uninterrupted. The PEWF/Placebo was given as "**add-on**" basis for one month.

Table number 3 summarizes the descriptive statistics of PEWF on Total Antioxidants Status in both pre and post drug analysis. In this table, serial numbers 01 to 04 are showing pre and post drug effects on Total Antioxidants Status for 50 participants with MI. The mean level of TAA and GPX (Sr.No.1 and 2) in pre drug analysis were 0.68 mMol/l and 2729 U/L and in post drug analysis these were 1.31 mMol/l and 4673.4 U/L. Serial numbers 3 and 4 highlight the effects Total Antioxidants Status in pre and post drug effects. The mean levels of GSH and SOD in pre drug analysis were 24.79 U/L and 118.63 U/L. The mean levels of GSH and SOD in post drug analysis were 65.30 U/L and 295.95 U/L. The study results indicate that after consumption of

PEWF; Total Antioxidants levels in blood are increased. This is a good sign of prognosis.

Table 4 summarizes the Descriptive statistics for placebo in both pre and post drug effects. Serial numbers 1 to 4 present post drug effects of placebo on Total Antioxidants Status in patients with MI. The mean levels of TAA, GPX, GSH and SOD in post drug effects were increased. The reason behind this is that all the patients were taking their prescribed allopathic medicines regularly.

Table number 5 and 7 summarize the Z and chi square test of PEWF in both pre and post drugs analysis. In table 5; pair number 1 to 4 showed that $p < 0.05$, which indicates that Total Antioxidants profile such as TAA, GPX, GSH and SOD were improved in post drug analysis after active medication. This indicates the prognosis. Statistical significance ($p < 0.05$) indicates the rejection of Null Hypothesis (H_0), which means that Alternative Hypothesis (H1) will be implemented.

Table number 6 and 8 show the z and chi square test of placebo in post drug analysis for Total Antioxidants Profile with MI. In pair 1 to 4 1 to 4 shows that $p < 0.05$, which indicates that Total Antioxidants profile such as TAA, GPX, GSH and SOD are improved in post drug analysis after Placebo. Statistical significance ($p < 0.05$) indicates the rejection of Null Hypothesis (H_0), which

means that Alternative Hypothesis (H1) will be implemented.

Table 9 shows the difference in coefficient of variation (C.V.) of post drug analysis after PEWF (active) and placebo medication. The C.V. of TAA, GPX, GSH and SOD on consumption of PEWF were 0.41, 0.47, 0.27 and 0.19. The C.V. of TAA, GPX, GSH and SOD on consumption of placebo were 0.22, 0.27, 0.13 and 0.12. This highlights that C.V. of all above parameters after active medications are higher than placebo. This shows that active medicine has much higher prognostic effect.

In figure 1, when C.V. for post drug effect of active medicine and placebo are compared with each other by graphical analysis, it is highlighted that the C.V. of active medicine are higher in all parameters. This indicates the improvement of Total Antioxidants Profile after consumption of PEWF.

Discussion

Oxidative stress leads to generation of ROS and Reactive Nitric Species (RNS). Thus generation of ROS and RNS are lethal for body. These lethal ROS/RNS are categorized as superoxide anion radical (dioxide or O_2^-), hydroxyl radical (OH) and peroxynitrite anion ($ONOO^-$). The ROS are generated from oxygen and RNS are derived from NO_2, ONOO, N_2O_3 and HNO_2.

These ROS and RNS may cause alterations in DNA by different mechanisms like nicking, base pair mutations, rearrangement, deletions, sequence amplifications, nitration, nitrosation and deamination. The mutated DNAs or nucleic bases are responsible for synthesis of mutated proteins and are cause for pathogenesis of many diseases such as atherosclerosis, cancer, diabetes mellitus, rheumatoid arthritis, post-ischemic perfusion injury and myocardial infarction[13]. For healthy leaving; levels of ROS and RNS should be on lesser side. The key mechanisms for reducing ROS and RNS are by changing sedentary life style, removal of stress or by antioxidant rich supplementations. Many natural and artificial supplements are available in market. Apart from these; pomegranate fruit has the most potent antioxidant capacity[14]. These natural Polyphenols and Antioxidants are safe and do not posses any toxicity because pomegranate is consumed as fruit since ages.

Literature reviews suggest that polyphenols and antioxidants supplementations improve blood antioxidants levels by activation of transcription factor like Nrf2. Nrf2 (nuclear factor (erythroid-derived 2) was identified in 1994. Nrf2 is an activator of antioxidant response element (ARE). ARE activates the expression of NAD (P) H: quinone oxidoreductase 1 (NQO1). Activated NQO1 is a key factor for synthesis and improvement of blood Antioxidants and Antioxidative enzymes [15].Another mechanism states that ROS induces a suppressor protein like Keap1. Activated form of Keap1 binds to Nrf2. By this Nrf2 become inactivated. This inactivated Nrf2 cannot enter in to the nucleolus and ARE will remain inactivated. Inactivated ARE cannot stimulate the synthesize antioxidants and thus level of ROS/RNS increases in blood (Figure 2) [16].

Our present trial indicates that consumption of polyphenols and antioxidants rich food items such as PEWF has the potential to increase Antioxidative status in blood. These antioxidants also reduce the generated ROS and RNS. These food items should be included as an integral part of human diet and may be given as food supplements.
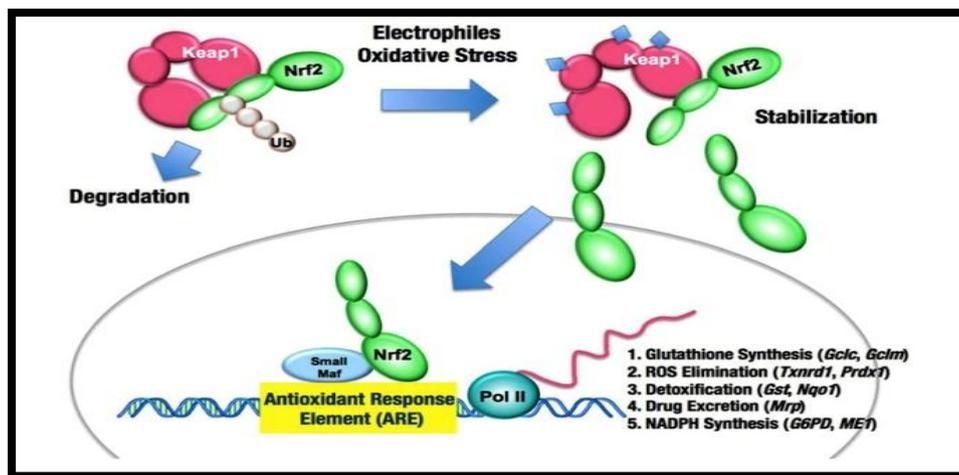
Figure: 02

Conclusion

Major risk factors of MI are ROS, RNS and CAD. Our findings suggest that consumption of antioxidant and polyphenols rich food supplements such as PEWFs for one month reduces the ROS and RNS by increasing Total Antioxidants status in blood. These antioxidants will neutralize the toxic and lethal effects of ROS and prevents the progression of CAD. In conclusion, polyphenols and antioxidants rich fruit supplements like pomegranate should be taken in diet for healthy living.

Ethical Considerations

Ethical clearance was obtained from Institutional Ethics Committee (IEC) of VCSGGIMS&R, Srikot, Pauri Garhwal-Uttarakhand and SMC, Meerut, Uttar Pardesh. The trial was registered with Clinical Trial Registry India (CTRI) before the initiation of the study. This trial was conducted according to Good Clinical Practices and the Declaration of Helsinki.

Acknowledgments

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