Bar-Ilan University
אוניברסיטת בר-אילן

הפקולטה לרפואה בגליל

נושא העבודה:

השפעת מיץ רימונים כמונע פגיעה באנדותל

Thesis subject:

Influence of Pomegranate Juice in Preventing Endothelial Injury

פרטי המנחים:

ד"ר שפרה סלע
המעבדה למיקרוביולוגיה ומעבדת
המחקר
המרכז הרפואי לגליל

ד"ר ריקרדו אלמולי
מעבדת המחקר
המרכז הרפואי לגליל

פרטי הסטודנט:

בועז אלעד

הצעת מחקר

Influence of Pomegranate Juice in preventing endothelial injury

1) Abstract (תקציר)

The endothelium, a layer of cells lining the intimae of blood vessels, constitutes a multifactorial endocrine organ, which among its many functions, synthesizes inflammatory mediators and substances that maintain the vascular tone, hemostasis and coagulation.
When endothelial function is impaired, there is a decreased secretion of vasodilators (i.e nitric oxide) and regulation of coagulation and platelets adhesion is altered. Non-traditional, however important causes for endothelial dysfunction and atherosclerosis formation are oxidative stress (OS) and inflammation.
Pomegranate juice (PJ) is one of the most powerful antioxidant among nutrients. It was found to have an effect, three times higher than red wines and green teas. In our previous studies PJ has been shown to reduce OS and inflammation in hemodialysis patients, however the mechanism of its action is not clear yet.
We hypothesize that PJ can attenuate/abolish endothelial injury conferred by hydrogen peroxide *in-vitro*, enabling to study the mechanism of PJ action.
The aims of this study are: i. to study the deleterious effect of hydrogen peroxide (H_2O_2, an oxidant) on endothelial cells *in-vitro;* ii. To study the beneficial effects of PJ on these injured endothelial cells by examining endothelial survival and several genes' expression in relation with OS and inflammation.
Endothelial survival will be monitored by a colorimetric assay and by levels of total RNA extracted. We will use human umbilical vein endothelial cells (HUVEC) grown in culture, treat them with PJ and H_2O_2, extract RNA and reverse transcribe it. The differences in gene expression will be tested with real time PCR. Several key genes which are known to be responsive to OS, inflammation and related to the atherosclerotic process will be tested: interlukin (IL)-6, monocyte chemotactic protein (MCP)-1, heme oxygenase (HO)-1, endothelial nitric oxide synthase (eNOS) and Sertuin (Sirt)-1.
We imply that PJ will confer anti-oxidative and anti-inflammatory effects, showing a protective effect on endothelial cells under OS (treated with H_2O_2). We suggest that changes in the expression of this genes will be indicative of their pivotal role in the initiation of the atherosclerotic process.
Key words: endothelial dysfunction, oxidative stress, inflammation, Pomegranate Juice.

2) Introduction (מבוא)

Atherosclerosis is associated with a variety of cardiovascular diseases (CVD) which are the major cause of morbidity and mortality worldwide. Endothelial injury/dysfunction which underlies atherosclerosis and CVD, starts long before these clinical manifestations.
The endothelium- a layer of cells lining the intimae of blood vessels, constitutes a multifactorial endocrine organ which among its many functions synthesizes inflammatory mediators and substances that maintain the vascular tone, hemostasis and coagulation.
These complex endothelial functions explain why alteration of endothelial functions constitutes the critical initial event in the atherosclerotic process.
Oxidative stress (OS), endothelial dysfunction and their role in atherosclerosis- OS is a non-traditional risk factor for atherosclerosis. It arises from an imbalance between oxidant production and antioxidant defense. In the process of normal cellular metabolism, oxygen undergoes a series of univalent reductions, leading to the production of reactive oxygen species (ROS) such as $O_2^{\bullet-}$ and H_2O_2. Potential enzymatic sources of ROS include components of the mitochondrial electron transport chain, xanthine oxidase, cytochrome p450 monooxygenases, lipoxygenase, nitric oxide

synthase (NOS) and the NADPH oxidase. $O_2^{\cdot-}$ is dismutated by SODs to H_2O_2 that is catalyzed to H_2O by catalase, peroxiredoxins or glutathione peroxidases. Excessive $O_2^{\cdot-}$ and H_2O_2 can stimulate cellular responses (hypertrophy, proliferation, migration of monocytes and lymphocytes into vascular wall) via oxidation of signaling molecules or via oxidative inactivation of proteins. ROS can also activate redox sensitive transcription factors which contribute to vascular inflammation[1]. OS plays a major role in clinical states associated with atherosclerosis such as diabetes mellitus (DM), hypercholesterolemia, hypertension, smoking and chronic and end stage kidney diseases. Endothelial dysfunction is a common denominator in pathologies such as dyslipidemia, DM and hypertension, where endothelial cells are injured, resulting in an unbalanced regulation of their functions mentioned above[2].

Pomegranate juice (PJ)- a natural and promising nutrient fighting endothelial damage- Many fruits and vegetables, with their natural flavonoid/polyphenol contents have anti-oxidative and anti-inflammatory properties, influencing cell signaling. PJ is known to scavenge ROS or inhibit their formation in a rate higher than that of red wines and green teas[3]. Kaplan, Fuhrman et al, have reported lower uptake of oxLDL in macrophages treated with PJ[4,5]. A decrease in oxidation-sensitive gene expression and an increase in eNOS activity were found in response to PJ supplementation in hypercholesterolemic mice and human coronary artery endothelial cells exposed to high shear stress[6]. However, the beneficial effect and the mechanism of PJ action on endothelial cells is not clear yet.

Since PJ's anti-oxidative and anti-inflammatory effects may be due to its regulation of cells signaling, we will focus on the expression of two groups of marker genes:

Inflammatory markers- **Monocyte chemotactic protein (MCP)-1**-An early feature of inflammation in stressed tissues. It causes increased expression of interstitial and vascular cellular adhesion molecules and attracts monocytes and other immunocytes towards the stressed site, which lead to pro-inflammatory gene activation producing cytokines like TNFα, IL-1, IL-6, interferon-γ etc. MCP-1 can also stimulate T cells to enter lesions[7], a key component in plaque formation.

Interleukin (IL)-6- appears to have a distinct messenger cytokine role, being the most important stimulator of C-reactive protein (CRP) production. Produced by numerous types of immune cells[8]. IL-6, CRP, and TNFα which are markers of inflammation were founded to be elevated in cardiometabolic abnormalities, hypertension and to be associated with cardiovascular disease.

Oxidant/anti-oxidant markers- **Sertuin (Sirt)-1**- a deacetylase that removes acetyl groups from histones and acts on regulatory factors such as nuclear factor (NF)-κB and eNOS[9], by that regulates the pathogenesis of chronic diseases, including CVD and chronic renal diseases. It is highly expressed in the vasculature, which plays a critical role in regulating endothelial cell-mediated vascular homeostasis and remodeling[9]. **Heme oxygenase (HO)-1**- a rate limiting enzyme in heme catabolism, a process which leads to the generation of anti-oxidants and anti-inflammatory agents. Upon OS its expression is induced, leading to generation of carbon monoxide (CO), Fe^{2+} and biliverdin and therefore represents an effective and cooperative strategy to intervene in protection against inflammatory processes and oxidative tissue injury[10]. **Endothelial nitric oxide synthase (eNOS)**- eNOS is a nitric oxide synthase that generates NO in blood vessels. It regulates vascular tone by inhibiting smooth muscle contraction and platelet aggregation. It keeps blood vessels dilated and by that controls blood pressure.

3) HYPOTHESIS (השערת מחקר)

We hypothesize that PJ can attenuate/abolish endothelial injury conferred by hydrogen peroxide *in-vitro*, enabling to study the mechanism of PJ action.

4) EXPECTED RESULTS & IMPORTANCE (משמעות)

Based on our previous studies and the proposed herein, we expect to find that:

PJ will confer a strong anti-oxidative and anti-inflammatory effect and therefore will protect endothelial cells exposed to H_2O_2, from injury. These effects can be explained by our expected results: increased eNOS and Sirt-1 and decreased HO-1, MCP-1, IL-6 gene expression, counteracting the effects conferred by OS to endothelial cells.

New biomarkers associated with endothelial dysfunction will arise from this study, serving as a tool to closely monitor the aggravation of atherosclerosis. This study will promote the use of PJ as a safe, not - expensive natural treatment modality in clinical states associated with atherosclerosis and CVD.

5) METHODS AND STUDY DESIGN (שיטות)

This basic research is based on an *in-vitro* model to study injury and recovery of endothelial cells.. We will use human umbilical vein endothelial cells (HUVEC) taken after delivery from healthy women. These cells will be cultured and then exposed to OS in the absence and presence of PJ. We will extract RNA and reverse transcribe it, study mRNA levels corresponding to the genes: eNOS, IL-6, MCP-1, HO-1 and Sirt-1, which changes will serve as markers for endothelial injury.

Cell culture from human umbilical vein endothelial cells (HUVEC)

The cells are cultured as described by Jacobi et al[11]. Endothelial cell specificity (>95%) is confirmed by flow-cytometry with anti-human CD31. Cells at a sub-confluence state will be treated with PJ before or after treatment with H_2O_2. HUVECs will be rinsed in PBS, after the addition of each H_2O_2 or PJ, thus H_2O_2 and PJ will never be present at the same time, to avoid direct scavenging of H_2O_2 by PJ.

Measurement of gene expression

Total RNA will be extracted from HUVEC with RNA mini prep kit. Quantities and integrity of RNA will be followed by Nanodrop and gel electrophoresis. Reveres transcriptase reaction will be done with the High Capacity cDNA Reveres transcriptase Kit (ABI), PCR with TaqMan gene expression system including the specific set of primers and probe for each gene analyzed. Reaction will be done in the ABI 7300 Prism real time PCR. The PCR conditions for all genes will be as follows: 50^0C for 2 min; preheating, 95^0C for 10 min; followed by 40 cycles of denaturation (95^0C for 15s) and annealing/elongation (60^0C for 60s). Each sample will be run in triplicate. Expression will be normalized to GAPDH as an internal control.

Endothelial cell survival

HUVEC will be seeded at 20,000 cells per well in a 12 well plate and grown until sub-confluence. Cells will be treated with PJ (Primor) for 3hrs prior to OS created by 250μM H_2O_2 for 1 hr. Cultures will be recovered for 20 hours in complete medium, rinsed twice with PBS and tested with 40ug/ml Neutral Red for 90min, again rinsed twice with PBS and lysed with lysis solution (50%EtOH, 1%Acetic acid in water). Endothelial survival results will be measured by a colorimetric reaction (neutral red) and by levels of total RNA extracted.

Statistical methods: All samples will be carried out in triplicate and each point will be an average of these. We hope to reach statistical significance, $p<0.05$, in these settings. Since this is a preliminary study the number of repetitions can not be determined yet, as we do not know the extent of PJ's effect.

6) THE STUDENT'S PRACTICLE PART

Extraction of RNA, preparation of cDNA, running RT-PCR, viability experiments (evaluation of the number of cells after insult), data analysis and writing the study.

7)References

1. Fukai T, Ushio-Fukai M. Superoxide dismutases: Role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal*. 2011;15:1583-1606
2. Victor VM, Rocha M, Sola E, Banuls C, Garcia-Malpartida K, Hernandez-Mijares A. Oxidative stress, endothelial dysfunction and atherosclerosis. *Curr Pharm Des*. 2009;15:2988-3002
3. Shema-Didi L, Sela S, Ore L, Shapiro G, Geron R, Moshe G, Kristal B. One year of pomegranate juice intake decreases oxidative stress, inflammation, and incidence of infections in hemodialysis patients: A randomized placebo-controlled trial. *Free Radic Biol Med*. 2012

4. Kaplan M, Hayek T, Raz A, Coleman R, Dornfeld L, Vaya J, Aviram M. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. *J Nutr*. 2001;131:2082-2089
5. Fuhrman B, Volkova N, Aviram M. Pomegranate juice inhibits oxidized ldl uptake and cholesterol biosynthesis in macrophages. *J Nutr Biochem*. 2005;16:570-576
6. de Nigris F, Williams-Ignarro S, Lerman LO, Crimi E, Botti C, Mansueto G, D'Armiento FP, De Rosa G, Sica V, Ignarro LJ, Napoli C. Beneficial effects of pomegranate juice on oxidation-sensitive genes and endothelial nitric oxide synthase activity at sites of perturbed shear stress. *Proc Natl Acad Sci U S A*. 2005;102:4896-4901
7. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res*. 2002;91:281-291
8. Papanicolaou DA, Vgontzas AN. Interleukin-6: The endocrine cytokine. *J Clin Endocrinol Metab*. 2000;85:1331-1333
9. Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of sirt1 in cellular functions: Role of polyphenols. *Arch Biochem Biophys*. 2010;501:79-90
10. Zhu X, Fan WG, Li DP, Kung H, Lin MC. Heme oxygenase-1 system and gastrointestinal inflammation: A short review. *World J Gastroenterol*. 2011;17:4283-4288
11. Jacobi J, Sela S, Cohen HI, Chezar J, Kristal B. Priming of polymorphonuclear leukocytes: A culprit in the initiation of endothelial cell injury. *Am J Physiol Heart Circ Physiol*. 2006;290:H2051-2058