

**APOPTOTIC EFFECTS OF PUNICALAGIN
ON PARTIALLY HEPATECTOMIZED RAT LIVER**

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Abstract

The antioxidant potential of pomegranate is attributed to its polyphenols, including punicalagin. In this study, we searched the effects of punicalagin on 70% partially hepatectomized rats. Animals were subjected to partial hepatectomy (PH), immediately after receiving 15 mg/kg punicalagin. They were dissected at different time points (3 h, 6 h) following surgery. NF-kappaB (NFκB), caspase-3 gene expression results were evaluated by RT-PCR and protein expression fold changes were evaluated according to the qPCR method. Apoptotic index results were compared between groups.

Gene expression levels of experimental groups necessarily correlate with protein expression levels. Results of RT-PCR ($p < 0.05$), qPCR ($p < 0.05$) and apoptotic index ($p < 0.05$) which belong to Caspase-3 gene region, have shown that 15 mg/kg of punicalagin induced apoptosis in a time-dependent manner despite the partial hepatectomy (group V–VI). Six hours after PH in group VI, apoptosis reached highest level with the injection of punicalagin. Activation of NFκB, at the end of the 6th hour in group IV, has demonstrated a significant increase compared to the other groups. After PH, 15 mg/kg of punicalagin has time-dependently suppressed regeneration and induced apoptosis. The results of the study showed that apoptosis induction could be associated with the caspase-3 increased activation. The liver, apoptotic index values triggered in group VI at the end of the 6th hour after PH. According to the results, 15 mg/kg intraperitoneally (IP) punicalagin application induced liver apoptosis despite the induction of liver regeneration (by partial hepatectomy).

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1. Introduction. The treatment alternatives in curing cancer, which has seriously threatened human life, fail to satisfy expectations. Therefore, protection from cancer has been a vital strategy [1]. Epidemiological studies show that consumption of fruits and vegetables with high phenolic contents correlates with reduced cardiovascular, cerebrovascular diseases, and reduced risk of cancer [2-4]. Anticancer effects of phenolic contents include antioxidant, anti-inflammatory, and antiproliferative properties [4-6].

Punica granatum L. (Pomegranate) has been traditionally used as medicine for the treatment of various diseases in lots of countries [3]. Pomegranate is a rich polyphenol source that takes its red colour from hydrolyzable tannins such as anthocyanins, ellagic acid, and punicalagin [5]. Pomegranate has also antioxidant [2], anti-inflammatory [6], anti-arteriosclerotic [7-9], anti-tumour activity [7], antiproliferative, pro-apoptotic effects [4]. Punicalagin is the major antioxidant polyphenol in pomegranate [5,6,10].

The liver is one of the organs of the body with the most crucial function. Any functional disorder that may occur in the liver influences all the systems in the body. Various factors such as chemical contaminators, medicines, alcohol, physical damage, liver tumours, viral infections, tumours, and surgical applications can lead to injuries in the liver tissue. PH in the liver is generally implemented in massive lesions (metastasis, liver tumours, hemangioma, etc.) and liver transplantation to any other living being. The liver has the potential to be able to rejuvenate itself entirely against the cell losses after hepatectomy.

Apoptosis begins with the production of the apoptotic signal within the cell which starts to undergo biochemical and morphological modifications. PH is a well-regulated model for the evolution of apoptosis and cell proliferation together during liver regeneration [11]. After PH, mitotic activity rises in the liver tissue. Most residual hepatocytes enter the cell cycle simultaneously. In the first phase, hepatocytes undergo a transition from resting state (G_0) to (G_1). The first phase lasts approximately 4-6 h after PH. DNA synthesis starts around 18 h and peaks at 24 h after PH [1,11,12]. The growth of the liver due to the increase in mitotic activity continues until the liver lobes reach the dimensions of an adult's liver lobes. Between 24 and 30 h after 70% hepatectomy, it has been observed that mitosis has commenced, and the regeneration has been completed in 7-10 days [13-15].

Rejection of 70% of the mass of the liver in rats is considered an optimum ratio in terms of regenerative induction, and it is supposed that a PH to be realized in a lower or upper rate compared to this optimum ratio can inhibit the regeneration [1,11,14,16]. After PH, the remaining hepatocytes synchronously enter the cell cycle. Those hepatocytes pass to the G_1 phase, which has the potential mitosis characteristic from the G_0 phase, and this period ends approximately 4-

6 h after hepatectomy. Around 18 h after PH cells commence DNA synthesis and it is completed about 24 h after PH [1].

Induction of liver regeneration with PH (70%) and upon this induction, histological and genomic investigation of the apoptotic effect of punicalagin on the rat liver constitutes the basis for the experiment model. Changes in mRNA expressions of Caspase-3 and NFκB genes have been figured out on rat livers at which regeneration has been induced by the method of PH. The effects of probable changes in mRNA levels on liver histology have been researched by the tunnel (terminal deoxynucleotidyl transferase-mediated nick end-labeling) method.

TaqMan Protein Assay allows relative quantitation of specific proteins. The method indicates three main steps: binding, ligation, and amplification. The first step starts with the incubation of protein samples with oligonucleotide probe pair. The protein assay probes are protein-specific antibodies conjugated to oligonucleotides with a biotin-streptavidin linkage. The second step indicates ligation of the oligonucleotide probes with the help of DNA ligase. The last step indicates the amplification of oligonucleotides and analyses by real-time PCR with the TaqMan probe. The ligation product is used as a template in the TaqMan real-time PCR assay [17].

2. Material and methods. 2.1. Chemicals. Punicalagin ($\geq 98\%$ HPLC) was purchased from Sigma. PureLink RNA Mini Kit, Paris Kit, and TRIzol were obtained from Ambion. High Capacity cDNA Reverse Transcription Kit and TaqMan Gene Expression Master Mix were obtained from Applied Biosystems. ApopTag Plus Peroxidase InSitu Apoptosis Detection Kit was obtained from Millipore.

2.2. Animals. Wistar albino male rats weighing 190–250 g were used for the experiment. Animals were housed in polycarbonate cages, kept in an air-conditioned atmosphere at 22 °C with a 12 h light/dark cycle. All animals were fed the standard commercial diet with tap water. Wistar albino male rats were obtained from the T. R. Ministry of Health, Refik Saydam National Public Health Agency, Experiment Animals Production Laboratory. At the end of 7-day acclimatization rats were randomly divided into six groups, and each group consisted of seven rats. The experiments were approved by the Institutional Ethical Committee for Animal Care and Use at the Eskişehir Osmangazi University, Eskişehir, Turkey.

Punicalagin was dissolved (15 mg/kg body weight) in distilled water and applied intraperitoneally immediately after the operation to the partially hepatectomized rats. All animals were submitted to a 70% PH and dissected at 3 and 6 h after surgery. Hepatectomized rats were housed in separate cages with free access to water and chow until the dissection time. PH (70%) was performed, according to HIGGINS and ANDERSON [18].

2.3. Experimental design.

Group I: Sham-operated group, dissected 3 h after surgery.

Group II: Sham-operated group, dissected 6 h after surgery.

Group III: Hepatectomized and immediately after applied 0.09% NaCl solution (SF). Dissected 3 h after surgery.

Group IV: Hepatectomized and immediately after applied 0.09% NaCl solution (SF). Dissected 6 h after surgery.

Group V: Hepatectomized and immediately after applied 15 mg/kg punicalagin. Dissected 3 h after surgery.

Group VI: Hepatectomized and immediately after applied 15 mg/kg punicalagin. Dissected 6 h after surgery.

2.4. RNA extraction. Liver tissue pieces were immersed in RNAlater and then frozen. Snap-frozen tissues were homogenized. High-quality total RNA was extracted using Trizol (Applied Biosystems) according to the kit protocol.

2.5. TaqMan gene expression assay. RNA isolation Kit and TaqMan Gene Expression Assay Kit, TaqMan[®] Gene Expression Master Mix, High Capacity cDNA Reverse Transcription Kit, RNAlater were obtained from Applied Biosystems. Gene expression assays were performed according to the kit protocol, observing the amplification plots for all experimental groups. Gene expression levels between control and experimental groups were determined by using the $\Delta\Delta C_T$ values [17].

2.6. TaqMan protein assay. TaqMan[®] Protein Assays Open Kit, TaqMan Protein Expression Core Reagents Kit, TaqMan[®] Protein Assays Fast Master Mix, All TaqMan[®] were purchased from Applied Biosystems. Protein Assays were performed according to the kit protocol. High-quality total RNA was extracted from the liver tissue using Trizol (Applied Biosystems). qPCR assays were performed on a StepOnePlus[™] Real-Time PCR System (Applied Biosystems). TaqMan[®] Protein Assays with rat liver tissue samples were performed using 500 ng/ μ L protein in each well. Data were analyzed using Protein Assist TM 1.0 (Applied Biosystems, Foster City, CA).

2.7. TUNEL staining (analysis of apoptotic nuclei by immunocytochemistry). Apoptotic nuclei of rat liver sections were visualized by in situ nick-end labellings with the use of the ApopTag Plus in situ apoptosis detection kit (Apop Tag Peroxidase Kit) according to the kit protocol.

3. Results and discussion. 3.1. TaqMan[®] protein assay provides relative protein quantification of the sample. Proteins can typically be detected in reactions containing 500 ng μ L⁻¹. The TaqMan Protein Assay has a simpler and faster way of western blotting process [17].

TaqMan protein assay consists of antibody-protein binding with real-time PCR-based detection of oligonucleotide sequence [19]. The reaction contains three main steps: 1. Binding of an antibody linked oligonucleotide probes to a specific protein in the sample. 2. Ligation of oligonucleotide probes by DNA ligase. 3. Amplification of the template and analysis by real-time PCR with TaqMan probe [17].

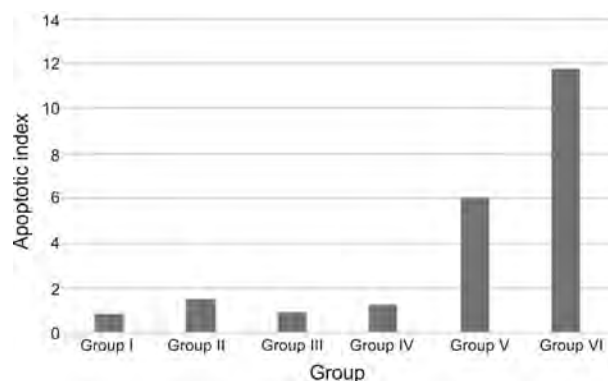


Fig. 1. Apoptotic index values of groups:
Asym Sig = 0.001; $P < 0.05$

3.2. Hepatocellular apoptosis index. In the sections of the liver where TUNEL staining was performed the apoptotic index was derived by proportioning the number of apoptotic cells in the randomly selected five-section areas to the number of cells having normal physiology. Changes in the apoptotic index between the experimental groups were determined. According to results, it was found out that the highest apoptotic index value was detected in group VI, and the lowest apoptotic index was detected in group I and III. After PH, it has been observed that exposure of liver cells to punicalagin has induced apoptosis. Group VI has shown a significant difference compared to group V ($P < 0.05$). In group VI, it has been seen that implementation of 15 mg/kg [2] punicalagin just after PH has induced apoptosis (Fig. 1).

3.3. RT-PCR. Analyzing the results of caspase-3 gene expressions, differences between groups have been found statistically significant. In group VI, caspase-3 gene expression has differed significantly when compared to group V and group IV. In groups V and VI, caspase-3 activity has displayed a significant increase in a time-dependent manner (Fig. 2).

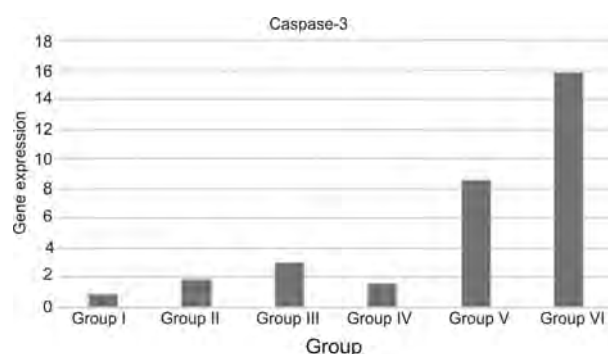


Fig. 2. Caspase-3 gene expression graph of groups ($n=7$)

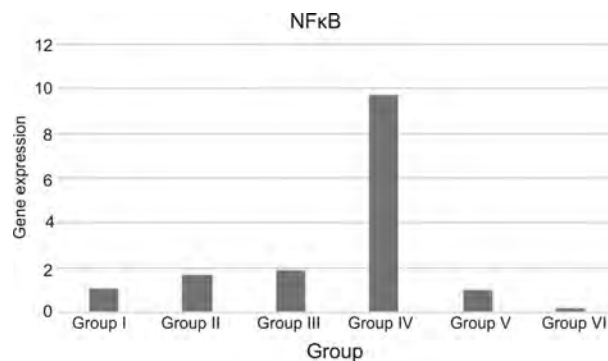


Fig. 3. NFκB gene expression graph of groups ($n = 7$)

As a result of the comparison of NFκB gene expression between the groups, in group III, there was an increase but the level of NFκB in group IV was quite high. NFκB gene expression level has a significant rise in group IV. In group V and VI, punicalagin application after PH has suppressed NFκB gene expression level time-dependently (Fig. 3).

3.4. qPCR. Caspase-3 protein expression has indicated an increase in group V compared to groups I, II, III, and IV. Caspase-3 protein expression has reached its peak value at the end of the 6th hour (group VI). As a result of application of IP punicalagin after PH, caspase-3 protein expression has increased reasonably at the end of the 3rd hour; however, at the end of the 6th hour, a considerable increase was observed (Fig. 4). NFκB protein expression in group IV has differed significantly compared to the other experimental groups. In group III, NFκB protein expression has started to increase at the end of the 3rd hour, and in group IV that value reached its maximum. In group V, NFκB protein expression was suppressed. In group VI, NFκB protein expression was further suppressed in a time-dependent manner (Fig. 5).

4. Conclusion. In this study, the apoptotic effects of punicalagin on rat liver after PH have been investigated in vivo. We used the TaqMan protein assay (a new method for relative protein quantification) and the TaqMan gene expression

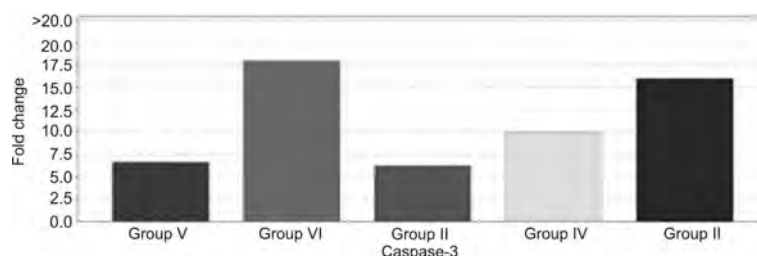


Fig. 4. Caspase-3 protein expression graph of groups ($n = 7$). Group I, accepted as a reference

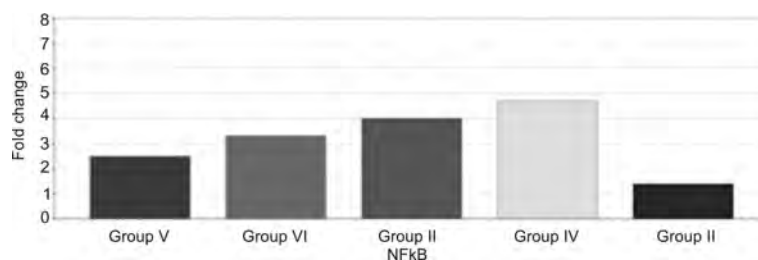


Fig. 5. NFκB protein expression graph of groups ($n = 7$). Group I, accepted as a reference

assay. The goal is to induce apoptosis with punicalagin injection on the rat liver, where regeneration was induced experimentally by PH.

We investigated whether punicalagin triggered apoptosis in the hepatocytes of hepatectomized liver tissue. Results demonstrated that the punicalagin application triggered apoptosis in hepatectomized rat liver in a short period of time (3 and 6 h). Punicalagin-induced apoptosis was associated with repression of the activity of NFκB and up-regulation of caspase-3 activity.

Caspase-3 is the essential killer and a sign of early apoptosis [20]. Caspase-9 and caspase-8 cleave procaspase-3 and generate the active caspase-3 that serves as the crucial executioner of apoptotic cell death. Caspase-3 activates the other caspases and breaks the cytoskeleton [21]. The activity of caspase-3 also reached the maximum level. TaqMan Protein analysis showed that NFκB protein expression level increased 6 h after PH without punicalagin injection. The increase in NFκB was followed by a decrease of caspase-3 6 h after PH in SF injected groups. While PH increased the NFκB activation, punicalagin induction triggered caspase-3 activation. Results revealed that punicalagin caused a significant induction of apoptosis in the hepatectomized liver section.

TUNEL-positive staining results showed typical morphological features of apoptotic nuclei in the punicalagin applied on hepatectomized rat liver. The identification of stained apoptotic bodies was confirmed by specific morphological features, including nuclear fragmentation, cytoplasmic shrinkage, and dissociation from other cells in the section [22]. The results of in situ end-labelling showed the appearance of apoptotic bodies in the regenerating liver, DNA fragmentation was observed as early as 3 h after PH with punicalagin. Apoptotic bodies in the regenerating liver sections increased significantly at the end of the 6th hour after PH.

Results revealed that caspase-3 activity was seen as a maximum level whereas NFκB activity was suppressed in group VI.

Finally, according to the results, punicalagin induced apoptosis and depressed cell proliferation despite the induction of regeneration (by partial hepatectomy) in the liver tissue. The apoptotic effect comparison of punicalagin against cancer cell lines and healthy cell lines will provide new aspects to develop novel approaches.

Statistical analysis. All data were evaluated using non-parametric tests (Kruskal–Wallis and Kolmogorov–Smirnov). SPSS 20.0 (SPSS: Statistical Package for the Social Sciences) was used for assessing the significance of differences between groups. Differences were considered significant $P < 0.05$.

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