Research Article

Protective effect of ellagic acid against angiotensin II-induced cardiomyocyte hypertrophy in H9c2 myocardial cells: role of calcineurin/NFAT pathway

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Abbreviations: Ang II, angiotensin II; ANP, atrial natriuretic peptide; NFAT, nuclear factor of activated T cell

Keywords: Ellagic acid, Cardiomyocyte hypertrophy, Angiotsenin II, Calcineurin, H9c2 cells

Background: Ellagic acid, a major ellagitannin found in pomegranate extract, might be an attractive natural and safe bioactive compound for prevention of cardiac hypertrophy in many pathological conditions that are associated with elevated circulating angiotensin II (Ang II). Ang II stimulates multiple signal transduction pathways involved in hypertrophy including calcineurin/nuclear factor of activated T cell (NFAT). Objective: The present study aimed to explore the possible anti-hypertrophic activity of ellagic acid against Ang II-induced cardiomyocyte hypertrophy and the role of calcineurin/NFAT signaling pathway in this action. Methods: H9c2 myocardial cells were treated with different concentrations of ellagic acid one hour before exposure to Ang II. Biological markers of cardiac hypertrophy including changes in cell size and protein content, and atrial natriuretic peptide (ANP) protein expression were assessed using light microscopy, Bradford method and western blotting, respectively. The effects of ellagic acid on the protein expression of calcineurin and nuclear localization of NFATc4 were also investigated using western blotting and immunofluorescence assay, respectively. Results: The results showed that pretreatment with ellagic acid could efficiently prevent Ang II-induced hypertrophic response which was associated with changes in hypertrophy-related biomarkers including increase in cell size and protein content, and ANP overexpression. Moreover, ellagic acid inhibited Ang II-induced calcineurin upregulation and nuclear localization of NFATc4. Conclusion: In summary, our findings showed that ellagic acid effectively inhibited Ang II-induced cardiomyocyte hypertrophy. This is the first report demonstrating the role of calcineurin/NFAT pathway inhibition in this protective effects. Future in vivo studies are required to elucidate if ellagic acid could ameliorate cardiac hypertrophy and its transition to heart failure.

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1. Introduction

Myocardial hypertrophy is a compensatory mechanism in which cardiomyocytes respond to stress or injury [1]. To accommodate alterations in its workload, the heart undergoes pathological enlargement which is characterized by an increase in the size of individual cardiac myocytes, protein synthesis, extracellular matrix accumulation and up-regulation of fetal genes such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain, and skeletal α-actin1 expression. Indeed, cardiac hypertrophy is a powerful risk factor which eventually causes significant cardiovascular disorders such as arrhythmias, heart failure and sudden death [2, 3].

Angiotensin II (Ang II) have long been determined as a potent factor in pathogenesis and regulation of cardiac hypertrophy [4]. Ang II promotes hypertrophy of cardiac myocytes by stimulating multiple signal transduction pathways involved in hypertrophy including calcineurin/nuclear factor of activated T cell (NFAT). As a serine/threonine protein phosphatase, calcineurin activates NFAT transcription factor, by dephosphorylating it. The activated NFAT, then, translocates into the nucleus that is a critical step to promote the expression of hypertrophic genes such as ANP and BNP [5, 6]. There is a considerable body of evidence indicating that calcineurin/NFAT pathway plays a major role in the process of cardiac hypertrophy [7-11]. For example, a previous report demonstrated that cardiac-specific overexpression of calcineurin or NFATc4 leads to the progression of significant hypertrophic responses and heart failure [12]. Furthermore, administration of calcineurin inhibitors has inhibited the hypertrophic response of cardiomyocytes to various pro-hypertrophic stimuli such as phenylephrine and Ang II [13].

Classic calcineurin inhibitors, such as cyclosporine A, are associated with severe side effects [11], thus, finding new therapeutic strategies that target this pathway and have no or minimal side effects can be helpful against cardiac hypertrophy and prevent fatalities.

In recent years, correlation between polyphenolic compound consumption and lower prevalence of certain diseases, such as cancer and cardiovascular disease, has received particular attention [14-17]. Ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6,2′,6′-dilactone) is one of these compounds that can be found naturally in many foods (Table 1) such as seeds, fruits and fruit products, some beverages [18]. Previous studies on ellagic acid have suggested that it possesses cardio-protective, antihypertensive, antioxidant and antihyperlipidemic activities [19]. It has been shown that oral pretreatment with ellagic acid ameliorates pathological ventricular hypertrophy, arrhythmias, altered lipid profile and myocardial necrosis in the isoproterenol-induced myocardial infarction in rats [20]. Interestingly, reduction of NFAT transcriptional activity has been recently reported for ellagic acid and punicalagin, the major polyphenol components of pomegranate extract [21]. Such evidence may indicate the potential of ellagic acid as a protective agent against cardiac hypertrophy. The present study was designed to evaluate the experimental therapeutic effects and the underlying mechanisms of ellagic acid in Ang II-induced cardiomyocyte hypertrophy in vitro using H9c2 myocardial cell line.

2. Methods

2.1. Drugs and Chemicals

Ellagic acid, Ang II and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA).
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Dulbecco’s modified Eagle’s medium (DMEM), antibiotic-antimycotic and trypsin-EDTA were supplied from Bioidea Co. (Tehran, Iran). Fetal bovine serum (FBS) was purchased from Gibco (life technologies, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany).

<table>
<thead>
<tr>
<th>Table 1. Natural sources of ellagic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Food</strong></td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Chestnut, raw</td>
</tr>
<tr>
<td>Blackberry, raw</td>
</tr>
<tr>
<td>Black raspberry, raw</td>
</tr>
<tr>
<td>Walnut</td>
</tr>
<tr>
<td>Pomegranate, juice from concentrate</td>
</tr>
<tr>
<td>Japanese walnut</td>
</tr>
<tr>
<td>Cloudberry</td>
</tr>
<tr>
<td>Walnut, dehulled</td>
</tr>
<tr>
<td>Red raspberry, raw</td>
</tr>
<tr>
<td>Pomegranate, pure juice</td>
</tr>
</tbody>
</table>

2.2. Cell culture

H9c2 cells were obtained from Pasteur Institute of IRAN. H9c2 cells were fed with medium consisting of DMEM supplemented with 10 % FBS, 100 U/ml of penicillin and 100 U/ml of streptomycin at 37 °C in a humidified incubator with 95 % O₂ – 5 % CO₂. All experiments were carried out on H9c2 cells between the third and sixth passages. Cultured cells at about 80% confluence were treated with 100 nM Ang II in serum free fresh DMEM media for 24 h. Two different concentrations of ellagic acid (10 and 100 μM) were added one hour prior exposure to Ang II and throughout the experimental period.

2.3. Cell viability assay

The MTT assay was used to determine possible toxicity of ellagic acid on H9c2 cells. We followed the method described previously [22]. H9c2 cells were seeded at a density of 5 × 10³ cells per well in 96-well plates. Ellagic acid was dissolved in dimethylsulfoxide (DMSO) (final concentration below 0.1 %) and then diluted in DMEM media just prior to use. An overnight after seeding, cells were incubated with different concentrations of ellagic acid for 24 h. The culture medium was removed from the cells, 100 μl of fresh culture medium and 10 μl of MTT solution (5 mg/ml) were added to each well and incubated for 3 h. The medium was aspirated and formazan precipitate in the viable cells dissolved in 100 μl of DMSO. Eventually, viability of cells was determined using an ELISA reader (Dynex MRX) by measuring absorbance at 570 nm (test) and 690 nm (reference).

2.4. Measurement of cell size

According to our previous work [22], plated cells were detached after different treatments and viewed using a light microscope, equipped with a digital camera and 40X objective lens. The surface area of individual cells was measured by analyzing the image using ImageTool 3.0 software (UTHSCSA, San Antonio, Texas, USA). At least 100 cells in each experimental group were randomly selected.

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2.5. Measurement of protein content

Determination of total protein content was performed based on the spectrophotometric assay using Bradford method. H9c2 cells were subjected to trypsinization and washed twice with phosphate buffered saline (PBS). Then, cells were lysed using 15 µl of lysis buffer containing a cocktail of protease inhibitors (Roche Inc. Basal, Switzerland). The lysate was incubated on ice for 30 min, and then centrifuged at 1,100 g for 30 min at 4 ºC (Beckman TL100 rotor and ultracentrifuge). Bradford method was used to determine the total content of protein in the lysates which was standardized with bovine serum albumin (BSA). The total protein per cell was calculated by dividing content of protein by number of cells prior to lysis.

2.6. Western blot analysis

This assay was performed in accordance with methods reported previously [22]. Based on total protein concentrations calculated from the Bradford assays, 25 µg of protein were loaded on 10 % acrylamide / bisacrylamide gel for each group. Then, separated proteins transferred onto nitrocellulose membrane using a wet system overnight at 20 V. Non-specific binding sites were blocked with the 2 % W/V non-fat dry milk in Tris-buffered saline containing Tween 20 (TBST) solution (10 mM Tris-HCl (pH = 7.4), 150 mM NaCl, 0.01 % V/V Tween 20) for 1 h. Immunoblotting was carried out overnight with rabbit anti-rat calcineurin (sc-9070; Santa Cruz Biotechnology, 1:200), ANP (SC-20158; Santa Cruz Biotechnology, 1:200) or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (sc-25778; Santa Cruz Biotechnology, 1:1000) antibodies at 4 ºC. Subsequently, membrane was probed with horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2004; Santa Cruz Biotechnology, 1:5000) for one hour at room temperature. After washing, blots were detected using enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). The density of each band was analysed using ImageJ software (Bethesda, MD, USA) in a semi-quantitative manner and the density ratio of each blot was assessed and normalized to the corresponding band of actin.

2.7. Immunofluorescence assay

The method was followed as described earlier [22]. In brief, cultured cells grown on coated-coverslips were subjected to various treatments as mentioned earlier and then washed three times with PBS and fixed in 4 % formalin for 30 min at room temperature. After washing (5 min for each wash), fixed cells were blocked with PBS containing 10 % goat serum at 37 ºC for 30 min to help reduce non-specific binding. Cells were then incubated with NFATc4 primary antibody (SC-13036; Santa Cruz Biotechnology), at a concentration of 1 µg/ml diluted in 1.5 % blocking serum in PBS in a humidified chamber for 60 min at room temperature, and washed with PBS three times (5 min for each wash). The slides were then covered with biotinylated secondary antibody diluted in PBS with 1.5 - 3 % blocking serum. After three times washing with PBS, an optimized concentration (10 µg/ml) of fluorescein isothiocyanate (FITC)-conjugated antibody (sc-2012; Santa Cruz Biotechnology, USA) was added for 15 min in a dark room. Following extensive rinsing with PBS (three times, 15 min for each wash), the cells were
2.8. Statistics
Data are presented as mean ± standard error of mean (mean ± SEM). Statistical analysis was carried out using one-way ANOVA (v. 20; SPSS Inc., Chicago, IL, USA). Differences between the groups were calculated by Tukey’s post hoc test, and P < 0.05 was considered as statistically significant.

3. Results
3.1. Effect of ellagic acid on cell viability
The possible cytotoxicity of ellagic acid was evaluated at different concentrations for 24 h using MTT assay. Ellagic acid, at 1-100 µM concentration range, did not show significant toxic effects while incubation with a higher concentration, i.e., 1 mM, led to a significant reduction in cell viability (P < 0.01) (Data not shown). Therefore, we selected two non-toxic concentrations, 10 and 100 µM, of ellagic acid for the next series of experiments.

3.2. Effect of ellagic acid on cell size
To explore the protective effects of ellagic acid against Ang II-induced hypertrophy in H9c2 myocardial cells, cell size was determined using ImageTool software. After incubation with Ang II (100 nM) for 24 h, the size area of H9c2 cells markedly increased when compared with the control group (P < 0.001). Incubation with two concentrations of ellagic acid prevented Ang II-induced H9c2 cell hypertrophy (P < 0.001 for both). Prevention of cell size enlargement was greater, although non-significant, at 100 µM than that observed with 10 µM ellagic acid. No significant difference was found between cell size in 100 µM ellagic acid and untreated control groups although the difference between 10 µM ellagic acid and control groups was statistically significant (Fig. 1).

3.3. Effect of ellagic acid on protein content
As shown in Table 2, treatment with 100 nM Ang II for 24 h resulted in a drastic rise in total protein content. H9c2 cells were pretreated with two concentrations of ellagic acid (10 and 100 µM) for one hour followed by the incubation of Ang II for 24 h. Pretreatment with ellagic acid exhibited inhibitory effect on the protein increase induced by Ang II in a concentration-dependent manner.

3.4. Effect of ellagic acid on ANP protein expression
To further delineate the effects of ellagic acid on Ang II-induced cellular hypertrophy, the expression of pathologic hypertrophy marker ANP was analyzed using a western blotting procedure. Findings indicated that Ang II increased ANP protein levels after 24 h of treatment as compared with control. Pretreatment with 100 µM ellagic acid significantly inhibited Ang II-induced ANP overexpression (P < 0.05). However, lower concentration of ellagic acid (10 µM) did not affect significantly on ANP protein level (Fig. 2).
3.5. Effect of ellagic acid on calcineurin protein expression

To investigate possible cellular mechanisms of antihypertrophic property of ellagic acid, we evaluated the alterations in calcineurin signal transduction. The results showed that Ang II significantly increased calcineurin protein expression. Pretreatment with two concentrations of ellagic acid for one hour before an additional 24 h treatment with Ang II, caused a significant prevention of calcineurin overexpression (P < 0.01) (Fig. 3).

3.6. Effect of ellagic acid on NFATc4 nuclear localization

NFAT dephosphorylation and its localization to the nucleus represent calcineurin phosphatase activity. We analyzed activation of transcription factor NFATc4 using immunofluorescence assay. As shown in Fig. 4, Ang II promoted the nuclear localization of NFATc4 as compared with control. We observed that Ang II-induced nuclear localization of NFATc4 was reduced when H9c2 cells were treated with 100 µM ellagic acid. This inhibition was not observed in the presence of 10 µM ellagic acid.

![Cell size graph](image)

**Fig. 1.** The effect of ellagic acid on cell size. Cell size was determined in H9c2 cells 24 h after treatment with 100 nM angiotensin II (Ang II) in the presence or absence of ellagic acid (10 or 100 µM). Both concentrations of ellagic acid significantly prevented Ang II-induced hypertrophy. Ang II, angiotensin II; EA, ellagic acid. Data are shown as means ± SEM (n=3). *P < 0.05; ***P < 0.001 vs control and ###P < 0.001 vs Ang II.

**Table 2.** Effect of ellagic acid on total protein content. 100 µM ellagic acid significantly prevented angiotensin II-induced increase in protein content. Values are expressed as mean total protein (µg /10⁶ cells) ± SEM (n=3). Ang II, angiotensin II; EA, ellagic acid. *P < 0.05; **P < 0.01 vs control and #P < 0.05 vs Ang II.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Ang II</th>
<th>EA 10 µM</th>
<th>EA 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>314.7 ± 25.8</td>
<td>442.4 ± 22.1**</td>
<td>404.3 ± 12.7*</td>
<td>354.3 ± 12.4#</td>
</tr>
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</table>
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Fig. 2. The effect of ellagic acid on ANP protein level in angiotensin II-treated H9c2 cells. Ellagic acid significantly reduced angiotensin II-induced ANP overexpression at 100 µM concentration. Western blot data were normalized to GAPDH. Ang II, angiotensin II; EA, ellagic acid. Data are shown as means ± SEM (n=3). **P < 0.01; ***P < 0.001 vs control and #P < 0.05 vs Ang II.

Fig. 3. The effect of ellagic acid on calcineurin protein expression in angiotensin II-treated H9c2 cells. Application of ellagic acid one hour before exposure to angiotensin II significantly prevented increase in calcineurin protein expression. Ang II, angiotensin II; EA, ellagic acid. Data are shown as means ± SEM (n=3). *P < 0.05; **P < 0.01; ****P < 0.0001 vs control and #P < 0.01 vs Ang II group.
Fig. 4. The effect of ellagic acid on nuclear localization of NFATc4 in angiotensin II (Ang II)-treated H9c2 cells. The cells were pretreated with ellagic acid (10 μM or 100 μM) for one hour followed by exposure to 100 nM Ang II for 24 h. Then, nuclear localization of the calcineurin substrate NFATc4 was determined by immunofluorescence staining and images were obtained using a confocal fluorescence microscope. Horizontally: first panels (a, e, i), control; second panels (b, f, j), cells treated with Ang II; third panels (c, g, k), cells pretreated with ellagic acid (10 μM); fourth panels (d, h, l), cells pretreated with ellagic acid (100 μM). Vertically the left panels show immuno-stained cells for NFATc4, the middle panels show DAPI-stained nuclei and the right panels show DAPI-stained nuclei merged with NFATc4. Ellagic acid at 100 μM concentration inhibited Ang II-induced nuclear localization of NFATc4. Arrows, nuclear localization of NFATc4. The original magnification was × 400. Ang II, angiotensin II; EA, ellagic acid.
4. Discussion

The results obtained in the present study demonstrated that exposure to ellagic acid may prevent the features of Ang II-induced pathological cardiomyocyte hypertrophy, including increased cell size and protein content, and also overexpression of ANP. To the best of our knowledge, this is the first study that demonstrates the ability of ellagic acid to inhibit overexpression of calcineurin as a signaling enzyme. The protective effect of ellagic acid in pathological cardiomyocyte hypertrophy was also associated with inhibition of NFATc4 nuclear localization.

In the present study, an in vitro model of cardiomyocyte hypertrophy was established via incubation of H9c2 myocardial cell line with Ang II for 24 h. The size of myocytes, total protein content and expression of ANP protein were remarkably increased in the presence of Ang II. These findings fit well with previous experimental evidence indicating characteristics of cardiomyocyte hypertrophy [4, 22]. Our previous study also revealed that these features could be useful markers in evaluation of protective effects of new agents against cardiac hypertrophy [22]. Increased expression of ANP is considered a prognostic parameter in pathological cardiac hypertrophy and heart failure [23]. In addition, Ang II-induced cardiomyocyte hypertrophy has also reported to be associated with ANP overexpression [4]. Pretreatment with ellagic acid prevented the increase in myocardial cell size and protein content as well as ANP protein expression induced by Ang II. Anti-hypertrophic effect of ellagic acid has been previously reported [20] in an animal model. In their study, administration of ellagic acid reduced the ratio of left ventricle weight/whole body weight, an index of cardiac hypertrophy, in isoproterenol-induced myocardial infarcted rats.

Calcineurin/NFAT signaling has been shown as a key player in coordination of cardiac hypertrophy following pathological stimulation [6]. Binding of Ang II to its receptor AT1 triggers Ca<sup>2+</sup> overload and subsequently activates calcineurin. Activation of calcineurin is followed by NFAT dephosphorylation and its translocation to nucleus, causing enhanced expression of hypertrophic-related genes [6]. In our study, Ang II significantly increased calcineurin protein expression. It should be noted that increased expression of calcineurin protein is associated with increased calcineurin activity [24, 25]. Therefore, activity of calcineurin enzyme was reliably confirmed by assessment of its protein expression in the present study. Further analysis of cells treated with Ang II revealed increased translocation of NFATc4 into the nucleus, which was correlated with overexpression of ANP protein. According to the fact that NFAT transcription factor is only dephosphorylated by calcineurin [26], NFAT nucleus localization and, therefore, its activity could be regarded as another way to identify calcineurin activity [5]. These findings are consistent with our previous work by Asadi et al [22] which showed that inhibition of overexpression of this signaling pathway induced by different hypertrophic stimulus, namely in vitro glucotoxic condition, could be a promising strategy to prevent cardiac hypertrophy.

It is noteworthy that ellagic acid has been reported to affect NFAT transcription factor activity in previous studies. A previous study [21] found that NFAT transcriptional activity and tumor necrosis factor-α concentration were decreased after 3 months feeding with pomegranate extract in brains of transgenic mouse model of Alzheimer’s disease. Moreover, they showed that punicalagin and ellagic acid, major polyphenol components found in
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pomegranate extract, significantly attenuated NFAT activity in a HeLa/NFAT-luc cell line. In another study, punicalagin inhibited production of interleukin-2 from anti-CD3/anti-CD28-stimulated murine splenic CD4+ T cells. These immunosuppressant effects of punicalagin was associated with inhibition of NFAT transcription factor activity [27].

In this context, it should be noted that an earlier investigation showed the effect of dietary natural polyphenols, punicalagin and ellagic acid, on human colon adenocarcinoma Caco-2 cells. They found that punicalagin was spontaneously hydrolyzed in the cell medium, facilitated by physiological pH, to yield ellagic acid. On the other hand, no apoptotic effects could be observed in colon cancer cells until punicalagin was hydrolyzed to ellagic acid and relative accumulation of ellagic acid in culture medium occurred. Accordingly, their study suggested ellagic acid as the actual active component [28]. This is also supported by the observation that plasma concentration of ellagic acid, but not punicalagin, increased after pomegranate extract consumption in human [29].

The results of this study demonstrated that pretreatment with ellagic acid at 100 µM concentration effectively prevented Ang II-induced changes in both calcineurin expression and nuclear localization of NFATc4 transcription factor. Inhibitory effect of ellagic acid may arise from directly binding to calcineurin or upon endogenous immunophilin/cyclophilin-mediated binding similar to classic calcineurin inhibitors. Direct inhibition of NFAT activity may also be possible. The underlying mechanism(s) of protective action of ellagic acid remains to be elucidated.

Classic calcineurin inhibitors represent potential agents for prevention of cardiac hypertrophy [30], but they have serious adverse effects [31]. In contrast, it has been reported that ellagic acid, at the concentration range of 10 - 100 µM, did not affect the viability of normal human fibroblast cells [32]. Furthermore, a 90 - day subchronic toxicity study showed that oral administration of different doses of ellagic acid did not cause any significant treatment-related clinical signs or mortality in F344 rats during the experimental period. Also, there were no obvious histopathological changes in any of the dosage groups [33]. It is noteworthy to mention that according to the results of MTT assay in the present study, incubation with ellagic acid concentration range of 1 µM to 100 µM did not show significant cytotoxic effect on H9c2 myocardial cells.

In summary, our findings revealed, for the first time, that ellagic acid effectively inhibited Ang II-induced cardiomyocyte hypertrophy, at least in part, through inhibition of calcineurin/NFAT signaling pathway. Future in vivo studies will be required to elucidate if ellagic acid, as a natural and safe component, could ameliorate cardiac hypertrophy and its transition to heart failure in many pathological conditions that are associated with elevated circulating level of Ang II.

Author contributions
M. Sh. designed research and managed the project; F. A. and A. R. performed experiments; F. A. and F. Gh. analyzed data and wrote the manuscript with contributions from all other authors.

Conflict of Interest
Authors declare that there is no conflict of interest.

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References


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Protective effect of ellagic acid …


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مقدمه: الازیک اسید (آنزیمات) مهم در عصاره آناتورامی می‌باشد. افزایش سطح عصاره آنزیمات در بسیاری از شرایط انتقال خون از نظر انتقال خون به انتقال خون می‌باشد. الازیک اسید ۲ فاز انتقال خون انتقال خون و کلسینورین که از این فاز و انتقال خون انتقال خون می‌باشد. الازیک اسید همچنین در این فاز انتقال خون انتقال خون در دمای پریونی عصاره می‌باشد.

نتایج: نشان داد که نشان داد که الازیک اسید ۲ فاز انتقال خون نشان داد که الازیک اسید ۲ فاز انتقال خون در دمای پریونی عصاره می‌باشد.

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