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The possible hepatoprotective effect of apricot against acrylamide induced hepatotoxicity in rats

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Abstract

Objective: The aim of this study was to investigate the possible toxic effects of sub-chronic doses of acrylamide taken with drinking water on liver tissue and to test the preventive role of 5% organic dried apricot as diet supplement, in female Sprague Dawley rats.

Materials and Methods: Forty female Spraque Dawley rats were divided into 4 equal groups as follows: control group (C) animals were fed with normal rat chow and tap water, apricot group (A) animals were fed with chow contain %5 apricot and tap water, acrylamide group (AA) animals were fed with normal rat chow and acrylamide at approximately 500 µg/kg/day via tap water, acrylamide+apricot group (AA+A) animals were fed with chow contain 5% apricot and approximately 500 µg/kg/day acrylamide via drinking water. The study procedure was maintained during 12 weeks experiment period. At the end of the study, samples of liver tissue were collected for biochemical, histopathological and molecular analyses.

Results: In this study, comparison of acrylamide group liver tissue GSH levels and GSH-Px activities were found lower when compared to the control group ($p < 0.05$). There were no significant differences between the groups based on GST activity, histopathological results and GST-Pi gene expression mRNA levels ($p < 0.05$).

Conclusion: It could be stated that acrylamide, at approximately 500 µg/kg/day, ingested by rats were detoxified by the liver without resulting any liver tissue damage and application of acrylamide with sun-dried organic apricot did not change any significant molecular, histopathological and biochemical parameters in the liver.

Keywords: Acrylamide; Apricot; Antioxidant Enzymes; GST-Pi; Liver.

INTRODUCTION

Malatya is located in eastern Turkey and provides 80% of the world's apricot production. Apricot *(Prunus armeniaca L.)* is a fruit with powerful antioxidant properties and high flavonoid, carotenoid, vitamin A and C and fiber content (1, 2). Apricot, with its high antioxidant properties, plays a role in protection of cellular components such as protein, lipid and DNA and it reduces oxidative stress (3). Furthermore, carotenoids, flavonoids and vitamins, in apricot, have protective functions against hepatotoxicity (4,5).

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Acrylamide is an α-β-unsaturated carbonyl compound, which does not exist in the nature and produced via chemical synthesis. It has prevalent use in industrial production and laboratory studies. While cooking food in high temperatures (over 120 °C), acrylamide is formed as a result of Maillard Reaction that occurs between carbohydrates and proteins (6–8). Studies conducted with experimental animals demonstrated that acrylamide has neurotoxic, gene-toxic and carcinogenic properties based on the dose (9,10). Reduced glutathione (GSH) is the most significant non-enzymatic antioxidant molecule in the body. Glutathione protects the tissues from the effects of oxidative stress by detoxifying free oxygen radicals (ROS) in a non-enzymatic manner. Furthermore, it is a coenzyme, which is drastically needed for glutathione S-transferase (GST) and glutathione peroxidase (GSH-Px) activities that are among the most important enzymatic antioxidants in the tissue (11). Under physiological conditions, there is equilibrium between oxidants and antioxidants in all tissues and this equilibrium neutralizes naturally produced ROS and protects the tissues against damage caused by radicals (12).

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The objective of the present study is to analyze possible damages in the liver due to acrylamide applied with drinking water to female Sprague Dawley rats and to test whether this damage could be prevented by organic apricot.

MATERIALS and METHODS

Animals and experimental design

Approval of Inonu University, Faculty of Medicine Experimental Animals Ethics Committee was obtained for this study. 99% pure acrylamide (C_3H_5NO) (Sigma) was applied to the rats in this study. The rats procured from Inonu University Experimental Animals Breeding and Research Center. In the study, 40 female 8-12 weeks old Sprague Dawley rats with a mean live weight of 200 ± 20 g were used. 40 Spraque Dawley rats were divided into 4 equal groups as follows: control group (C) animals were fed with normal rat chow and tap water, apricot group (A) animals were fed with chow contain %5 apricot and tap water, acrylamide group (AA) animals were fed with normal rat chow and acrylamide at approximately 500 µg/kg/day via tap water, acrylamide+apricot group (AA+A) animals were fed with chow contain 5% apricot and approximately 500 µg/kg/day acrylamide via drinking water. The study procedure was maintained during 12 weeks experiment period. The rats were kept in rooms that received light for 12 hours and were under darkness for 12 hours with an ambient temperature of 21°C and humidity of 55-60%. At the end of the study, animals were decapitated under xylazine and ketamine anesthesia and liver samples were dissected from all groups.

Diet

In the present study, we preferred Kabaaşı cultivar as experimental apricot diet supplement material. The Nutrient composition of normal rat chow and chow containing 5% apricot (Sun-Dried Organic Apricot; SDOA) were analysed in Department of Animal Feeding, Faculty of Veterinary Medicine, University of Fırat, Elazıg, Turkey. The average nutrient content of normal rat chow and chow containing 5% SDOA which was used in the present study are given in Table 1 (13). Apricots were purchased from local market with having organic certificates. Except control and acrylamide groups animals, all other animals were fed with chow containing 5% SDOA.

Table 1. Nutrient Compositions of Normal Rat Chow and Chow contain 5% Sun-Dried Organic Apricot (SDOA).

Nutrient	Normal Rat Chow	Chow containing 5% SDOA
Protein (%)	24.3±1.2	23.3±0.9
Fat (%)	5.2±0.3	4.6±0.2
Carbohydrate(%)	54.8±0.4	62.1±0.6
Ash (%)	8.6±0.3	7.6±0.2
Energy (kcal/g)	2.56±0.06	2.60±0.07

Biochemical analyses

GSH-Px, GST activity levels and GSH and protein levels were measured on supernatants obtained by centrifuging liver tissue homogenates 30 minutes at 5000 rpm under +4°C. GSH-Px activity, GST activity, GSH levels were analyzed by spectrophotometric measurement methods.

Paglia and Valentine method was used to identify the GSH-Px activity (14). 50 mM potassium phosphate buffer (pH 7.0, 5 mM ethylene diamine tetraacetic acid), NADPH (1 mM), GSH (5 mM), sodium azide (1 mM), glutathione reductase (1 IU), and a supernatant of tissues were mixed in the final reaction tube. The mixture was incubated for 30 minutes and the reaction was started by the addition of H_2O_2 (1 mM), which was used as the substrate. A spectrophotometer was used to measure the absorbance values at 340 nm, which were recorded at the end of a 5-min period. The enzyme activity level was calculated and presented as U/mg protein.

Ellman method used to identify serum GSH levels (15) was applied with slight modifications. Trichloroacetic acid precipitate of the tissue extract was centrifuged at 3000 rpm under 4°C for 20 minutes. GSH reacts with 5,5-dithiobis-2-nitrobenzoic acid producing a yellow product with a maximal absorbance at 410 nm. As a blank, distilled water was utilized. GSH levels are presented as nmol/g wet tissue (g.w.t).

Habig method was used to determine GST activity (16). Enzyme amount catalyzing 1 µmol of S-(2,4 dinitrophenyl)-glutathione within 1 min by GSH and 1-chloro-2,4-dinitrobenzene at 340 nm and under 37 °C was measured to determine GST enzyme activity. GST (Sigma-Aldrich) product was dissolved in normal saline and serial enzyme standards containing 5, 3.75, 2.5, 1.25, and 0.63 units of enzyme per milliliter were prepared. Each standard was considered as a sample for spectrophotometric measurements. GST standard graph was plotted and GST activity was presented in terms of U/mg protein.

Afterwards, Lowry method with minor modifications was used to identify tissue protein concentrations (17).

Total RNA extraction and RT-qPCR

A lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarkosyl, and 1% beta-mercaptoethanol) was utilized to homogenize 100 milligrams of liver tissue for 40 s at 13,500 rpm. The specimens were stored at –80 °C until the analyses, the homogenate was melted on ice and 600 µL was transferred to another tube and centrifuged for 10 min at 18,000 rpm. The supernatant was transferred into a total RNA isolation filter (74104, RNeasy Mini Kit, QIAGEN, Hilden, Germany) according to the manufacturer's directions. The total extracted RNA was run on 1% agarose gel and 18S and 28S ribosomal bands were visualized over a UV trans-illuminator by ethidium bromide staining for integrity check. Nanodrop spectrophotometer was used to measure the purified total RNA concentration. SuperScript III reverse transcriptase kit (11732-088, Invitrogen, Carlsbad, CA, USA) was used to reverse transcribe GST-Pi and GAPDH mRNA.

Real-time PCR (Roche LC480) was used for quantitative PCR (qPCR). 0.2 µL of cDNA, 0.2 µL of forward (100 pmol/µL) and 0.2 µL of reverse primers (100 pmol/µL),

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and 10 µL of SYBR Green I Master assay solution was mixed (Roche 04707516001) in the PCR tubes. PCR amplification was conducted at 95 °C for 5 min, followed by 50 cycles of denaturation under 95 °C for 30 s, and annealing under 55 °C for 40 s, and extension under 72 °C for 40 s. All qPCRs were performed in three wells in the same plate in the presence of the housekeeping gene. GAPDH and GST-Pi primer sequences were obtained by Wang et al. and Fatemi et al. respectively (Table 2) (18, 19). GST-Pi relative mRNA expression levels were computed according to GAPDH housekeeping gene with the $2^{\Delta\Delta}$Ct method (20).

Table 2. Primer sequences of GAPDH and GST-Pi.

Primer	Primer Sequences	Amplicon Size, bp
GAPDH-F	CAAATTCAACGGCACAGTCA	540
GAPDH-R	ACACATTGGGGGTAGGAACA	
GST-Pi-F	CCTCACCCTTTACCAATCTA	462
GST-Pi-R	TTCGTCCACTACTGTTTACC	

Histopathological analyses

Animals were killed by ketamine anaesthesia at the end of 3 months. The livers were rapidly removed by laparotomy. Tissue samples were placed in 10% formalin and prepared for routine paraffin embedding. Paraffin blocks were cut at 5mm thick, mounted on slides, stained with haematoxylin–eosin (H–E), peri-odic acid Schiff (PAS) and Masson's trichrome (MT). Sections were examined for histopathological evaluated in terms of intracellular edema and vacuolization Sections were examined using a Leica DFC280 light microscope and a Leica Q Win and Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical analyses

Statistical analyses were conducted using SPSS 21.0 Windows software package. Normal distribution of the data was tested with Shapiro Wilk method. As a result of Shapiro Wilk test, it was determined that GSH-Px and GST data demonstrated normally distribution ($P < 0.05$), however, GSH data did not ($P > 0.05$).

Normally distributed data were presented as mean ± SD and not normally distributed data were presented as median (min – max). Inter-group comparisons for normally distributed data were performed with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The Kruskal–Wallis analysis of variance and the Mann–Whitney U test were performed on the not-normally data. P values smaller than 0.05 were considered statistically significant.

RESULTS

In the present study, the liver tissue of rats was analyzed for biochemical and molecular parameters after treatment with acrylamide. GSH-Px activity was found to be significantly reduced in acrylamide group and the addition of dried organic apricot did not change the result ($p < 0.05$; Figure 1). Similarly, GSH levels were also found to be significantly reduced in acrylamide group, in addition to the apricot group ($p < 0.05$; Figure 2). Statistically significant differences could not be observed between the groups of control and acrylamide in terms of GST activities ($p < 0.05$; Figure 3). For molecular analysis, total RNA was extracted from rat liver tissues and they were run on the agarose gel to check RNA integrity. All extracted RNA samples exhibited ribosomal bands without any smear (data not shown). All total RNA extracts were reverse transcribed to obtain cDNA and they were amplified with real time PCR for comparison analysis. All cDNA samples were successfully amplified (Figure 4A). Melting analysis of the amplified products of GAPDH and GST-Pi genes revealed distinct intact peaks (Figure 4B). Gene expression profiles of GST-Pi gene were normalized according to GAPDH gene and they were compared between different groups. According to mRNA expression analysis, acrylamide ingestion did not alter the expression of GST-Pi gene (Figure 5) ($p < 0.05$) and histopathological results (Figure 6).

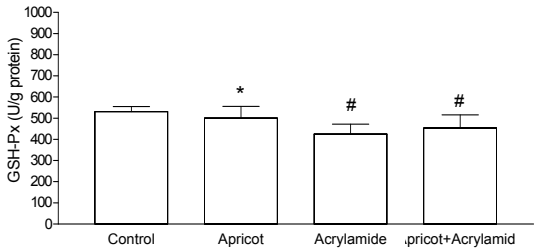

Figure 1. Effects of acrylamide and apricot on liver GSH-Px activity. The values are shown as mean ± SD. *significantly different from the acrylamide group ($P < 0.05$), # significantly different from the control group ($P < 0.05$).

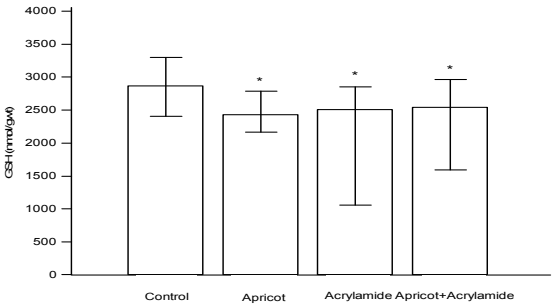

Figure 2. Effects of acrylamide and apricot on liver GSH levels. The values are shown as median (min-max). *significantly different from the control group ($P < 0.05$)

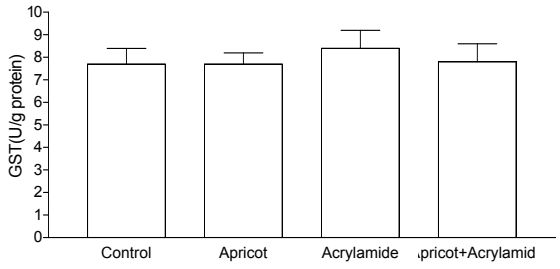

Figure 3. Effects of acrylamide and apricot on liver GST activity. The values are shown as mean ± SD. No significant difference was found among groups ($P > 0.05$).

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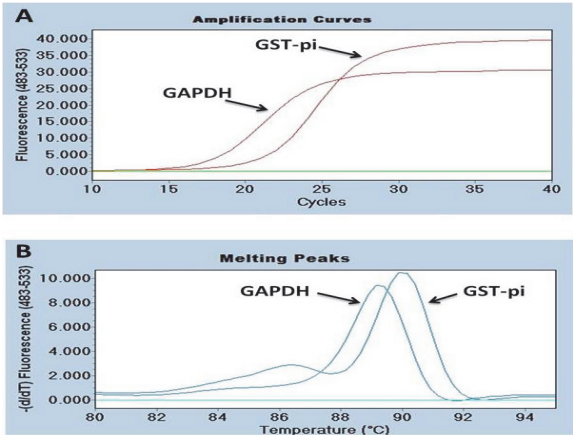

Figure 4. Real Time PCR Amplification and Melting Analysis. Total RNA was extracted from rat liver tissues and they were reverse transcribed to obtain cDNA which later was subjected to real time PCR analysis. The amplification plot (A) and melting peaks (B) of GAPDH and GST-pi genes are displayed.

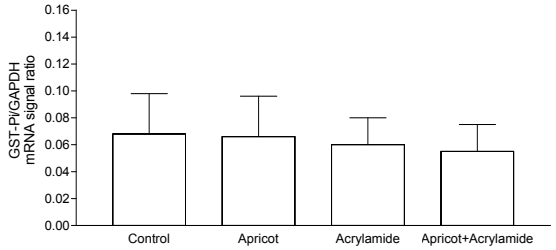

Figure 5. Effects of acrylamide and apricot on liver GSTPi/GAPDH mRNA ratio. The values are shown as mean±SD. No significant difference was found among groups (P > 0.05).

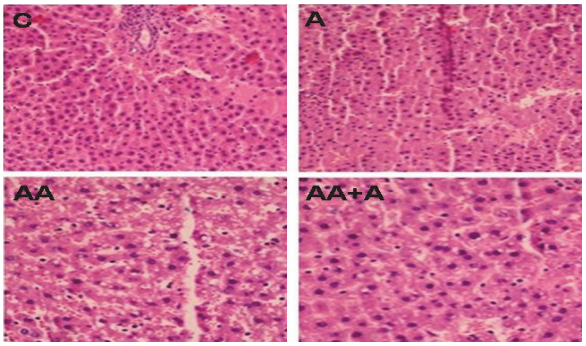

Figure 6. The liver tissues from the groups were evaluated in terms of intracellular edema and vacuolization. (C) Control group; intracellular edema (H&E: x200), (A) Apricot group; intracellular edema (H&E: x200), (AA) Acrylamide group; intracellular edema (H&E: x400), (A+AA) Apricot+Acrylamide group; intracellular edema and slight vacuolization (H&E: x400). It was not observed a significant histopathologic difference between the groups.

DISCUSSION

The objective of the present study is to analyze possible damages in the liver of female Sprague Dawley rats due to acrylamide ingestion and to test whether this damage could be prevented by dried organic apricot intake. Liver tissue was examined for the change of GSH-Px activity, GSH level, GST activity and GST-Pi mRNA level and histopathology. It was found that GSH-Px activity decreased as a result of acrylamide application and the apricot application did not fully ameliorate the effect of acrylamide. Interestingly, both apricot and acrylamide significantly decreased the levels of GSH in liver tissues. Under normal physiological conditions ROS are produced continuously and the amount of ROS increases considerably under toxic substance intake which finally increases cellular damage in the body. These toxic substances are destroyed by a series of antioxidant enzyme actions (such as superoxide dismutase, catalase, glutathione S-transferase, glutathione peroxidase, and antioxidant glutathione and vitamin A, vitamin E, and vitamin C) and an equilibrium is established between the oxidants and antioxidants at the cellular level. However, activities and gene expression of the enzymes that function in these mechanisms could be altered due to the exposure to the toxic substances (21–24).

Since, acrylamide is highly water soluble xenobiotic, it reaches to all tissues quickly after taken orally. In the presence of GST enzyme, acrylamide is conjugated with GSH throughout the digestive tract (esophagus, stomach, small and large intestines) and transformed into acrylamide-glutathione complex and protects the digestive tract tissues from the harmful effects of acrylamide (25 – 28). In mammals, cytosolic GSTs are divided into eight groups (alpha (α), mu (μ), pi (π), theta (θ), omega (Ω), kappa (κ), sigma (σ) and zeta (ζ)) based on their chemical properties, immunologic reactivity and amino acid sequences (29). Although a certain portion of the acrylamide taken with foods orally is rendered ineffective in the digestive tract as explained above, its effective conjugation throughout the digestive tract is largely dependent on the tissue GST activity and GSH levels. Lieshout et al. fed the rats with a diet that included antioxidants and anti-carcinogenic substances such as α-tocopherol, β-carotene, lycopene, flavonoid and limonene for a period of time, and observed that GST activity significantly increased in esophagus, stomach, small and large intestines throughout the digestive tract as a result. Lieshout et al. argued that sufficient consumption of fruits and vegetables rich in antioxidant and anti-carcinogen substances would facilitate the conjugation of toxic and carcinogen substances taken orally with GSH throughout the digestive tract and their excretion via urine, feces, bile and sweat, therefore protecting the body from their harmful effects (30).

Different results were obtained in previous experimental animal studies that scrutinized whether acrylamide caused oxidative stress. It was reported that these differences were due to differences in application method and dose of acrylamide (31 – 33). Several xenobiotics could affect GST activity in different ways such as induction, activation or inhibition. These studies were conducted mostly with liver enzymes and it was determined that phenobarbital and polycyclic aromatic hydrocarbons induced GSTs (34), certain herbicides

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(such as trifluralin, molinate) increased GST activity (35), and certain hypolipidermic drugs such as ciprofibrate decreased GST activity (36). It was reported that long term low dose acrylamide application (0.5 – 500 µg acrylamide/kg in drinking water for 10 weeks) caused lipid peroxidation in rat liver tissues and reduced GSH concentration (30). In contrast, it was observed that short term sub lethal dose (50 mg/kg IP for 5 days) acrylamide application did not cause any variations in liver GSH concentrations (33). After the application of 0.5, 5, 25, 50, 250 and 500 µg/kg/bw acrylamide addition to drinking water for 10 weeks, Yousef and El-Demerdash found that plasma protein levels and creatine kinase activities decreased significantly, plasma phosphatase levels increased, transaminase and phosphatase activities significantly decreased in liver and testes, and no changes were observed in lactate dehydrogenase levels. They identified increases parallel to the increase in acrylamide dose in thiobarbituric acid – reactive substances, GST activity levels and plasma, liver, testis, brain and kidney tissue superoxide dismutase levels and a serious decrease in GSH levels. Results of that study demonstrated that acrylamide caused oxidative stress and irregularities in enzyme activities due to its toxic effects, thus results in tissue damage (31). In another study, Erdemli et al. reported that acrylamide administered in 500 µg/kg/bw daily doses for 3 months increased large intestine tissue GST levels in rats (37).

In the present study, acrylamide application did not cause any change in liver tissue GST activity. However, the fact that GSH levels demonstrated a decrease with acrylamide application compared to the control group was consistent with the findings of other studies. Catalgol et al. treated human erythrocytes with 0.10, 0.25, 0.50 and 1.00 mM acrylamide solutions under 37 °C for 1 hour and determined that high acrylamide doses decreased GSH-Px activity and acrylamide caused deterioration of antioxidant enzymes due to oxidative stress (38). A study by Zhu et al. demonstrated that sciatic nerve GSH-Px activity increased and glutathione reductase activity decreased in rats treated with 40 mg/kg acrylamide intraperitoneally 3 times a week for 10 weeks. This result showed that acrylamide was neurotoxic, increased lipid peroxidation and deteriorated antioxidant defense system (39). In the current study, it was identified that GSH-Px activity in acrylamide group was lower than the control and apricot groups, confirming the findings of other studies. However, it was also observed that apricot did not have any effects on GSH-Px activity. Mei et al. identified that 500 mg/L acrylamide addition to drinking water of mice for 3 weeks resulted in 2.4 times decrease in GST-Pi gene expression at the end of the experiment (40). In a study conducted to investigate the effects of sub-chronic acrylamide application on brain hypothalamus and pituitary GST-Pi gene expression in male rats, it was found that application of high dose 2.5, 10 and 50 mg/kg/day acrylamide to drinking water did not have any effects on hypothalamus GST-Pi gene expression, however, pituitary GST-Pi mRNA gene expression was significantly affected (41). In a similar study, it was reported that 50 mg/kg/day acrylamide applied to the drinking water of male rats did not cause any difference in brain striatum and substantia nigra regions GST-Pi mRNA gene expressions (42). Similarly, in the present study, as a result of the comparison of liver GST-Pi mRNA levels in acrylamide groups, no significant difference was observed in GST-Pi mRNA levels ($P > 0.05$). Similar findings were also obtained with activity of GST enzyme. Thus, it could be stated that the applied acrylamide dose in the present study did not affect GST-Pi mRNA level in the transcription stage and enzyme activity in the translation stage.

CONCLUSION

It was determined that acrylamide applied to rats in 500 µg/kg/day doses for 3 months could be detoxified by the liver without causing any damage. Since 3 months 500 µg/kg/day acrylamide dose applied with 5% organic dried apricots in feed did not cause a significant difference in liver biochemical, histopathological and molecular parameters, it was not possible to conclude the protective role of apricot in liver tissues. Finally in a future study, higher doses of acrylamide could be investigated to scrutinize the effects of dried apricot on prevention of acrylamide induced hepatotoxicity.

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