RADIOPROTECTIVE EFFECTS OF LINDEN HONEY IN RAT PERIPHERAL BLOOD

by

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Radiotherapy affects not only malignant, but also a healthy tissue adjacent to tumor by increasing reactive oxygen species generation, with consequent damage to biomolecules, such as the oxidation of membrane lipids, known as lipid peroxidation. The end product of lipid peroxidation is malondialdehyde. Radioprotectors are compounds that could significantly protect normal cells from radiation, without changing the tumor cell radiosensitivity. Synthetic radioprotectors usually have side effects and are toxic. Natural radioprotectors exert protection without adverse effects. In this study, we examined the radioprotective ability of linden honey in rat blood, by detecting alterations in the activities of antioxidant enzymes catalase and glutathione peroxidase and malondialdehyde concentration after the exposure to a therapeutic dose of gamma rays. Sixteen rats were randomly divided into Control and Honey groups. Honey group received honey (1.5 mL(kgd^{-1})) orally for four weeks, while at the same time Control group were given distilled water. After four weeks, blood was sampled from all animals. Samples were halved, and one series of samples were gamma irradiated (2 Gy). Radiation induced decreased glutathione peroxidase activity and increased malondialdehyde level, while honey treatment attenuated those alterations, keeping glutathione peroxidase and malondialdehyde at physiological levels. These findings confirm radioprotective properties of linden honey.

Key words: radiotherapy, radioprotection, antioxidant enzyme, malondialdehyde, linden honey

INTRODUCTION

Radiotherapy is widely used for treating cancer. It has been estimated that 80 % of cancer patients need radiotherapy either for curative or palliative purpose [1]. However, radiation affects not only malignant, but also a healthy tissue adjacent to tumor. Ionizing radiation induces biochemical changes in living tissue through various molecular events, such as direct reaction with macromolecules (nucleic acids, proteins and lipids) and production of aqueous free radicals due to the action of radiation on water. The main free radicals resulting from the radiolysis of water are hydroxyl (OH·) and hydrogen radical (H·) [2]. In the presence of oxygen, hydrated electrons and H atoms react with molecular oxygen producing hydroperoxyl (HOO·), superoxide anion radical (·OO$^-$) and non-radical reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [3]. Even low doses of radiation induce ROS production and may eventually increase the risk of malignancy [4-6]. The ROS can react with all cellular macromolecules including nucleic acids, proteins, lipids *etc*. changing their functions and structures and causing cell dysfunction and mortality. To counteract overproduction of ROS cells have developed enzymatic, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR)) and non-enzymatic antioxidants (glutathione (GSH), vitamins C, A, E, which maintain a dynamic balance between pro- and antioxidant (AO) processes in normal physiological conditions. Radiotherapy increases ROS generation in both malignant and healthy tissue leading to oxidative stress, with consequent damage to biomolecules. One of the first consequences of oxidative stress is the oxidation

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of membrane lipids, a chain reaction known as lipid peroxidation (LPO). The end product of the oxidation of biological membranes is malodialdehyde (MDA), which is considered a good biomarker of oxidative stress and LPO. Previous studies have shown that radiotherapy induced oxidative damage to membrane lipids [7, 8]. In the last few decades, a great effort has been made to discover radioprotective agents that will reduce harmful effects of radiotherapy. Radioprotectors are compounds that could significantly protect normal cells from radiation induced damage without changing the tumor cell radiosensitivity [9]. However, the use of synthetic radioprotectors is limited due to their side effects and toxicity. Therefore, more attention has recently been paid to natural radioprotectors that exert protective actions against radiation, without adverse effects. Garlic, green tea, apples, citrus and ginger are examples of food containing natural radioprotective agents [9].

Honey is well known for its health effects as a natural food supplement. Previous studies have reported numerous beneficial effects of honey: antibacterial, anti-inflammatory, antiviral, immunomodulatory, antimutagenic and antioxidant [10, 11]. Furthermore, honey contains many active agents, such as enzymes (glucose oxidase, catalase, diastase, invertase etc.), ascorbic acids, phenolic acids, carotenoid derivates, amino acids, proteins [12-14]. The total antioxidant capacity of honey originates from phenols, flavonoids, phenolic acids, proteins and amino acids, but depends not only on its chemical composition, but also on the complex interaction between particular compounds [15]. Although antioxidant properties of honey are well documented [16, 17], radioprotective role of honey and honey-bee products, such as propolis has only lately been investigated [18-21].

Linden honey is particularly known to be rich in vitamins (B1, B2, B3, B5, B6, C, E, and K), minerals (potassium, phosphor, magnesium, sodium, and iron), trace elements (manganese, chromium, selenium, cobalt, zinc, copper *etc*.) [22]. It is also very rich in flavonoids and other polyphenols known for their antioxidant activity [23, 24]. Nevertheless, the research on its radioprotective potential is lacking. Therefore, in this study, we examined the radioprotective ability of linden honey from Fruška Gora Mountain in rat peripheral blood, by detecting alterations in the activities of antioxidant enzymes CAT and GPx and concentration of MDA after the exposure to a therapeutic dose of gamma rays.

MATERIALS AND METHODS

Animals

Eleven-week old male Wistar rats weighing 300-350 g were used in this study. Animals were housed in a temperature and climate-controlled room (22 1 °C; 45-65 % humidity) with reversed day/night cycle (lights on from 19:00 to 7:00) and food and water ad libitum. They were treated as ethically as possible according to the recommendations of the Ethical Committee for the care and use of laboratory animals of the Vinča Institute of Nuclear Sciences, which follows the guidelines of the Serbian Society for the Use of Animals in Research and Education. Since the Serbian Law on Animal Protection prohibits testing radiation directly on animals, we opted for ethically and legally acceptable alternatives and irradiated rat blood samples, keeping the animals alive and safe. The Ethical Committee of the Vinča Institute approved the study (approval number 06/2023).

Experiment design

Sixteen animals were randomly divided into two groups, each with eight individuals. The control group was raised under standard conditions, while the Honey group received honey dissolved in distilled water in a ratio 1:1 and in a dose of 1.5 mL(kgd)$^{-1}$. Honey was administered by oral syringe, once a day, always at the same time, for four weeks, as previously described [25]. Linden honey used in this study originated from the linden forest located on the slopes of Fruška Gora, an isolated mountain in the Pannonian Plain and was collected in 2022 in the apiary of Dr. V. Stanić. After four weeks blood was sampled from rat tails into tubes containing lithium heparin as the anticoagulant and animals were returned to their cages. All animals survived the experiment without signs of illness. Blood samples (approximately 1 mL) were divided in half. One series of samples was labeled as C for Control group and H for Honey group and kept at 4 °C until use. The other series of samples, labeled as C1 or H1 for Control and Honey group, respectively, was taken to the Department of Radiation and Environmental Protection, Vinča Institute of Nuclear Sciences, where it was placed on the radiation bench and gamma irradiated with a dose of 6 Gyh^{-1} for 20 minutes. The absorbed dose was 2 Gy, which is a therapeutic dose that can be given in one session without significant damage to the adjacent healthy tissue. Throughout the handling samples were kept on wet ice to prevent degradation.

Sample preparation

After irradiation of C1 and H1 all blood samples were centrifuged at 1500 g, 4 °C for 10 minutes. The plasma (supernatant) was stored at –80 °C for MDA assay. The blood cells were washed three times in cold saline and then lysed in 20 packed cells volumes of ice-cold distilled water. Lysates were centrifuged at 8500 g, 4 °C for 10 minutes to remove the blood cell stroma and supernatants were stored at –80 °C for CAT and GPx assays.

Assays

Antioxidant properties of linden honey are investigated in the Laboratory for investigation of natural resources of pharmacologically and biologically active compounds, Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad. Lipid peroxidation inhibition capacity (LPIC), hydroxyl radical scavenging capacity (HRSC) and superoxide anion radical scavenging capacity (SARSC) are determined by the spectrophotometric method proposed by Pintać *et al*. [26].

Assays for CAT, GPx and MDA were performed using Perkin Elmer Lambda 25 Spectrophotometer (Perkin Elmer Instruments, Norwalk, CT, USA).

CAT activity was determined by the method of Beutler [27]. The decomposition of hydrogen peroxide (H_2O_2) by CAT from the samples was monitored through a decrease in absorbance at 230 nm as a function of time. One CAT activity unit is defined as 1 mol of H_2O_2 decomposed per minute under the assay conditions. The specific enzyme activity of CAT was expressed as kilounits per gram of hemoglobin ($kUg^{-1}Hb$).

The GPx activity was determined by the method of Paglia and Valentine [28] using a commercial kit Ransel® (Randox Laboratories Ltd., Crumlin, UK). Reduction of organic peroxide by the GPx from the sample is accompanied by concomitant oxidation of glutathione. In the presence of GR, oxidized glutathione is immediately recycled to its reduced form, with simultaneous oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. The rate of NADPH oxidation accompanied by a decline in absorbance at 340 nm as a function of time is directly proportional to the GPx activity. One GPx activity unit is defined as 1 mol of NADPH decomposed per minute under the assay conditions. The specific enzyme activity of GPx was expressed as units per gram of hemoglobin ($Ug^{-1}Hb$).

The MDA concentration was determined by the method of Siddique *et al*. [29]. In the reaction of MDA from the sample with a chromogenic agent 1-methyl-2-phenylindole at 45 °C, a colored compound with maximal absorbance at 586 nm is formed. MDA concentration is directly proportional to the absorbance at 586 nm and is expressed as micromoles per liter of plasma ($\mu molL^{-1}$).

Statistics

The data were presented as mean standard deviation. Normality of data was assessed by Shapiro- Wilk test. Effects of honey administration and gamma radiation were tested by two-way ANOVA with repeated measurements on one factor. Differences between independent samples (*i. e*., C *vs*. H, C *vs*. H1, C1 *vs*. H, and C1 vs. H1) were tested by Tukey post-hoc test, while Pair-sample *t*-test was used for testing paired samples (C *vs*. C1, H *vs*. H1). Statistical analyses were carried out using the Origin Pro 9 data analysis software (OriginLab Corporation, Northampton, MA, USA). A P value below 0.05 was considered significant.

RESULTS AND DISCUSSION

Antioxidant characteristics of linden honey, measured by LPIC, HRSC and SARSC are shown in tab. 1. The results demonstrate that the honey preserved the antioxidant properties even after being irradiated with gamma rays. The LPIC is higher at lower concentrations (below 1 $mg(mL)^{-1}$). The HRSC is also preserved after radiation with almost unchanged half-maximal inhibitory concentration (IC50), while for SARSC IC_{50} is even lower after radiation. Our results confirm antioxidant properties of linden honey. Furthermore, we demonstrated that antioxidant capacity of linden honey remained well preserved after gamma radiation. These findings are in accordance with the results of Saxena *et al*. [30] who found that honey retained its antimutagenic, antioxidant and

Table 1. Antioxidant capacity of linden honey from the region of Fruška Gora mauntain before and after the irradiation by 2 Gy of gamma rays

Sample	Concentration $[mg(mL)^{-1}]$	Lipid peroxidation inhibition capacity [%]	Hydroxyl radical scavenging capacity IC_{50}* $[mg(mL)^{-1}]$	Superoxide anion radical scavenging capacity IC_{50} $[mg(mL)^{-1}]$
Honey	0.179	24.5 3.2	1.116 0.157	1.890 0.112
	0.359	28.4 5.3		
	0.717	26.9 4.2		
	2.152	4.4 12.2		
	4.303	8.1 11.8		
Honey irradiated	1.171	30.2 1.4	1.165 0.084	1.587 0.233
	0.343	23.8 8.3		
	0.685	15.9 3.8		
	2.06	2.0 4.8		
	4.11	11.1 9.4		

* IC_{50} – half maximal inhibitory concentration

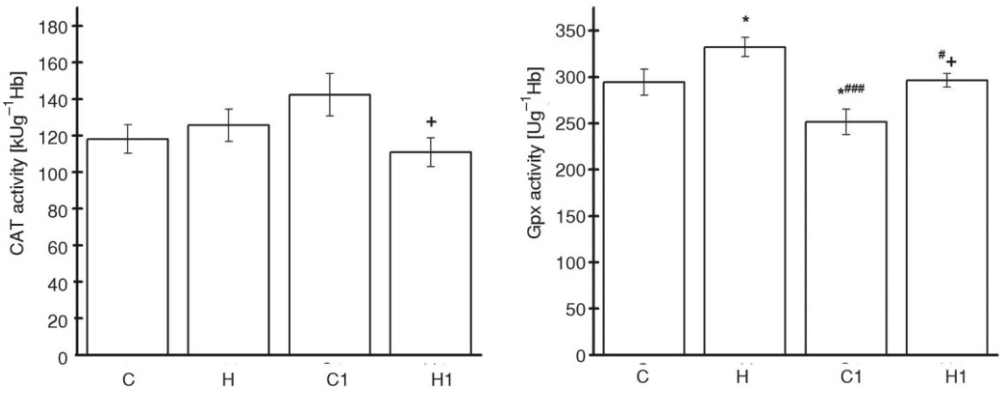

Figure 1. Effects of linden honey and gamma radiation on CAT and GPx in rat blood; C - blood samples from Control group, H - blood samples from Honey group, C1 - blood samples from Control group irradiated with 2 Gy of gamma radiation, H1 - blood samples from Honey group irradiated with 2 Gy of gamma radiation, * P < 0.05 significantly different from C, # P < 0.05, ### P < 0.001 significantly different from H, and + P < 0.05 significantly different from C1

radioprotective properties after gamma irradiation aimed to achieving microbiological safety.

Enzyme activities of CAT and GPx are presented in fig. 1. For CAT activity no significant main effect was found for either honey or radiation treatment or interaction between the two ($F = 2.48$, $F = 0.2$, $F = 3.44$, $P > 0.05$ for honey, radiation and interaction, respectively). Tukey test revealed significant difference between C_1 and H_1 ($t = 2.24$, $P < 0.05$). For GPx activity significant main effects were found for both honey and radiation, but not for their interaction ($F = 10.61$, $P < 0.01$, $F = 14.12$, $P < 0.01$, $F = 0.11$, $P > 0.05$, for honey, radiation and interaction, respectively). Honey treatment significantly increased GPx activity both before (C *vs*. H, $t = –2.18$, $P < 0.05$) and after irradiation (C_1 *vs*. H_1, $t = –2.88$, $P < 0.05$). On the contrary, gamma radiation significantly decreased GPx activity in both Control (C *vs*. C_1, $t = 2.85$, $P < 0.05$) and Honey group (H *vs*. H_1, $t = 2.46$, $P < 0.05$). Major difference was found between H and C_1 group ($t = 4.76$, $P < 0.001$).

The MDA concentrations are shown in fig. 2. Significant main effects were found for radiation and interaction between honey and radiation ($F = 6.77$, $P < 0.05$; $F = 5.99$, $P < 0.05$, for radiation and interaction, respectively). Radiation provoked significant increase in MDA concentration only in Control group (C *vs*. C_1, $t = –2.62$, $P < 0.05$). Irradiated blood from Control group also had higher MDA concentrations comparing with Honey group before H *vs*. C_1, $t = –2.21$, $P < 0.05$) and after irradiation (C_1 *vs*. H_1, $t = –2.18$, $P < 0.05$).

Changes in antioxidant enzyme activities and MDA concentrations found in our study suggest that both honey treatment and gamma radiation have influenced antioxidant status in rat blood. Since radiation induces ROS overproduction, increased activities of AO enzymes should be an adequate response. However, we found only slightly increased CAT activity (about 16 %) in irradiated blood obtained from control animals, but this was not statistically significant, while

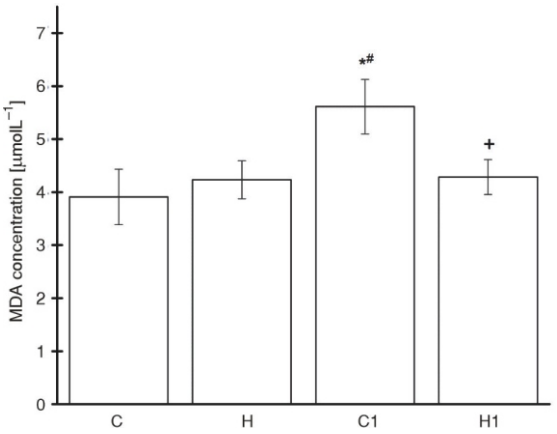

Figure 2. Effects of linden honey and gamma radiation on MDA concentrations in rat blood; C – blood samples from Control group, H– blood samples from Honey group, C1 – blood samples from Control group after 2 Gy of gamma radiation, H1 – blood samples from Honey group after 2 Gy of gamma radiation, * *P* < 0.05 significantly different from C; # P < 0.05 significantly different from H, and + *P* < 0.05 significantly different from C1

GPx activity was decreased. The study of Focea et al. [31] reported increased CAT activity after low- and medium dose of ionizing radiation in various animal tissues. Up-regulation of CAT activity could be a protective response to increased oxidative stress due to irradiation. Honey treatment had no effect on CAT activity in non-irradiated blood, but decreased it in irradiated blood. Since linden honey used in our research is proven to have the ability to neutralize hydroxyl and superoxide anion radicals, we may presume that overproduction of H_2O_2, the substrate of CAT, is prevented before it started. The CAT is a key enzyme for neutralization of H_2O_2 through its decomposition to molecular oxygen and water. However, due to its high Michaelis-Menten constant, CAT is not highly effective at low (physiological) levels of H_2O_2, thus maintaining optimum level of this important signaling molecule [32]. Another H_2O_2 detoxifying enzyme, GPx, is more efficient at lower concentrations

of the substrate [33]. In this study decreased GPx activity in the blood of control and honey treated animals were detected after gamma irradiation. Lowered GPx activity may be explained by free radical induced damage to hydrogen bonds that ensure the active site of the enzyme. Decreased GPx activity and expression after radiation have previously been reported in human fibroblasts [34]. Since GPx depends on GSH availability, depletion of GSH, as a consequence of increased ROS level due to gamma radiation may also contribute to reduced enzyme activity. The GSH is one of the most important cellular antioxidants, enzyme cofactor and free radical scavenger, capable of breaking chain reaction of lipid peroxidation and recycling other antioxidants [35]. Inactivation of GPx by irradiation, as well as exhaustion of GSH content leads to impaired GSH redox cycle, metabolic oxidative stress and prolonged cell injury [36]. Impaired mechanism of peroxide detoxification due to GPx inactivation is reflected in an increased level of MDA in the irradiated rat blood found in our study. The application of honey significantly increased GPx activity both in non-irradiated and irradiated blood. Furthermore, MDA level in irradiated blood of animals treated with honey remained stable. Similar results are reported by Al-Shemary and Abdulatif [37], who found elevated MDA and decreased GSH concentration in rabbit testicles after exposure to X-rays, while those changes were attenuated by pre- or post-treatment with honey, suggesting radio-protective properties of honey. Antioxidant compounds from honey may contribute to the strengthening of the antioxidant defense system by neutralizing ROS and preventing inactivation of GPx and GSH depletion. Besides, since GPx function depends on Se availability, Se from honey can also reinforce its activity. Furthermore, a recent study has demonstrated that honey bees can produce melatonin [38], which can then be transferred to honey. Melatonin has been detected in some honey samples from Australia, USA and Poland [39]. Melatonin is a pluripotent molecule with a wide range of biological functions, including antioxidant. Pre-administration and post-administration of melatonin have been recently reported to maintain normal GPx activity in human fibroblasts after radiation [40]. There is still no evidence about the presence of melatonin in linden honey from Fruška Gora used in our study.

CONCLUSION

Our results confirm radioprotective properties of linden honey. The application of honey invigorated the antioxidant capacity in rat blood by enhancing GPx activity and preventing lipid peroxidation, which is reflected in physiological level of MDA in the irradiated blood of animals treated with honey. Further investigations, including detection of particular antioxidants, should provide better understanding of processes underlying radioprotective effects of linden honey. However, our results suggest that linden honey may be beneficial for patients undergoing radiotherapy.

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AUTHORS' CONTRIBUTIONS

V. R. Stojiljković performed statistical analysis, drafted the manuscript, helped with animal handling and participated in the experiments. Lj. V. Gavrilović designed the experiment, carried out animal handling, participated in the experiments and helped to draft the manuscript. V. D. Stanić participated in the experiments and helped to draft the manuscript. S. J. Stanković performed blood samples irradiation and helped to draft the manuscript. D. M. Nikolić participated in statistical analysis and helped to draft the manuscript. S. A. Pejić helped with statistical analysis. S. B. Pajović critically revised the manuscript.

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РАДИОПРОТЕКТИВНО ДЕЈСТВО ЛИПОВОГ МЕДА У ПЕРИФЕРНОЈ КРВИ ПАЦОВА

Радиотерапија утиче не само на малигно, већ и на суседно здраво ткиво, повећавајући стварање реактивних врста кисеоника, уз оштећења биомолекула, као што је оксидација липида у мембранама, позната као липидна пероксидација. Крајњи производ липидне пероксидације је малондиалдехид. Радиопротектори су једињења која могу да заштите нормалне ћелије од зрачења, без промене радиосензитивности туморских ћелија. Синтетички радиопротектори често имају споредне ефекте и токсични су. Природни радиопротектори испољавају заштиту без негативних ефеката. У овом истраживању испитивали смо радиопротективну способност липовог меда у крви пацова, одређујући промене у активностима антиоксидативних ензима каталазе и глутатион пероксидазе и концентрацији малондиалдехида након излагања терапеутским дозама гама зрака. Шеснаест пацова је по принципу случајности подељено на Контролну и Мед групу. Мед група је добијала мед (1,5 $mLkg^{-1}$ дневно) орално у току четири недеље, док је Контролна група уместо тога добијала дестиловану воду. Након четири недеље узети су узорци крви од свих животиња. Узорци су преполовљени и једна серија је била озрачена гама зрачењем (2 Gy). Радијација је изазвала смањену активност глутатион пероксидазе и повећан ниво малондиалдехида, док је третман медом ублажио ове промене, одржавајући глутатион пероксидазу и малондиалдехид на физиолошком нивоу. Ови резултати потврђују радиопротективна својства липовог меда.

Кључне речи: радиотерапија, заштита од зрачења, антиоксидативни ензим, малондиалдехид, липов мед