The effects of royal jelly on the oxidant-antioxidant system in rats with N-methyl-N-nitrosourea-induced breast cancer

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Objectives: To determine the effect of royal jelly (RJ) on the oxidant-antioxidant balance in rats with N-methyl-N-nitrosourea (MNU) induced breast cancer and to compare this with the chemotherapeutic agent paclitaxel.

Material and methods: Fifty-six female Sprague Dawley rats were divided into five groups. Except control group \( (n=8) \), others received MNU (50 mg/kg, a single dose, i.p.) to develop breast cancer: Group II \( (n=8) \) untreated, Group III \( (n=7) \) treated with paclitaxel (15 mg/kg/week, 3 times, i.p.), Group IV \( (n=7) \) with RJ (by oral gavage, 100 mg/kg/day, for 30 days), and Group V \( (n=7) \), with paclitaxel + RJ. At the end of 30 days, histopathological and biochemical parameters were evaluated in breast tissues.

Results: Levels of protein carbonyl (PC) and 8-hydroxy-deoxyguanosine (8-OHdG) were higher in Group V than in Group II while malondialdehyde (MDA) levels were lower in groups IV and V compared to Group II \( (p<0.05) \). Levels of catalase (CAT) in Group V and glutathione (GSH) in Group III were higher than Group II \( (p<0.05) \). Glutathione peroxidase (GPx) and superoxide dismutase (SOD) levels did not significantly different. Decreasing effect of RJ on CA15-3 levels was relevant to histopathological results.

Conclusion: Although RJ (with or without paclitaxel) had increasing effect of antioxidant status it was insufficient to reduce oxidative stress in breast cancer.

Keywords: Royal jelly; Breast cancer; Oxidative stress; Antioxidant activity; MNU.
Introduction

Breast cancer is one of the types of cancer with the highest morbidity and mortality worldwide [1]. Reactive oxygen species (ROS) that increase in carcinogenesis and various diseases are associated with steps of the redox signaling that generally leads to DNA injury [2]. Increased oxidative stress leads to lipid peroxidation, protein oxidation, DNA mutations and breaks, cytotoxic effects and compromise of signal transmission [3]. Patients with breast cancer receive adjuvant radiotherapy, chemotherapy, and hormone and immune therapy after surgical procedures [4]. Chemotherapeutic drugs used in the treatment of breast cancer exhibit anti-tumor effects by compromising specific cell structures or metabolic pathways in normal cells, as well as cancer cells [5].

Paclitaxel is a chemotherapeutic drug widely used in the treatment of ovarian, lung, bladder, prostate and esophageal cancer, melanoma and other solid tumor cancer types [6]. The side-effects of the drug include neutropenia, bone marrow suppression, hair loss, gastrointestinal system disorders, severe allergic reactions, cardiovascular problems and infections associated with white blood cell insufficiency [7]. New agents capable of preventing tumor growth and protecting against chemotherapy-induced toxicities in normal tissue are therefore needed.

Royal jelly (RJ) is a nutrient used to feed the queen bee and young larvae that is released from the hypopharyngeal and mandibular glands on the heads of worker bees (Apis mellifera) [8]. It consists of 50%–70% water, 7%–18% carbohydrate, 9%–18% protein, 3%–8% lipids, and 1.5% mineral salts, polyphenols, enzymes, hormones and vitamins [9]. The bioactive component of RJ consists of large quantities of peptides, sterols and fatty acids such as 10-hydroxy-2-decenonic acid. The proteins and peptides in RJ are reported to be effective in scavenging ROS such as anion and hydroxyl radical [10, 11]. As a result, RJ exhibits various pharmacological activities including vasodilative and hypotensive activities, increase in growth rate, disinfectant action, antitumor activity, anti-inflammatory, antioxidative and scavenging ability, hypoglycemic and wound healing activity, immunomodulatory, and estrogenic activity [12].

There is an increasing emphasis on alternative natural products, due to their fewer side-effects and lower toxicity, in improving the quality of life of breast cancer patients [13]. To the best of our knowledge, there was no in vivo study about the effect of RJ on breast cancer. Therefore we aimed to determine the effect of RJ on the oxidant-antioxidant balance in rats with MNU induced breast cancer and to compare that effect with that of the chemotherapeutic agent paclitaxel.

Materials and methods

Chemicals

N-methyl-N-nitrosourea [MNU; (Sigma-Aldrich, Taufkirchen, Germany)], paclitaxel (100 mg/17 mL, Koçak Pharma, Drug and Chemistry Industry and Commercial Incorporation, Tekirdağ, Turkey) and RJ [the Muğla Beekeepers’ Association (MAYBİR) in the Aegean region of Turkey] were purchased commercially. We used RJ that analyzed chemical composition in previous studies [14].

Experimental design

Fifty-six Sprague-Dawley rats (140–170 g) aged 8–12 weeks were used in this study. All animals were kept under standard laboratory conditions in a controlled environment in a 12-h dark: light cycle. Standard laboratory chow and water were provided ad libitum throughout the experiment. The study was approved by the Institutional Animal Ethical Committee (reference 2014/22).

MNU (50 mg/kg i.p.) solution (dissolved in 0.9% NaCl adjusted to pH 4 with acetic acid) was prepared fresh in order to induce breast cancer in all rats, with the exception of the control group (Group I, n = 8) and was administered immediately. Four weeks after MNU administration, the animals were followed daily and palpated every 3 days to detect the induction of the tumors until 150 days. Tumor development was observed in only 29 rats. These rats were randomly assigned into one of four groups: untreated cancerous rats (Group II, n = 8), cancerous rats treated with paclitaxel (15 mg/kg, i.p., once a week for 3 weeks [15]) (Group III, n = 7), cancerous rats treated with RJ (100 mg/kg, by oral gavage for 30 days
(Group IV, n=7) and cancerous rats treated with both paclitaxel and RJ (Group V, n=7). After the experimental period, the animals were sacrificed by euthanasia with ether after overnight food deprivation. Blood samples collected from each animal were placed into serum separator tubes. Breast tissues were excised immediately. A part of breast tissues was used for histopatologic examination and the other part was stored −80°C to analyze biochemical parameters.

**Histopathological examination**

Tissues were fixed in a 10% formalin solution. For light microscopy, they were dehydrated using 70% and 100% alcohol solutions, processed in an autotechnicon, and embedded in paraffin. Sections 4–5 μm in thickness were cut with a microtome and stained with hematoxylin-eosin (HE). The stained specimens were then examined by a blinded pathologist using an Olympus BX51 (Olympus, Tokyo, Japan) light microscope.

**Biochemical analysis**

**Determination of serum CA15-3 levels**

Serum samples were obtained by centrifugation of blood in tubes without anticoagulant at 2000 × g for 10 min. Levels of serum CA 15-3, a tumor marker mainly used for breast cancer, were determined using an enzyme linked immunosorbent assay (ELISA) kit (SunRed Chemical Company, Cat. No. DZE 201110851) in line with the manufacturer’s instructions. Absorbance of samples was measured at 450 nm using a VersaMax tunable microplate reader (Molecular Devices, CA, USA). Results were expressed as μU/mL.

**Determination of tissue MDA levels**

Level of the lipid peroxidation product malondialdehyde (MDA) was measured spectrophotometrically using the thiobarbituric acid (TBA) method [16]. Firstly, 30 mg breast tissues were homogenized with 1 mL of 1.15% KCl buffer containing detergent 0.05% Triton X-100 using a mechanically driven homogenizer (OMNI-Tissue Master 125, USA). The homogenate was then centrifuged at 3200 × g for 10 min (Eppendorf Centrifuge 5810, USA). The mixture of 500 μL supernatant and 1 mL TBA (0.375% in 0.25 M HCl) was heated at 100°C for 15 min in a hot water bath. After cooling, absorbance readings of this mixture were carried out at 535 nm against a reaction blank. Tetramethoxypropane was used as a standard. Tissue MDA levels were calculated as nmol/g wet tissue.

**Determination of PC levels**

Levels of tissue homogenate protein carbonyl (PC) were determined using an enzyme linked immunosorbent assay (ELISA) kit (SunRed Chemical Company Cat. No. DZE 20111700), in line with the manufacturer’s instructions. Absorbance of samples was measured at 450 nm using a VersaMax tunable microplate reader (Molecular Devices, CA, USA). Results were expressed as ng/g tissue.

**Determination of 8-OHdG levels**

The preparation of tissue homogenates and determination of 8-OHdG levels were determined using a commercial ELISA kit (SunRed Chemical Company Cat. No. DZE 201110032) in line with the manufacturer’s instructions. Absorbances of samples were measured at 450 nm using a VersaMax tunable microplate reader (Molecular Devices, CA, USA). Results were expressed as ng/g wet tissue.

**Determination of GSH levels**

Breast tissue glutathione (GSH) levels were measured as described previously by Garcia et al. [17] using high-performance liquid chromatography (HPLC, Agilent 1100 Series HPLC Systems, Waldbronn, Germany). For GSH measurement, 50 mg of breast tissue homogenate in 500 μL ice-cold buffer (4 g NaCl, 0.1 g KCl, 0.8 g Na₂HPO₄ · 2H₂O, 0.135 g KH₂PO₄ and 750 μL Triton X-100, 0.219 g EDTA in 500 mL distilled water, pH 7.4) was centrifuged at 1500 × g for 5 min at 4°C. One milliliter of supernatant under the fat layer was taken to which was added 300 μL chloroform. The mixture was vortexed and centrifuged at 10,000 × g for 15 min. The supernatant was used for the derivatization procedure, in which 240 μL of supernatant was added to 100 μL of 15% trichloroacetic acid (m/v) and centrifuged at 13,000 × g for 10 min at +4°C. The derivatization reaction was carried out using 130 μL of the supernatant, 500 μL of 0.5 M Tris–HCl buffer (pH 8.9) and 350 μL of 10 mM DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) in of 0.5 M K₂HPO₄ (pH 8.0). After being kept in an ice bath for 5 min, this was acidified by the addition of 100 μL of 7 M H₃PO₄ and centrifuged at 3000 × g for 10 min. Derivatized samples were
filtered through a 0.22 μm membrane, and 20 μL from each filtrate was injected into the HPLC column (ZORBAX Eclipse XDB-C18; 4.6 × 150 mm; 5 μ, Agilent Technologies). The mobile phase consisted of a mixture of 25 mM KH₂PO₄ (pH 3.8) and methanol. The gradient of mobile phases was used in elution. The amount of GSH in the samples was calculated with the aid of 0.25, 0.125, 0.0625 and 0.0312 mM GSH standards (SIGMA). The derivatization procedure was applied to the prepared standards. The results were expressed as mM/g tissue.

Determination of GPx levels

Tissue homogenization and measurement of glutathione peroxidase (GPx) levels were performed using an ELISA kit (SunRed Chemical Company Cat. No. DZE 201115104), in line with manufacturer’s instructions. Absorbance of samples was read at 450 nm using a VersaMax tunable microplate reader (Molecular Devices, CA, USA). The results were expressed as ng/g tissue.

Determination of CAT and SOD activities

In order to determine the activities of catalase (CAT) and superoxide dismutase (SOD), first of all, tissue homogenization was performed according to a modified method [18]. Briefly, 50 mg tissue was homogenized with 2 mL of a solution containing 500 μL/L Triton X-100 and 50 mM Tris–HCl buffer (pH: 7.4). Supernatant was collected after centrifugation at 3200 × g for 10 min (Eppendorf Centrifuge 5810, USA). The underlying supernatant was collected after removing the top layer. After vortexing of the mixture of 0.8 mL supernatant with 400 μL of ethanol:chloroform (2:3), this was centrifuged at 10,000 × g for 30 min at 4°C. The obtained supernatants were used in activity analyses.

CAT and SOD activities were determined spectrophotometrically according to the method of Goth [19], and of Sun et al. [20], respectively. Percentage inhibition of the reaction catalyzed by SOD was calculated based on the decrease occurring in optical density as a result of removal of superoxide radicals in the SOD environment. The amount of SOD corresponding to 50% inhibition was regarded as one unit.

During determination of antioxidant activities in breast tissues, protein levels of the relevant homogenates were measured using the Bradford method [21] with bovine serum albumin (SIGMA, Lot 117K7415, USA) as a standard.

The results of CAT and SOD activities were given as U/mg protein.

Statistical analysis

The analysis results were expressed as median (interquartile range for 25%–75%). The distributions of oxidant-antioxidant parameters in each group were calculated by Kolmogorov-Smirnov test. Kruskal-Wallis analysis was used in comparison of independent parameters and the Mann-Whitney U-test was used for post-hoc comparisons. p < 0.05 was considered statistically significant.

Results

Histopathological results

The normal breast tissue in control group composed of acini which were covered with two layers of epithelium and ductus where acini terminated (Figure 1A). In Group II which received only MNU but no other treatment most of the rats developed either benign or malignant neoplastic lesions which were generally well circumscribed (Figure 1B). Lesions identified as ductal papilloma occurred in all groups (Figure 1B–E). Papilloma obliterating the dilated cystic ductus lumen was composed of glandular and papillary structures on hyalinized fibrous stroma. In situ carcinoma foci were mostly developed inductal papilloma and could be identified by increased mitotic figures and solid appearance of ductus due to increased cellular proliferation. In Group II, the presence of necrosis in the center of ductal carcinoma in situ areas indicated high grade in situ carcinoma (Figure 2B). In other groups except the control group, there were both low grade in situ ductal carcinoma areas with monotonous appearance, and also high-grade areas with mitotic figures and evident necrosis (Figure 1B–E). Low grade invasive tumors which were characterized with solid-cribriform areas invading the adipose tissue were only demonstrated in Group II (Figure 1B). Invasive tumors with ducal carcinoma morphology was not evident in any of the study groups. Tumors were mostly benign in nature in treatment groups often being ductal papilloma. Invasive tumors were not observed in treatment groups (Figure 1C–E). However, there were also plenty specimens of ductal papilloma demonstrating in situ carcinoma foci in treatment groups.

Serum CA15-3 levels

Serum CA15-3 levels were shown in Figure 2. CA15-3 increased in Group II, the untreated cancer-induced group,
compared to the control animals, although the increase was not statistically significant ($p > 0.05$). In Group IV and Group V, CA15-3 levels decreased significantly compared to Group II ($p < 0.05$).

**Levels of oxidant/antioxidant parameters in breast tissue**

The levels of oxidant parameters, MDA, PC and 8-OHdG in breast tissues were shown in the Table 1. While PC levels were not significantly different between Group II and Group I, MDA and 8-OHdG levels increased in Group II significantly. Statistical analysis revealed a significant increase in PC and 8-OHdG levels in Group V compared to the untreated Group II. MDA levels decreased significantly in Group IV, and in Group V, compared to Group II ($p < 0.05$).

The levels of antioxidant parameters, CAT, SOD, GPx and GSH in breast tissues are shown in Table 1. The levels of these parameters in Group II were decreased but GPx decreasing was not significant when compared to Group I. The levels of antioxidant parameters were higher in Group III, IV and V than Group II but only CAT levels increased significantly in Group V compared to Group II ($p < 0.05$). GSH levels were lower in Group II

**Figure 1:** Histopathological findings in breast tissues.

(A) Normal breast tissue in Group I (black arrow: acinus, blue arrow: ductus) (HEx200). (B) Necrosis (blue arrow) in the carcinoma in situ area in Group II (HEx200). (C) Papilloma morphology in Group III (HEx100). (D) Ductal papilloma morphology in the Group IV (HEx40). (Papilloma structures marked on the figures.) (E) Ductal papilloma containing areas of low-grade ductal carcinoma in Group IV. Mitotic figures are often seen (arrows) (HEx400).
Discussion

Chemotherapeutic agents used in the treatment of cancer exacerbate oxidative stress by causing an increase in ROS. However, the oxidative stress that damages biological molecules and thus compromises mechanisms in cancer treatment needs to be reduced [22]. Murrell [23] reported that chemical carcinogens such as free oxygen radicals can lead to injury to the breast epithelium, fibroblastic proliferation, epithelial hyperplasia, cellular atypia and eventually to breast cancer. Natural products with antioxidant capacities may be recommended as supplementary products in the diets of cancer patients in order to prevent free radical-related injury.

RJ is a natural product with a high antioxidant and anti-inflammatory capacity containing bioactive substances such as amino acids (aspartic acid, cysteine, tyrosine, glycine, lysine, leucine, valine and isoleucine), peptides such as royalisin and jellein, fatty acids such as 10-hydroxy-2-decanoic acid, and phenolic molecules [10]. In view of the beneficial bioactive components described above, this study was intended to investigate how RJ affected the oxidant/antioxidant balance when used in the treatment of breast cancer, alone or together with a chemotherapeutic agent, paclitaxel.

Histopathological investigation revealed no invasive tumor with a morphology indicating ductal carcinoma in Group IV and treated with RJ. The absence of malignant tumors in the treated groups suggests that RJ and paclitaxel may have beneficial effects. In agreement with the histopathological findings, serum CA15-3 levels increased in the cancerous group treated with paclitaxel compared to the non-cancerous group, but decreased in the groups receiving RJ and paclitaxel. CA15-3 is a protein that enters the blood from cancer cells and that is used as a tumor marker of response to treatment and recurrence in patients with breast cancer. Decreased CA15-3 levels particularly in the groups receiving RJ alone or with paclitaxel suggests that RJ has an ameliorating effect. Yu et al. [24] showed that RJ exhibits an antitumor effect by inhibiting N acetylation of 2-aminoflourene in human liver tumor cells (J5). Orsolić et al. [25] reported that when RJ was given intraperitoneally or subcutaneously, it did not affect metastasis formation but when given intravenously before tumor-cell inoculation it inhibited metastasis.

Increased free radicals as a result of chemotherapeutic agents using in cancer treatment may cause the

![Figure 2: The levels of serum CA15-3 in experimental groups.](image)

Table 1: The levels of oxidant-antioxidant parameters in breast tissues in experimental groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I n=8</th>
<th>Group II n=8</th>
<th>Group III n=7</th>
<th>Group IV n=7</th>
<th>Group V n=7</th>
<th>p-Value</th>
</tr>
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<tbody>
<tr>
<td>SOD (U/mg protein)</td>
<td>7.85 (7.15–8.85)</td>
<td>3.24* (2.76–3.83)</td>
<td>6.26* (5.91–6.40)</td>
<td>7.13 (6.65–8.01)</td>
<td>5.74* (5.25–6.07)</td>
<td>0.001</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>83.3 (78.3–88.8)</td>
<td>31.6* (29.4–34.1)</td>
<td>25.0* (21.2–34.6)</td>
<td>38.4* (36.9–39.9)</td>
<td>49.5* (45.9–53.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>GPx (ng/g tissue)</td>
<td>957 (668–1072)</td>
<td>636 (561–684)</td>
<td>703 (419–988)</td>
<td>751 (563–1033)</td>
<td>1055 (808–1302)</td>
<td>0.101</td>
</tr>
<tr>
<td>GSH (mM/g tissue)</td>
<td>2.08 (1.88–2.28)</td>
<td>1.47* (1.05–1.89)</td>
<td>2.15* (1.85–2.45)</td>
<td>1.62 (1.24–2.01)</td>
<td>1.77 (1.36–2.18)</td>
<td>0.013</td>
</tr>
<tr>
<td>MDA (nmol/g tissue)</td>
<td>307 (292–325)</td>
<td>345* (325–417)</td>
<td>330 (303–460)</td>
<td>246* (217–307)</td>
<td>256* (177–334)</td>
<td>0.018</td>
</tr>
<tr>
<td>8-OHdG (ng/g tissue)</td>
<td>67.7 (56.4–73.6)</td>
<td>84.1* (71.8–98.6)</td>
<td>107* (84.4–116)</td>
<td>123* (81.4–141.0)</td>
<td>141* (120–162)</td>
<td>0.003</td>
</tr>
<tr>
<td>PC (ng/g tissue)</td>
<td>1493 (1070–1614)</td>
<td>1467 (1030–1904)</td>
<td>1900 (1690–2096)</td>
<td>2212* (1651–2727)</td>
<td>2506* (1988–2834)</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Group I: healthy control, Group II: MNU induced (untreated), Group III: treated with paclitaxel, Group IV: treated with RJ, Group V: treated with paclitaxel and RJ. Data were expressed as median (interquartile range for 25%–75%), p < 0.05. p-Values according to Kruskal-Wallis test. Post-hoc in-group analyses were performed using “Mann Whitney U” tests. *Significantly different from Group I. **Significantly different from Group II.
oxidation of protein, lipid and DNA and may trigger cell death [26]. Malekinejad et al. [27] showed that RJ reduced lipid and protein oxidation occurring in the liver with paclitaxel induction. In the present study, the levels of the lipid peroxidation product MDA were higher in breast tissues with induced cancer but were significantly lower in the groups receiving RJ with and without paclitaxel compared to the untreated cancer-induced group in this study. The levels of PC (showing the protein oxidation of numerous amino acids, such as histidine, proline, arginine and lysine, and of peptide conformation [28]) raised in all the treatment groups, but significant difference was determined in the group receiving paclitaxel in combination with RJ. Similarly, levels of 8-OHdG, the most important marker of oxidative injury in DNA increased in the groups receiving RJ alone or with paclitaxel, and were particularly high in the group receiving RJ with paclitaxel. In summary, although RJ had a lowering effect on MDA in breast tissue when administered alone or in combination with paclitaxel, 8-OHdG and PC levels increased in combination with paclitaxel. The observation of significant increases in DNA and protein oxidation levels in the group treated with RJ in combination with paclitaxel is indicative of side-effects. In contrary to our study, Inoue et al. [29] showed that dietary RJ extended the mean life span of C3H/HeJ mice, possibly by reducing levels 8-OHdG.

The levels of antioxidants, GSH and GPx, in cancerous breast tissue decreased significantly compared to the control group in this study. This decrease in tumorous tissue probably derives from decreased synthesis or increased consumption. Although these levels were higher in all the treatment groups compared to the untreated cancer group, this elevation was only significant in GSH levels in the paclitaxel group (p < 0.05). GSH is one of the most important non-enzymatic defense systems protecting cells against the deleterious effects of ROS. GSH measurements can be used as a marker in malignancies, in monitoring the effects of chemotherapeutic agents against these cancers and lymph node metastasis [30]. The GPX enzyme reduces H₂O₂ to water by using GSH as a substrate, plays an important role against free oxygen radicals and prevents tumor formation by altering lipooxygenase and cyclooxygenase pathways [31]. On the other hand, SOD detoxifies superoxide anions and converts them into H₂O₂ and oxygen [32]. Padmavathi et al. [33] had shown that SOD and CAT levels decrease in breast cancer. Our findings showed that levels of CAT and SOD decreased, in agreement with the literature, compared to the Group I due to increased ROS in the untreated cancer group. But RJ treatment increased SOD and CAT enzyme levels. These findings show that using RJ as a supplementary product increased the levels of antioxidant enzymes but that this increase was insufficient. This is because the effectiveness of cancer treatment and the antioxidants administered may vary depending on the type and stage of cancer and the amount of antioxidant given [34, 35]. Shirzad et al. [36] reported that RJ enhances immune system but needs to time for its antitumor activity, and advised that RJ may be used at least 10 days before tumor inoculation.

Conclusion

Although our histopathological findings showed that RJ has a therapeutic effect on breast cancer, when given together with a chemotherapeutic agent used in treatment, it is inadequate in reducing the developed oxidative stress. Further studies are needed to determine that RJ administration may affect the breast cancer tissue oxidant-antioxidant balance when given before cancer formation.

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