Evaluation of Therapeutic Effect of Chrysin against 5-Fluorouracil-Induced Ovarian Damage in Rats

Elif Ayazoglu Demir¹, Ahmet Mentese², Selim Demir³⁴, Hatice Kucuk⁵, Nihal Turkmen Alemdar⁶, Yuksel Aliyazicioglu⁷

1Karadeniz Technical University, Macka Vocational School, Department of Chemistry and Chemical Processing Technologies, 61750 Trabzon, Turkey.
2Karadeniz Technical University, Faculty of Medicine, Department of Medical Biochemistry, 61080 Trabzon, Turkey.
3Karadeniz Technical University, Faculty of Health Sciences, Department of Nutrition and Dietetics, 61080 Trabzon, Turkey.
4University of Health Sciences, Kamuni Training and Research Hospital, Department of Pathology, 61250 Trabzon, Turkey.
5Karadeniz Technical University, Graduate School of Health Sciences, Department of Medical Biochemistry, 61080 Trabzon, Turkey.
6Recep Tayyip Erdogan University, Vocational School of Health Services, Department of Medical Sciences and Techniques, 53100 Rize, Turkey.

*Corresponding author e-mail: selim-demir@hotmail.com

Abstract

5-fluorouracil (5-FU) is an effective and widely used chemotherapeutic agent to treat various malignancies, but its therapeutic use is limited due to dose-related tissue toxicity. Many studies have confirmed that oxidative stress and inflammation play a major role in the pathogenesis of 5-FU-induced damage in the various tissues. Chrysin (CHS), a natural flavone, exhibits various beneficial activities, including antioxidant, anti-inflammatory and anticanccer. The aim of this study was to determine the therapeutic effect of CHS against 5-FU-induced oxidative stress and inflammation in the ovary tissue of rats for the first time. Thirty female rats were divided into 5 groups: control, 5-FU (100 mg/kg), 5-FU+CHS (1 mg/kg), 5-FU+CHS (2 mg/kg) and CHS (2 mg/kg). 5-FU treatment was administered intraperitoneally (i.p.) on the first day and CHS (i.p.) were applied for the following 3 days. The ovarian tissue levels of malondialdehyde (MDA), total oxidant status (TOS), total antioxidant status (TAS), 8-hydroxy-2'-deoxyguanosine (8-OHdG), catalase (CAT) and interleukin-6 (IL-6) were determined using spectrophotometric methods. MDA, TOS, 8-OHdG and IL-6 levels were significantly higher (p<0.05) and TAS and CAT levels were significantly lower (p<0.05) in the 5-FU group than in the control group. CHS treatments significantly restored the levels of oxidative stress and inflammation parameters in a dose-dependent manner (p<0.05). Our results suggest that CHS can have a therapeutic effect against 5-FU-induced ovarian damage and therefore the use of CHS after chemotherapy may be beneficial in abolishing 5-FU-induced reproductive toxicity.

Keywords: 5-fluorouracil, Chrysin, Inflammation, Ovarian damage, Oxidative stress, Rat

Özet

5-fluorourasil (5-FU), çeşitli maligniteleri tedavi etmek için etkili ve yaygın olarak kullanılan bir kemoterapötik ajandır, ancak doza bağlı doku toksitesi nedeniyle terapötik kullanımı sınırlıdır. Biz çalışımızda, 5-FU nedeniyle oksidatif stres ve inflamasyonun etkisini ilk kez belirlemektedik. Doğal bir flavon olan krisin (CHS), antioksidan, anti-inflamatuar ve antikanşer de dahil olmak üzere çeşitli faydaları sergilebilmektedir. Çalışmanın amacı, sıçanların yumurtalık dokusunda 5-FU ile indüklenen oksidatif stres ve inflamasyonun krisinin terapötik etkisini ilk kez kez belirlemektir. 30 adet dişi sıçan, kontrol, 5-FU grubunda ve 5-FU+CHS (2 mg/kg) ve CHS (2 mg/kg) olarak 5 gruba ayrıldı. 3 gün CHS (i.p.) uygulandı. Yumurtalık dokularında 8-OHdG düzeyi anlamlı olarak yüksek (p<0.05), TAS ve CAT düzeyleri ise anlamlı olarak düşüktü (p<0.05). Sonuçlarımız, CHS'Inin 5-FU ile indüklenen yumurtalık hasarına karşı terapötik bir etkiye sahip olabileceğini ve bu nedenle kemoterapötik sonradan CHS kullanımının 5-FU ile indüklenen üretme toksisitesini ortadan kaldırıma faydali olabileceğini düşündürülmektedir.

Anahtar Kelimeler: 5-fluorourasil, İnflamasyon, Krisin, Oksidatif stres, Rat, Yumurtalık hasarı
INTRODUCTION

Cancer is a disease characterized by the uncontrolled growth and proliferation of abnormal cells and an important public health problem worldwide. Chemotherapy is one of the most widely used methods of cancer treatment. 5-fluorouracil (5-FU) is a widely used antineoplastic agent in the treatment of breast, gastrointestinal, head and neck cancers. The anticancer effect of 5-FU is due to its inhibition of thymidylate synthase enzyme, which is responsible for DNA and RNA synthesis in cancer cells. However, 5-FU not only kills cancer cells, but also acts on rapidly dividing normal cells, causing side effects as with other chemotherapeutics. Common intolerable and serious side effects of 5-FU-based chemotherapy are mucositis, hepatoareal toxicity, diarrhea, myelosupression, carditoxicity, dermatitis and reproductive toxicity. These toxic effects of 5-FU limit its clinical use. Since 5-FU is generally used in combination with other chemotherapeutics, information about its harmful effects on the ovaries is limited. However, experimental studies have revealed that 5-FU administration causes ovarian dysfunction, decreased reproductive hormones and follicle numbers in rodents in recent years. It has been suggested that 5-FU-induced tissue toxicity is associated with increased oxidative stress and inflammation due to increased formation of reactive oxygen species (ROS), lipid peroxidation and decreased glutathione levels. It is therefore suggested that post-chemotherapy undesirable effects in the body can be eliminated by the treatment of chemopreventive agents with antioxidant and anti-inflammatory effects.

Flavonoids are secondary metabolites found in natural products, especially plants, and are phytochemicals that have an important place in the human diet. Chrysin (CHS, 5,7-dihydroxyflavone) is a phytochemical belonging to the flavonoid class, plants containing CHS has been used in traditional medicine since ancient times. CHS has been shown to be one of the main ingredients of some medicinal plants, fruits, mushrooms, honey and propolis. CHS has wide variety of pharmacological activities, including antioxidant, anti-allergic, anti-asthmatic, anti-aging, antihypertensive, antimicrobial, hepatoprotective, neuroprotective, cardioprotective, renoprotective, anti-inflammatory, anticaner, anti-angiogenesis, antihyperlipidemic and anti diabetic. There are increasing evidences that CHS reduce the toxicity of various chemotherapeutic agents, such as cyclophosphamide, cisplatin, 5-FU, methotrexate and doxorubicin in different tissues through its antioxidant and anti-inflammatory potential. Although the protection of female reproductive health against 5-FU toxicity in chemotherapy is crucial for the maintenance of fertility, to our knowledge, there are no studies of the therapeutic effect of CHS on 5-FU-induced ovarian damage in an experimental rat model. The aim of this study was therefore to examine whether CHS has a therapeutic effect against 5-FU-induced ovotoxicity within the framework of oxidative stress and inflammation, for the first time.

METHODS

Chemicals

Phosphate buffered saline (PBS) tablet, phosphoric acid, thiobarbituric acid, 1,1,3,3-tetramethoxypropane, sodium carbonate, dimethyl sulfoxide (DMSO), 5-FU and CHS were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents used were of analytical grade and of the highest purity.

Animals

The thirty female Sprague-Dawley rats (150±15 g) were obtained from Surgical Practice Research Center of Karadeniz Technical University (Trabzon, Turkey). Rats were housed at room temperature (25°C) with 12 h light/dark cycles and free access to standard pellet diet and tap water. Animals received humane care in accordance with the guidelines of the US National Institutes of Health and prior permission was sought from the Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol No: 2021/66). Before treatment, rats were allowed to acclimate for 7 days. The estrus stages of the rats were determined using staining the vaginal smear sample according to the Papanicolaou staining procedure and examining the cell types under the microscope, and only rats whose estrus stage was confirmed were included in the study.

Experimental design

After the familiarization period, the rats were randomly divided into 5 groups with 6 animals in each group. The rats of Group I (control) received physiological saline in first day and DMSO for three consecutive days. The rats of Group II (5-FU) received 5-FU (100 mg/kg) in first day and DMSO for three consecutive days. The rats of Group III and IV (5-FU+CHS groups) received 5-FU...
(100 mg/kg) in first day and CHS (1 and 2 mg/kg) for three consecutive days, respectively. The rats of Group V (CHS per se) received physiological saline in first day and CHS (2 mg/kg) for three consecutive days. 5-FU and CHS were dissolved in physiological saline and DMSO, respectively. All drugs were administered intraperitoneally (i.p.). Doses of 5-FU\textsuperscript{21,22} and CHS\textsuperscript{20,23} were selected based on previous studies. The animals were fasted overnight after the final treatment and sacrificed by cervical dislocation on the 5\textsuperscript{th} day, after which the ovaries were removed from the animals in each group.\textsuperscript{24} The ovarium tissues were excised and stored at -80°C for subsequent biochemical analysis.

**Biochemical analysis**

The tissue samples were homogenized at 9500 rpm in 2 mL of PBS using a homogenizer (IKA, T25 UltraTurrax, Staufen, Germany). The supernatant portions were separated by means of centrifugation at 18000xg for 10 min at 4°C and used in the biochemical analysis. Protein levels of the supernatants were determined using a commercial kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) according to the manufacturer's instructions and calculated as mg/mL bovine serum albumin equivalent. All biochemical parameters measured in the supernatants were proportioned to the amount of protein and expressed as per mg protein.

Malondialdehyde (MDA) levels of tissue samples were determined according to the method developed by Mihara and Uchiyama.\textsuperscript{25} 1,1,3,3-tetramethoxypropane was used as a standard and tissue MDA levels were expressed as nmol/mg protein.

Tissue total oxidant status (TOS) and total antioxidant status (TAS) levels were determined using commercial colorimetric kits (Rel Assay Diagnostics, Gaziantep, Turkey) according to the manufacturer's recommendations. The TOS/TAS ratio was used as the oxidative stress index (OSI) and was calculated using the formula\textsuperscript{26},

\[
\text{OSI (arbitrary unit)} = \frac{\text{TOS (µmol hydrogen peroxide equivalent/L)}}{\text{TAS (µmol trolox equivalent/L)}} \times 100
\]

Tissue catalase (CAT), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and interleukin-6 (IL-6) levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Fine Biotech Co. Ltd, Wuhan, China) according to the manufacturer's recommendations. CAT, 8-OHdG and IL-6 levels were expressed mIU/mg protein, ng/mg protein and pg/mg protein, respectively.

**Statistical analysis**

Data were analyzed with Statistical Package for the Social Sciences (Version 23.0, NY, USA). The compliance of the data to normal distribution was evaluated with the Kolmogorov-Smirnov test. Comparisons of the groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. Statistical significance was set at p<0.05.

**RESULTS**

As shown in Table 1, there were significant increase in the levels of MDA, TOS, OSI, 8-OHdG and IL-6 in the 5-FU-treated group compared with the control group (p=0.016, p=0.0001, p=0.0001 and p=0.0001, respectively). Treatment with CHS (1 mg/kg) decreased only the levels of TOS, OSI, 8-OHdG and IL-6 compared with only 5-FU-treated rats (p=0.0001, p=0.0001, p=0.045 and p=0.002, respectively). However, there were marked reductions in MDA, TOS, OSI, 8-OHdG and IL-6 levels in case of group treated with CHS (2 mg/kg) as compared to the only 5-FU-treated group (p=0.033, p=0.0001, p=0.0001 and p=0.0001, respectively).

The TAS and CAT levels were significantly depleted in the 5-FU-treated group compared to the control group (p=0.027 and p=0.001, respectively). However, the TAS and CAT levels in the CHS (2 mg/kg)-treated group were significantly increased as compared to the only 5-FU-treated group (p=0.033 and p=0.006, respectively).

In addition, treatment with CHS (2 mg/kg) alone did not show any significant change in the any biochemical parameter levels compared with the control group (p>0.05) (Table 1).
The increased MDA, TOS, OSI and 8-OHdG levels and decreased TAS levels in 5-FU-treated rats indicates that 5-FU toxicity is mediated by ROS-induced oxidative cell damage. These findings are consistent with data from previous studies demonstrating that 5-FU increases oxidative stress and DNA damage. Antioxidants, which act as the main defense systems of the body, work in a complex and multistage phenomenon. It involves a number of pathological processes, such as overproduction of ROS, alteration of various signaling pathways and increased inflammation. It is therefore suggested that the use of agents with antioxidant and inflammatory potential may be beneficial in eliminating 5-FU-related tissue toxicity. This study therefore aimed to evaluate the therapeutic efficacy of CHS against 5-FU-induced ovarian damage for the first time.

5-FU-induced lipid peroxidation and free radical generation leading to cell membrane damage are considered as the main mechanism behind its toxic effects. MDA is a lipid peroxidation end product and accepted as a direct indicator of the degree of oxidative stress. It is well known that two of the crucial parameters for evaluating redox balance in biological systems are TAS and TOS. While TAS determines the overall ROS scavenging ability in a biological sample, TOS can be defined as the cumulative amount of total oxidants in the sample. For the quantitative assessment of redox homeostasis disorders, the OSI, which is called the "gold indicator of oxidative stress", is used. Oxidative stress also increases DNA damage and 8-OHdG is one of the main products of DNA oxidation.

### Table 1. Comparison of the levels of biochemical parameters of all experimental groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>5-FU</th>
<th>5-FU+CHS (1 mg/kg)</th>
<th>5-FU+CHS (2 mg/kg)</th>
<th>CHS (2 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>29.3±9.3</td>
<td>75.9±41.9</td>
<td>45.3±23.4</td>
<td>33.5±17.9</td>
<td>25.7±7.9</td>
</tr>
<tr>
<td>TOS (µM H₂O₂ equivalent/L)</td>
<td>10.8±1.6</td>
<td>58.3±14.1</td>
<td>23.2±13.5</td>
<td>11.1±1.7</td>
<td>11.1±4.3</td>
</tr>
<tr>
<td>TAS (mM trolox equivalent/L)</td>
<td>0.93±0.37</td>
<td>0.30±0.13</td>
<td>0.63±0.32</td>
<td>0.92±0.42</td>
<td>0.95±0.39</td>
</tr>
<tr>
<td>OSI (arbitrary unit)</td>
<td>1.3±0.5</td>
<td>24.8±9.9</td>
<td>3.8±1.0</td>
<td>1.5±0.8</td>
<td>1.3±0.6</td>
</tr>
<tr>
<td>8-OHdG (ng/mg protein)</td>
<td>20.5±19.4</td>
<td>100.9±27.2</td>
<td>70.2±14.3</td>
<td>22.6±12.5</td>
<td>22.9±10.5</td>
</tr>
<tr>
<td>CAT (mIU/mg protein)</td>
<td>142.5±26.0</td>
<td>71.1±18.5</td>
<td>109.1±25.0</td>
<td>128.8±24.4</td>
<td>135.4±32.7</td>
</tr>
<tr>
<td>IL-6 (pg/mg protein)</td>
<td>111.8±33.1</td>
<td>432.4±155.7</td>
<td>224.5±98.0</td>
<td>117.9±21.7</td>
<td>118.6±21.5</td>
</tr>
</tbody>
</table>

P-values according to one-way ANOVA test, post-hoc Tukey test. Data were expressed as mean±SD.

**DISCUSSION**

Ideal chemotherapy aims to cause minimal damage to healthy cells while killing cancerous cells. However, there is no chemotherapy protocol that does not harm healthy cells at all in practice. 5-FU is one of the most widely used chemotherapeutics in the world, and ovarian tissue is one of the tissues most affected by 5-FU chemotherapy. 5-FU-induced tissue toxicity is a complex and multistage phenomenon. It involves a number of pathological processes, such as overproduction of ROS, alteration of various signaling pathways and increased inflammation. It is therefore suggested that the use of agents with antioxidant and anti-inflammatory potential may be beneficial in eliminating 5-FU-related tissue toxicity. This study therefore aimed to evaluate the therapeutic efficacy of CHS against 5-FU-induced ovarian damage for the first time.
injury. Increasing evidences point to the role of increased inflammation in 5-FU-induced tissue damage. IL-6 is a very important cytokine involved in the pro-inflammatory process and there is a positive correlation between increased IL-6 levels and the degree of inflammation. Our findings revealed that higher IL-6 levels appeared in the ovarian tissue of rats exposed only to 5-FU than control group and CHS treatments significantly reduced these values in a dose-dependent manner. This improvement appears to be due to the anti-inflammatory property of CHS, which has often been demonstrated. Consistent with our results, CHS has previously been shown to prevent chemotherapeutic-induced tissue damage by inhibiting inflammation in experimental models.

Flavonoids are secondary metabolites originating from natural products and it is suggested that regular and balanced intake of flavonoids is associated with a lower risk of cancer, neurodegenerative and cardiovascular diseases. The antioxidant activities of flavonoids are due their ability to scavenge free radicals, chelate metal ions, and modulate antioxidant enzymes. CHS is a popular member of the flavonoid family, and its antioxidant activity has been reported to be mainly due to the hydroxyl and keto groups in its rings. Therefore, it is thought that the therapeutic effect of CHS on 5-FU-induced ovarian damage is mainly due to its antioxidant properties.

CONCLUSION

CHS could attenuate 5-FU-induced ovarian toxicity by decreasing oxidative stress and inflammation and increasing antioxidant status. This study supports the hypothesis that CHS is a potential therapeutic compound that can be used for the alleviation of 5-FU-induced ovarian injury.

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Authorship contribution statement
Consept and deing: EAD.
Acquisition of data: EAD, HK, SD and NTA
Analysis and interpretation of data: EAD, AM, SD and YA.
Drafting of the manuscript: EAD and SD.

Critical revision of the manuscript for important intellectual content: YA.
Statistical analysis: AM.

Declaration of competing interest

None of the authors have potential conflicts of interest to be disclosed.

Ethical approval
This study was approved by the Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol no: 2021/66) and performed according to the animal research reporting of in vivo experiments (ARRIVE) guidelines.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

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REFERENCES


