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**ARAŞTIRMA / RESEARCH**

# Effect of curcumin on rat sublingual gland exposed to cyclophosphamide

## Siklofosfamide maruz kalmış sıçanların dilaltı bezi üzerine kurkuminin etkisi

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**Abstract**

**Purpose:** This study investigated the effect of cyclophosphamide (CY) on the sublingual glands of 12 weeks old rats, as well as possible efficacy of curcumin (CR) on morphometrical change in these tissues.

**Materials and Methods:** Thirty-five adult male Wistar albino rats were randomly selected and divided into five group of seven rats as control (Con), olive (OV), CY, CR, and CY+CR. The mean volumes of sublingual gland structures and the number of mucous and serous cells were estimated using stereological method.

**Results:** We found that the number of mucous and serous cells was significantly decreased in the CY group than the Con group. The total volume of mucous acini, serous acini, and intercalated ducts as well volume fraction ratio of mucous acini to stroma was significantly decreased in the CY group than the Con group. By the contrary, there was a significant increase in the total volume of stroma in the CY group (when compared with the Con group). In the CY+CR group, the number of mucous cells and serous cells was significantly higher than the CY group.

**Conclusion:** We speculated that CY treatment caused a detrimental effect on the sublingual gland tissues, and that administration of CR also ameliorated the changes induced by CY.

**Keywords:**. Cyclophosphamide, curcumin, sublingual gland, rat

**Öz**

**Amaç:** Bu çalışma, siklofosfamidin (CY) 12 haftalık sıçanların dil altı bezleri üzerindeki etkisi ve ayrıca kurkuminin (CR) bu dokulardaki olası morfometrik değişimi üzerindeki olası etkinliğini araştırdı.

**Gereç ve Yöntem:** Otuz beş yetişkin erkek Wistar albino sıçan rastgele seçilerek aşağıdaki gibi yedi sıçandan oluşan beş gruba ayrıldı: kontrol (Con), zeytin (OV), CY, CR ve CY + CR. Dil Dil altı bez yapılarının ortalama hacimleri ve müköz ve seröz hücre sayısı stereolojik yöntemi kullanılarak tahmin edildi.

**Bulgular:** CY grubundaki mükös hücre ve seröz hücre sayısı, Con grubuna göre anlamlı olarak azaldığı bulduk. Ayrıca CY grubundaki mükös asinüsler, seröz asinüsler ve interkalat kanalların toplam hacmileri ile mükös asinüslerin stromaya hacim fraksiyon oranı, Con grubuna göre anlamlı olarak azalmıştı. Buna ek olarak, Con grubuna kıyasla CY grubunda toplam stroma hacminde anlamlı ölçüde bir artış gözlemlendi. CY+CR grubundaki müköz hücre ve seröz hücre sayısı, Con grubuna göre anlamlı olarak artmıştır.

**Sonuç:** CY tedavisinin dil altı bezleri üzerinde zararlı bir etkiye neden olduğunu ve CR uygulamasının da CY'nin neden olduğu değişiklikleri iyileştirdiğini düşündük.

**Anahtar kelimeler**: Dilaltı bezi, kurkumin, rat, siklofosfamid

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## INTRODUCTION

Anticancer drugs are used in chemotherapy cancer treatment to slow down, regress or stop the process of neoplastic disease. The increased use of chemotherapeutic drugs may induce cytotoxic effect on the body organisms. On other words, anticancer drugs not only destroy cancer cells that grow pathologically in the body, but also normal cells. This drug toxicity is one of the conditions that should be considered to prevent unexpected health problems. Many of these drugs have severe side effects such as neurotoxicity, nephrotoxicity, hepatotoxicity, and lung toxicity[1-4]. The reason that has increased public concerns about the usage of chemotherapeutic drugs is their toxic effect.

Alkylating agents are known as the most efficiency on cancer cells. Cyclophosphamide (CY) as an alkylating drug is a powerful drug commonly used in cancer treatment, but it has thought-provoking toxic effects[5]. Two active metabolites of cyclophosphamide are phosphoramide mustard and acrolein[6]. The antineoplastic effect of cyclophosphamide is related to phosphoramide mustard. It is thought that phosphoramide mustard suppresses cell division by binding to DNA and mediates immunosuppressive and antitumor effects of cyclophosphamide. Also, active metabolite acrolein causes oxidative toxicity by interfering with tissue antioxidant defence system. Therefore, excessive formation of reactive oxygen species (ROS) contributes to vital cell damage. Oxidative balance refers to balance between the formation and elimination of ROS. Disruption of this balance results in oxidative stress that is responsible for unwanted complications in biosystem. Sublingual glands are one of the major salivary glands located at the bottom of the oral cavity and under the tongue. This exocrine gland is mainly composed of major mucous acini and minor serous acini. Morphology and function of the sublingual gland may be affected by the cytotoxicity of chemotherapeutic agents used in cancer treatment. To avoid toxic effects of cyclophosphamide on health cells and organs, the usage of antioxidant agents may be beneficial.

Curcumin (CR), the major ingredient in turmeric spice, is obtained from the Curcuma longa plant. CR as a dietary supplementation has been reported to be pharmacologically safe and nontoxic[7]. There are studies that document the effectiveness of curcumin on human cancers such as colorectal, prostate pancreatic, and breast cancers[8-11]. CR can also improve oxidative damage to vital organs via antioxidant activity. Akomolafe et al. reported a relationship between the administration of CR and decrease in oxidative stress induced by cyclophosphamide[12].

There are fewer studies focusing on the toxicity of chemotherapeutic drugs and approach that reduces the side effect of chemotherapy in salivary glands. The aim of this study was to experimentally investigate the possible protective effects of CR supplementation on structural changes caused by CY in the sublingual glands of Wistar albino rats.

## MATERIALS AND METHODS

The ethical approval was granted by Laboratory Animal Ethics Committee of Gazi University (26.08.2020, E.19891). In the present study, thirty-five adult male Wistar albino rats, 250-300 g body weight and 10-12 weeks old, were utilized. All rats were purchased from the Experimental Animal Research and Application Centre of Pharmacy Faculty of Gazi University, Ankara. Animals were maintained in plastic cages under 12-12 h light/dark cycles at a temperature of 22 ± 2 °C and humidity of 50 ± 5% with free access to food and tap water. The experimental period was applied for 10 days. After the rats were randomly divided into four groups (n = 7), the experimental procedure was followed as [illegible]:

1. Control (Con) group: This group consisted of healthy rats.
2. Olive oil (OV) group: Rats were orally administered 150 mg/kg OV for 10 days.
3. Cyclophosphamide (CY) group: Rats were administered a single intraperitoneal injection of 150 mg/kg CY on the first day of the experiment [13].
4. Curcumin (CR) group: Rats were orally administered 150 mg/kg/day CR (Sigma-Alderich, C1386-5G) for 10-day experimental period [14].
5. Cyclophosphamide + curcumin (CY+CR) group: Rats were not only administered a single intraperitoneal injection of 150 mg/kg CY on the first day of the experiment, but also given orally 150 mg/kg/day CR for 10 days.

Lastly, rats were anesthetized intraperitoneally by giving ketamine (80 mg/kg; Sigma-Alderich Chemical Comp, St. Louis, MO, USA) and xylazine

(5 mg/kg; Sigma-Alderich Chemical Comp, St. Louis, MO, USA), followed by perfusion with 10% formalin. Subsequently, sublingual glands were dissected for stereological examination.

## Histology

We used 10% formalin (Merck, 104002.2500) to fix dissected sublingual glands [15]. Samples then underwent a routine tissue processing including dehydration, impregnation, embedding, and blocking [16]. Thin sections (7 µm thickness) were cut from each tissue blocks based on the systematic random sampling method, followed by haematoxylin (Sigma-Alderich, H3136)-eosin (Sigma-Alderich, E4009-5G) staining [17]. Images of each section were used for morphometric analysis.

## Stereology

The Cavalieri technique was utilized to calculate the mean volume of the regions of interest in the sublingual gland tissues. A pilot study was determined whether the point-counting grid was appropriate for the present work. This grid was overlaid on images, and the number of points hitting sublingual glands was counted. The area of sublingual gland was calculated as :(18)

$$\text{Area}(A) = a(p) \cdot \sum P$$

Where, "a(p)" is the area of point interval, and "ΣP" is the point number counted in all sections. The total volume of interest regions was computed as:

$$\text{Volume}(V) = t \times A$$

Where, "t" is the sum of section thickness and interval, and "A" is the total area of the interest regions.

We used the physical disector method for estimation of serous and mucous cells[19]. Particles were counted using systematic random sampling technique. A pilot study was executed to identify the sampling and counting strategy in small scale. Briefly, the sublingual gland tissues were cut into pairs of consecutive sections, first section reference and the other look up. The pairs were photographed, and then a counting frame was randomly overlaid on images. The numerical density of interest particles was calculated as follow[20]:

$$Nv = \frac{\sum Q -}{\sum V \text{ disector}}$$

where, "ΣQ-" is the number of particles counted in sampling fields, and "ΣV disector" is the total volume of disector frames. Finally, the particle number was calculated as:

$$N = N_V \times V_{ref}$$

where, "N" is the particle number, "$V_{ref}$" is the mean sublingual gland volume, and Nv is the numerical density of particles.

The coefficient of error (CE) and coefficient of value (CV) confirmed sufficient cells counted in each animal and group, respectively[21]. Also, CV showed that the number of animals in each group was enough[20].

## Statistical analysis

SPSS software (IBM version 23.0; SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. Statistical analysis of stereological data (the cell numbers and structure volume) was done by One-Way ANOVA and the Tukey's post hoc test. Mean ± standard deviation (SD) was used for result expression. P value was statistically significant at less than 0.05.

## RESULTS

The mucous cell numbers are given in Figure 1. Stereological analysis showed that the number of mucous cells was significantly less in the CY group when compared with the Con group ($p < 0.05$). There was no significant difference between the Con group and the OV, CR or CY+CR groups. In the CY+CR group, the mucous cell number was significantly increased when compared with the CY group ($p < 0.05$).

The serous cell numbers are given in Figure 2. The number of serous cells was significantly less in the CY group than the Con group ($p < 0.05$). To the contrary, the serous cell number in the CY+CR group was observed to be significantly higher when compared with the CY group ($p < 0.05$). No significant difference was revealed between the Con group and the OV, CR or CY+CR groups.

The total volumes of intercalated ducts are given in Figure 3. Volumetric results indicated that the total volume of intercalated ducts was significantly less in the CY group when compared with the Con group ($p < 0.05$). In the CY+CR group, there was observed to be significantly higher than the CY group ($p < 0.05$).

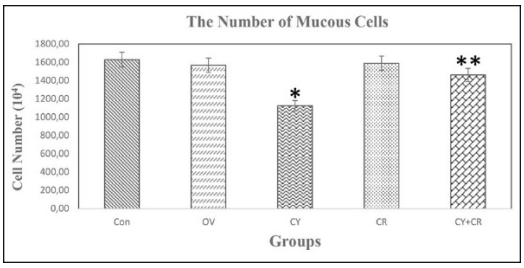


**Figure 1. The numbers of mucous cells in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

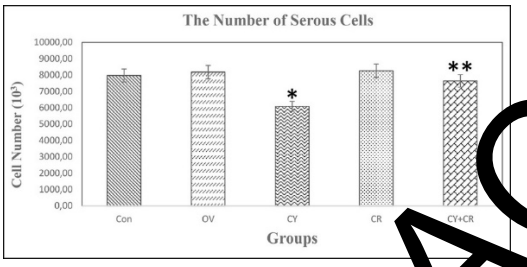


**Figure 2. The numbers of serous cells in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

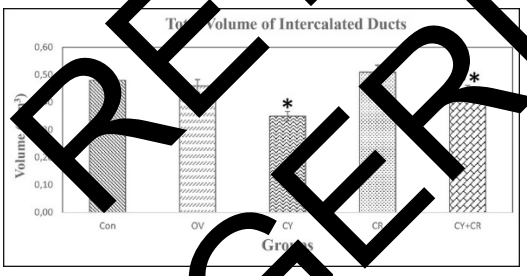


**Figure 3. The total volumes of intercalated ducts in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

The total volumes of striated ducts are given in Figure 4. We found that the total volume of striated ducts was not significant in the CY group when compared with the Con group. Also, significant difference was not detected among groups.

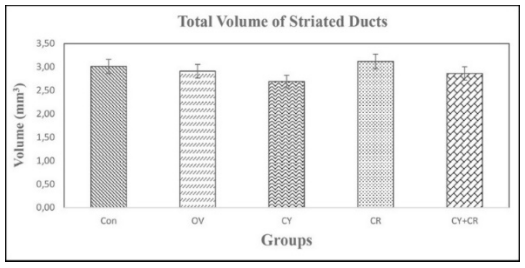


**Figure 4. The total volumes of striated ducts in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

The total volumes of mucous acini are given in Figure 5. The total volume of mucous acini was significantly less in the CY group when compared with the Con group ($p < 0.05$). By contrast, a significant increase in the CY+CR group was detected when compared with the CY group ($p < 0.05$).

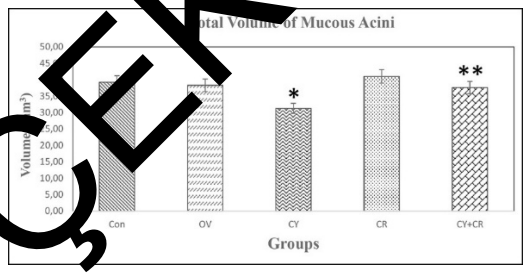


**Figure 5. The total volumes of mucous acini in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

The total volumes of serous acini are given in Figure 6. Our results revealed in the CY group that the total volume of serous acini was significantly decreased when compared with the Con group ($p < 0.05$). In the CY+CR group, the total volume of serous acini was significantly higher when compared with the CY group ($p < 0.05$). No difference was detected between the Con group and the OV, CR or CY+CR groups.

The total volumes of stroma are given in Figure 7. We found that the total volume of stroma was significantly higher in the Cy group when compared

with the Con group ($p < 0.05$). In the CY+CR group, there was a significant reduction in the stroma volume when compared with the CY group ($p < 0.05$).

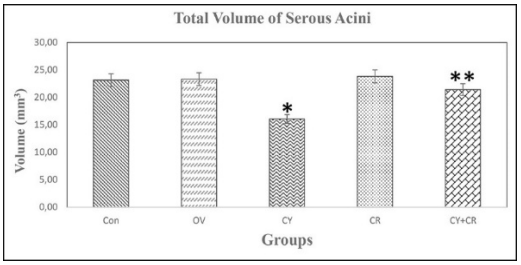


**Figure 6. The total volumes of serous acini in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

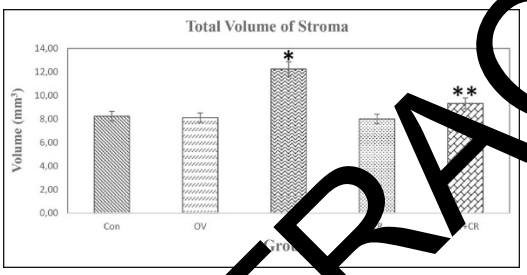


**Figure 7. The total volumes of stroma in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

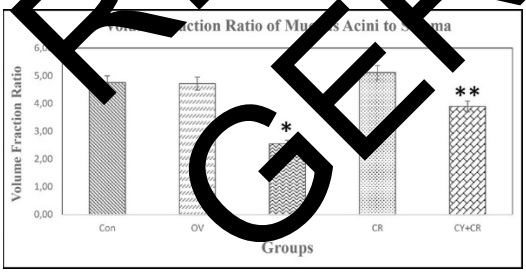


**Figure 8. The volumes fraction ratio of mucous acini to stroma in the Con, OV, CY, CR, and CY+CR groups.**

*, there is a significant difference between the CY and Con groups; **, there is a significant difference between the CY+CR and CY groups. Control, Con; Olive, OV; cyclophosphamide, CY, curcumin, CR; cyclophosphamide + curcumin, CY+CR.

No significant difference was observed in the OV, CR, and CY+CR groups when compared with the Con group. The volume fraction ratios of mucous acini to stroma are given in the Figure 8. This ratio was significantly less in the CY group when compared with the Con group ($p < 0.05$). In the CY+CR group, it was found to be significantly higher when compared with the CY group ($p < 0.05$).

## DISCUSSION

The use of high-dose cytotoxic drugs and the prolonged survival of cancer patients have increased the side effects of these drugs. Although CY is a valuable chemotherapeutic agent used in the treatment of many neoplastic tumours, its toxicity is an important limiting factor[22]. While studies have focused mostly on CY's effect on salivary gland carcinoma, its side effects have not been investigated in this organ tissues.

Unbiased stereological methods utilize accurate tool for estimating quantitative parameters. In the present study, we used the Cavalieri and physical disector methods to determine structure volume and cell number in the sublingual gland tissues. We found that CY significantly reduced the number of mucous cells and serous cells in the CY group when compared with the Con group. These findings showed the toxic effect of CY on the sublingual gland tissues. Main cause of cytotoxicity of CY was possibly due to oxidative damage to the sublingual gland tissues, which is consistent with a study that reported a relationship between CP treatment and increased oxidative stress in biosystem[23]. Increased oxidative stress causes lipid peroxidation in the cell membranes [24]. Hanukoglu documented that oxidative stress was associated with biomolecule damage in the vital cell and other have suggested damage to DNA and alteration in gene expression due to oxidative stress [25,26]. In the CY+CR group, administration of CR significantly increased the mucous cell and serous cell number than the CY group. In fact, CR attenuated the cytotoxicity of CY in the sublingual gland tissues. This increase may have derived from antioxidant efficacy of CR. The widespread use of CR is thought to be due to its biological activity, safe substance, and lack of side effects [27,28]. It has been reported that CR not only decrease caspase-3 expression and cellular degeneration caused by CY, but also improves activity of antioxidant enzyme [12]. Avci et al. suggested

that CR caused a significant increase in Bcl-2-positive cells following exposure to CY[29].

Our volumetric findings showed that CY treatment significantly reduced the total volume of intercalated ducts, serous acini, and mucous acini, as well as the volume fraction ratio of mucous acini to stroma in the CY group when compared with the Con group. Furthermore, the total volume of stroma in the CY group was significantly higher than the Con group. These volume changes revealed the detrimental effect of CY on the sublingual gland tissues, which is a novel result. Moreover, increased stroma volume was possibly derived from inflammatory effect of CY[30]. The studies regarding the side effect of CY on sublingual glands was lacking, so we benefited from the results of research on other tissues. Some studies have suggested the cytotoxic effect of CY on sublingual gland. CY treatment can damage genetic material, followed by programmed cell death[30]. Patwa et al. also reported a significant increase in oxidative stress and apoptotic activity[31]. In the CY+CR group, we found the total volume of intercalated ducts, serous acini, and mucous acini, as well as the volume fraction ratio of mucous acini to stroma was significantly higher when compared to the CY group. Furthermore, there was a significant decrease in the total volume of stroma in the CY+CR group when compared with the CY group. These findings exhibited antioxidant and anti-inflammatory potential of CR on CY-induced toxicity in the sublingual gland tissues. Giordano and Tommonaro suggested therapeutic ameliorative properties of CR via anti-inflammatory and antioxidant activity[32]. They also noted that administration of CR could contributed to the modulation of inflammatory cytokines.

Our study limitation is related to dose-dependent efficacy of CR has not been surveyed. Hence, additional CR doses should be examined to provide the valuable data and utilize the appropriate dosage.

In conclusion, we found that CY treatment caused toxic effect on the number of serous and mucous cells, as well as the total volume of stroma, intercalated ducts, serous acini, mucous acini, and the volume fraction ratio of mucous acini. Moreover, administration of CR significantly improved such morphometrical change in sublingual gland tissues following exposure to CY. We suggest that further studies should be carried out to reveal unknown details regarding the ameliorative effect of CR on human organs exposed to anticancer drug toxicity.

**Yazar Katkıları:** Çalışma konsepti/Tasarımı: AY; Veri toplama: AY; Veri analizi ve yorumlama: AY; Yazı taslağı: AY; İçeriğin eleştirel incelenmesi: AY; Son onay ve sorumluluk: AY; Teknik ve malzeme desteği: AY; Süpervizyon: AY; Fon sağlama (mevcut ise): yok.
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**Author Contributions:** Concept/Design : AY; Data acquisition: AY; Data analysis and interpretation: AY; Drafting manuscript: AY; Critical revision of manuscript: AY; Final approval and accountability: AY; Technical or material support: AY; Supervision: AY; Securing funding (if available): n/a.
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