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# Effectiveness of radiotherapy + ozone on tumoral tissue and survival in tongue cancer rat model

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ABSTRACT

*Objective:* The objective of this study is to examine therapeutic effect of the combination of radiotherapy and ozone which features increasing of the destruction of cancer cells by increasing oxygen level of the body on advanced tongue cancer induced in rats.

*Methods:* A total of 36 female rats were included in this study as 4 groups.

Group 1 (Cancer, n = 8): 4NQO.
Group 2 (Cancer + Radiotherapy, n = 10): 4NQO + Radiotherapy.
Group 3 (Cancer + Ozone + Radiotherapy, n = 10): 4NQO + Ozone + Radiotherapy.
Group 4 (Cancer + Ozone, n = 10): 4NQO + Ozone.
Group 5 (Control, n = 8): Physiological saline solution.

At the end of the week 20, rats in Groups 1 and 5 were sacrified. The rats in Groups 2,3 and 4 were waited until oral food intake was disrupted. The necessary applicatione were then carried out and survivals were evaluated. Each rat was sacrified after death. Tongues of the rats were excised, stained with hematoxylin & eosin and histopathologically evaluated.

*Results: Histopathologic evaluation*: The model that we applied was seen to achieve success in Group 1 in which 7 of the rats developed squamous cell carcinoma and one rat developed dysplasia at the end of the week 20. In Group 2 squamous cell cancer was seen in 6 and dysplasia in 4 rats. Six rats presented normal tongue tissues and 4 rats developed hyperplasia in Group 3.

In Group 4; 3 rats had squamous cell cancer, 2 rats dysplasia, 3 rats hyperplasia and 2 rats had normal tissue. In Group 5, normal tongue tissues were observed in all rats. A significant histopathological improvement was observed in Group 3 compared to Group 2 ($p < 0.05$). Histopathologic scorings were similar in Groups 3 and 5 ($p > 0.05$). No statistically significant difference was found in histopathologic scorings between Group 1 and Group 2 ($p > 0.05$). A significant improvement was observed in Group 4 compared to Group 1 ($p < 0.05$). Group 3 showed a significant histologic improvement compared to Group 4 ($p < 0.05$).

*Evaluation of survival*: Survival times were found as $3.4 \pm 1.3$ days, $76.4 \pm 14.9$ days and $76.4 \pm 14.9$ days in the Groups 2, 3 and 4; respectively. Survival was significantly longer in Group 3 than in Groups 2 and 4 ($p < 0.05$). Survival was significantly longer in Group 4 compared to Group 2 ($p < 0.05$).

*Conclusion:* In this study, demonstrated that radiotherapy plus ozone application both provided histopathological improvement and prolonged survival in advanced tongue cancer rat model.



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## 1. Introduction

According to World Health Organization (WHO), oral cavity cancers the six most common cancer in men following lung, prostate, colorectal, gastric and bladder cancers and the tenth most common cancer in women [1]. The tongue is the most involved cancer localization in the intraoral region with squamous cell cancer being the most common subtype [2].

There are several effectors for tongue cancer including smoking, alcohol use, syphilis, sunlight, history iron deficiency and systemic malnutrition [3,4]. It is known for a long time that cancer cells gain autonomy with effects of internal and external factors. There is strong evidence about the relationships between the cancer cell and surrounding microenvironment have effects in the development and progression of cancer [5]. With various factors, the development and progression of oral cancers are in a form of hyperplastic epithelial lesions – dysplasia – invasive carcinoma [6].

Primary goal of the treatment of tongue cancer is to treat the primary tumor in the tongue, to take nodal metastasis under control and to maintain the functions of the tongue as much as possible [7]. Various treatment modalities are used for treatment of tongue cancer including surgery, chemotherapy, radiotherapy (intensity modulated radiotherapy (IMRT), continuous, hyperfractionated, accelerated radiotherapy (CHART)), other biological therapies, gene therapy, reovirus applications and phototherapy (PDT or photodynamic therapy) [8]. Most of these methods provide satisfactory results in early stage, although are of limited efficiency in treatment of advanced tongue carcinomas of the tongue.

Medical ozone therapy is the application of ozone and oxygen mixture to body fluids and the cavity. Pure ozone application is not performed, because it is toxic. Ozone and oxygen are mixed with a ozone generator and used in clinical practice [9]. Ozone rapidly increases reactive oxygen species in a short period and than a rapid decrease occurs in reactive oxidative species (ROS) within 5 min. This increase upregulates antioxidant and immune systems [10]. Success of the intraperitoneal ozone applications has been proven in numerous clinical and experimental trials. Bocci et al. [11] demonstrated that ozone therapy shows cytotoxic effect against malignant peritoneal cells and increases antioxidant capacity in tumor cells. In their experimental studies, Schultz et al. [12] showed that ozone therapy significantly decreases metastasis. Some preclinical studied demonstrated that the use of medical ozone therapy prevented the resistance due to chemotherapy. Cytotoxic and radiosensitive effects of ozone have been demonstrated in in vitro lung, breast, uterus and ocarian cancer trials [13–16].

In the present study, we evaluated effects of medical ozone + radiotherapy on tumor treatment and survival in an experimentally induced advanged stage tongue cancer model.

## 2. Material & methods

### 2.1. Animals, diets and chemicals

The study was started after receiving approval from Bezmialem Vakıf University Experimental Research Ethics Committee. A total of 36 Sprauge Dawley female rats were included. All the animals were kept in cages as 3 or 4 rats in each at a 12-h light-dark cycle and 21 ± 1 °C temperature with basal diet and water were available ad libitum. The animals were randomized to five groups.

*Group 1 (Cancer, n = 8)*: 4NQO (20 ppm into drinking water, 20 weeks).

*Group 2 (Cancer + Radiotherapy, n = 10)*: 4NQO (20 ppm into drinking water, 20 weeks) + Radiotherapy (administered after the week 22, 500 cGy, 4 sessions, with 5 day intervals).

*Group 3 (Cancer + Ozone + Radiotherapy, n = 10)*: 4NQO (20 ppm into drinking water, 20 weeks) + Radiotherapy (administered after the week 22, 500 cGy, 4 sessions, with 5 day intervals) + Ozone (after the week 22, 1 ml at a concentration of 15 mcg/ml, rectal, 4 sessions, 5 days).

*Group 4 (Cancer + Ozone, n = 10)*: 4NQO (20 ppm into drinking water, 20 weeks) + Ozone (after the week 22, 1 ml at a concentration of 15 mcg/ml, rectal 4 sessions, 5 days).

*Group 5(Control, n = 8)*: Physiological saline solution (1 cc gavage, 20 weeks).

### 2.2. Tongue cancer rat model

An recognized experimental method with proven efficiency in numerous studies conducted to induce tongue cancer was used in our study. In this method, carcinogenic agent was used together with drinking water in a similar way of the formation of tongue cancer. The most accepted agent in the literature to produce tongue cancer is 4-NQO [17–21]. Tongue cancer occurs in the chronic process when this agent is added to the drinking water of rats in a certain amount (20–50 ppm) [17–21]. While 4-NQO causes hyperplasia and dysplasia at the 12th week depending of the administration duration, it produces carcinoma at the 20th week [17–21].

At the end of the week 20, rats in Groups 1 and 4 were sacrified. The rats in Groups 2–4 were waited until oral food intake was disrupted. Here, we defined the progression of tumor of the tongue so as to prevent oral intake as 'advanced tongue cancer'. The necessary applicatione were then carried out and survivals were evaluated. Each rat was sacrified after death. Tongues of the rats were excised, stained with hematoxylin & eosin and histopathologically evaluated.

### 2.3. Radiotherapy

Radiotherapy was performed using a Crius cobalt-60 teletherapy machine (CIS Bio International, Bagnois-sur-Ceze, France). The neck fields of the rat were treated by external irradiation. The fractionation was 500 cGy per day for 4 days, 5 seance.

### 2.4. Ozone application

For the medical ozone therapy, an ozone generator (Longevity, Sydney, British Columbia, Canada) produced an oxygen–ozone mixture (95% O, and 5% $O_3$).

The average volume of the oxygen/ozone gas mixture containing $O_3$ gas with 15 mcg/ml dosage was identified to be

1 ml for rats weighing between 200 and 240 g. An equivalent volume (5 ml) of saline was rectal injected into the control group rats.

### 2.5. Histopathological analysis

The tongue was cut in half longitudinally and each tissue specimen was fixed in 10% buffered formalin and embedded in paraffin blocks. Each tissue was totally submitted to multiple transverse sections for histological processing.

Histopathological evaluation was performed by light microscopy. Tongue sections were graded as normal, hyperplasia, dysplasia, and carcinoma per animal, as modified from Ribeiro et al. [22].

## 3. Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences version 13.0 software for Windows (SPSS Inc, Chicago, Illinois, USA). All quantitative variables were estimated using measures of central location (i.e. mean and median) and measures of dispersion (i.e. standard deviation (SD)). Data normality was checked using the Kolmogorov–Smirnov tests of normality.

Comparison of histopathological data between the groups was performed with Kruskal Wallis variance analysis ($p < 0.05$ was considered statistically significant). The groups causing significant difference were determined with Tukey HSD post-hoc test. Bonferroni corrections were made ($p < 0.008$ was considered statistically significant). Comparison of survival between Group 2, 3 and 4 was performed with Kruskal Wallis variance analysis ($p < 0.05$ was considered statistically significant). The groups causing significant difference were determined with Tukey HSD post-hoc test. Bonferroni corrections were made ($p < 0.016$ was considered statistically significant).

## 4. Results

### 4.1. Histopathologic evaluation

Histopathologic distribution of the tongue tumors in the group is shown in Table 1. At the end of 20 weeks, in Group 1 (Cancer, n = 8) 7 of the rats developed squamous cell carcinoma (Fig. 1) and one rat developed dysplasia. In Group 2 (Cancer + Radiotherapy, n = 10) squamous cell cancer was seen in 6 and dysplasia in 4 rats (Fig. 2). Six rats presented normal tongue tissues and 4 rats developed hyperplasia in Group 3 (Cancer + Ozone + Radiotherapy, n = 10) (Fig. 3). In Group 4 (Cancer + Ozone, n = 10); 3 rats had squamous cell cancer, 2 rats dysplasia, 3 rats hyperplasia and 2 rats had normal tissue. In Group 5 (Control, n = 8), normal tongue tissues were observed in all rats. A significant histopathological improvement was observed in Group 3 compared to Group 2 ($p < 0.05$). Histopathologic scorings were similar in Groups 3 and 5 ($p > 0.05$). No statistically significant difference was found in histopathologic scorings between Group 1 and Group 2 ($p > 0.05$).

A significant improvement was observed in Group 4 compared to Group 1 ($p < 0.05$). Group 3 showed a significant histologic improvement compared to Group 4 ($p < 0.05$) (Fig. 4).

### 4.2. Evaluation of survival

The mean survival after treatment was found as $3.4 \pm 1.3$ days in Group 2 (Cancer + Radiotherapy, n = 10). The mean survival after treatment was found as $76.4 \pm 14.9$ days in Group 3 (Cancer + Ozone, n = 10). Mean post-treatment survival in was found as $17.3 \pm 6.2$ + Radiotherapy days in Group 4 (Cancer + Ozone, n = 10). Survival was significantly longer in Group 3 than in Groups 2 and 4 ($p < 0.05$) (Fig. 5). Survival was significantly longer in Group 4 compared to Group 2 ($p < 0.05$) (Fig. 5).

## 5. Discussion

Tongue cancer is the most common type of oral cavity cancers [23]. The most frequently seen type of tongue cancer is squamous cancer cell carcinoma of the tongue [24]. Smoking, alcohol and chewing tobacco are shown among the most common possible causes of tongue cancers [23]. Surgery, radiotherapy and chemotherapy are used in the conventional treatment of tongue cancer. Location of the surgical area in treatment of tongue tumors depends on the size, localization, type and location of the tumor [25]. Whereas chemotherapy is used in combination with radiotherapy in the stages with metastasis of tumor to the lymph nodes and other organs (stages 2–4) [26]. Radiotherapy is used as an alternative to surgery at early stage and medium sized tumors, in order to maintain functions of the tongue and minimize functional losses [27]. Despite all advancements, today survival from higher stage squamous cell carcinomas remains 50%–59% [8].

Ozone ($O_3$) is a reactive gas molecule which occurs after exposure of oxygen to electrical current and/or UV radiation [28]. Medical ozone therapy is application of a mixture of oxygen and ozone gases. Medical ozone features disinfectant, immuno-

**Table 1**
Comparison of histopathological data of the groups.

| GROUPS | Normal | Hyperplasia | Dysplasia | SCC |
|---|---|---|---|---|
| Group 1 (n = 8) (Cancer) | 0 | 0 | 1 (12,5%) | 7 (87,5%) |
| Group 2 (n = 10) (Cancer + Radiotherapy) | 0 | 0 | 4 (40%) | 6 (60%) |
| Group 3 (n = 10) (Cancer + Radiotherapy + Ozone) | 7 (70%) | 3 (30%) | 0 | 0 |
| Group 4 (n = 10) (Cancer + Ozone) | 2 (20%) | 3 (30%) | 2 (20%) | 3 (30%) |
| Group 5 (n = 8) (Control) | 8 (100%) | 0 | 0 | 0 |

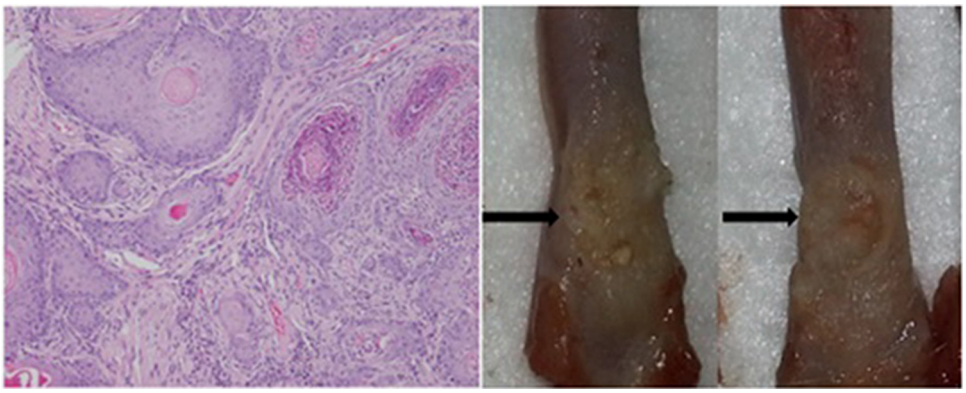

**Fig.1.** A macroscopic and histopathologic appearance from Group 1 (SCC).

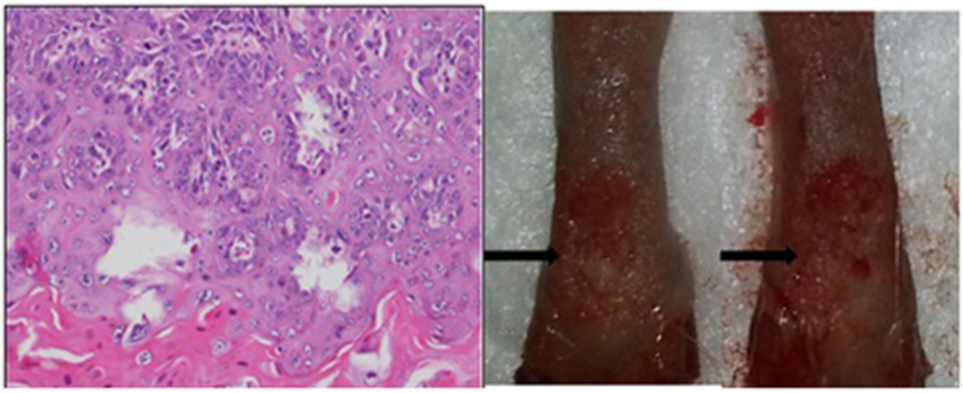

**Fig. 2.** A macroscopic and histopathologic appearance from Group 2 (Dysplasia).

modulatory, antioxidant, enzyme inducing, metabolism increasing effects, and stem cell activator in neovascularization and tissue reconstruction [29]. It is reported that medical ozone application increases microcirculation, regulates oxidative stress and is an important indicator in physiologic reactions [30]. Effectiveness of ozone–oxygen mixture has been demonstrated in cerebrovascular diseases, immundeficiency, cardiac ischemia and autoimmune diseases [31]. Furthermore, studied have shown that the medical use ozone both is effective against primary tumors and leads to significant decreases in metastasis [11,12].

In order to increase efficiency of radiotherapy (RT) in head & neck tumors, tumor related hypoxia and ischemia must be reduced. Hypoxic feature of tumor increases the tumoral condition by 2.5–3 folds [32]. In addition, tumors being hypoxic increase physological selection of cancer cells, resulting in tumor cells becoming more aggressive. Additionally, this decrease the potential of cells to progress toward apoptosis [33]. Furthermore, hypoxic state increase resistance of the tumor to RT and chemotherapy (CT) [33]. According to a meta-analysis by Overgaard and Horsman, increased tumor oxygenation provides a better local control as well as prolonges survival following RT [34]. Ozone therapy has been shown to provide healing even in the most hypoxic tumors [35].

In our study, Group 1 was given 4NQO (20 ppm) which is used in the literature to experimentally induce tongue carcinoma for 12 weeks. Of 8 rats in Group 1, seven developed

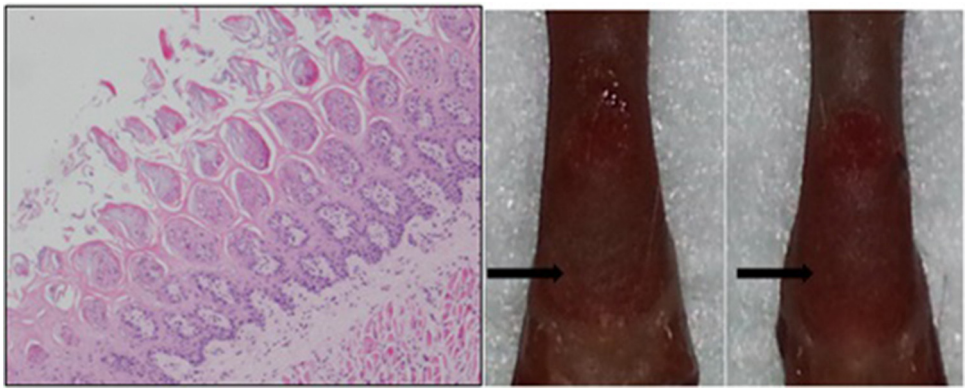

**Fig. 3.** A macroscopic and histopathologic appearance from Group 3 (Hyperplasia).

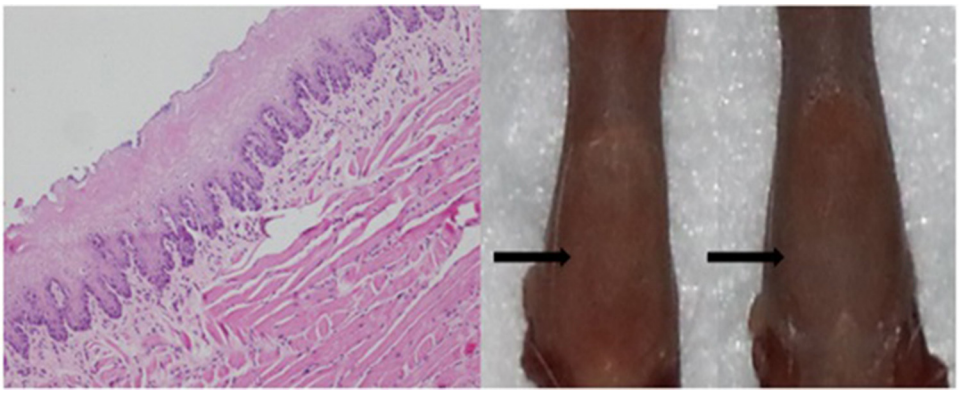

**Fig. 4.** A macroscopic and histopathologic appearance from Group 4 (Normal).

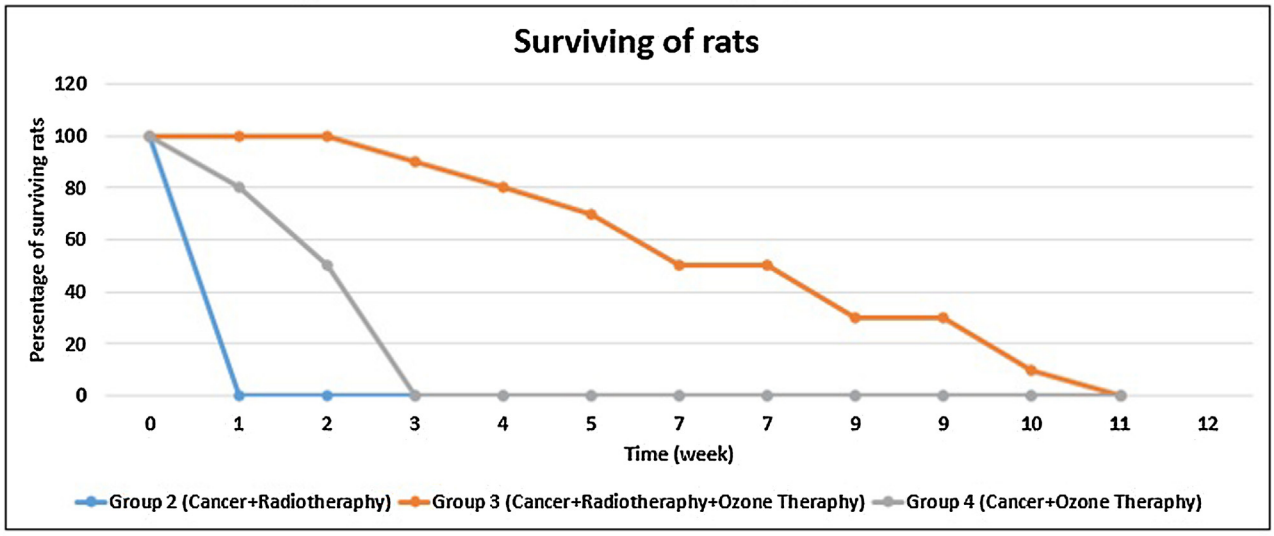


**Fig. 5.** Survivals of rats in Groups 2–4.

squamous cell carcinoma and one rat developed dysplasia (Fig. 1). These data are consistent with the literature [17,19], showing that our cancer model was successfully created.

In Group 2 which we administered radiotherapy following induction of tongue tumor; six rats developed squamous cell cancer and four dysplasia (Table 1). No significant difference was found between Group 1 and Group 2 in terms of histopathological results. This suggested us that RT administered was not sufficiently effective in tumoral regression. Moreover, all the rats in this group died within a week following radiotherapy which was given after discontinuation of oral intake. Radiotherapy had no effect on survival.

In Group 3 which was administered radiotherapy and ozone after the induction of tongue tumor; normal tongue tissue was observed in 6 rats and hyperplasia in 4 rats (Table 1). No significant difference was found between Group 3 and Group 5 (Control) in terms of histopathologic scorings. Histopathologic condition of Group 3 was significantly better than Groups 1 and 2. This indicate that RT + ozone application created significantly tumoral regression.

In our study, ozone therapy was applied after cancer induction in Group 4 and a significant histopathologic improvement was obtained in this group compared to Group 2. However, rate of improvement was significantly better in Group 3 than in Group 4 (Table 1). In the study by Kızıltan et al. [36], medical ozone application in peritoneal carcinoma has been shown to be effective on both tumor regression and survival time. The mean survival was found as 17.3 days in Group 4 and was significantly higher compared to Group 2 and significantly lower compared to Group 3. Studies in the literature have demonstrated that medical ozone application provided tumoral regression owing to antioxidant effect [11,12,36]. In our study, we obtained results supporting these data. We believe that, more effective results could be achieved with longer medical ozone applications.

Furthermore, following RT + ozone therapy applied after discontinuation of oral intake, mean survival was found as 76.4 days in Group 3 which was significantly longer than in Group 2. This information suggested us that, ozone therapy increased radiosensitivity by increasing tumor oxygenation, resulting in a significant tumoral regression. In a study by Clavo et al. [37], blood flow and rate were showed to increase following ozone therapy. Similarly we believe that increased blood flow in tumor area was effective by increasing efficiency of RT.

Ozone therapy causes transient oxidative stress at proper concentration. This in turn induces increase of antioxidants in the circulation. Therefore, medical ozone application shows antioxidant effect as well. This effect occurs both in autohemotransfusion [38]. and rectal applications [39]. In our study, we applied ozone therapy via the rectal route.

In a study by Hernuss et al., ozone + RT application was demonstrated to have potential therapeutic effects in gynecological cancers [40]. In another study, modification of the changes in hypoxia levels have been observed to exert improving effects on therapeutic outcomes of radiotherapy [34]. In the present study, RT + ozone therapy was applied for the first time in the literature in an experimental tongue cancer model. Owing to the treatment which we have applied, both survival was increased and significant regressions were observed in the tumor.

Limitations of our study include lack of immunohistochemical evaluation, a tumor + ozone group and, evaluation of the therapeutic efficiency in early stage tongue cancer. Since our study was the first experimental application of this treatment modality in experimental tongue cancer, we could not increase the number of study groups. We planned further studies based on the results of the current study.

## 6. Conclusion

This study demonstrated that radiotherapy plus ozone therapy produced tumoral regression and loner survival compared to radiotherapy alone in experimentally induced advanged stage tongue cancer model. Our study is the first in the literature using radiotherapy + ozone therapy in advanced tongue cancers and further clinical and experimental trials are warranted in order to provide support and contribution.

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