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RESEARCH ARTICLE

An Immunohistochemical Investigation of The Effect of Sambucus Nigra on Chymase-, Tryptase- and Ghrelin- Positive Cells in Rat Lung

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ABSTRACT

Sambucus nigra (S.nigra) is used in the treatment of many diseases and disorders thanks to its antioxidant, anticarcinogenic, immunostimulating, antiallergic, antiviral, and antibacterial properties. Ghrelin has anti-inflammatory effect on oxidative damage in various organs and cell types. The aim of this study was to immunohistochemically examine the chymase, tryptase, and ghrelin in rat lung after S. nigra administration. A total of 16 male rats were used in the study. The rats were assigned to two groups, control and S. nigra. 1st control group (n=8): No application was made. 2nd S. nigra group (n=8): S. nigra extract was administered at 15 mg/kg by oral gavage for 14 days. Tryptase, chymase, and ghrelin-positive cells were found in the lung tissue in a spindle-shaped, round, or oval shape. When the groups were evaluated within themselves, a significant increase in the number of tryptase, chymase, and ghrelin positive cells was observed in the S. nigra treated group. This study showed that S. nigra, which has an immunomodulatory and antioxidant effect, increases the expression of chymase-, tryptase- and ghrelin-positive cells in the lungs. Additionally, based on our findings, it can be said that mast cells can produce, store and release ghrelin.
Keywords: Chymase, ghrelin, lung, Sambucus nigra, tryptase

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Sıçan Akciğerinde Sambucus Nigra'nın Kimaz, Triptaz ve Ghrelin Pozitif Hücrelerin Üzerindeki Etkisinin İmmünohistokimyasal Olarak İncelenmesi

ÖZ

Sambucus nigra (S. nigra) antioksidan, antikanserojenik, immün sistemi uyarıcı, antialerjik, antiviral ve antibakteriyel özellikleri sayesinde birçok hastalık ve rahatsızlığın tedavisinde kullanılmaktadır. Ghrelin, çeşitli organlarda ve hücre tiplerinde oksidatif hasar üzerinde anti-inflamatuar etkilere sahiptir. Bu çalışmanın amacı, S. nigra uygulamasından sonra sıçan akciğerinde kimaz, triptaz ve ghrelin immünopozitif hücrelerinin immünohistokimyasal olarak incelenmesidir. Çalışmada toplam 16 erkek sıçan kullanıldı. Sıçanlar kontrol ve S. nigra olmak üzere iki gruba ayrıldı. 1. kontrol grubu (n=8): herhangi bir uygulama yapılmadı. 2. S. nigra grubu (n=8): S. nigra ekstresi 14 gün süreyle oral gavaj yoluyla 15 mg/kg dozunda uygulandı. Akciğer dokusunda yuvarlak, oval veya mekik şeklinde triptaz, kimaz ve ghrelin pozitif hücreler bulundu. İki grup kendi içinde değerlendirildiğinde S. nigra uygulanan grupta triptaz, kimaz ve ghrelin pozitif hücre sayısında önemli artış gözlendi. Bu çalışma, immünomodülatör ve antioksidan etkiye sahip olan S. nigra'nın akciğerde kimaz, triptaz ve ghrelin pozitif hücrelerin ekspresyonunu arttırdığını göstermiştir. Ayrıca mast hücrelerinin diğer bağışıklık hücreleri gibi ghrelin üretebildiği, depolayabildiği ve serbest bırakabildiği açıklanmaya çalışılmıştır.
Anahtar kelimeler: Akciğer, ghrelin, kimaz, Sambugus nigra, triptaz

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INTRODUCTION

Sambucus nigra (S. nigra) is a widespread species of the Caprifoliaceae family that grows in most of Europe, western Asia, northern Africa, and the United States (Fazio et al. 2013). Because of its antioxidant, anticarcinogenic, immune system boosting, antiallergic, antiviral, and antibacterial characteristics, S. nigra has been used in folk medicine for generations to treat a variety of ailments and problems (Oniszczuk et al. 2016). It contains high levels of polyphenols, especially flavonols, phenolic acids and anthocyanins. At the same time, these compounds are known as radical scavengers, which protect the body against oxidative stress and lipid peroxidation (Duymuş et al. 2014). Herbal supplements, including S. nigra, have been known to be used to boost immunity against respiratory diseases (Wieland et al. 2021). Moreover, it has been suggested that S. nigra may help in the treatment of upper respiratory tract symptoms and shorten the duration of the common cold or flu (Hawkins et al. 2018).

Mast cells (MCs) are tissue-resident sentinel cells with densely packed secretory granules. (Metcalfe et al. 1997). MCs are capable of secreting a variety of biologically active mediators, cytokines, and chemokines. MC-derived mediators can affect the biological activities of adjacent cells and tissues (Mukai et al. 2018). These cells are multifunctional effector cells involved in innate immunity, host defense, hypersensitivity, and allergic disease (Da Silva et al. 2014). MCs exhibit substantial heterogeneity based on their granule content and protease expression patterns (Dwyer et al. 2016). Based on protease content, two types of MCs have been identified immunohistochemically: tryptase positive mast cells (MC_T) and chymase positive mast cells (MC_{TC}) (Tütüncü et al. 2020). Tryptase is stored in the secretory granules of MCs, from which it is released after degranulation following cell stimulation (Schwartz et al. 1981). Under various physiological and pathological settings, tryptase is involved in the activation, proliferation, and migration of many mesenchymal cells, including endothelial cells and fibroblasts (Sonneck et al. 2006). Chymase is an intracellular, granular-associated, neutral serine protease produced mainly by MCs. It plays a role in regulating extracellular matrix proteolysis, which promotes tissue remodeling (Hamada et al. 1999). In addition, MC_{TC} can promote vascular proliferation, atherosclerosis and tissue fibrosis (Miyazaki et al. 2006). In experimental studies, chymase has been shown to reduce fibrosis in lung tissue (Tomimori et al. 2003, Sakaguchi et al. 2004).

Ghrelin is an endogenous peptide that interacts with the growth hormone secretagogue receptor 1a (GHSR1a) (Kojima and Kangawa, 2005). The presence of ghrelin and its receptor in a wide range of tissues has been determined by gene expression studies in humans and rats (Akalu et al. 2020). Ghrelin regulates growth hormone secretion, cell proliferation, appetite increase, and inflammation through GHSR1a (Nakazato et al. 2001). It has anti-inflammatory effects on oxidative damage in various organs and cell types (Raghay et al. 2020). The expression of ghrelin can be modulated by factors such as peptide hormones, neurotransmitters, glucose, fatty acids, neurotransmitters and enzymes (Akalu et al. 2020). It has been reported that ghrelin is expressed in immune tissues and modulates immune function (Chowen and Argente. 2017). Furthermore, it is stated that ghrelin may have an effect on hematological parameters, which may increase lymphocyte count by stimulating lymphopoiesis (Narin and Çetin, 2010). Moreover, Stefanov et al. (2017) suggested that MCs in rat stomachs can produce, store, and release ghrelin like other immune cells.

The aim of this study was to investigate the effect of S. nigra on the immunohistochemical distribution of chymase-, tryptase- and ghrelin-positive cells in rat lung. Also, the lack of data on the ability of MCs to express, store and release ghrelin motivated this study.

MATERIAL and METHODS

Animals

All procedures were approved by the Ethical Committee of Ondokuz Mayıs University (Decision no: 11.03.2020, number 15).

In this study, 16 male rats, weighing 250-300 g, that were used. The rats were kept in a standard cage with 12 hours of light and 12 hours of darkness in a 22°C ambient temperature environment and were given ad libitum and tap water.

Experiment Groups

1st control group (n=8): there was no application made. 2nd S. nigra group (n=8): S. nigra extract was administered at a dose of 15 mg/kg by oral gavage for 14 days (Bidian et al. 2021). Then, cervical dislocation was performed under anesthesia and lung tissue samples were collected for immunohistochemistry. The lung tissue samples were fixed for 24 h in a 10% formaldehyde solution, and tissue sections were cut from the prepared paraffin blocks with a thickness of 5 μm.

Immunohistochemistry

The lung sections were stained immunohistochemically with Streptavidin biotin complex. Immunopositive cells were determined using anti-rabbit polyclonal chymase (1/200 dilution, Biorbyt, orb11030), mouse monoclonal tryptase

(1/200 dilution, Abcam, ab2378), and rabbit polyclonal anti-ghrelin antibody (1/400 dilution, Abcam, ab129383) (True, 1990). As a secondary antibody, Histostain Plus (Zymed kit: 85-6743) was used. Following deparaffinization, sections were heated in a 700-watt microwave oven in a citrate buffer (pH=6) solution for proteolysis. The tissues were treated in a 3 % hydrogen peroxide solution to inhibit endogenous peroxidase activity. To prevent nonspecific protein binding in sections, serum in the kit was instilled after washing with phosphate buffer solution (PBS). The primary antibody was applied to the samples, which were then kept at +4 0C overnight. The negative control group's tissues were treated with only PBS solution. Following the washing procedure, sections were treated with a biotinylated secondary antibody and incubated with streptavidin-horseradish peroxidase complex. Finally, 3, 3'-diaminobenzidine (DAB) was utilized as chromogen, and the samples were counterstained with hematoxylin and then coated with entellan.

Microscopical Evaluation and Positive Cell Counts

Following histochemical and immunohistochemical staining, tissue samples were examined under an microscope (Nikon Eclipse 50i) in terms of immunoreactivity. Chymase-, tryptase- and ghrelin-positive cell distribution was evaluated semiquantitatively. The following criteria were employed in semiquantitative evaluation: no positive cell in the scanned area (-), 1-2 cells (±), 3-4 cells (+), and 5-6 cells (++) (Ertuğrul et al. 2021).

The immunopositive cells were scored from 0 to 3 semi-quantitatively (Samrao et al. 2012) as follows. A histoscore was derived from the immunopositive cell distribution, 0: no positive cell in the scanned area (-), +1: 1-2 cells (±), +2: 3-4 cells (+), +3: 5-6 cells (++).

Statistical Analysis

The IBM SPSS Statistics Version 22.0 statistical software program was used for all statistical analyses. The Shapiro-Wilk W test was used to determine whether the distribution was normal. Depending on the normality of the data, comparisons between control and S. nigra groups were performed by independent Student's t-test for parametric data. The findings were presented as mean ± SEM (standard error of the mean), and statistical significance was accepted at $p < 0.05$.

RESULTS

Tryptase- and Chymase- Immunopositive Cells

In the light microscopic examination, MC_T and MC_{TC} with positive immune reactions were clearly distinguished in brown color. Tryptase and chymase positive cells were found in the lung tissue in a spindle-shaped, round, or oval shape. (Figures 1A, 2A). MC_T and MC_{TC} were observed around the sacculus alveolaris, in the visceral pleura of the lung, the bronchial wall, and connective tissue in the terminal and respiratory bronchioles' walls (Figures 1B, 2B). Also, MC_{TC} and MT_C were observed to be mostly located near the blood vessels and bronchus-associated lymphatic tissue (BALT). When the two groups are evaluated among themselves, a significant increase in the number of MC_T and MC_{TC} was observed in the S. nigra treated group. MC_{TC} and MT_C were seen individually or in groups in the lung tissue (Table 1).

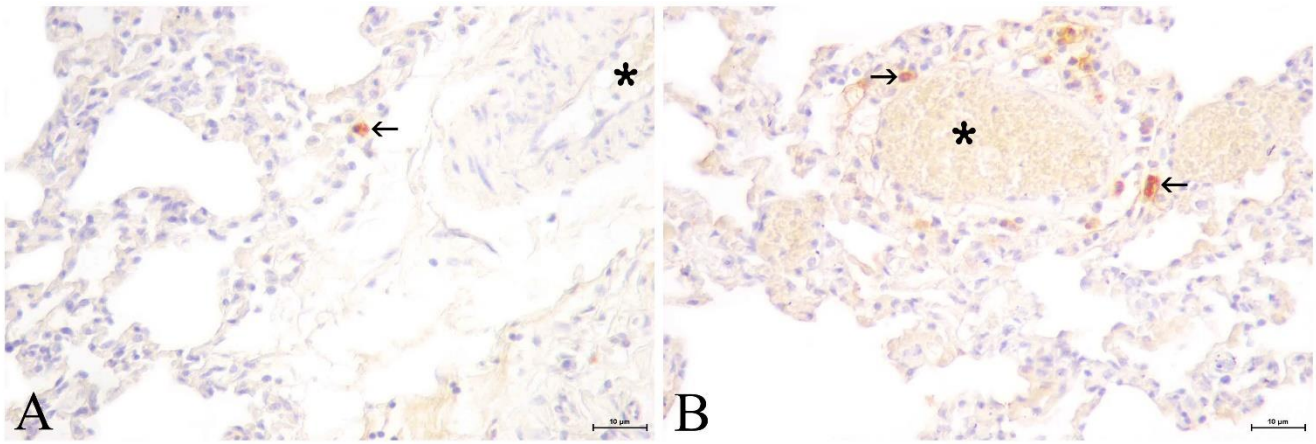

Figure 1: Lung tissue immunostained with antibodies against tryptase; (A) Control group, (→): tryptase immunopositive cell, (asterix): blood vessel, (B) S. nigra group, (→): tryptase immunopositive cell, (asterix): blood vessel, original magnification X40; range bar, 10 µm.

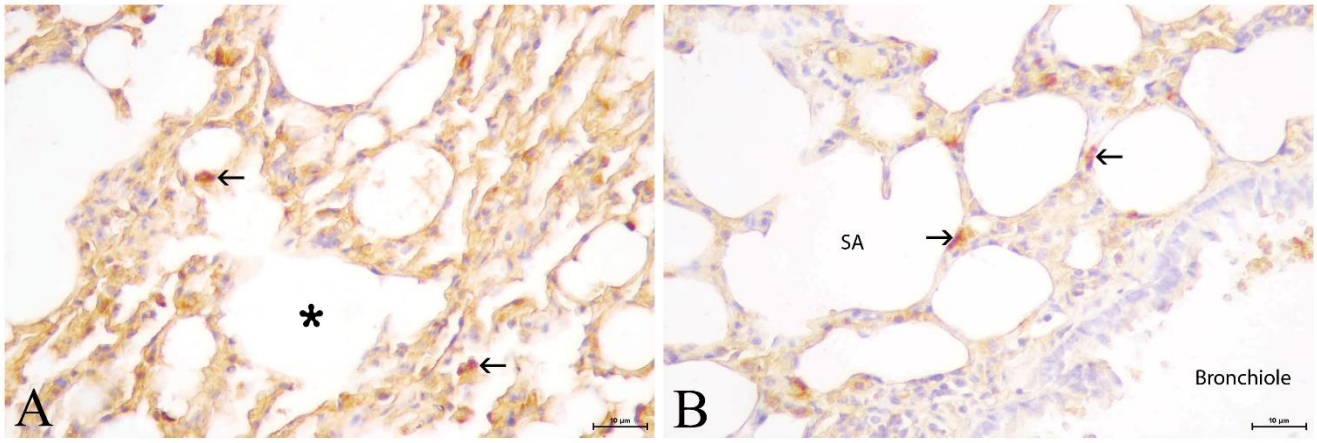

Figure 2: Lung tissue immunostained with antibodies against chymase; (A) Control group, (→): chymase immunopositive cell, (asterix): alveolar space, (B) S. nigra group, (→): chymase immunopositive cell, (SA): sacculus alveolaris, original magnification X40; range bar, 10 μm.

Table 1. Tryptase-. chymase- and ghrelin- immunopositive cell numerical density

	Control group (mean ± SEM)	S. nigra group (mean ± SEM)
Tryptase immunopositive cell	0.73 ± 0.06	1.28 ± 0.09**
Chymase immunopositive cell	0.72 ± 0.05	1.31 ± 0.11**
Ghrelin immunopositive cell	1.82 ± 0.07	2.15 ± 0.11*

* $p<0.05$ and ** $p<0.001$

Ghrelin- Immunopositive Cells

Immunohistochemistry revealed that brown ghrelin-positive cells were scattered throughout the lung tissue, including perivascular areas. In lung tissue, ghrelin-positive cells were spindle-shaped, round, or oval. Ghrelin-positive cells were observed around the sacculus alveolaris, in the interalveolar septal connective tissue, on the periphery of the bronchi, and around blood vessels (Figure 3A). Ghrelin-positive cells were also observed inside and at the margin of the bronchus-associated lymphatic tissue in the lungs. In the lung tissue, ghrelin-positive cells were mostly seen separately or in groups (Figure 3B).

A significant increase in ghrelin positive cells was observed after S. nigra administration compared to the control group (Table 1).

When we compared ghrelin-positive cells with MC_T and MC_{TC}, we also found that ghrelin-positive cells had similar morphology (similar size and shape) and localization to MC_T and MC_{TC}, which reacted positively to immune staining. In addition, ghrelin-positive cell density increase after S. nigra application allowed us to suggest that MCs can produce, store and release ghrelin in rat lung.

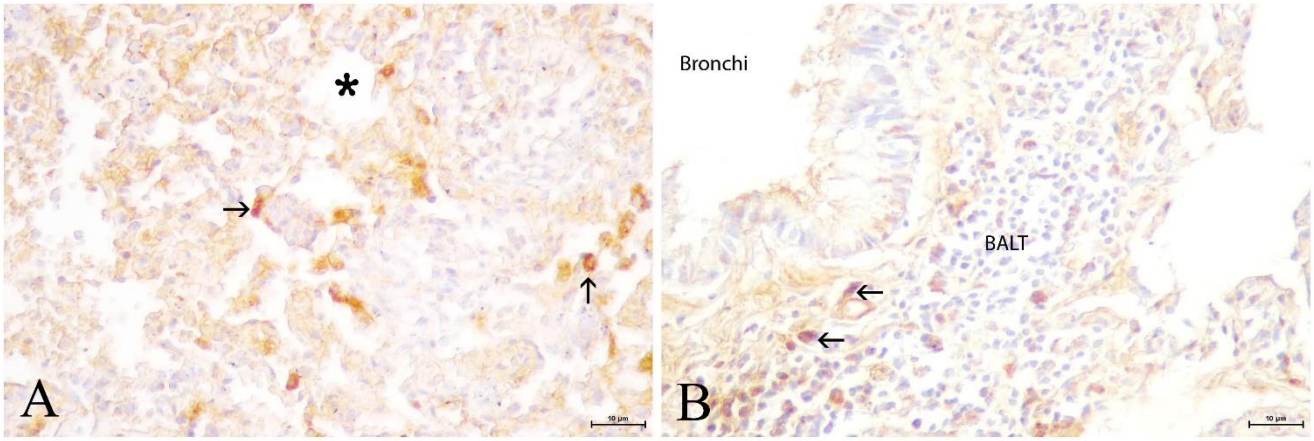

Figure 3: Lung tissue immunostained with antibodies against ghrelin; (A) Control group, (→): ghrelin immunopositive cell, (asterix): alveolar space, (B) S. nigra group, (→): ghrelin immunopositive cell, (BALT): bronchus-associated lymphatic tissue, original magnification X40; range bar, 10 μm.

DISCUSSION

Numerous food components, due to their immunomodulatory properties, have been shown to activate MCs as well as modulate the synthesis of MC mediators (Uranga et al. 2020). Cells of the immune system are highly sensitive to changes in metabolism status. They can affect by changes in circulating hormones, which can affect immune responses and cytokine expression (Baatar et al. 2011).

Chymase plays an important role in the regulation of coagulation by activating and catalyzing the degradation of thrombin and plasmin (Dell'Italia LJ and Husain, 2002). MC_{TC} has been shown to contribute to tissue remodeling, fibroblast mitogenicity, and angiogenesis in lung tissue (Mitani et al. 1999). Increased numbers of MC_{TC} have been found in chronic asthma (Van der Velden et al. 2012), lungs with interstitial pneumonia (Hirata et al. 2007), and blunt lung trauma (Tütüncü at al. 2020). It is also known to cause an increase in MC_{TC} in many conditions such as viral infections, asthma, chronic obstructive pulmonary syndrome, pulmonary hypertension, and fibrosis in the lungs (Kosanovic et al. 2015). However, although there are studies on some active substances with protective effects on the organism in the literature, we have not found any studies on the effects of S. nigra on chymase expression in the lung. Because of this, our study is the first one conducted in this field. There are studies in the literature on the effects of other active ingredients on the number of MC_{TC}s. For example, in a study evaluating the effects of thymoquinone (TQ) application method and dose on the expression of the cytokines in the rat spleen, it was observed that TQ, which has an immunomodulatory effect, did not directly affect the number of MC_{TC} (Ertuğrul et al. 2021). However, Hayıroğlu et al. (2016) emphasized that chymase expression increases in metabolic diseases such as diabetes and hormonal changes. Moreover, it was found in the study conducted by Tütüncü et al. (2020) that MC counts were closely related to polyphenolic antioxidants with anti-inflammatory effects. It was reported that there is a close relationship between increased chymase expression and resveratrol application. In this study, we found that S. nigra, an immune system stimulating product, may have positive effects on MC_{TC} in the lung. It is well known that MCs play a key role in lung pathophysiology. We think that increased MC_{TC} with S. nigra application may have a beneficial effect on possible lung disorders.

Tryptase is a neutral protease that plays an important role in allergic diseases, cytokine release, adhesion molecule expression, and smooth muscle bronchi contraction (Pejler et al. 2010). Also, tryptase is a known potent growth factor for epithelial and smooth muscle cells of the airways in lung tissue (Payne and Kam, 2004). It has been observed that the number of MC_{T}s increases in diseases acute lung injury, sepsis, pneumonia (Zhao et al. 2014). Montelukast (Çetinel et al. 2011), which is used in the treatment of asthma, and ketamine (Li et al. 2014), which can be used for pain relief and sedation, have been shown in studies to cause an increase in the number of MC_{T}s. Furthermore, resveratrol, an active element in the structure of many plants that has an antibacterial effect and can be utilized against infections, has been shown to increase MC_{T}s (Tütüncü et al. 2020). Additionally, it was demonstrated that capsaicin increased the density of tryptase-positive cells. (Tutuncu and Ertuğrul, 2019). Immunostaining against tryptase found in the granules of MCs is one of the most effective methods for identifying MCs. Parallel to the above studies, it has been demonstrated that MC_{T}s behavior can vary according to different active substances. In this study, it was observed that S. nigra, effectiveness was investigated recently can also affect the number of MC_{T}s. It is known that MCs are multifunctional

effector cells involved in host defense. Based on the study's findings, it can be said that S. nigra may be effective in protecting the lung tissue indirectly by increasing the number of MCTs.

Ghrelin regulates many cellular functions and physiological processes, including apoptosis, vascular permeability, and both innate and adaptive immunity. It also contributes to the healing of various lung diseases such as pulmonary edema, emphysema, cystic fibrosis, and pneumonia (Chen et al. 2008, Schwenke et al. 2008). Ghrelin is known to regulate the expression of inflammatory cytokines and plays an important role in immune cell function (Xia et al. 2004). Ghrelin-producing cells are found in various tissues such as the hypothalamus, pituitary, stomach, heart, lung, pancreas, intestine, kidney, testes, and ovaries (Gnanapavan et al. 2002). Volante et al. (2002) observed by immunochemistry using a specific antibody that ghrelin-producing cells have a polygonal or elongated shape and are found in small clusters in fetal, infant, and adult human lungs. It has also been shown that ghrelin-positive cells in the lung are found mainly around the bronchi, in the bronchiolar wall, and in the alveolar septa (Ivanova et al. 2021). Stefanov et al. (2017) reported that in double immunofluorescence staining, both ghrelin and tryptase are expressed by the same MCs in rat stomachs. Additionally, it was demonstrated that ghrelin-positive cells have a similar morphology and location to MCTs. Moreover, co-localization with tryptase immunoreactivity in serial sections from the porcine bile duct suggested that most ghrelin-positive cells might also be MCTs (Stefanov, 2021). Stefanov et al. (2021) used immunohistochemistry to investigate the distribution of ghrelin cells and MCTs in the domestic pig and found that the numbers of ghrelin and tryptase immunoreactive positive cells both varied in parallel to the tunicas of the common hepatic duct. Furthermore, it was shown that the percentages of ghrelin- and tryptase-positive cells increased in parallel with age in the interalveolar septa of the rat lung (Ivanova and Stefanov, 2021). Our data showed that S. nigra increased ghrelin immunopositive cell expression. In addition, an increase in tryptase immunopositive cells was also observed in our study. Also, we found that ghrelin- and tryptase-positive cells were morphologically similar to each other. This approach does not allow for precise identification but considering in our study, ghrelin, tryptase positive cells, which increased in density with S. nigra application, and previous studies, it might be concluded that MCs can produce and secrete ghrelin.

CONCLUSION

This study showed that S. nigra, which has an immunomodulatory and antioxidant effect, increases the expression of chymase-, tryptase- and ghrelin-positive cells in lung. Also, it has been tried to explain that MCs can produce, store and release ghrelin like other immune cells. In conclusion, we think that the findings we obtained in this study will contribute to the literature on the potential role of S. nigra, the MC, and ghrelin in the respiratory and immune systems.

Conflict of Interest: The authors declare that there is no conflict of interest for this article and no financial support has been received.

Ethics Committee Information: The tissue samples used in our study were obtained from the project named "Sambucus Nigranın diyabetli rat dalağında mast hücre ve Vasküler Endotelial Büyüme faktörü (VEGF) üzerine etkilerinin histokimyasal ve immunohistokimyasal olarak incelenmesi" which is approved by the Animal Ethics Committee of Ondokuz Mayıs University (Decision no: 11.03.2020, number 15).

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Description: --

Authors Contribution Rate: TE:%65, GS%35

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