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# Expression patterns of natriuretic peptides in pre-hibernating and hibernating anatolian ground squirrel (*Spermophilus xanthoprymnus*) lung

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ABSTRACT

Anatolian ground squirrel (*Spermophilus xanthoprymnus*) is a true hibernator. This animal transiently reduces pulmonary function during hibernation. Continuance of pulmonary function is very important to survive ground squirrels during the hibernation. Natriuretic peptides may be key players in the modulation of pulmonary hemostasis. However, NPs' role in pulmonary function during hibernation remains unclear. We aimed to investigate the localization and distribution of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) in squirrel lungs during pre-hibernation and hibernation periods using immunohistochemistry. Our immunohistochemical data indicate that ANP, BNP, and CNP were produced by the mucosal epithelium of terminal and respiratory bronchioles, smooth muscle cells in the lamina propria of terminal bronchioles and vascular smooth muscle cells, alveolar type II cells, and macrophages. ANP immunoreactivity was weaker than BNP and CNP immunoreactivities in these cells. The results also demonstrate that the number of ANP, BNP and CNP positive alveolar type II cells tended to increase, although statistically non-significant, during the hibernation period, but the expression of NPs in other pulmonary cells is unaffected by hibernation. This study firstly investigates ANP, BNP and CNP distribution in the Anatolian ground squirrel lung. However, further studies are required to dissect their functional roles during the hibernation.

## 1. Introduction

Hibernation is a physiological adaptation to limited food availability and low temperatures during winter in many mammalian species including squirrels. It consists of torpor and arousal in small rodents. Torpor is represented by a drastic fall in heart rate, respiratory rates and body temperature (Geiser, 2004). Torpor is interrupted by sporadic short arousal periods that restore body temperature and many physiological functions to normal euthermic values (Kortner and Geiser, 2000).

Anatolian ground squirrels are diurnal, obligate hibernators belonging to the rodent order (Gür and Gür, 2010). Ground squirrels entering hibernation drop substantially their core temperature, metabolic rate, respiratory rate and heart rate when compared to euthermic values, thereby saving a significant amount of energy (Kortner and Geiser, 2000; Geiser, 2004). In hibernating ground squirrels, respiration makes up of one to two deep sighs followed by apnea of variable duration at an average of 1–3 breaths per minute (Landau and Dawe, 1958). Respiration increases from 1 to 3 breaths per minute to ˜200 per minute during the arousal period. For example, the respiratory rate ranges between 100 and 125 breaths per minute in the aroused 13-lined ground squirrel (Landau and Dawe, 1958). In these torpor-arousal cycles, ground squirrels are exposed to prolonged periods of immobility, hypometabolism, hypothermia, repetitive stress of cooling-rewarming, and hypoxia during hibernation. However, these animals do not exhibit gross indications of organ damage, which would lead to functional impairment (Drew et al., 2001; Dave et al., 2006; Lindell et al., 2005; Larson et al., 2014).

Although lungs are important organs in the regulation of ventilation during hibernation, there are only a few reports examining how

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hibernation affects pulmonary function. These reports have concentrated mainly on surfactant production, immune function, tissue remodeling, and ventilation of lungs (McArthur and Milsom, 1991; Zimmer and Milsom, 2001; Ormond et al., 2003; Talaei et al., 2011). In recent years, studies in the Syrian golden hamster have revealed that remodeling is one of the mechanisms that provide adaptation to the hibernation (Talaei et al., 2011, 2012). Talaei et al. (2011) demonstrated that low temperature leads to considerable modification in hibernating hamster lung structure. In their study, hamsters also showed noticeable lung remodeling during torpor. These researchers found that the torpor-related lung remodeling was completely restored during arousal and the changes in the hamster lungs in the torpor phase of hibernation resembled lung remodeling seen in asthma (Talaei et al., 2011). Furthermore, they suggested that hibernating animals could provide a model to explore mechanisms of reversible lung remodeling. Overall results of these reports on the lung of hibernators indicate the presence of protective mechanisms that remain poorly understood.

In the mammalian lung, a number of peptides are known to play important roles in respiratory and non-respiratory functions that are vital to normal physiology and respiratory system diseases. Among them, natriuretic peptides (NPs) are implicated in the pathogenesis of the allergic response to the respiratory mucosa and protect against the development of asthma by modulating the function of bronchial epithelium and airway smooth muscle (Mohapatra et al., 2004). As a family of peptide hormones with pleiotropic effects, NPs, namely atrial NP (ANP), B-type NP (BNP) and C-type NP (CNP), are genetically distinct yet structurally and functionally similar hormones. They are produced by various organs such as heart and lungs, then released into the circulation (Woodard and Rosado, 2008). NPs play an important role in regulating various biologic activities of the lung, including pulmonary permeability, bronchorelaxation, vasodilatation, and surfactant production and action (Sakamoto et al., 1986; Perreault and Gutkowska, 1995). ANP is mainly synthesized by and stored principally in mammalian atrial myocytes, then being delivered to the lung as the first target organ (Sakamoto et al., 1986; Perreault and Gutkowska, 1995). Even though the quantity of ANP produced by the lung is considerably less than that by the atria, the lung seems to be an important source of ANP because of its relatively larger mass (Bianchi et al., 1985). ANP has been observed in the lungs of human, pigs and rats, cattle, and lamb (Gutkowska and Nemer, 1989; Tallerico-Melnyk et al., 1992; Mohapatra et al., 2004; D'Angelis et al., 2008). BNP is expressed by multiple respiratory cells, such as type II alveolar cells, airway smooth muscle (ASM) cells and airway epithelial cells (Hellermann et al., 2004; Matera et al., 2011). Matera et al. (2011) found that BNP provides protection against bronchial hyperresponsiveness via an interaction between respiratory epithelium and ASM in patients with asthma. CNP was initially considered as a neurotransmitter expressed mainly in the central nervous system after its discovery (Sudoh et al., 1990). Since then, it has been detected in many tissues, notably heart and lung (Minamino et al., 1993). Accumulating evidence shows that CNP assumes a key role in circulatory and respiratory physiology because it is expressed predominantly in the endothelial cells that line the entire vascular system and are present in capillary beds surrounding the alveoli. CNP has vasorelaxant and antiproliferative actions and seems to regulate vascular tone and remodeling (Volpe et al., 2014). However, its exact role in the lung and associated structures remains elusive. Arjona et al. (1997) reported that it is a more potent antiproliferative agent for rat pulmonary arterial smooth muscle cells. Nevertheless, Klinger et al. (1998a) found that, in normoxic and hypoxia-exposed rats, CNP produces a weaker pulmonary vasodilatory effect than ANP does.

NPs play major regulatory roles in lung tissues and are involved in the development of asthma. Lung remodeling seen in asthma share similarities with the structural changes in the lungs during the torpor phase of hibernation (Talaei et al., 2012). Lung remodeling is a complicated process that requires all the airway components from the epithelium to the adventitia. Each structural change during lung remodeling possesses the potential to change airway physiology (Fixman et al., 2007). Therefore, the determination of presence and localization of NPs is a first-choice candidate to be explored in ground squirrel lung function during prehibernation and hibernation periods. However, it remains largely unknown to what extent and on which level the expression of NPs is affected by hibernation. When reviewing the literature, the expression of ANP, BNP and CNP has not been documented in the lung of hibernating ground squirrel so far. Here, we tested the hypothesis that during the hibernation, the presence and localization of NPs in the lung of ground squirrels may differ according to temperature fluctuations, the structural changes may occur in the lungs and, thus, NPs may have a role in remodeling of lung and adaptation to cold conditions. As a first approach, we evaluated the presence and localization patterns of ANP, BNP and CNP in different structures of the lung of Anatolian ground squirrel (*Spermophilus xanthoprymnus*) during prehibernation and hibernation period using immunohistochemistry.

## 2. Materials and methods

### 2.1. Animals ethics statement and experimental conditions

All the animal experiments were conducted pursuant to the guidelines on the Care and Use of Animals by the Ethical Committee, Erciyes University and approved by Erciyes University Local Ethics Committee for Animal Experiments (HADYEK) (approved number: 15/140), Kayseri. A total of 12 male Anatolian ground squirrels (*Spermophilus xanthoprymnus*), weighing 300–380 g, were included in the study. Animals were wild captured by Tomahawk Traps (Model #202, Tomahawk, WI, USA) from steppes in Develi, Kayseri, Turkey in late August. Therefore, the exact age of the animals is unknown. They were then transported to the laboratory. Immediately after capture, all of squirrels were kept in quarantine for at least 7 days in cages with a stable ambient temperature of 21 °C under a 12 h light: 12 h dark cycle until utilized for experimental purposes so as to be sure they were good health. Then, they were split up into two groups, each of which consists of six (6) animals. Animals in the pre-hibernation designated as the control group are those a few weeks before entrance into torpor. On the other hand, animals in the hibernation designated as the experimental group are those that still hibernate in the late phase of torpor. The prehibernating squirrels were anesthetized with intraperitoneal xylazine and ketamine (11.1 mg of xylazine/ml and 88.9 mg of ketamine/ml) (Olson and McCabe, 1986) and were euthanized with cardiac puncture. Then, the lung of each animal was quickly excised and dissected. The lung tissue samples were fixed within 10% formol-alcohol solution for 18 h, followed by dehydration in graded series of ethanol (70%, 80%, 96%, 100%: 1 h each). Following dehydration, the samples were cleared in methyl benzoate and benzol, and embedded in paraffin wax for histological and immunohistochemical observation.

For hibernation group, squirrels were fed up a standard rodent mix, sunflower seeds, fresh fruit and water ad libitum until body energy stores were enough for entrance into hibernation. The laboratory was kept at 21 ± 1 °C at the beginning and photoperiod of the artificial light-dark cycle (200-0 lx) was set to simulate the outside photoperiod from September 2016 until December 2016, the laboratory temperature was reduced to 6 °C and all the foods were taken away from the cages and the lights were switched off completely to ease a natural transition into torpor. In order not to interrupt torpid squirrels, a red safe light (3–5 lx) was utilized when going into the laboratory. Three months after first entrance into torpor while squirrels are in full torpid, hypothermic state, the above-mentioned sampling procedures were pursued for the experimental group.

### 2.2. Histology and immunohistochemistry

Paraffin-embedded sections cut at a thickness of 5 μm using a rotary

microtome (Leica RM2125RT, Nussloch, Germany) were collected on 3-aminopropyl-ethoxysilane (APES, Sigma–Aldrich, #440140) slides and overnight incubated in an oven at 37 °C. Five slides were obtained from each sample. In order to determine the general structure of the lung tissues of Anatolian ground squirrels during the pre-hibernation and hibernation periods, the first slide of each animal was stained with modified Mallory's connective tissue staining. The following adjacent slides were used for immunohistochemical staining.

The streptavidin-biotin-peroxidase technique was employed for immunohistochemical staining, as specified before (Özbek et al., 2018). After keeping in the oven at 55 °C for 30 min, sections were dewaxed with xylene and rehydrated by descending series of alcohol concentrations until 70% (100%, 96%,80%, and 70%). After rinsing in phosphate-buffered saline (PBS, Sigma-Aldrich, #P4417) pH 7.4 for 5 min, antigen retrieval was done by boiling in 0.01 M citrate buffer (Thermo Fisher Scientific, #AP-9003-500), pH 6.0, for 20 min in a microwave oven at full power. After cooling for 20 min, sections were kept in 0,2% Triton X-100 in PBS for 15 min to improve the permeability. Sections were incubated in 3% hydrogen peroxide ($H_2O_2$) in distilled water for 20 min to block endogenous peroxidase activity in a humidity chamber, then washed for 15 min in PBS and drawn around sections using Dakopen. Non-specific binding was inhibited with Ultra V Block (Thermo Fisher Scientific, #TA-125-UB) for 10 min. After draining excess serum from the slides, sections were overnight incubated with primary antibodies (Table 1) at 4 °C. Routine immunohistochemical procedures were then followed. The resulting signal was developed with 3,3′-Diaminobenzidine (DAB, Invitrogen, #34002) and 3-amino-9-ethylcarbazole (AEC, (Thermo Fisher Scientific, #TA-125-HA). Finally, sections were counterstained with Gill's hematoxylin and covered with entellan (Merck, #107961) and aqueous mounting medium (Thermo Fisher Scientific, # TA-060-UG). It was followed manufacturer's instructions for all dilutions. The specificity of the immunohistochemical staining was controlled by using negative and positive control sections. In the negative controls, normal rabbit IgG (Santa Cruz, #sc-2027) was used instead of the primary antibody. In positive controls, squirrel atrium and elastic artery were used. Immunoreactivity was determined as the presence of brown and red color. All the procedures were conducted in a humidity chamber at room temperature unless otherwise specified. Finally, slides were examined under BX51 microscopy, and photographs were taken from slides using DP74 camera attached to BX51 microscopy.

### 2.3. Quantitative assessment of immunohistochemical staining

The staining intensities of ANP, BNP and CNP were quantified based on the method of Jensen (2013) with slight modifications. The immunostaining intensity was measured for an entire image obtained from lung slides. The images of squirrel lungs during pre-hibernation and hibernation periods were taken at 400X magnification. For this purpose, 12 images were utilized per animal (n = 6) in each group. After submitting the images into the software ImageJ 1.51 version (Java 1.8.0_112, https://imagej.nih.gov/ij/), they were sent to the plug-in "color deconvolution" where the staining of hematoxylin and AEC was divided into 3 different panels with hematoxylin, with the only AEC image and background. Then, employing the only AEC images, threshold values were determined for obtained AEC images. Later on, parameters of area and area fraction (percentage of staining area, namely "staining intensity") were determined. Then, area and percentage of staining area were calculated for each image.

### 2.4. Counting of ANP, BNP and CNP positive ATII cells

In order to determine the abundance of ATII cells in immunostained tissue sections, we used a 40× objective and 10× eyepiece containing an ocular grid measuring 0.625 $mm^2$ of tissue section. In each of six serial sections per animal (n = 6) in both groups, the number of ATII

**Table 1**
Primary antibodies used for immunohistochemistry (IHC).

| Antibody | Host | Immunogen | Catalogue no* | Supplier | Application | Dilution | Species reactivity |
|---|---|---|---|---|---|---|---|
| CD68 mAb | Mouse | Subcellular fraction of human alveolar macrophages | MS-397 | Thermo Scientific | IHC | 1/50 | Human, Monkey, Cat and Rat |
| ANP (FL-153) pAb | Rabbit | Amino acids 1–153 representing full length ANP of human origin | sc-20158 | Santa Cruz | IHC | 1/100 | Human, rat, mouse |
| BNP (FL-134) pAb | Rabbit | Amino acids 27–134 mapping at the C-terminus of BNP of human origin | sc-20159 | Santa Cruz | IHC | 1/100 | Human |
| CNP (H-115) pAb | Rabbit | Amino acids 1–115 of CNP of human origin | sc-20952 | Santa Cruz | IHC | 1/100 | Human, rat, mouse |

Abbreviations: CD68: Cluster of Differentiation 68 (Macrophage Marker Ab-3, clone KP1, the mouse homolog is macrosialin); ANP: Atrial Natriuretic Peptide; BNP: Brain Natriuretic Peptide; CNP: C-type Natriuretic Peptide; mAb: monoclonal antibody; pAb: polyclonal antibody.

cells was counted by two blinded observers for 5 randomly selected microscopic fields. The data are presented as the mean number of ANP, BNP and CNP positive ATII cell per $mm^2$.

### 2.5. Statistical analysis

Data was analyzed using GraphPad Prizm 7 for Windows (Version 7.04) software. Data on percentage of staining area were submitted into GraphPad Prizm 7 for Windows (Version 7.04) software in order to evaluate data of staining intensity (% staining area). A *t*-test was used in order to compare whether there is any significant difference in staining intensity of NPs between the prehibernation and hibernation conditions and between ATII cells for each NP. Two-way ANOVA with Bonferroni's multiple comparisons test was used to test whether there is any significant difference between ANP, BNP and CNP in each group. All the values are displayed as the mean ± SEM. Statistically significance was taken as $P < 0.05$.

## 3. Results

### 3.1. The histology of the lung in the Anatolian ground squirrel during the prehibernation and hibernation periods

In pre-hibernation and hibernation periods, general structural features were similar (Fig. 1). The lung sections include bronchioles that fork off terminal and respiratory bronchioles with adjacent alveolar walls and small to medium pulmonary veins and arteries. However, there were also the larger (nonterminal) bronchioles in some sections. In addition, alveolar lumen demonstrably enlarged in hibernation (Fig. 1d–f). The wall of the bronchioles consisted of epithelium, smooth muscle cells and small amount of connective tissue without glands or cartilage. The epithelium was a simple columnar or cuboidal epithelium composed of ciliated and nonciliated Clara cells. The alveolar wall was lined with two different epithelial cell types, alveolar type I (ATI, also known as type I pneumocytes (pI)) and alveolar type II (ATII, also known as type II pneumocytes (pII)) cells. ATI cells were squamous cells which extend over the basal lamina as a thin sheet. ATII cells were cuboidal or polygonal cells. The interstitium was delimited by the basement membranes of the epithelium and endothelium. The interstitial space was wide, and contained fibroblasts and connective tissue fibers. In this study, the lung stromal cells were identified according to their morphology and localization employing histochemical and immunohistochemical techniques.

### 3.2. CD68 immunohistochemistry in the lung of Anatolian ground squirrels during the prehibernation and hibernation periods

In order to identify the macrophages, we used CD68 as a macrophage marker (Gibbings et al., 2017). CD68 positive-staining cells were localized particularly to the subepithelial stroma of bronchi and bronchioles, alveolar septa, and the bronchus-associated lymphoid tissue (BALT) (Fig. 2). As shown in Fig. 3, we observed CD68 positive macrophages in the lamina propria of terminal bronchioles, alveolar septa and lymphoid follicles of BALT. In addition, interstitial cells staining with BNP and CNP showed a strong cytoplasmic immunoreaction while interstitial cells staining with ANP exhibited a weak immunoreaction.

### 3.3. The immunolocalization of ANP, BNP and CNP in the lung of Anatolian ground squirrels during the prehibernation and hibernation periods

For ANP and BNP positive controls, we used Anatolian ground squirrel atrium in which immunoreactivity was observed granular reaction in atrial cardiomyocytes, and, for CNP positive control, we used squirrel elastic artery and observed staining in endothelial and smooth muscle cells (Öztop et al., 2019) (Fig. 4, left panel). In negative controls, lung sections were incubated with normal non-immune serum and no immunoreaction for each antibody was detected (Fig. 4, right panel). Immunohistochemistry demonstrated the cytoplasmic expressions of ANP, BNP and CNP proteins in various lung cells of the ground squirrel during the prehibernation and hibernation periods. When the staining scores of ANP, BNP and CNP were evaluated by image analysis, there was no statistically significant difference in staining intensity between pre-hibernation and hibernation periods ($t = 0.0386$; $p = 0.9694$ for ANP, Fig. 5a; $t = 1.419$; $p = 0.1628$ for BNP, Fig. 5b; $t = 1.967$; $p = 0.0553$ for CNP, Fig. 5c). Generally, in the lung, ANP immunoreactivity was quite weaker than BNP and CNP immunoreactivity in both pre-hibernating and hibernating groups. In both periods, there was a statistically significant difference between staining intensities of both BNP and CNP and that of ANP ($F(2, 69) = 137.10$; $p < 0,0001$), although there was no statistically significant difference between staining intentisies of BNP and CNP (Fig. 5d). The cellular localization patterns of the NPs in prehibernating and hibernating Anatolian ground squirrel lungs are shown in Figs. 6–8). ANP, BNP and CNP were localized in the same cell types of lung of ground squirrels.

### 3.4. Immunolocalization of ANP in the lung

Fig. 6 shows the localization patterns of ANP in structural components of the lung in Anatolian ground squirrels during pre-hibernation and hibernation period. During the pre-hibernation and hibernation periods, although weak cytoplasmic and nuclear ANP immunoreactivity were observed in the epithelial cells of the terminal and respiratory bronchioles (Fig. 6a, b, e and f), moderate cytoplasmic immunoreactivity was found in bronchial and vascular smooth muscle cells (Fig. 6a, e, d and h). During the pre-hibernation period, in alveoli, weak cytoplasmic immunostaining for ANP was observed in multifocal cuboidal cells lining and within the alveolar septa consistent with ATII cells, whilst ANP immunostaining was absent in ATI cells lining the alveolar septa (Fig. 6c). The immunolabelling intensity in ATII cells of hibernating animals' lung was weaker than that observed in the pre-hibernating animals' lung (Fig. 6g). However, the number of ATII cells tended to increase in the hibernation period, albeit not statistically significant ($t = 0.9102$; $p = 0.3726$; Fig. 9a). Clara cells had weak immunoreactivity for ANP during hibernation. Furthermore, weak immunostaining was observed in follicle-associated epithelial (FAE) cells overlying lymphoid follicles of bronchus-associated lymphoid tissue (BALT).

### 3.5. Immunolocalization of BNP in the lung

Fig. 7 shows the expression patterns of BNP in structural components of the lung in Anatolian ground squirrels during pre-hibernation and hibernation period. The cellular immunolocalization patterns of BNP in the lung during the prehibernation period were very similar to those of the hibernation period. In both periods, the ciliated cells of epithelia lining terminal and respiratory bronchioles, and bronchial and vascular smooth muscle cells displayed moderate to strong positive reactions for BNP (Fig. 7a–h). Generally, staining in the epithelial cells was cytoplasmic (Fig. 7a, b, e and f); however, strong immunostaining for BNP was also observed at the point of ciliary attachment on the apical surface throughout in epithelia of the terminal bronchioles (Fig. 7a). In both periods, in alveoli, positive BNP immunostaining was clearly visible as a granular precipitate within the cytoplasm of ATII cells, but was absent in ATI cells (Fig. 7c, f and g). Although the number of BNP-immunopositive ATII cells tended to increase during the hibernation period, there was no statistically signigicant difference in both periods ($t = 1.821$; $p = 0822$; Fig. 9b). The immunostaining intensity remained relatively stable during the hibernation period.

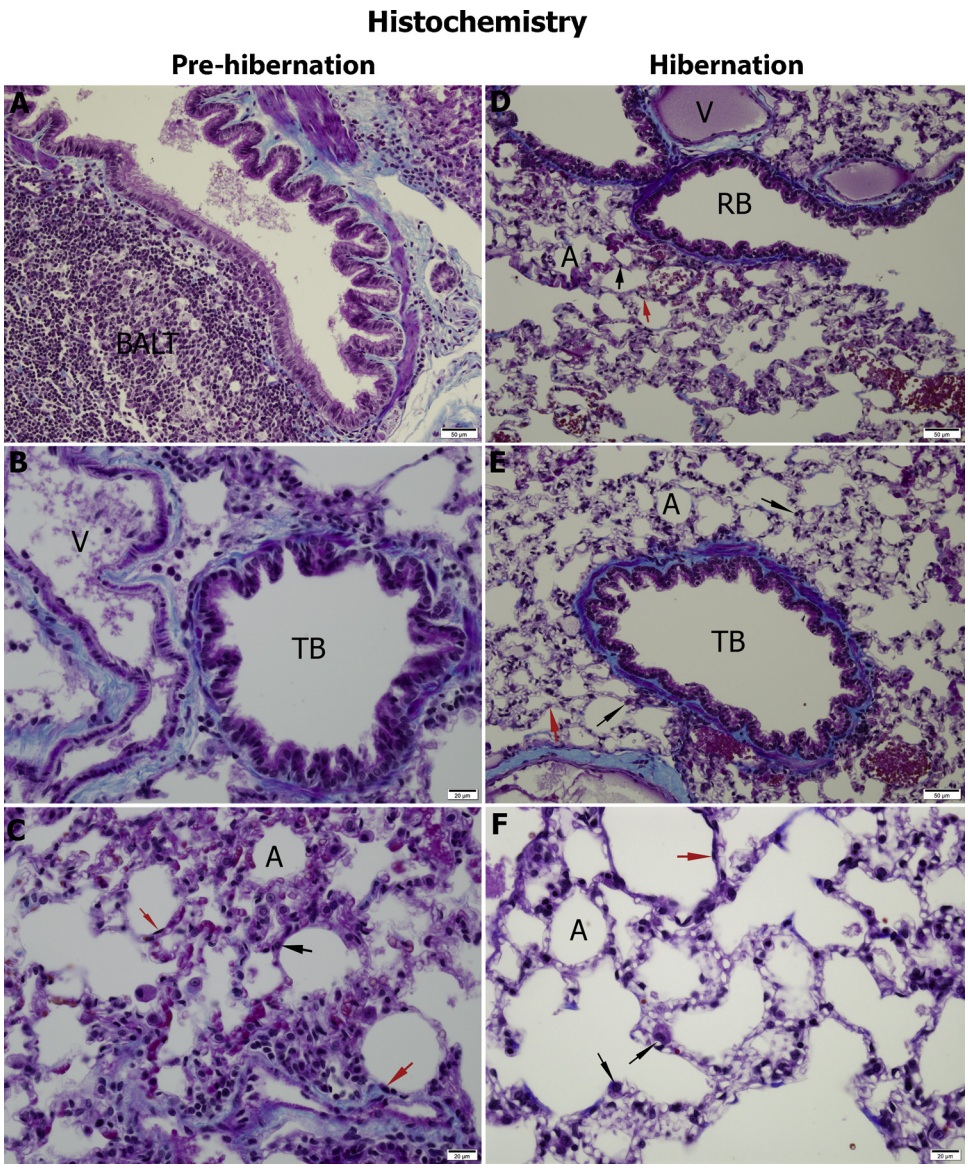



**Fig. 1.** Histology of pre-hibernating and hibernating Anatolian ground squirrel lungs. Section of lung from a pre-hibernating ground squirrel showing normal bronchiolar and alveolar structure (Fig. 1a–c). Sections of lungs from a hibernating ground squirrel (Fig. 1d–f). Enlarged airspaces are seen in the hibernating squirrel lungs. Red and black arrows indicate alveolar type I and II cells, respectively. v, vein; RB, respiratory bronchiole; TB, Terminal bronchiole; A, alveole. Modified Mallory's connective tissue stain. Scale bars, 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

### *3.6. Immunolocalization of CNP in the lung*

Fig. 8 shows the expression patterns of CNP in structural components of the lung in Anatolian ground squirrels during pre-hibernation and hibernation period. The bronchioli displayed CNP immunoreactivity in the nuclei and cytoplasm of the ciliated cells and the nuclei of non- ciliated Clara cells of the respiratory epithelium. In both periods, the cytoplasm of cilated cells in the epithelium of terminal and respiratory bronchioles was strongly positive for CNP (Fig. 8a, b, e and f). The bronchial smooth muscle cells exhibited weak immunostaining for CNP in pre-hibernation (Fig. 8a) and moderate in hibernation (Fig. 8e). In alveoli, positive immunoreactivity for CNP was also observed in the nuclei and cytoplasm of ATII cells. It was also detected that the number of CNP-immunopositive ATII cells tended to increase during the hibernation period, although not statistically significant ($t = 1,798$; $p = 0.0859$; Fig. 9c). The FAE of BALT displayed moderate immunostaining for CNP. In blood vessels, CNP immunoreactivity was weak in the endothelial cells and moderate to strong in smooth muscle cells.

## 4. Discussion

This study is the first to examine the localization patterns of natriuretic peptides (ANP, BNP and CNP) in the Anatolian ground squirrel lungs during pre-hibernation and hibernation periods. Our immunohistochemical study reveals two important findings in the lungs of Anatolian ground squirrels. The first is that ANP immunoreactivity was significantly weaker than BNP and CNP immunoreactivities in various structural components of ground squirrel lung during the pre-hibernation and hibernation periods, as demonstrated with image analysis. The second is that ANP, BNP and CNP were produced by the mucosal epithelium of terminal and respiratory bronchioles, smooth muscle cells in the lamina propria of terminal bronchioles and vascular smooth muscle cells, and alveolar type II cells, as well as macrophages. In addition, alveolar lumens were larger in hibernation than in pre-hibernation, as evidenced by histochemistry.

ANP has already been known to be released from the atrial cardiomyocytes into the circulation in response to the stretched or distended atrial wall. However, several studies have demonstrated that ANP is produced by different lung cells of many mammalian species (Matsubara et al., 1988; Sirois and Gutkowska, 1988; Tharaux et al., 1998; D'Angelis et al., 2008). In hamster lung, ANP expression was

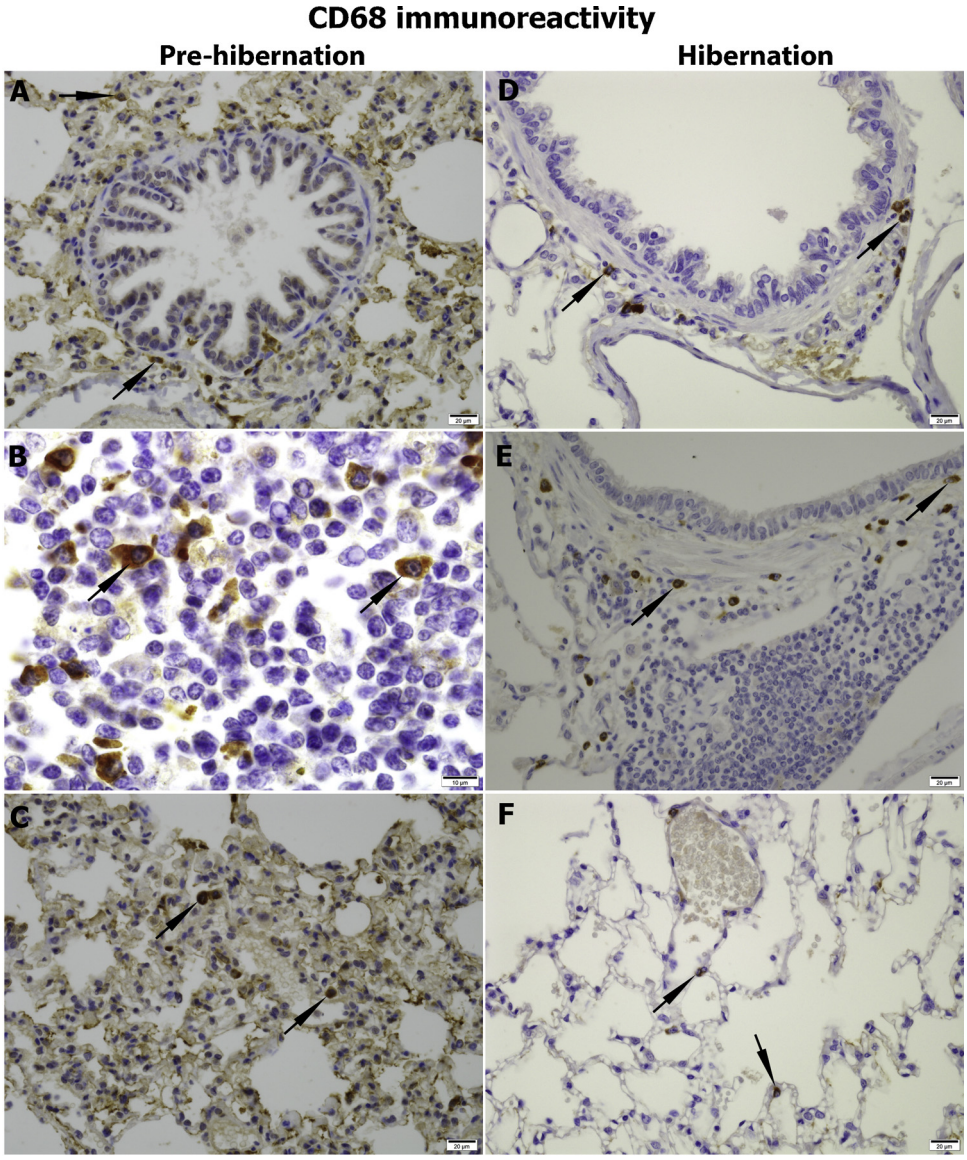



**Fig. 2.** Localization patterns of CD68 in the lung of Anatolian ground squirrels during the pre-hibernation and hibernation periods. Fig. 2a–c, Prehibernating squirrel lungs, Fig. 2d–f, Hibernating squirrel lungs. Lung sections were immunostained for CD68. CD68 positive cells were localized particularly to the subepithelial stroma of bronchi and bronchioles, and alveolar septa. Arrows indicate macrophages immunoreactive for CD68. Strept-ABC, AEC, Paraffin.

found mainly in the airway epithelium and smooth muscle, alveolar wall, and muscular media of the pulmonary vessels (Nardo et al., 1996). Furthermore, in rat lung, ANP immunoreactivity was largely found in bronchial and alveolar epithelial cells (Louzier et al., 2001). In agreement with these studies (Nardo et al., 1996; Louzier et al., 2001; D'Angelis et al., 2008), our findings corroborate the results of nuclear and cytoplasmic expression of ANP in the mucosal epithelium of terminal and respiratory bronchioles, bronchiole and vascular smooth muscle cells, Clara cells and ATII cells. Furthermore, the present immunohistochemical data demonstrates that ANP expression in the Anatolian squirrel lung was not significantly different between the pre-hibernation and hibernation periods.

A recent study has demonstrated that ANP may serve as an important epithelial-derived inhibitor of surfactant release in the fetal lung, and that downregulation of ANP following birth may make ATII cells sensitive to the effects of circulating catecholamines, thus easing surfactant secretion (Mathew et al., 2017). In lamb lungs, it was shown that ANP evokes surfactant release in isolated ATII cells from fetal lambs by a cGMP-dependent mechanism (D'Angelis et al., 2008). They reported that ANP expression in ATII cells is highest in late gestation and decreases sharply in the postpartum period. Hollenhorst et al. (2011) found that ATII cells participate in the synthesis and secretion of pulmonary surfactant and in the immune functions and play an important role in tissue repair process after lung injury by proliferation and migrating to injured areas. It has been demonstrated that the composition and surface properties of surfactants of the active and hibernating ground squirrels are largely different from porcine surfactant (Suri et al., 2013). These researchers suggested that the relatively more fluid surfactant films of hibernating squirrels may help to overcome collapse pressures and support low surface tension during the normal breathing cycle at low body temperatures. In the light of these literature reports, the presence of ANP immunoreactivity in ATII cells suggests that it may play a role in producing the surfactant during hibernation.

Talaei et al. (2011) demonstrated that structural changes occurring in the lung tissue of Syrian hamster during hibernation were reversible. These researchers found increased expression of α-smooth muscle actin (SMA), which is used as a marker for the proliferation of smooth muscle cells, in the hibernating hamster lungs in late torpor, suggesting that smooth muscle cells underwent hypertrophic process different from that under pathological conditions. Therefore, in the present study, the

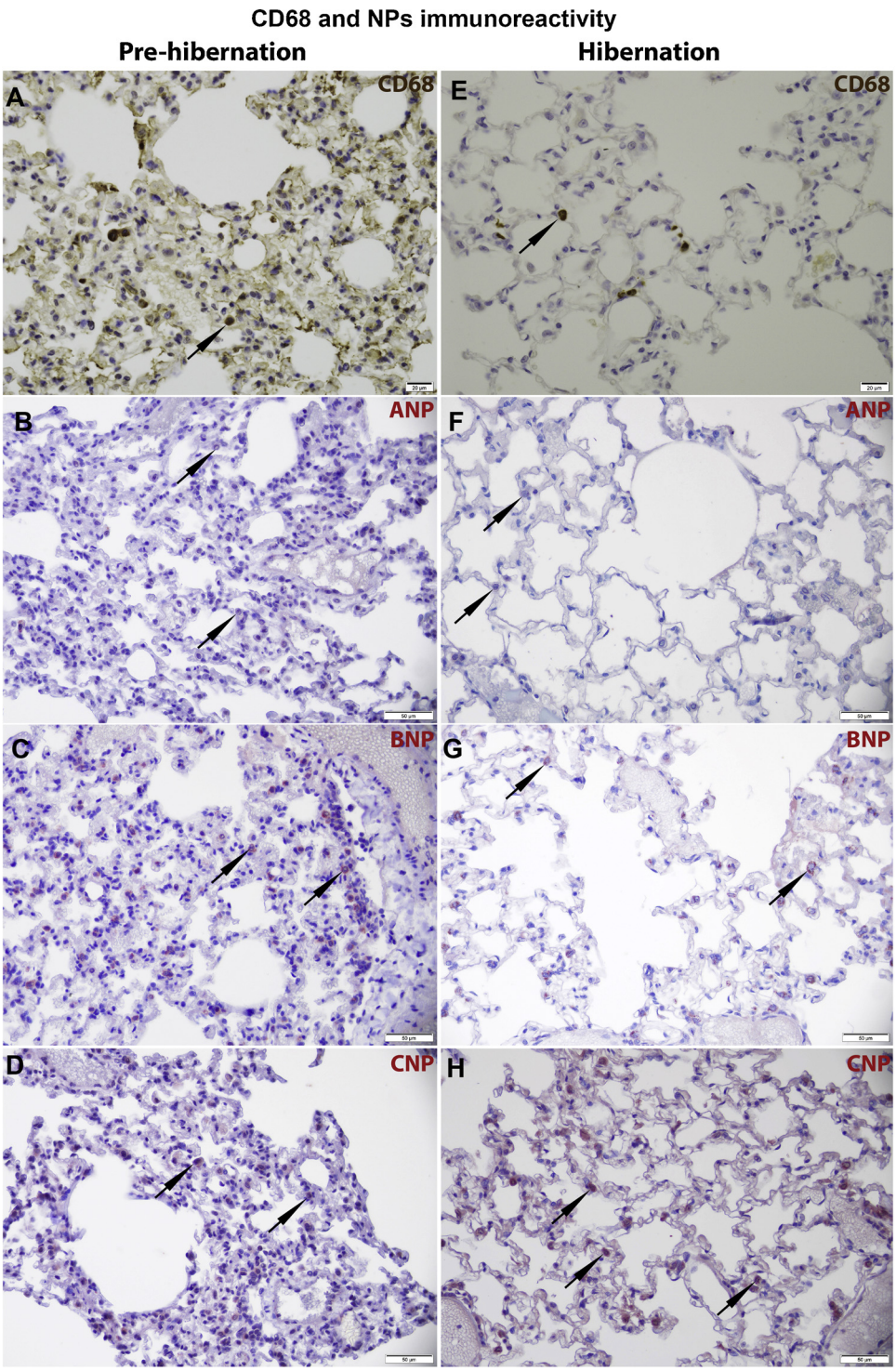



**Fig. 3.** Localization patterns of CD68, ANP, BNP and CNP in the lung of Anatolian ground squirrels during the pre-hibernation and hibernation periods. Fig. 3a–d, Prehibernating squirrel lungs, Fig. 3e–h, Hibernating squirrel lungs. Lung sections were immunostained for CD68, ANP, BNP and CNP. CD68 positive macrophages were observed in the lamina propria of terminal bronchioles, alveolar septa and lymphoid follicles of BALT. Interstitial cells staining with BNP and CNP showed a strong cytoplasmic immunoreaction while interstitial cells staining with ANP exhibited a weak immunoreaction. Arrows indicate positive staining cells. Strept-ABC, AEC, Paraffin.

presence of ANP protein in bronchiole and vascular smooth muscle cells of ground squirrel lung could point to its regulatory role in pre-hibernation and hibernation periods and, perhaps, it may have a dual role during entrance into torpor and active period in a way to facilitate lung function. However, it would be difficult to evaluate its role during hibernation in light of this information. Further experimental studies are needed.

BNP is a cardiac neurohormone synthesized by cardiomyocytes in the right and left ventricles (Mukoyama et al., 1991). However, the synthesis of BNP had also been found in rat (Dagnino et al., 1991), hamster (Cárnio et al., 1997) and human (Gerbes et al., 1994) lungs. Lung BNP may thus make a considerable contribution to the pool of circulating hormone. Ohsaki et al. (1999) reported that human small cell lung cancer cells produce BNP. However, no study has reported the localization of BNP in the squirrel lung tissues so far. Consistent with previous expression studies (Dagnino et al., 1991; Gerbes et al., 1994; Cárnio et al., 1997), our immunohistochemical results indicate that BNP protein is expressed in the mucosal epithelium of terminal and respiratory bronchioles, bronchiole and vascular smooth muscle cells, and ATII cells in ground squirrel lungs. Taken all together, one possible interpretation on these findings is that BNP is synthesized by ground squirrel lungs.

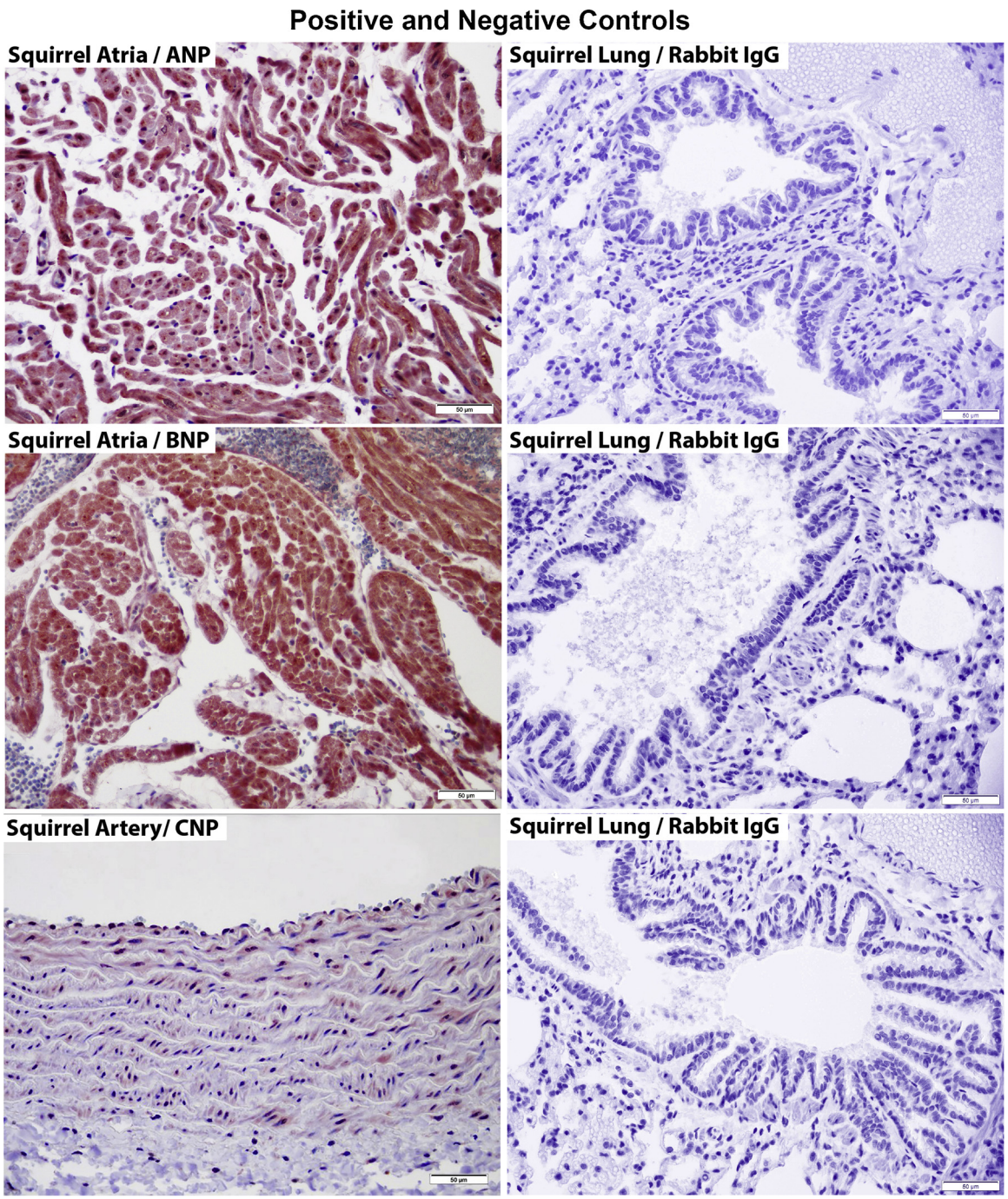



**Fig. 4.** Representative images of positive and negative control tissues. Positive immunostaining for ANP and BNP was detected in atrial cardiomyocytes (Fig. 4a and b), and positive CNP immunostaining was present in smooth muscle cells and endothelial cells (Fig. 4c). For negative controls, non-immune rabbit IgG is substituted for the primary antibody and no immunostaining for ANP (Fig. 4d), BNP (Fig. 4e) and CNP (Fig. 4f) was observed in any structure of lungs. Strept-ABC, AEC, Paraffin. Scale bars: 50 µm.

BNP has a key role in some biological functions, including bronchial relaxation. The levels of BNP have elevated in patients with pulmonary diseases such as chronic obstructive pulmonary disease (COPD), primary pulmonary hypertension, and thromboembolic pulmonary hypertension (Hargett and Tapson, 2005; Salerno and Marik, 2011). Elevated level of BNP in pulmonary disease has a regulatory effect on airway smooth muscle (ASM) (Matera et al., 2009). Therefore, BNP measurement is important for pulmonary hypertension recognition (Ruocco et al., 2015). Matera et al. (2013) found that cooperation between airway epithelium and smooth muscle cells is required BNP-dependent bronchorelaxant activity. In addition, Calzetta et al. (2014) demonstrated that BNP provides protection against bronchial hyperresponsiveness through an interaction between respiratory epithelium and ASM in asthmatic subjects. Akerman et al. (2006) reported that recombinant human BNP (nesiritide, which is used as a drug used for the treatment of decompensated heart failure) has potent bronchodilatory action in asthmatic patients. Our immunohistochemical data, which showed the presence of moderate to strong BNP immunoreactivity in the ciliated cells of epithelia lining terminal and respiratory bronchioles, and bronchiole and vascular smooth muscle cells, suggest that BNP produced by these lung cells may have a bronchodilatory and bronchorelaxant activity under hypoxic conditions of

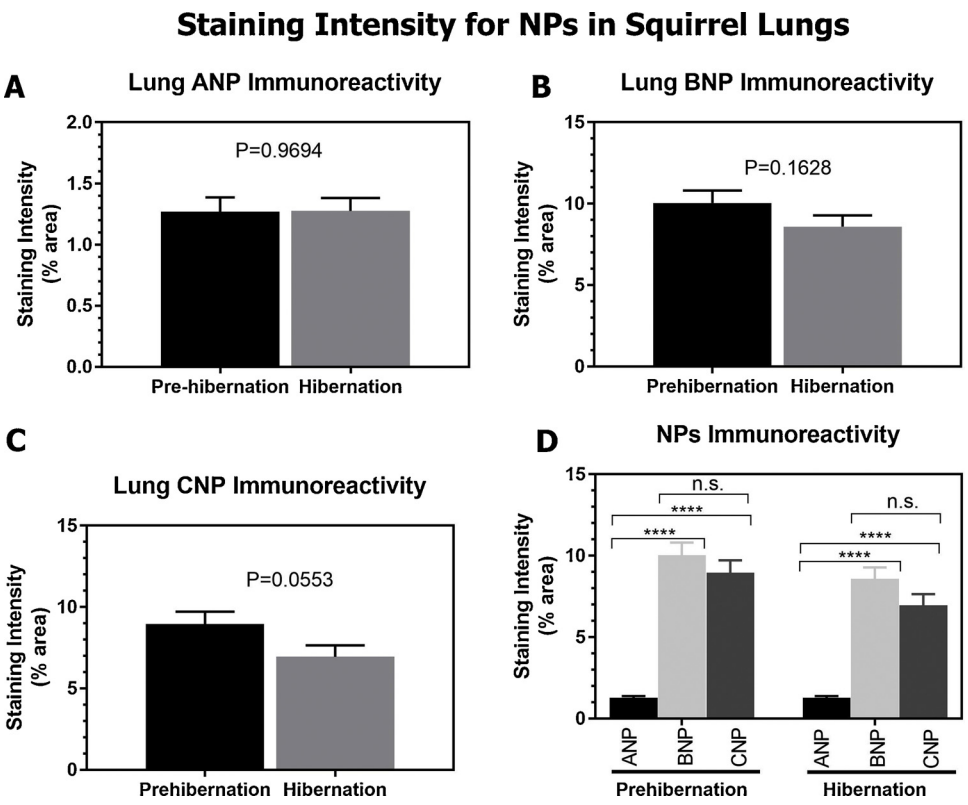



**Fig. 5.** Staining intensity of ANP, BNP and CNP in squirrel lungs. A, B and C indicate staining intensity of ANP, BNP and CNP as the percentage of staining area according to image analysis. D indicates comparisons between ANP, BNP and CNP in each period. Data are derived from 12 images per animal (n = 6) in each group. Error bar values represent SEM. A *t*-test was used in order to compare whether there is any significant difference in staining intensity of NPs between the prehibernation and hibernation conditions. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. n.s., not significant; ****p < 0.0001.

hibernation. However, our immunohistochemical findings cannot provide convincing evidence of how BNP functions in squirrel lungs. In addition, in the present study, we detected strong granular BNP immunostaining in the cytoplasm of ATII cells. This finding indicates that ATII cells have the capacity to synthesize BNP and they are an important source of BNP in the ground squirrel lung. Furthermore, we observed that while the number of BNP-immunopositive ATII cells tended to increase during the hibernation period, the immunostaining intensity remained relatively stable during the hibernation period. The simplest interpretation of these findings is that the amount of BNP synthesized by ATII cells in ground squirrel lung is on the rise, although not statistically significant, during hibernation. The enhanced amount of BNP may be important to increase the synthesis of surfactant during the normal breathing cycle at low body temperature throughout hibernation.

CNP is a neuropeptide found in a variety of tissues such as brain (Matsuo and Goeddel, 1991), heart, immune organs (Gerbes et al., 1993), endothelial cells and smooth muscle cells of blood vessels (Chen and Burnett, 1998), and intrapulmonary airway and alveolar epithelia of the lung (Nakanishi et al., 1999; Lakshminrusimha et al., 2001). Consistent with the above-mentioned literature reports, our immunohistochemical results indicate that the cell-specific localization of CNP protein in ground squirrel lung was similar to that of the BNP during prehibernation and hibernation periods. These findings provide strong evidence that various lung components in ground squirrel may all be capable of synthesizing CNP protein.

In the rat lung, CNP mRNA and protein were observed in the bronchial airway and alveolar epithelia and smooth muscle cells and endothelium of blood vessels during late gestation and on postnatal days 0–32. Furthermore, expression of CNP protein was found primarily in the non-ciliated Clara cells throughout the rat bronchiolar epithelium (Nakanishi et al., 1999). A study of fetal and newborn lamb lungs showed that immunostaining for CNP was lacking in all pulmonary epithelium at 100 days gestation, but marked CNP staining was seen in ATII cells, non-ciliated Clara cells of the distal airways, and ciliated epithelium of the upper airways at 136 days gestation (D'Angelis et al., 2006). Furthermore, these researchers demonstrated that, at both 3 days and 4 weeks following birth in the lamb lung, CNP immunostaining was absent in ATII cells and ciliated bronchial epithelium and was substantially diminished in Clara cells. When we looked at the terminal and respiratory bronchioles and alveolar localization of CNP protein, we observed that while the cytoplasm of ATII cells and the ciliated cells in the airway epithelium were strongly positive for CNP during both periods, the bronchiole smooth muscle cells exhibited weak immunostaining for CNP in pre-hibernation and moderate in hibernation. Furthermore, we found that the number of CNP-immunopositive ATII cells has a tendency to increase, if not statistically significant, during the hibernation period. These findings suggest that in ground squirrel lungs, the intensity of immunostaining for CNP can vary depending on cell type and life period. Furthermore, the observation that CNP is localized to alveolar type II cells and ciliated cells propounds that CNP synthesized by types of these cells is released into the alveolar space and acts on receptors on the epithelial cells of the terminal and respiratory bronchioles in ground squirrel lungs.

CNP serves as an endothelium-derived relaxing factor in the control of vascular tone. Additionally, it mediates vascular remodeling by inhibiting smooth muscle proliferation (Itoh et al., 1997). In line with the findings of Nakanishi et al. (1999) and Lakshminrusimha et al. (2001) presenting that CNP mRNA or protein was localized in pulmonary vascular endothelium and medial smooth muscle in the lungs of rats during late gestation and on postnatal days 0–32 and ovine fetuses during late-gestation, our immunohistochemical findings revealed weak CNP staining in the endothelium and its strong staining in the smooth muscles. This finding may indicate that vascular smooth muscles are the likely primary site of action of CNP. Lakshminrusimha et al. (2001) showed that exogenous CNP relaxes pulmonary veins and arteries and it is a more potent vasodilator in pulmonary veins than arteries in the fetal lamb lung at 135–150 days of gestation, but we did not compare the CNP immunoreactivity in the pulmonary arteries and veins. Therefore, based on our immunohistochemical data, we cannot assert whether exogenous CNP produces any effect on pulmonary vessels of ground squirrel lung. However, we observed that the intensity of

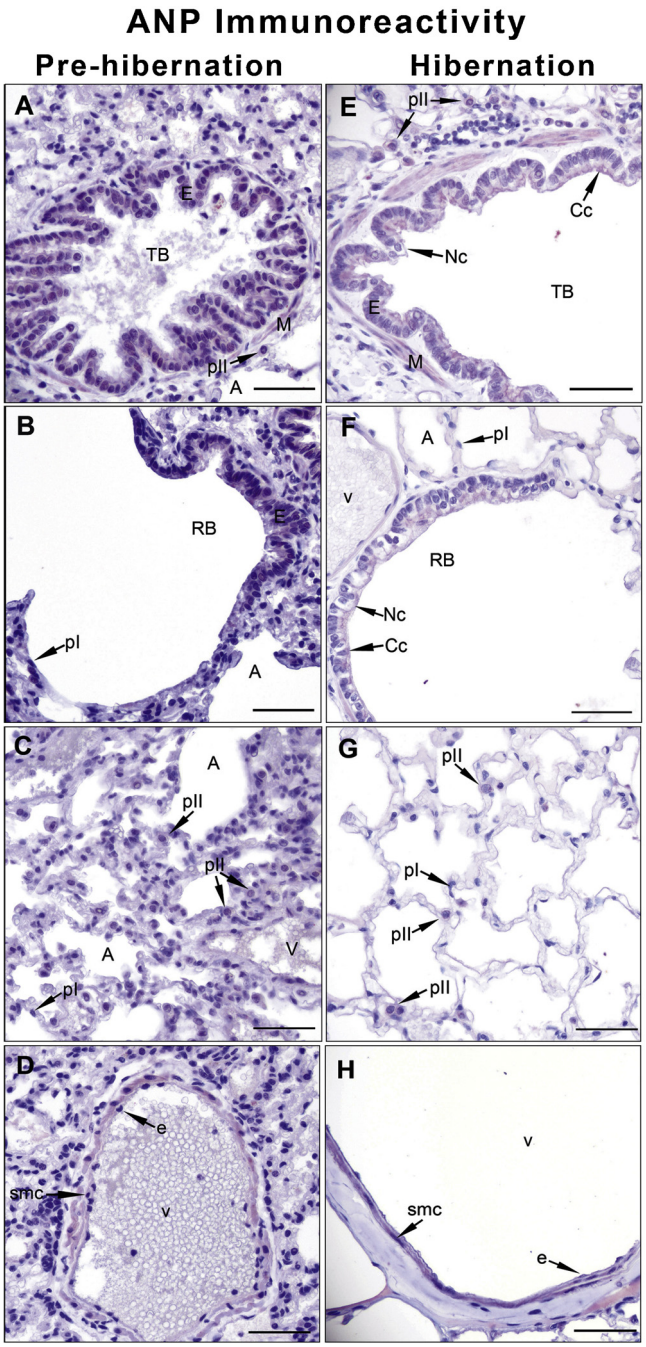



**Fig. 6.** Localization patterns of ANP in the lung of Anatolian ground squirrels during the pre-hibernation and hibernation periods. Fig. 6a–d, Prehibernating squirrel lungs, Fig. 6e–h, Hibernating squirrel lungs. Lung sections were immunostained for ANP. During the pre-hibernation and hibernation periods, weak cytoplasmic and nuclear ANP immunoreactivity was observed in the ciliated of the terminal (TB) and respiratory bronchioles (RB) (Fig. 6a, b, e, and f), while moderate cytoplasmic immunoreactivity was found in bronchial and vascular smooth muscle cells (Fig. 6a, e, d, and h). In alveoli (A) of lungs of prehibernating squirrels, ANP immunoreactivity was weak in the cytoplasm of ATII cells (pII), whilst it was absent in ATI cells (PI) (Fig. 6c). This immunoreactivity in ATII cells (pII) of hibernating animals' lungs was weaker than that observed in the pre-hibernating animals' lungs (Fig. 6g). Arrows indicate immunoreactive components. E, epithelium; M, muscle; Nc, Neuroendocrine cells; e, endothelium; v, vein; smc, smooth muscle cell; Cc, Clara cell. Strept-ABC, AEC, Paraffin. Scale bars: 50 µm.

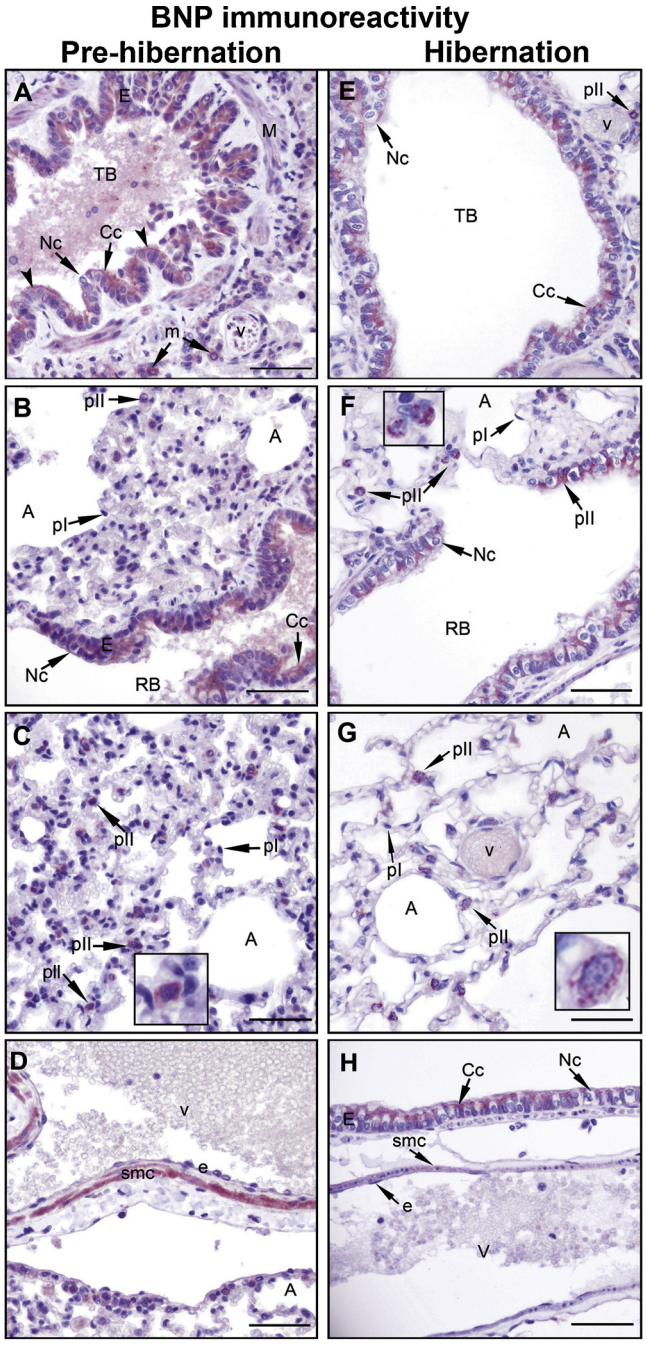



**Fig. 7.** Localization patterns of BNP in the lung of Anatolian ground squirrels during the pre-hibernation and hibernation periods. Fig. 7a–d, Prehibernating squirrel lungs, Fig. 7e–h, Hibernating squirrel lungs. Lung sections were immunostained for BNP. In both periods, the cilated cells of epithelia lining terminal and respiratory bronchioles (Fig. 7a, b, e, and f), and bronchial and vascular smooth muscle cells showed moderate to strong cytoplasmic immunoreactivity for BNP (Fig. 7d and h). Strong immunostaining for BNP was also observed at the point of ciliary attachment on the apical surface throughout in epithelia of the terminal bronchioles (Fig. 7a). In both periods, in alveoli, positive BNP immunostaining was clearly visible as a granular precipitate (insets) within the cytoplasm of ATII cells, but was absent in ATI cells (Fig. 7c, f and g). Although the number of BNP-immunopositive ATII cells increased during the hibernation period, the immunostaining intensity remained relatively stable during the hibernation period. Arrows indicate immunoreactive components. E, epithelium; M, muscle; Nc, Neuroendocrine cells; e, endothelium; v, vein; smc, smooth muscle cell; Cc, Clara cell; m, macrophage. Strept-ABC, AEC, Paraffin. Scale bars: 50 µm.

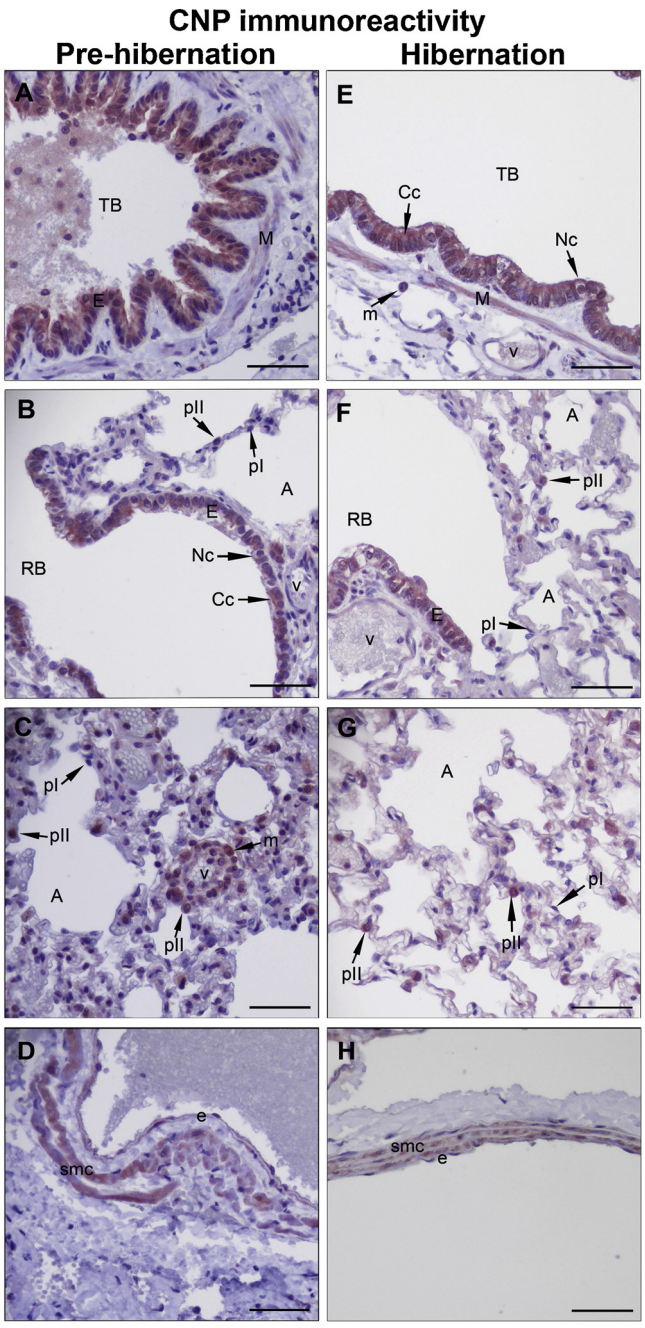



**Fig. 8.** Localization patterns of CNP in the lung of Anatolian ground squirrel during the pre-hibernation and hibernation periods. Fig. 8a–d, Prehibernating squirrel lungs, Fig. 8e–h, Hibernating squirrel lungs. Lung sections were immunostained for CNP. Strept-ABC, AEC, Paraffin. Scale bars: 50 µm. The bronchioli displayed CNP immunoreactivity in the nuclei and cytoplasm of the ciliated cells and the nuclei of non- ciliated Clara cells of the respiratory epithelium. In both periods, the cytoplasm of cilated cells in the epithelium of terminal and respiratory bronchioles was strongly positive for CNP (Fig. 8a, b, e, and f). The bronchial smooth muscle cells exhibited weak immunostaining for CNP in pre-hibernation (Fig. 8a) and moderate in hibernation (Fig. 8e). In alveoli, positive immunoreactivity for CNP was also observed in the nuclei and cytoplasm of ATII cells (Fig. 8c and g). In blood vessels, CNP immunoreactivity was weak in the endothelial cells and moderate to strong in smooth muscle cells (Fig. 8d and h). Arrows indicate immunoreactive components. E, epithelium; M, muscle; Nc, Neuroendocrine cells; e, endothelium; v, vein; smc, smooth muscle cell; Cc, Clara cell; m, macrophages. Strept-ABC, AEC, Paraffin. Scale bars: 50 µm.

CNP immunostaining in the blood vessels was not significantly changed during the pre-hibernation and hibernation periods. These results seem to support the notion that CNP activity in the blood vessel endothelial and smooth muscle cells in the ground squirrel lung has not already been induced during hibernation.

Previous studies demonstrated that ANP, BNP and CNP are important dilators of the pulmonary vessels during hypoxia, a condition that stimulates their synthesis (Klinger et al., 1997, 1998a, 1998b). Furthermore, it has shown that ANP might act on pulmonary arteries, and CNP might have an effect on pulmonary veins (Tolsa et al., 2002). In the present study, the presence of ANP, BNP and CNP proteins in the vascular smooth muscle cells in the lungs of ground squirrels may indicate that NPs are the most important factors causing vasodilation in pulmonary vessels of ground squirrels during pre-hibernation and hibernation periods.

In the lung, macrophages are divided into alveolar macrophages (AMs), which are found within the alveoli, and interstitial macrophages (IMs), which are found within the lung parenchymal tissue (Byrne et al., 2015). In order to identify macrophages in Anatolian ground squirrel lungs, we used the macrophage marker CD68 (Gibbings et al., 2017). We found that CD68 immunoreactivity was positive in the interstitial macrophages, but it was absent in alveolar macrophages. The quantity of pulmonary macrophages did not show marked difference between the pre-hibernation and hibernation periods of the Anatolian ground squirrel annual cycle. This finding is in agreement with previous report in the golden-mantled ground squirrel (Maniero, 2005). Sevareal studies demonstrated that macrophages express ANP, BNP and CNP and that all three peptides may be involved in modulation of immunological function (Vollmar and Schulz, 1995; Kiemer and Voolmar, 1997). Kiemer and Vollmar (2001) have reported that ANP regulated the production of inflammatory mediators in macrophages. In our study, we thought that CD68-positive macrophages, localized in the subepithelial stroma and follicles of BALT, could be the same cells immunoreactive for all NPs depending on their morphology and location.

It has been reported that cardiac ANP and BNP expression tends to increase in humans (Hassi et al., 1991) and rats (Mifune et al., 1993; Bordicchia et al., 2012) acclimatized to low environmental temperatures. If the same is true of hibernating conditions, an increase might be expected in pulmonary expression of these proteins. However, in the present study, NPs expression did not show a marked change in prehibernating and hibernating periods, supporting the notion that NPs production remained constant in cold conditions such as hibernation. In this regard, constant NP production may promote the hibernator lungs to acclimate to torpor conditions. On the other hand, maintenance of NP expression may aid rapid reversibility of lung remodeling caused by cold-exposure. It is most likely that this occurs through hypoxia-inducible factor 1 (HIF-1) whose expression increases depending on cold exposure (Rissanen et al., 2006) because hypoxia-response elements have been found in ANP (Chun et al., 2003) and BNP (Weidemann et al., 2008) gene promoter. This evidence confirms that at least ANP and BNP expression is controlled by HIF-1. Morin and Storey (2005) reported that HIF-1 mRNA and protein levels remain almost unchanged in the lungs of hibernating *S. tridecemlineatus* compared with pre-hibernating animals. This is consistent with our finding that ANP and BNP expression remained constant in both pre-hibernation and hibernation periods.

## 5. Conclusion

This study presents firstly the localization patterns of natriuretic peptides (ANP, BNP and CNP) in the Anatolian ground squirrel lungs during pre-hibernation and hibernation periods. Prehibernating ground squirrel lung morphology does not differ significantly from hibernating squirrel lung morphology. ANP, BNP and CNP are produced by the mucosal epithelium of terminal and respiratory bronchioles, smooth muscle cells in the lamina propria of terminal bronchioles and vascular

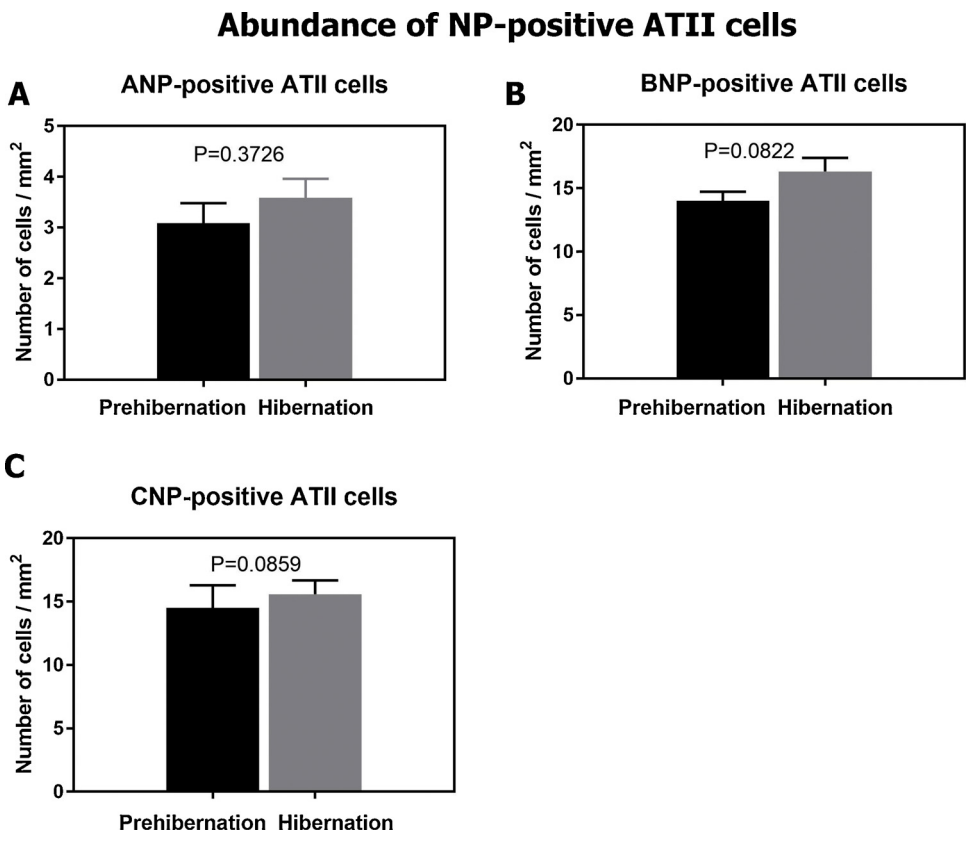



**Fig. 9.** Abundance of ATII cells positively-stained with ANP, BNP and CNP in squirrel lungs. In each of six serial sections per animal (n = 6) in both groups, the number of ATII cells was counted by two blinded observers for 5 randomly selected microscopic fields. A *t*-test was used in order to compare whether there is any significant difference in ATII cells stained with ANP, BNP and CNP between the pre-hibernation and hibernation conditions. The data are presented as the mean number of ANP, BNP and CNP positive ATII cell per $mm^2$. Error bar values represent SEM.

smooth muscle cells, and alveolar type II cells, as well as macrophages. The results of NPs expression patterns confirm that the expression of NPs is cell-specific, due to differences in localization and expression when compared to lung cell types during prehibernation and hibernation periods. These results also demonstrate that expression of NPs in the epithelium, bronchial smooth muscle cells, vascular endothelium and smooth muscle cells and macrophages is unaffected by hibernation. However, the number of ANP, BNP and CNP positive alveolar type II (ATII) cells tended to increase during the hibernation period. The present results have been encouraging enough to merit further investigation into the regulatory roles of NPs and their protective actions in the lungs during hibernation.

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## CRediT authorship contribution statement

M. Öztop, M. Özbek, F. Beyaz and N. Liman planned the study and performed the analysis of the results and interpretation of data. F. Beyaz, N. Liman, U.K. Kavraal and E. Ergen performed the animal experiments. M. Öztop, M. Özbek, E. Ergün and L. Ergün provided immunohistochemical reagents: M. Öztop and N. Liman prepared original draft and wrote the manuscript.

## Declaration of Competing Interest

All authors note no conflicts of interest relevant to this study.

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