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ORIGINAL ARTICLE

Localization profiles of natriuretic peptides in hearts of pre-hibernating and hibernating Anatolian ground squirrels (*Spermophilus xanthoprymnus*)

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Abstract

The Anatolian ground squirrel (*Spermophilus xanthoprymnus*) is a typical example of true mammalian hibernators. In order to adapt to extreme external and internal environments during hibernation, they lower their body temperatures, heart rates and oxygen consumption; however, pathological events such as ischemia and ventricular fibrillation do not occur in their cardiovascular systems. During the hibernation, maintenance of cardiac function is very important for survival of ground squirrels. Natriuretic peptides (NPs) are key factors in the regulation of cardiovascular hemostasis. Since NPs' role on the protection of heart during hibernation are less clear, the aim of this study was to investigate dynamic changes in NPs content in the cardiac chambers and to reveal the possible role of NPs on establishing cardiac function in ground squirrel during hibernation using immunohistochemistry. The immunohistochemical results indicate that cardiac NP expressions in atrial and ventricular cardiomyocytes were different from each other and were sex-independent. ANP and BNP were expressed in a chamber-dependent manner in female and male squirrel hearts. Furthermore, cardiac NPs expression levels in hibernation period were lower than those at the pre-hibernation period. During prehibernation period, ANP, BNP and CNP were expressed in the white and beige adipocytes of epicardial adipose tissue (EAT); while during hibernation period, the brown adipocytes of EAT were positive for BNP and CNP. These data suggest that the hibernation-dependent reduction in levels of NPs, particularly ANP, in cardiac chambers and EAT may be associated with low heart rate and oxygen consumption during hibernation. However, further studies are needed to better delineate the roles of NPs during the hibernation.

Keywords Natriuretic peptides · Hibernation · Anatolian ground squirrel · *Spermophilus xanthoprymnus* · Adipose tissue

Introduction

Hibernation is an energy saving strategy by various mammals to survive the winter under situations of low ambient temperatures and limited or no access to food (Hut et al. 2002; Melvin and Andrews 2009). Hibernating animals can remain dormant for months in a state called torpor, which is characterized by prominent changes in physiological parameters, such as a substantial reduction in the heart rate, ventilation rate, blood pressure, basal metabolic rate, body temperature and urinary output (Lyman and Chatfield 1955). During torpor, basal metabolic rate is reduced to 2–4% of normal rates and minimal body temperature falls to as low as −3 °C. Torpid states are regularly interrupted by shorter periods of full restoration of metabolism and body temperature, called arousals. Despite reduced body temperature, cardiac output, and tissue perfusion, data on hibernating animals have shown that the rapid rewarming during arousal did not lead to significant organ damage in the gut (Fleck and Carey 2005), kidneys (Zancanaro et al. 1999; Sandovici et al. 2004), brain (Arendt et al. 2003) or lungs (Talaei et al. 2011). Conversely, the reduced blood supply in humans and non-hibernating mammals leads to ischemia and organ injury in some clinical situations, including the organ transplantation, trauma, surgeries, cardiac arrest and stroke (Bouma 2013). Therefore, the hibernation is an excellent natural model of organ preservation. It poses an extreme

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physiological challenge for hibernators and confers a considerable survival advantage to them (Zancanaro et al. 1999; Green 2000). For this reason, the investigation of the molecular mechanisms of organ preservation during natural hibernation is extremely valuable (Ratigan and McKay 2016).

During the hibernation, the protection of heart is very important for the survival of hibernator animals. In hibernators, the cardiac muscle maintains a continuous and regular contraction function at low body temperature values of torpor, which contains extreme conditions, resembling ischemia and reperfusion (Milsom et al. 1999; Fedorov et al. 2005). In contrast, the hearts of non-hibernators are much more susceptible to cold. They exhibit arrhythmic patterns beginning at 32 °C, begin atrial fibrillation at 30 °C, and display increased cardiac ventricular excitability with asystole and cardiac arrest at 15 °C (Johansson 1996). Ground squirrel is one example of true mammalian hibernators and its heart exhibits tolerance of near 0 °C temperatures without falling into cardiac arrest (Luu et al. 2015). Typical ground squirrel hibernation is characterized by prolonged periods of torpor with significantly reduced heart rate, blood pressure, and blood flow. Therefore, it is an excellent model animal to study natural tolerance to myocardial ischemia and profound changes in cardiovascular function under unfavorable environmental conditions (Carey et al. 2003; Bonis et al. 2018). Ground squirrel has the ability to use lipids as metabolic fuel instead of burning carbohydrates, as humans do during surgeries. It gets ready for hibernation in autumn by overconsuming food with considerable weight gains of up to 40%, before shifting its metabolism towards oxidation of stored lipids throughout the long winter months when there is little or no food (Buck and Barnes 2000; Storey and Storey 2010). Many parameters, such as heart rate and body temperature, have been studied in this hibernating animal (Ignat'ev et al. 2001; Luu et al. 2015; Herinckx et al. 2017; Bonis et al. 2018).

The cellular and molecular mechanisms for the continued function of cardiac muscle at low body temperature values of torpor remain largely unknown. The heat production is an important physiological requirement for thermoregulation in a cold environment of hibernation (Cannon and Nedergaard 2004). Therefore, adipose tissue, which is a major metabolic organ, must remain responsive and retain its function at the low body temperature values of torpor, despite depressed metabolic activity. On the basis of the anatomical and developmental characteristics, the morphological structures and functions, the adipose tissue in mammals has been classified as white adipose tissue (WAT) or brown adipose tissue (BAT) (Giralt and Villarroya 2013). WAT contains the white adipocytes with unilocular lipid droplets and is the dominant metabolic fuel throughout the hibernation (Viscarra and Ortiz 2013). On the other hand, BAT consists largely of the brown adipocytes with multilocular lipid droplets and plays a role as a natural defense system against hypothermia in mammals (Lidell et al. 2014). It is a tissue responsible for adaptive non-shivering thermogenesis and is important in rewarming from deep torpor (Sidossis and Kajimura 2015). Recently, brown-like adipocytes, which are called beige or brite adipocytes, have been identified in WAT (Barbatelli et al. 2010; Lidell et al. 2014). There is no difference between the beige/brite and white adipocytes with unilocular lipid droplets, but they have a gene expression pattern distinct from that of either white or brown adipocytes (Lidell et al. 2014; Ballinger and Andrews 2018). The studies have also shown the presence of an adipose tissue called as epicardial adipose tissue (EAT) (Iacobellis et al. 2005), which demonstrates characteristics of beige adipocytes (Sacks et al. 2013). EAT is localized between the myocardial surface and visceral layer of the pericardium and it is a common histological component of the atrial myocardium. EAT exhibits thermogenic activity in a cold environment (Shabalina et al. 2013). In this way, it plays substantial roles in the protection of the myocardium against hypothermia (Sacks et al. 2009) and in energy supply to the myocardium (Iacobellis and Bianco 2011). It can also provide a mechanical protective role for coronary circulation (Prati et al. 2003; Fitzgibbons and Czech 2014). Recent studies have provided evidence that atrial EAT derives from adult atrial epicardial cells and the atrial natriuretic peptide (ANP), which is a cardiac natriuretic peptide (NP) secreted by atrial cardiomyocytes, is a major adipogenic factor (Suffee et al. 2017). Furthermore, it has recently been reported that cardiac natriuretic peptides stimulate a similar "browning" of human and mouse adipocytes (Bordicchia et al. 2012; Collins and Bordicchia 2013; Collins 2014; Suffee et al. 2017).

Natriuretic peptides (NPs) are structurally similar yet genetically distinct hormones which are mainly secreted from heart and participate in regulation of cardiovascular homeostasis and extracellular fluid volume (Woodard and Rosado 2008). The NP system consists of atrial natriuretic peptide (ANP), B-type natriuretic peptide (also known as brain natriuretic peptide, BNP) and C-type natriuretic peptide (CNP). These peptides exert their effects by binding to natriuretic peptide receptors (NPRs) including NPR-A, NPR-B and NPR-C on the surface of target cells in mammalian tissues. While ANP and BNP are primarily synthesized by the atrial and ventricular myocardium, respectively, in response to volume overload and increased tension in the wall (Nishikimi et al. 2006), both ligands specifically bind to NPR-A and exert hypotensive, diuretic, and natriuretic effects (Espiner et al. 1995). ANP and BNP is co-stored in specific atrial granules, whereas BNP is not stored in ventricular granules. Ventricular BNP production is transcriptionally regulated by the stretch of the cardiac wall (Potter et al. 2006). CNP is primarily expressed in neurons, endothelial cells and cardiac fibroblasts where it binds to NPR-B (Potter et al. 2006) and exerts its autocrine/paracrine roles for vascular tone and muscle cell growth (Woodard et al. 2002). CNP has been shown to be more potent than ANP for producing smooth muscle relaxation, but it has modest natriuretic and diuretic actions (Sudoh et al. 1990). It is likely that the cardiovascular effects of CNP are produced by local effects on blood vessels (Komatsu et al. 1992). Significant

progress has been made in understanding the distribution and localization of ANP and BNP in cardiac tissue of humans and various animals (Mifune et al. 1995, 1996, 2004; Biondo et al. 2003; Osman et al. 2004; Yamane et al. 2011), although little is known about the cardiac distribution and localization of CNP (Del Ry et al. 2011; Lu and Pan 2016; Öztop et al. 2018). Furthermore, there are a few reports on the presence of ANP in the plasma of active and hibernating marmots, *Marmota flaviventris* (Zatzman and Thornhill 1989) and in the heart of active and hibernating thirteen-lined ground squirrels, *Ictidomys tridecemlineatus* (Luu et al. 2015). However, it has not been carried out on any immunohistochemical research into the tissue localization of NPs in the heart of a hibernating mammal, Anatolian ground squirrel (*Spermophilus xanthoprymnus*).

Recent studies have shown the cardioprotective role of cardiac NPs, especially in heart disease (Rademaker and Richards 2005). The analysis of serum levels of ANP and BNP is used as biomarkers of cardiac health with diagnostic and prognostic applications in a variety of disorders (i.e. heart failure) affecting the cardiovascular system (Sagnella 1998; Sirithunyanont et al. 2003; Hu et al. 2015; Torres-Courchoud and Chen 2016). However, their plasma concentration does not always coincide with the quantitative characteristics of peptides in the heart (Mifune et al. 2012). Immunohistochemical analysis of ANP, BNP and CNP in the secretory granules of cardiomyocytes is one of the methods of determining synthesis intensity and release of NPs in a specific location. This method provides the possibility to evaluate the contribution of ANP, BNP and CNP to cardiovascular homeostasis in physiological and pathological conditions (Abrosimov et al. 2015).

The objectives of this study were to: 1) determine the distribution and localization patterns of NPs in the epicardial adipose tissue and different chambers of the Anatolian ground squirrel (*Spermophilus xanthoprymnus*) heart during the pre-hibernation and hibernation periods; 2) evaluate whether gender affects cardiac NPs expression, using immunohistochemistry. In the present study, determination of the distribution, localization and quantitative characteristics of ANP, BNP, and CNP immunoreactivity in ground squirrel heart will allow us to dissect the possible role of cardiac NPs in the maintenance of cardiovascular homeostasis under pre-hibernation and hibernation conditions.

Materials and methods

Animals ethics statement and experimental conditions

All the experimental protocols on animals were carried out in accordance with 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 24 female and male Anatolian ground squirrels (*Spermophilus xanthoprymnus*), which weighed 300–380 g, were used equally in the study. The exact age of the animals is unknown since animals were wild captured by Tomahawk Traps (Model #202, Tomahawk, WI, USA) from steppes in Develi, Kayseri, Turkey in late August. They were then transferred to Erciyes University, the laboratory of the Department of Histology and Embryology. Directly after capture, ground squirrels were quarantined a minimum of 7 days in cages with a constant ambient temperature of 21 °C under a 12 h light: 12 h dark cycle until used for experimental purposes in order to ensure they were good health. Then, they were divided into pre-hibernation [female ($n = 6$) and male ($n = 6$)] and hibernation (late torpor) [female ($n = 6$) and male ($n = 6$) experimental] groups. After anesthetizing with intraperitoneal xylazine and ketamine (11.1 mg of xylazine/ml and 88.9 mg of ketamine/ml) (Olson and McCabe 1986), the female and male ground squirrels in the pre-hibernation group were euthanized with cardiac puncture and the heart of each animal were quickly removed. In order to examine the localization of NPs in the atria and ventricles and epicardial adipose tissue, the heart samples were sectioned completely across the frontal plane. The samples from each heart were immersed into 10% formol-alcohol solution for 18 h at room temperature and were dehydrated through graded alcohols. After dehydration, the samples were cleared in methyl benzoate and benzol, and embedded in paraffin wax for immunohistochemical evaluation.

For hibernation group, squirrels were fed a standard rodent mix, sunflower seeds, fresh fruit and water ad libitum until body lipid stores were sufficient to enter 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

Table 1 Primary antibodies used for immunohistochemistry (IHC)

Antibody	Host	Immunogen	Catalogue no*	Application	Dilution	Species reactivity
ANP (FL-153) pAb	Rabbit	Amino acids 1–153 representing full length ANP of human origin	sc-20,158	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-20,159	IHC	1/100	Human
CNP (H-115) pAb	Rabbit	Amino acids 1–115 of CNP of human origin	sc-20,952	IHC	1/100	Human, rat, mouse

*All antibodies listed were obtained from Santa Cruz Biotechnology

ANP Atrial Natriuretic Peptide, *BNP* Brain Natriuretic Peptide, *CNP* C-type Natriuretic Peptide, *pAb* polyclonal antibody

Table 2 Semiquantitative scoring for specific immunostaining of NPs in the Anatolian ground squirrel (*Spermophilus xanthoprymnus*) heart during the pre-hibernation and hibernation periods

NPs	Periods	Sexes	Right atria	Left atria	Right ventricle	Left ventricle
ANP	Pre-hibernation	Female	+++	+++	+	+
		Male	+++	+++	+	+
	Hibernation	Female	+	++	–	–
		Male	+	+	–	–
BNP	Pre-hibernation	Female	+++	+++	++	+++
		Male	+++	++	++	+++
	Hibernation	Female	++	+	+	+
		Male	++	+	+	++
CNP	Pre-hibernation	Female	+++	+++	+++	+++
		Male	+++	+++	++	+++
	Hibernation	Female	+	++	+	+
		Male	+	+	+	+

Staining intensity: -, negative (no staining even at high magnification, 400X); +, weak staining (only visible at high magnification, 400X); ++, moderate staining (readily visible at low magnification, 100X); and +++, strong staining (strikingly positive at low magnification, 100X)

outside photoperiod from September 2016 until December 2016, the laboratory temperature was fallen to 6 °C and all the foods were removed from the cages and the lights were turned off completely to facilitate a natural transition into torpor. So as not to disturb torpid squirrels, a red safe light (3–5 lx) was used when entering the laboratory. The 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) three months after first entrance into torpor while they were in full torpid, hypothermic state. After sampling, the same tissue processing steps were followed for the experimental hibernating group.

Immunohistochemistry

Paraffin embedded sections cut at a thickness of 5 μm using a rotary microtome (Leica RM2125RT, Nussloch, Germany) were mounted on poly-L-lysine coated slides (Menzel Microscope slide Polysine, Thermo Fisher Scientific, Fremont, CA, USA) and overnight incubated in an oven at 37 °C. The streptavidin-biotin-peroxidase technique was performed for immunohistochemical staining, as previously described (Özbek et al. 2018). After incubating in the oven at 55 °C for half an hour next day, sections were deparaffinized with xylene and rehydrated through graded alcohols. After washing in phosphate-buffered saline (PBS, P4417, Sigma-Aldrich, Germany) pH 7.2 for 15 min, antigen retrieval was performed by boiling in 0.01 M citrate buffer (AP-9003-500, Thermo Fisher Scientific Lab Vision, Fremont, USA), pH 6.0, for 20 min in a microwave oven at full power. After cooling for 20 min, sections were incubated in 0,2% Triton X-100 in PBS for 15 min to increase the permeability. Sections were incubated in 3% H_2O_2 in distilled water for 20 min to quench endogenous peroxidase activity in a dark chamber, then rinsed for 15 min in PBS and drawn around sections using Dakopen. Non-specific binding was blocked with Ultra V Block (TA-125-UB, Ultra V Block®, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 10 min. After draining excess serum from the slides, sections were overnight incubated with primary antibodies (Table 1) at 4 °C. After washing in PBS for 15 min, the sections were incubated with biotinylated secondary antibody (TP-125-BN, Biotinylated Goat anti-Mouse & Rabbit IgG, Ready to Use, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 30 min at room temperature (RT) and rinsed again with PBS for 15 min. The resulting signal was developed with 3-amino-9-ethylcarbazole (TA-125-HA, AEC Chromogen & Substrate System, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA). Finally, sections were counterstained with Gill's hematoxylin and covered with aqueous mounting medium (TA-125-UG, Vision Mount, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA). All dilutions were as specified by the respective manufacturer's instructions. Normal rabbit IgG (sc-2027, Santa Cruz Bio Inc., Dallas, TX, USA) was substituted for primary antibody as the negative control. Rat heart was used as the positive control. Immunostaining was defined as the presence of red color on the periphery of a hematoxylin stained cell nucleus. All protocols were performed in a humidity chamber at room temperature unless stated otherwise.

Qualitative assessment of immunohistochemical staining

The stained sections were examined and photographed using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP74, Olympus, Tokyo, Japan). The immunostainings were assessed qualitatively using a four-points intensity score (IS). The staining intensities of NPs in

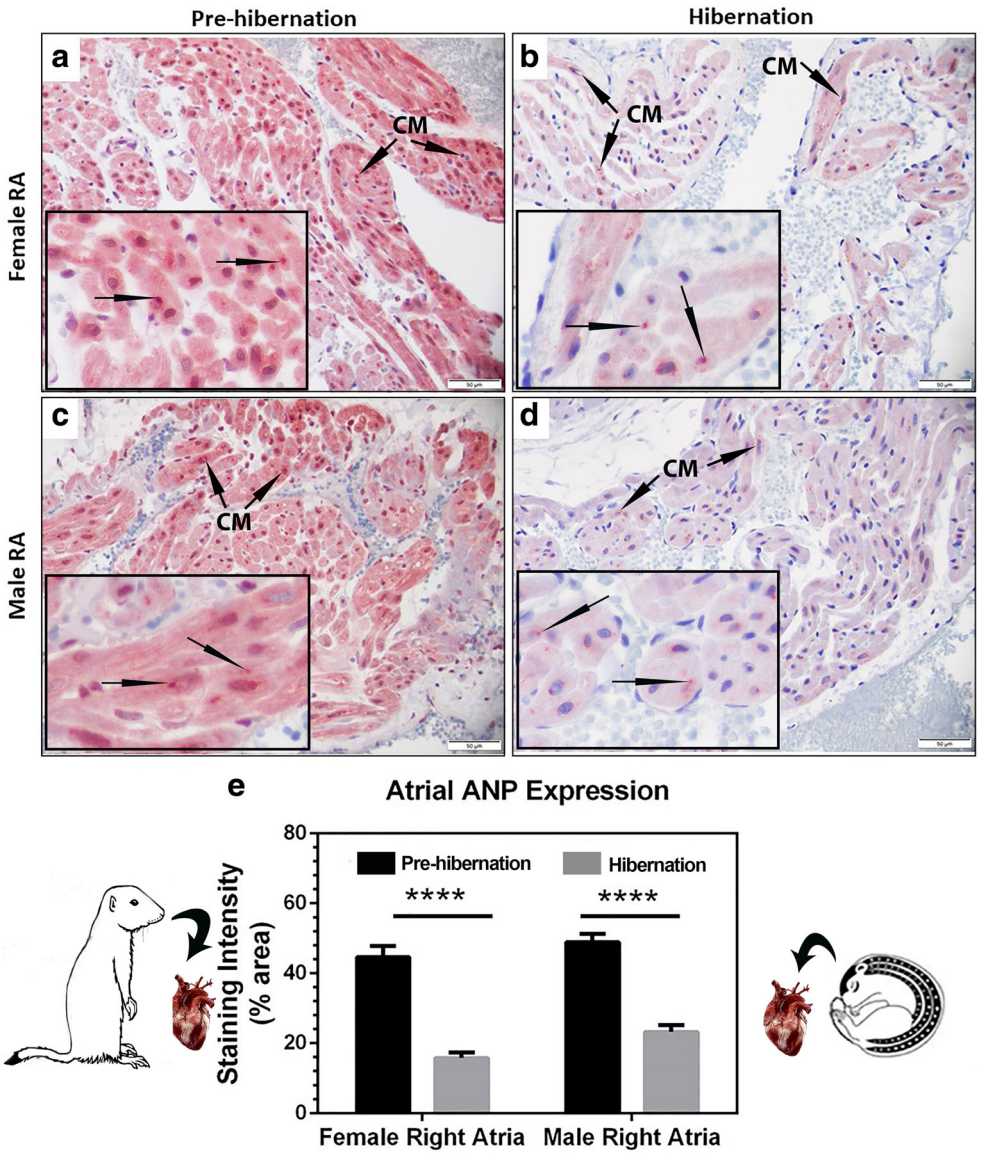


Fig. 1 The localization patterns (**a**–**d**) and staining intensity (**e**) of ANP in the right atria of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. ANP immunoreactivity was observed in the atrial granules (arrows) which are located near the nuclear poles. Insets: ANP immunolocalization can be seen in the atrial granules (arrows) of cardiomyocytes (CM). Both immunohistochemical results and image analysis data confirm that in the right atria of both sexes, ANP staining was more intense in the pre-hibernation period than in the hibernation period. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. ****$P < 0.0001$. Bars: 50 μm

the heart chambers were evaluated microscopically at 100x, and 400x magnifications. For this method, staining intensities of ANP, BNP and CNP were scored in the following manner: -, negative (no staining even at high magnification, 400X); +, weak staining (only visible at high magnification, 400X); ++, moderate staining (readily visible at low magnification, 100X); and +++, strong staining (strikingly positive at low magnification, 100X) (Liman 2017). Results of qualitative assessment are presented in Table 2.

Quantitative assessment of immunohistochemical staining

In this study, the staining intensities of ANP, BNP and CNP were determined quantitatively according to the method described by Jensen (2013). Quantitative analyzes of staining intensities for NPs were performed only in the images obtained from atria and ventricles. The immunostaining intensity was measured for an entire image. The images of atria and ventricles of the male and female squirrels during pre-hibernation and hibernation periods were captured at 400X magnification. For this, twelve images were used for each group. After importing relevant images into the software ImageJ 1.51 version (Java 1.8.0_112, https://imagej.nih.gov/ij/), they were submitted to the plug-in "color deconvolution" where the staining of hematoxylin and AEC was separated into 3 different panels with hematoxylin, with the only AEC image and background. Then, using the only AEC images, threshold values were set for acquired AEC images. After that, parameters of area and area fraction (percentage of staining area, namely "staining intensity") were set. Then, area and percentage of staining area were measured for each image.

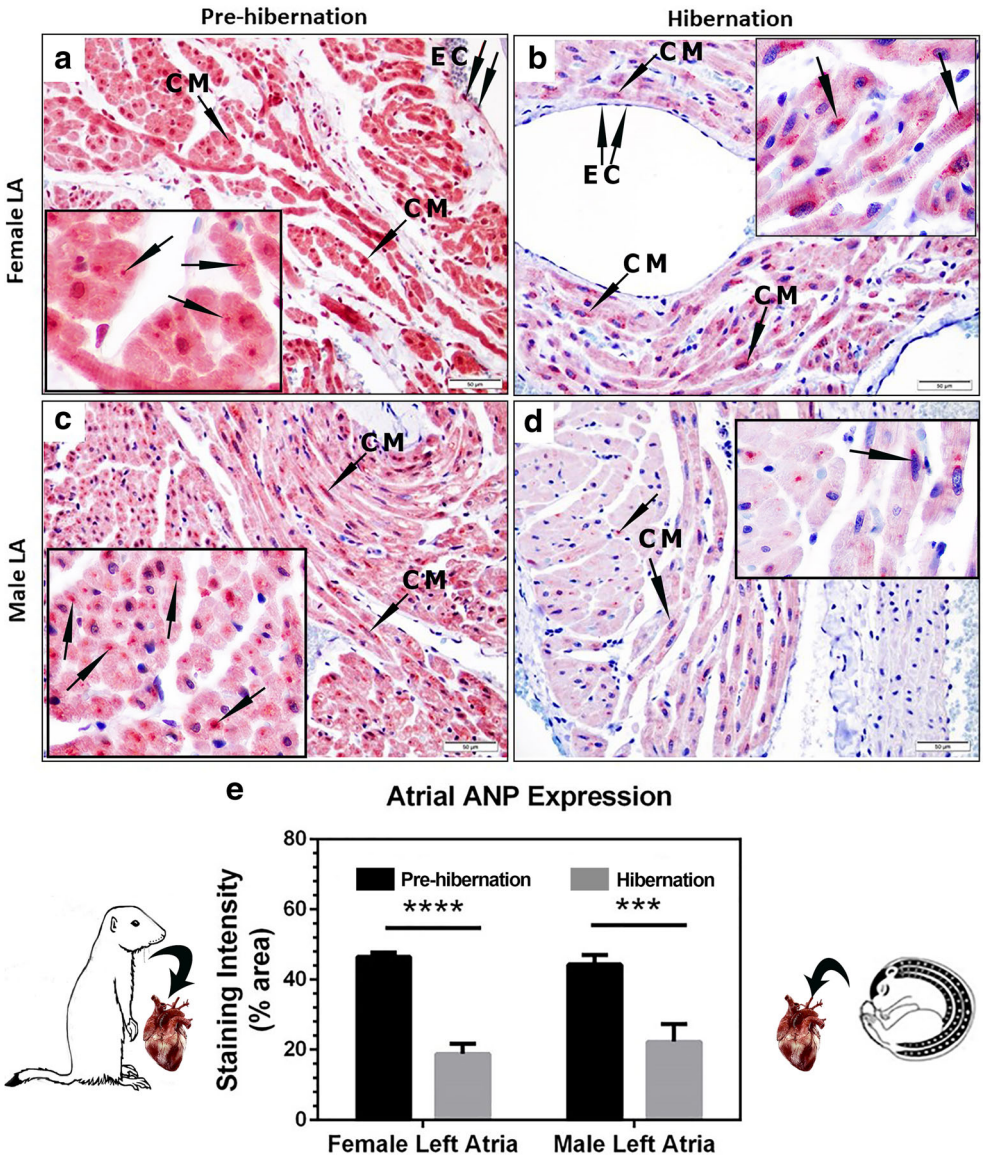


Fig. 2 The localization patterns (**a**–**d**) and staining intensity (**e**) of ANP in the left atria of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. ANP immunostaining (arrows) was observed in both the atrial granules of cardiomyocytes (CM) and endothelial cells (EC) during the prehibernation period, while it was only found in the atrial granules of cardiomyocytes during the hibernation period. The localization of ANP in the atrial granules is presented in the insets. Both immunohistochemical results and image analysis data confirm that in the left atria of both sexes, ANP staining was more intense in the pre-hibernation period than in the hibernation period. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. ***$P < 0.001$, ****$P < 0.0001$. Bars: 50 μm

Statistical analysis

Statistical analysis was performed using GraphPad Prizm 7 for Windows (Version 7.04) software. Results of the obtained percentage of staining area were imported into GraphPad Prizm 7 for Windows (Version 7.04) software in order to analyze quantitative data of staining intensity (% staining area). After that, two-way ANOVA with Bonferroni's multiple comparisons test was applied to determine whether there were any significant differences in image analysis quantifications for NPs between pre-hibernating and hibernation groups and between female and male animals in each period. All values are shown as the mean ± SEM. $P < 0.05$ was accepted as statistically significant.

Results

No immunostaining was detected in negative control sections when normal rabbit serum was substituted for the primary antibodies. The localization patterns of ANP, BNP, and CNP in the atria and ventricles of hearts of male and female Anatolian ground squirrels during the pre-hibernation and hibernation periods are summarized in Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The scores of immunostaining intensity for each NP in the atrial and ventricular cardiomyocytes of the heart are presented in Table 2. The localization patterns of all NPs in epicardial adipose tissue (EAT) and blood vessels are shown in Figs. 13 and 14, respectively.

Immunohistochemical staining of pre-hibernating and hibernating ground squirrel heart tissue of both male and female

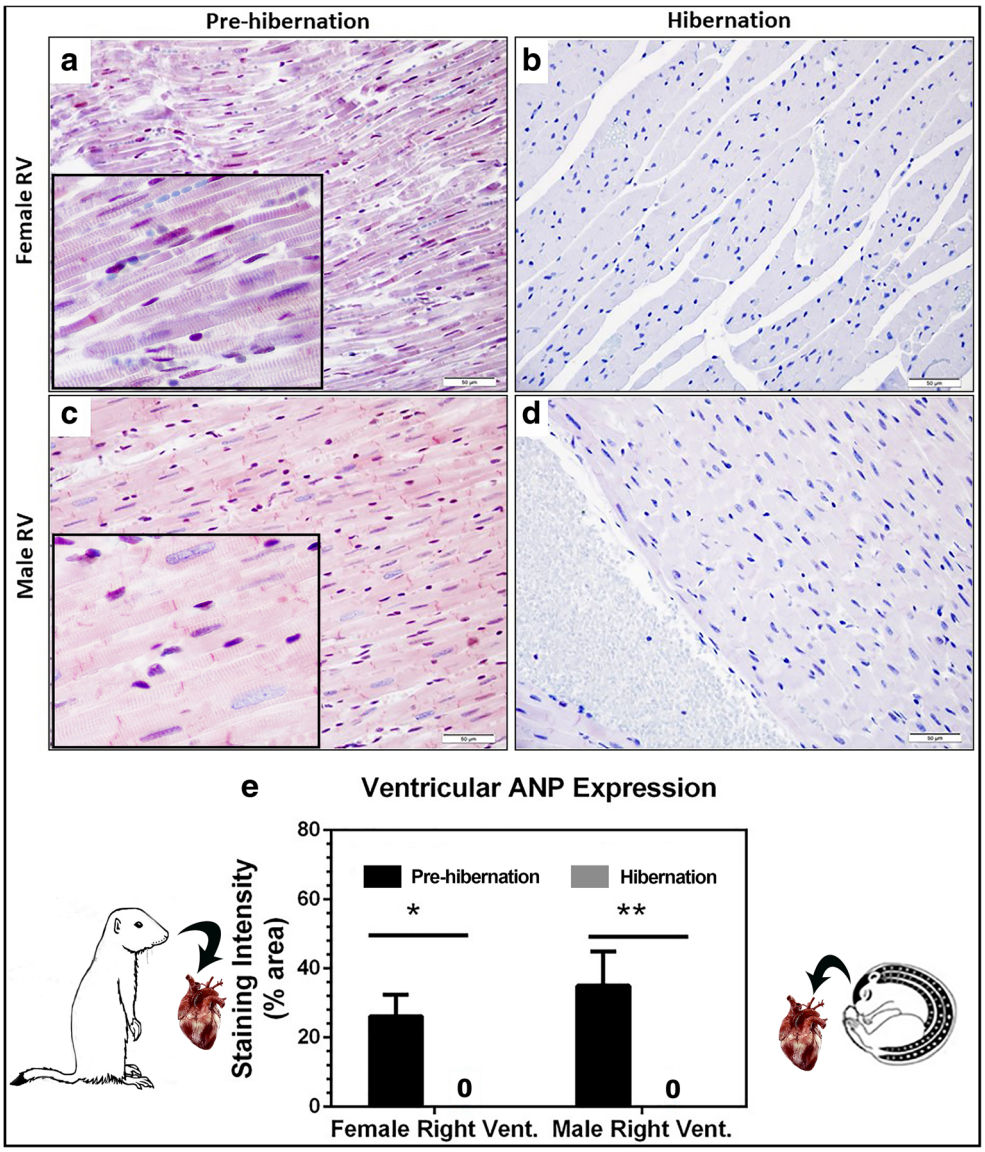


Fig. 3 The localization patterns (**a**–**d**) and staining intensity (**e**) of ANP in the right ventricles of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. Although weak ANP immunoreactivity was observed in right ventricles of pre-hibernating animals from both sexes (**a**, **c**), immunostaining hardly ever existed in those of hibernating animals (**b**, **d**). The localization of ANP in the ventricular cardiomyocytes is presented in the insets. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. 0 denotes almost no immunoreactivity. n.s., not significant; $*P < 0.05$, $**P < 0.01$. Bars: 50 μm

genders revealed different distribution patterns of ANP, BNP and CNP in the cardiomyocytes (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12), adipocytes (Fig. 13) and endothelial and smooth muscle cells of blood vessels (Fig. 14). In the atria of female and male animals, all of the investigated NPs were expressed in the cytoplasm of cardiomyocytes during pre-hibernation and hibernation periods. In atrial cardiomyocytes, ANP (Figs. 1 and 2) and BNP (Figs. 5 and 6) immunoreactivity was observed in the atrial granules which were located near the nuclear poles, whereas CNP immunoreactivity was in the nuclei and cytoplasm (Figs. 9 and 10). These immunostainings were more pronounced in pre-hibernating animals than in hibernating animals. On the other hand, the ventricular cardiomyocytes of pre-hibernating and hibernating ground squirrels exhibited cytoplasmic staining pattern for all NPs since secretory granules were not found in ventricular cardiomyocytes (Figs. 3, 4, 7, 8, 11 and 12).

The immunostaining scores of ANP, BNP and CNP in the atrial and ventricular cardiomyocytes were comparatively evaluated for the following: (a) right and left atrial cardiomyocytes in the pre-hibernation versus hibernation periods; (b) right and left ventricular cardiomyocytes in the pre-hibernation versus hibernation periods; (c) right and left atrial cardiomyocytes in female versus male in each period of life; (d) right and left ventricular cardiomyocytes in female versus male in each period of life; (e) atrial cardiomyocytes versus ventricular cardiomyocytes in male and female animals in each period of life. The results obtained for each of the proteins investigated are described below.

In the atrial and ventricular cardiomyocytes, ANP, BNP and CNP immunoreactivities were significantly higher during the pre-hibernation period than the hibernation period ($P < 0.05$) (Figs. 1e, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12e). However, the immunostaining scores of ANP, BNP, and

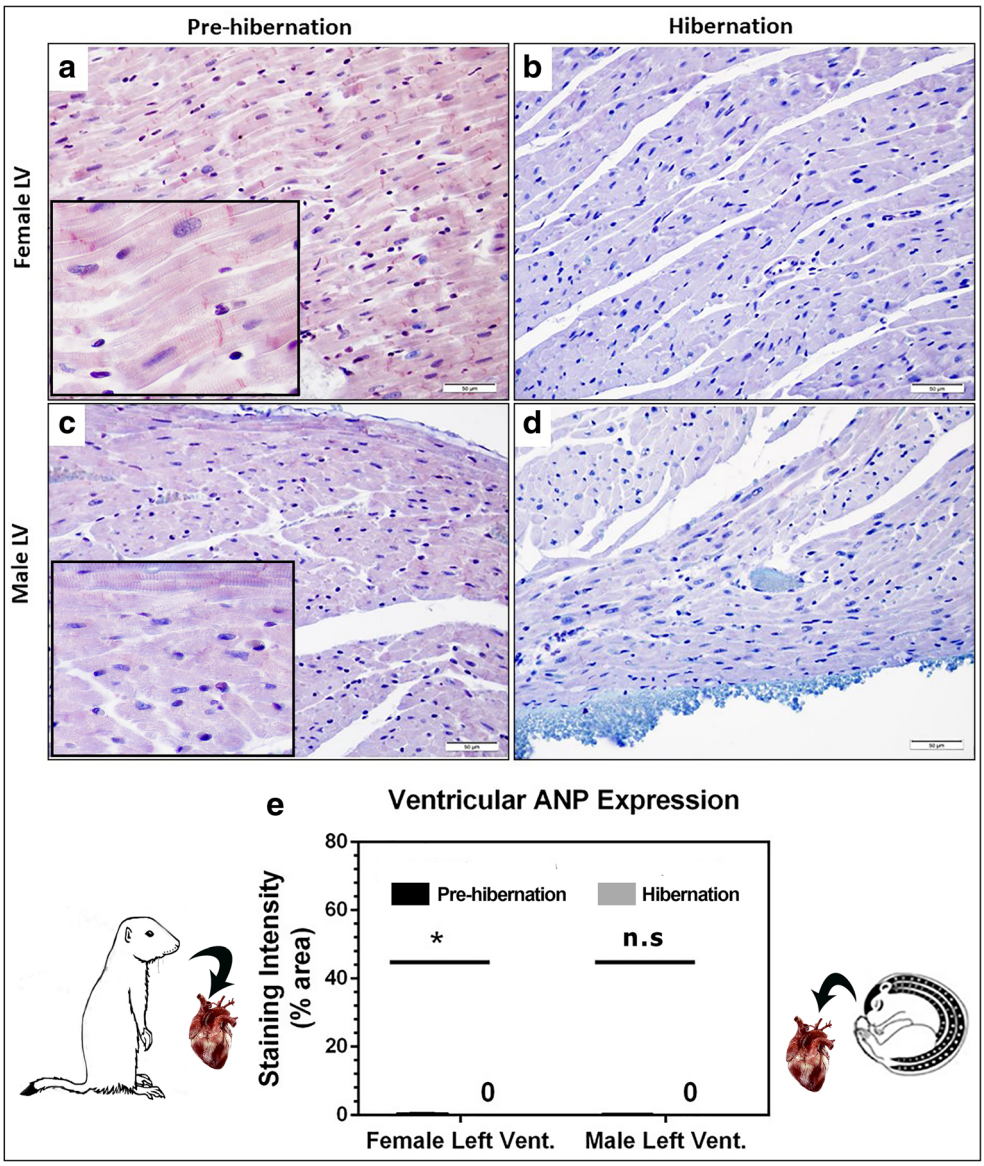


Fig. 4 The localization patterns (**a**–**d**) and staining intensity (**e**) of ANP in the left ventricles of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. Although weak ANP immunoreactivity is observed in left ventricles of pre-hibernating animals from both sexes (**a**, **c**), immunostaining hardly ever existed in those of hibernating animals (**b**, **d**). The localization of ANP in the ventricular cardiomyocytes is presented in the insets. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. 0 denotes almost no immunoreactivity. n.s., not significant; *$P < 0.05$. Bars: 50 μm

CNP in the atrial and ventricular cardiomyocytes did not differ between the female and male animals in each period ($P > 0.05$).

Immunolocalization of ANP in atria and ventricles

In atrial cardiomyocytes of female and male animals, ANP immunoreactivity was stronger during the pre-hibernation period (Figs. 1a, c and 2a, c) than the hibernation period (Figs. 1b, d and 2b, d). When the staining scores were evaluated by image analysis, it was found that the differences between pre-hibernation and hibernation periods were statistically significant in right (RA) ($P < 0.0001$) (Fig. 1e) and left atria (LA) of female ($P < 0.0001$) and male ($P < 0.001$) animals (Fig. 2e). Although weak ANP immunoreactivity was observed in ventricles of pre-hibernating animals from both sexes (Figs. 3a, c, 4a and c), immunostaining hardly ever existed in those of hibernating animals (Figs. 3b, d and 4b, d). These differences were statistically significant in female and male right ventricle (RV) ($P < 0.01$) (Fig. 3e) and in female left ventricle (LV) ($P < 0.05$) (Fig. 4e). However, the immunostaining score of ANP in the LV of male animals did not differ between the pre-hibernation and hibernation periods ($P > 0.05$) (Fig. 4e). Furthermore, the immunostaining intensity of ANP in the atrial cardiomyocytes was stronger than that in the ventricles in the pre-hibernating period.

Immunolocalization of BNP in atria and ventricles

The BNP exhibited moderate to strong cytoplasmic immunostaining pattern in the atrial cardiomyocytes of male and

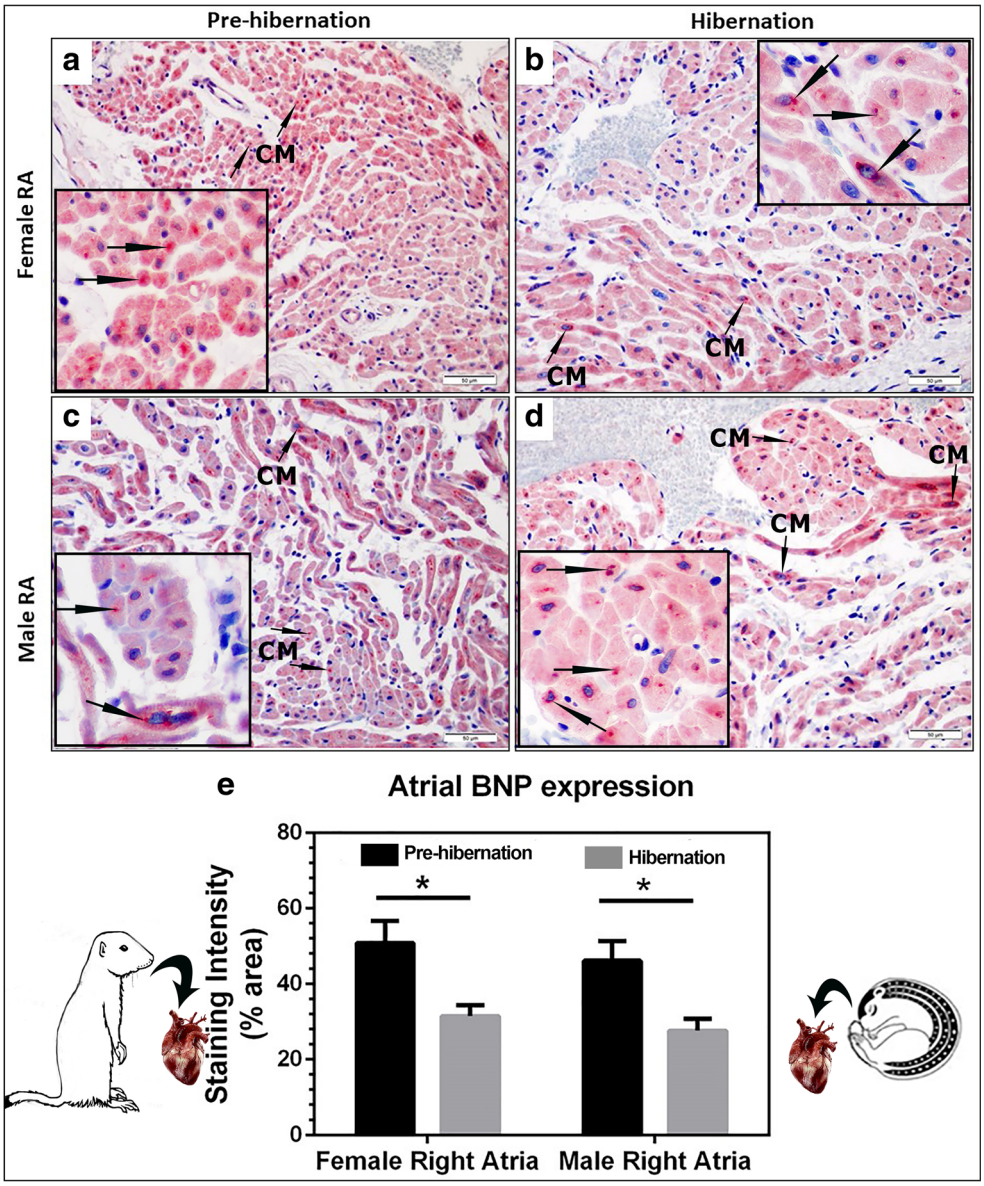


Fig. 5 The localization patterns (**a**–**d**) and staining intensity (**e**) of BNP in the right atria of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. BNP immunoreactivity was found in the atrial granules (arrows) which are located near the nuclear poles. Insets: BNP immunolocalization can be seen in the atrial granules (arrows) of cardiomyocytes (CM). Both immunohistochemical results and image analysis data indicate that in the right atria of both sexes, BNP staining was more intense in the pre-hibernation period than in the hibernation period. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. *$P < 0.05$. Bars: 50 μm

female animals (Figs. 5 and 6). In the right and left atrial cardiomyocytes of both sexes, the immunostaining score of BNP was significantly higher during the pre-hibernation period than the hibernation period ($P < 0.05$ for female and male RA, and for female LA; $P < 0.01$ for male LA) (Figs. 5e and 6e). In pre-hibernating female and male animals, BNP staining was moderate in the cardiomyocytes of RVs (Fig. 7), while it was strong in the cardiomyocytes of LVs (Fig. 8). Furthermore, in the right and left ventricular cardiomyocytes of male and female ground squirrels, BNP immunoreactivity was significantly higher during pre-hibernation period than the hibernation period ($P < 0.05$ for female and male RV, and for male LV; $P < 0.01$ for female LV) (Figs. 7e and 8e). BNP immunoreactivity was prominent in the epicardial regions of both pre-hibernating (Fig. 8c) and hibernating (Fig. 8d) male animals.

Immunolocalization of CNP in atria and ventricles

In the atrial cardiomyocytes of both sexes, cytoplasmic CNP staining was stronger in pre-hibernating squirrels (Figs. 9a, c, 10a and c) than the hibernating animals (Figs. 9b, d and10b, d), while nuclear CNP immunostaining was absent during the hibernation period. Furthermore, immunostaining score of CNP in atrial cardiomyocytes showed important difference between the pre-hibernation and hibernation periods in the male and female animals ($P < 0.05$ for male RA and female LA; $P < 0.01$ for female RA; $P < 0.001$ for male LA) (Figs. 9e and 10e). In the female and male squirrels, CNP immunolabelling in the ventricular cardiomyocytes was stronger during the pre-hibernation period (Figs. 11a, c, 12a, and c) than the hibernation period (Figs. 11b, d and 12b, d) and the differences in the immunostaining intensity for CNP between

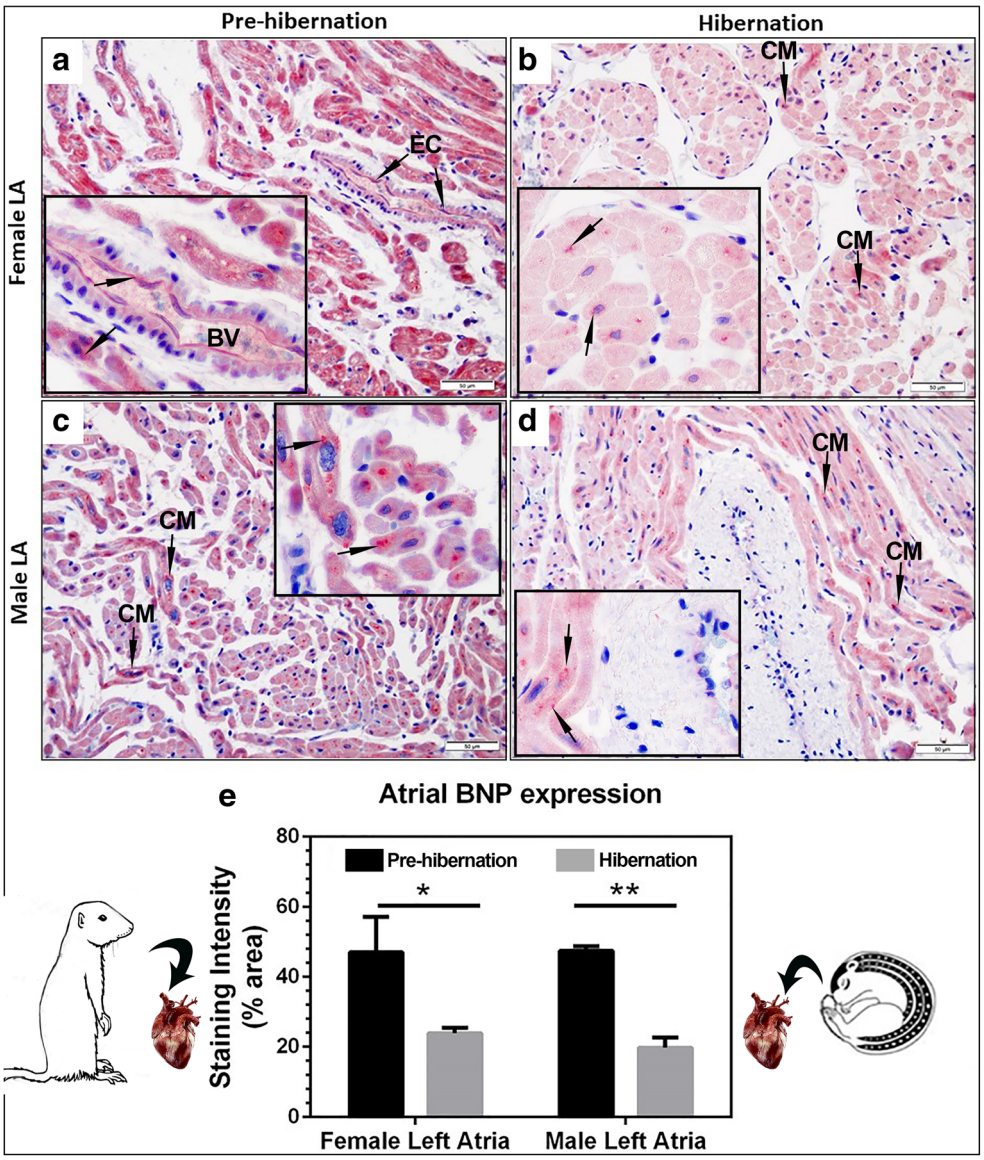


Fig. 6 The localization patterns (**a**–**d**) and staining intensity (**e**) of BNP in the left atria of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. The localization of BNP in the atrial granules and endothelial cells is presented in the insets. BNP immunostaining (arrows) was observed in both the atrial granules of cardiomyocytes (CM) and endothelial cells (EC) of blood vessels during the prehibernation period, while it was only found in the atrial granules of cardiomyocytes during the hibernation period. Both immunohistochemical results and image analysis data confirm that in the left atria of both sexes, BNP staining was more intense in the pre-hibernation period than in the hibernation period. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. *$P < 0.05$, **$P < 0.01$. Bars: 50 μm

pre-hibernating and hibernating animals were statistically significant ($P < 0.001$ for male RV; $P < 0.0001$ for female RV and for female and male LV) (Figs. 11e and 12e). Weak CNP staining was observed in the endocardial endothelial cells of pre-hibernating animals, but not of hibernating animals.

Immunolocalization of ANP, BNP and CNP in epicardial adipose tissue (EAT)

In the present study, we observed that female and male ground squirrel hearts have EAT, localized between the atrial and ventricular chambers (the atrial–ventricular groove) on the dorsal to dorsolateral sides of the heart. Histologically, this tissue was subepicardial and contained three different types of adipocytes (white, beige/brite and brown adipocytes) and blood vessels. The white adipocytes possessed unilocular lipid droplets with an eccentric nucleus, while the beige/brite and brown adipocytes had multilocular lipid droplets with a central nucleus. In the pre-hibernation period, the EAT was composed of white adipose tissue (WAT) that contains the white and beige adipocytes, while it consisted of brown adipose tissue (BAT) that contain the brown adipocytes in the hibernation period. Furthermore, in the hibernation period, brown adipose tissue was characterized by rich vasculature (Fig. 13b, d and f). In the pre-hibernation period, the epicardial white and beige adipocytes surrounding the left and right atria of both sexes exhibited strong immunostaining for ANP (Fig. 13a), while they displayed moderate to strong BNP immunolabelling (Fig.13c). However, variations for CNP immunolabelling were observed in the epicardial adipocytes of the right and left atria of both sexes. CNP immunolabelling in the epicardial white and beige adipocytes surrounding the right atria of both sexes was weak to moderate, although it was moderate to strong in these adipocytes surrounding the left atria (Fig. 13e). In the hibernation period, there was no ANP staining (Fig. 13b),

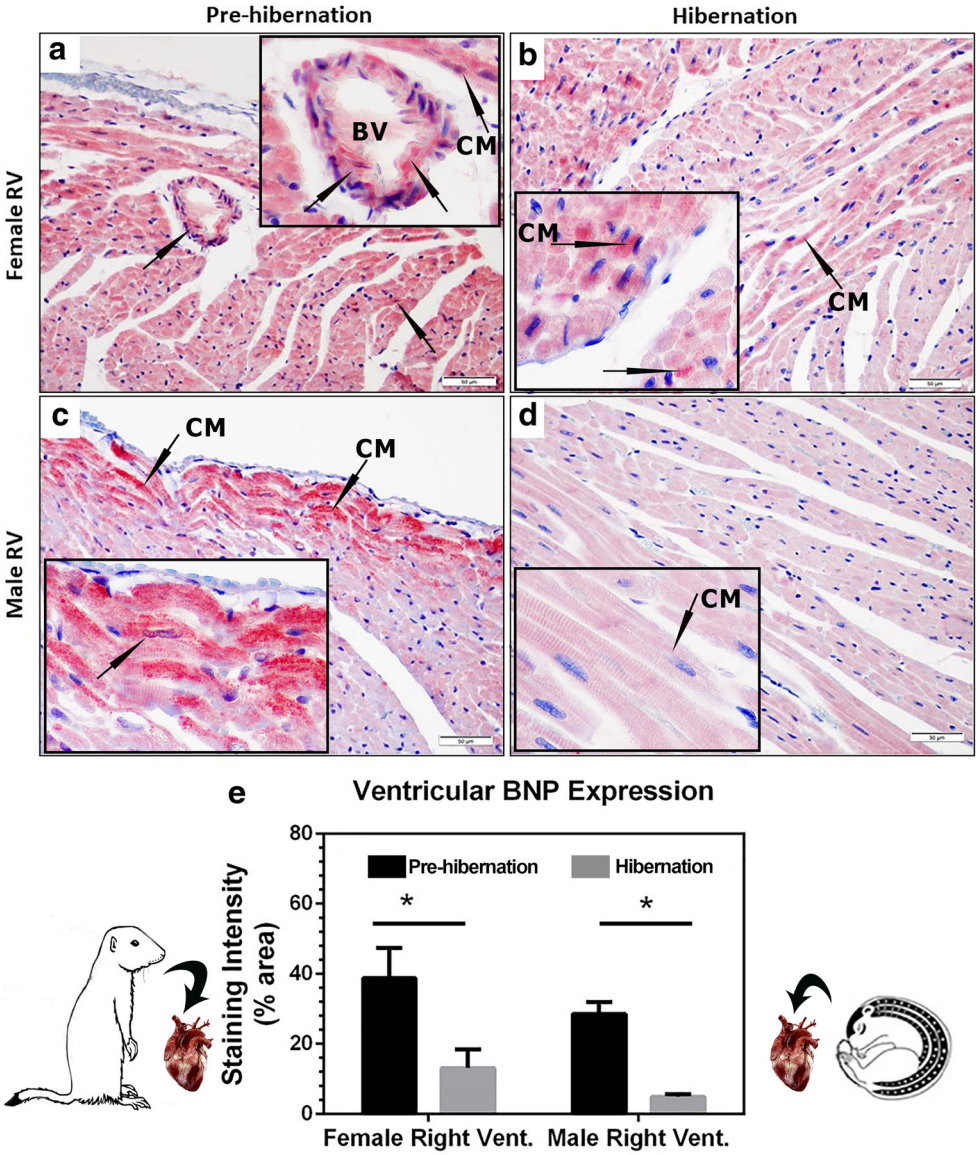


Fig. 7 The localization patterns (**a**–**d**) and staining intensity (**e**) of BNP in the right ventricles of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. The localization of BNP in the ventricular cardiomyocytes is presented in the insets. The ventricular cardiomyocytes exhibited cytoplasmic staining pattern for BNP. In general, this immunostaining was moderate in the cardiomyocytes (CM) of the right ventricles. However, epicardial cardiomyocytes showed a strong BNP immunoreactivity (inset of C). Furthermore, BNP immunostaining was observed in the vascular wall (inset of A). Both immunohistochemical results and image analysis data confirmed that in the right ventricles of both sexes, BNP staining was more evident in the pre-hibernation period than in the hibernation period. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. *$P < 0.05$. Bars: 50 μm

while moderate BNP (Fig. 13d) and CNP (Fig. 13f) immunolabelling was observed in the epicardial brown adipose tissue surrounding the left and right atria from both sexes.

Immunolocalization of ANP, BNP and CNP in blood vessels

In the pre-hibernation period, weak immunostaining for ANP and BNP was observed in the endothelial and smooth muscle cell layers of the elastic artery (Fig. 14a, c). In the hibernation period, there was no immunoreaction to ANP (Fig. 14b), while moderate BNP staining was observed in the endothelial cells, but not in the smooth muscle cell layers of the elastic artery (Fig. 14d). Interestingly, moderate CNP staining was observed in the endothelial and smooth muscle cell layers of the elastic artery in both pre-hibernating and hibernating animals (Fig. 14e and f). None of the cells of tunica adventitia showed any immunoreactivity for each NPs in both pre-hibernation and hibernation. Generally, it was not observed any sex-dependent difference between NPs immunoreactivity in both endothelial cells and smooth muscle cells during pre-hibernation and hibernation (not shown data).

Discussion

This study is the first to investigate the sex-, cardiac chamber- and hibernation- dependent changes in the amount and localization of natriuretic peptides - ANP, BNP and CNP - in the hearts of pre-hibernating and hibernating female and male Anatolian ground squirrels. Our immunohistochemical findings indicated that there were no significant differences in the immunostaining intensity and localization pattern of NPs among the female and male ground squirrel atria and

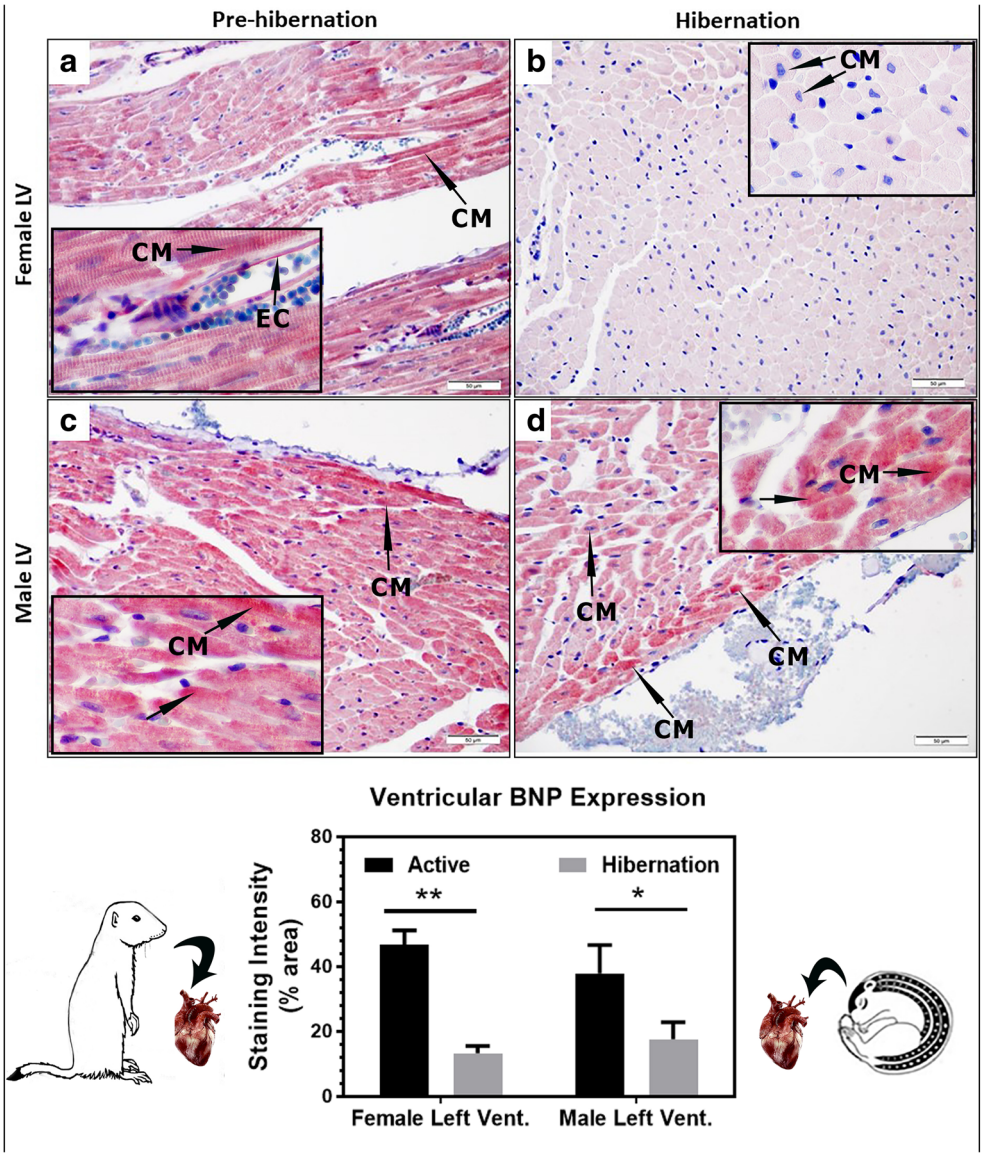


Fig. 8 The localization patterns (**a**–**d**) and staining intensity (**e**) of BNP in the left ventricles of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. The localization of BNP in the ventricular cardiomyocytes is presented in the insets. The ventricular cardiomyocytes exhibited strong cytoplasmic staining pattern for BNP during pre-hibernation period. Both immunohistochemical results and image analysis data confirmed that in the right ventricles of both sexes, BNP staining was more evident in the pre-hibernation period than in the hibernation period. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. $^{*}P < 0.05$, $^{**}P < 0.01$. Bars: 50 μm

ventricles. Furthermore, our immunohistochemical data indicate that ANP, BNP and CNP were differentially localized in atrial and ventricular cardiomyocytes of both female and male squirrels during the pre-hibernation and hibernation periods. In agreement with the reports available for rat (Nemer et al. 1986), our results showed that ANP is localized in atrial cardiomyocytes in the form of granular deposits and in the cytoplasm of ventricular cardiomyocytes of Anatolian ground squirrels. This contrasts with the distribution of ANP in healthy mammalian species, wherein ANP is only restricted to the cytoplasmic granules of atrial cardiomyocytes (including the auricles) (Chapeau et al. 1985; Colbatzky et al. 1993; Mifune et al. 1995, 1996; Cameron et al. 2000; Biondo et al. 2003; Osman et al. 2004). Our findings also indicated that the immunostaining intensity of ANP in the atria was stronger than the ventricles in the prehibernating period. This finding is in agreement with the information that the atrium is the main site of synthesis for ANP within the heart (Chapeau et al. 1985; Federico 2010). However, ventricular ANP production in ground squirrel hearts may also be physiologically significant in view of the much larger ventricular mass. Our results have also shown that, in hibernating ground squirrel hearts, ANP immunostaining in atria was reduced compared to the prehibernating animals and it was not found in ventricles. Although the physiological importance of this finding cannot be fully explained, the decreases in atrial and ventricular ANP immunostaining may be particularly important in preventing cardiac tissue damage during hibernation.

Previous studies have shown that the localization of BNP in the heart of human and animals is different. With the exception of ventricular cardiomyocytes of mice (Nakagawa et al. 1995) and rats (Ogawa et al. 1991; Thibault et al. 1992), BNP was not found in the ventricle of most species that have been studied (Mifune et al. 1995; Cameron et al.

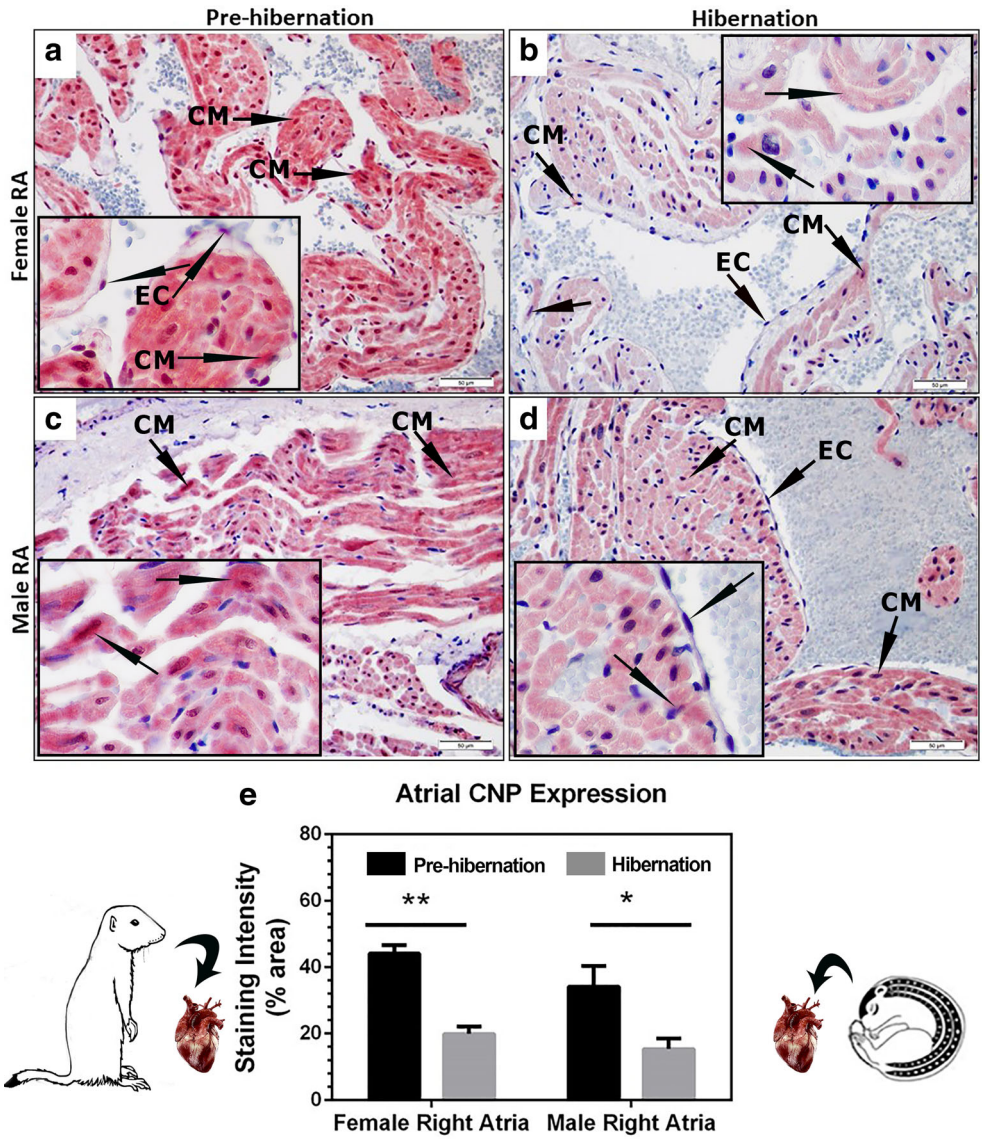


Fig. 9 The localization patterns (**a**–**d**) and staining intensity (**e**) of CNP in the right atria of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. The localization of CNP in the atrial cardiomyocytes (CM) and endothelial cells (EC) is resented in the insets (arrows). The atrial cardiomyocytes (CM) exhibited nuclear and cytoplasmic staining patterns for CNP (arrows). Both immunohisto-chemical results and image analysis data indicate that in the right atria of both sexes, cytoplasmic CNP staining was more intense in the pre-hibernation period than in the hibernation period. Furthermore, during hibernation period, CNP immunoreactivity was absent in the nucleus of the cardiomyocytes and in the cytoplasm of endothelial cells. **e** indicates staining intensity as the percentage of staining area according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. $*P < 0.05$, $**P < 0.01$. Bars: 50 μm

2000; Biondo et al. 2003). Its localization in humans is still controversial (Yasue et al. 1994; Luchner et al. 1998). However, it has been supposed that the left ventricle exhibits strong BNP expression under normal conditions in humans (Mukoyama et al. 1991) and rats (Ogawa et al. 1991). In contrast to these reports (Mukoyama et al. 1991; Ogawa et al. 1991), we observed that the cardiomyocytes of both atria and the left ventricle of ground squirrels during the pre-hibernation period exhibited strong immunostaining for BNP, while the cardiomyocytes of the right ventricle showed moderate immunostaining. These results demonstrate that BNP is predominantly synthesized and secreted from the atria and ventricles of Anatolian ground squirrel hearts.

Recent studies have shown that CNP is also expressed within myocardium and is an important local mediator in the heart (Del Ry et al. 2011; Öztop et al. 2018). Similarly, our observations indicate that CNP was localized in the cytoplasm and nuclei of cardiomyocytes in the atria and ventricles and CNP immunoreactivity did not change depending on chambers. To sum up, these immunohistochemical results reveal that the cellular expression patterns of ANP, BNP and CNP in the Anatolian ground squirrel heart chambers were different from each other and ANP and BNP, but not CNP, were expressed in a cardiac chamber-dependent manner in female and male squirrel hearts.

A few studies in the hibernating mammals have shown that NPs are expressed in a hibernation-dependent manner. For example, Luu et al. (2015) have examined changes in the transcript and protein levels of ANP in the heart of thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*, over the torpor-arousal cycle including euthermic in the cold room (EC), entrance into torpor (EN), early torpor (ET), late torpor (LT), early arousal (EA), and interbout arousal (IA). These researchers have reported that transcript and protein levels of

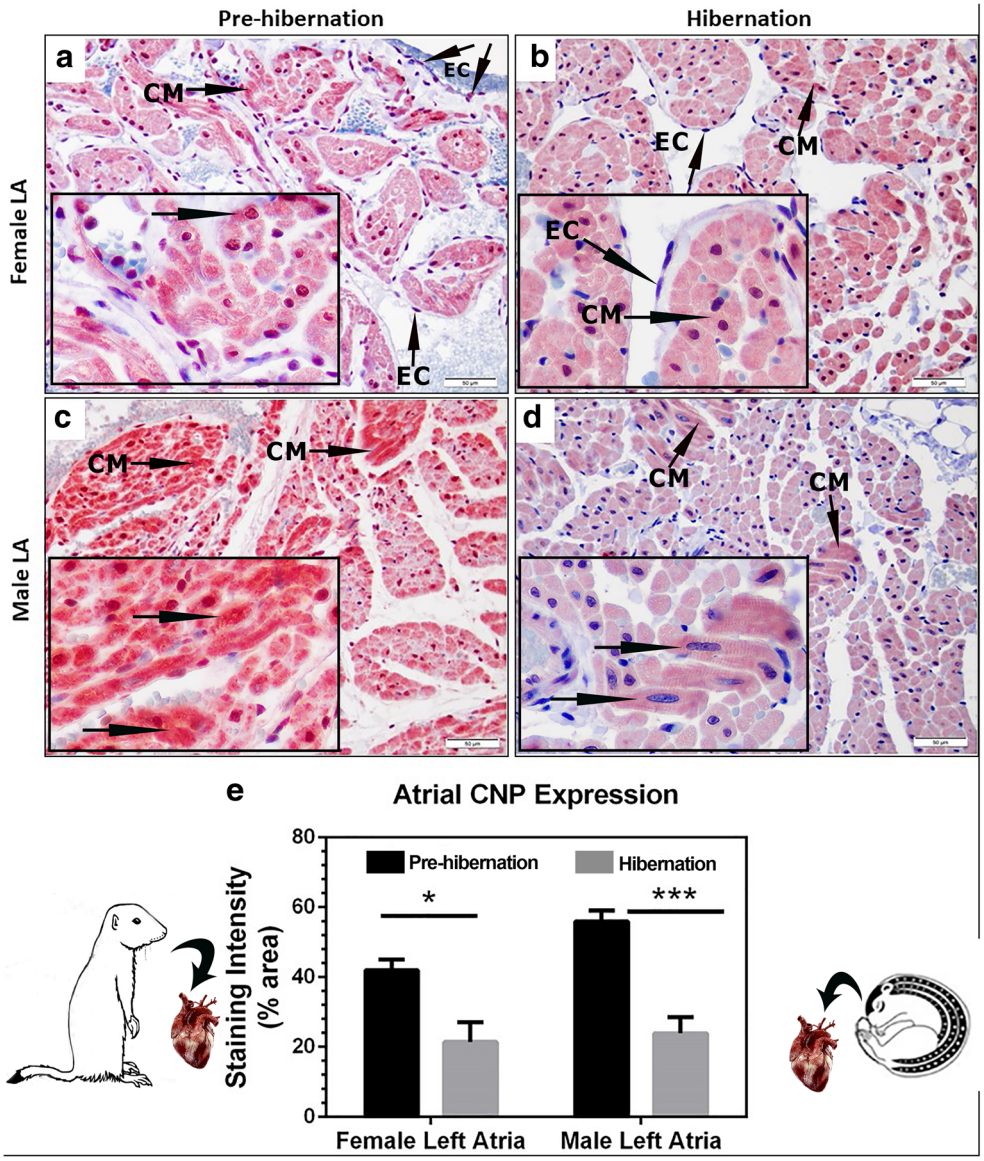


Fig. 10 The localization patterns (**a**–**d**) and staining intensity (**e**) of CNP in the left atria of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. The localization of CNP in the atrial cardiomyocytes and endothelial cells (EC) is presented in the insets. The atrial cardiomyocytes (CM) exhibited nuclear and cytoplasmic staining patterns for CNP (arrows) during the pre-hibernation period, while the nuclear CNP immunostaining was absent and cytoplasmic CNP staining was weak in the cardiomyocytes during the hibernation period. Both immunohistochemical results and image analysis data confirmed that in the left atria of both sexes, CNP staining was more intense during the pre-hibernation period than during the hibernation period. **e** indicates staining intensity as the percentage according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. $*P < 0.05$, $***P < 0.001$ Bars: 50 μm

ANP rose entrance into torpor, with respect to EC. However, they found that transcript levels of ANP decreased below control values during ET, while its protein levels were not significantly different from EC values throughout the remainder of the torpor/arousal course. These researchers have suggested that changes in the relative expression levels of ANP may represent adaptive mechanisms which will facilitate the expected physiological changes such as shifts in heart contraction rate and coping mechanisms to deal with changes in blood pressure. Similarly, Bordicchia et al. (2012) found that plasma levels of BNP and cardiac gene expression levels of ANP and BNP significantly increased in mice kept at 4 °C compared to counterparts maintained at room temperature. In the present study, in order to determine the synthesis intensity and release of NPs in a specific location, we used immunohistochemistry. Our results showed that the immunostaining intensities of ANP and BNP in hibernation period were lower than those in the pre-hibernation period. In this regard, our results seem to contradict the information in some previous studies (Bordicchia et al. 2012; Luu et al. 2015). These inconsistencies in many species may result from differences in sensitivity of immunohistochemistry to the uneven tissue distribution of the peptide. Furthermore, another reason for this discrepancy may be that different hibernation or cold exposure durations have been investigated in the studies. In the Anatolian ground squirrels, which have the ability to undergo deep hibernation, beginning hibernation is the month of August (11 August in female, 31 August in male) and hibernation period is 168 days in males and 212 days in females (Gür 2010). Therefore, in this study, the adult female and male Anatolian ground squirrels in the pre-hibernating group were euthanized in 30 August 2016, while the animals in the hibernation group were euthanized in about 90 days of hibernation (mid-December 2016) during which they were in full torpid,

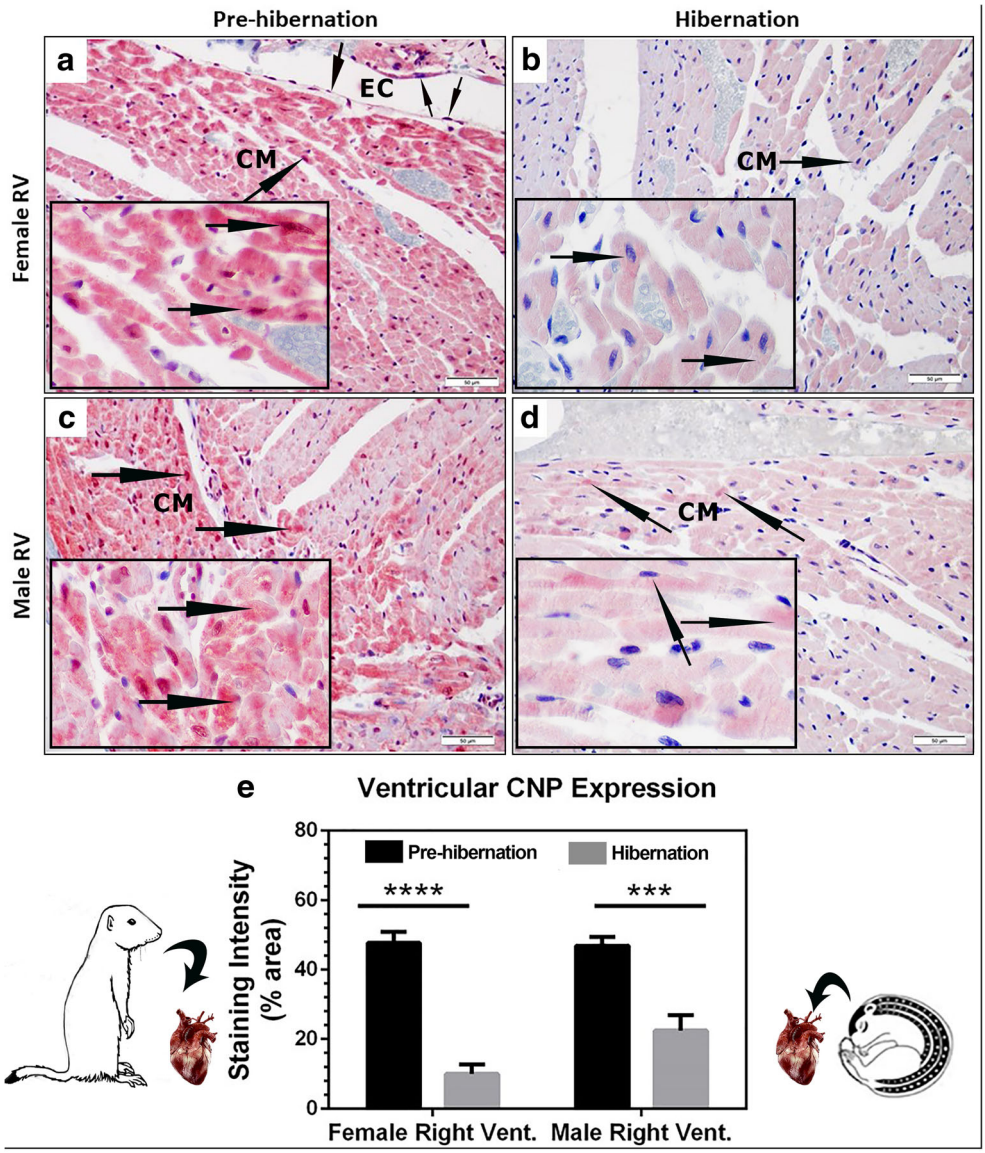


Fig. 11 The localization patterns (**a**–**d**) and staining intensity (**e**) of CNP in the right ventricles of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. CNP immunoreactivity was observed in the cardiomyocytes (CM) and endothelial cells (EC). The ventricular cardiomyocytes (CM) exhibited nuclear and cytoplasmic staining patterns for CNP (arrows) during the pre-hibernation period, while the nuclear CNP immunostaining was absent and cytoplasmic CNP staining was weak in the cardiomyocytes during the hibernation period (insets). Both immunohistochemical results and image analysis data confirmed that in the right ventricles of both sexes, CNP immunostaining was more intense during the pre-hibernation period than during the hibernation period. **e** indicates staining intensity as the percentage according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. $^{***}P < 0.001$, $^{****}P < 0.0001$. Bars: 50 μm

hypothermic state. In a recent study, maintaining the body temperature of the animals between 5 and 8 °C for at least 5 days has been defined as the late torpor (Luu et al. 2015), whereas in the present study, the hibernation group was considered as the prolonged or deep torpor because the animals kept their body temperature between 5 and 8 °C for a long time. However, there are some studies that support the findings of our study. For example, Aoki et al. (1989) have reported that fewer cardiomyocytes are labelled with ANP in hibernating frog *Bufo arenarum* while most atrial cardiomyocytes are able to produce ANP in active frogs. In addition, Addario et al. (2004) have demonstrated that in the atrial cardiomyocytes, ANP immunostaining was moderate in the active frogs, *Rana esculenta*, while it was very weak in the hibernating frogs, with rather weak ventricular immunoreactivity. Results of frogs are consistent with our immunohistochemical findings in Anatolian ground squirrel, and the decline in the content of ANP during hibernation could be crucial in three species.

Previous studies have demonstrated that plasma levels of both ANP and BNP are elevated in patients with heart failure, while their concentrations in the atrial tissue are decreased (Yamaji et al. 1987; Satoh et al. 1989; Moe et al. 1993, 1996). These studies have also demonstrated that ANP and BNP respond differently to changes in heart rate and atrial pressures. For example, increased heart rate and arterial pressures contributed to the increased plasma ANP levels (Moe et al. 1991), whereas the elevated BNP plasma levels is mediated by other mechanisms rather than increased atrial pressure (Yamaji et al. 1987; Moe et al. 1996). The conditions that increase the oxygen consumption or change the oxygen diffusion of cardiomyocytes, such as stretch, pressure or metabolic challenges, initiate the synthesis of NPs and enhance their release from intracellular locations (Arjamaa 2014). It is

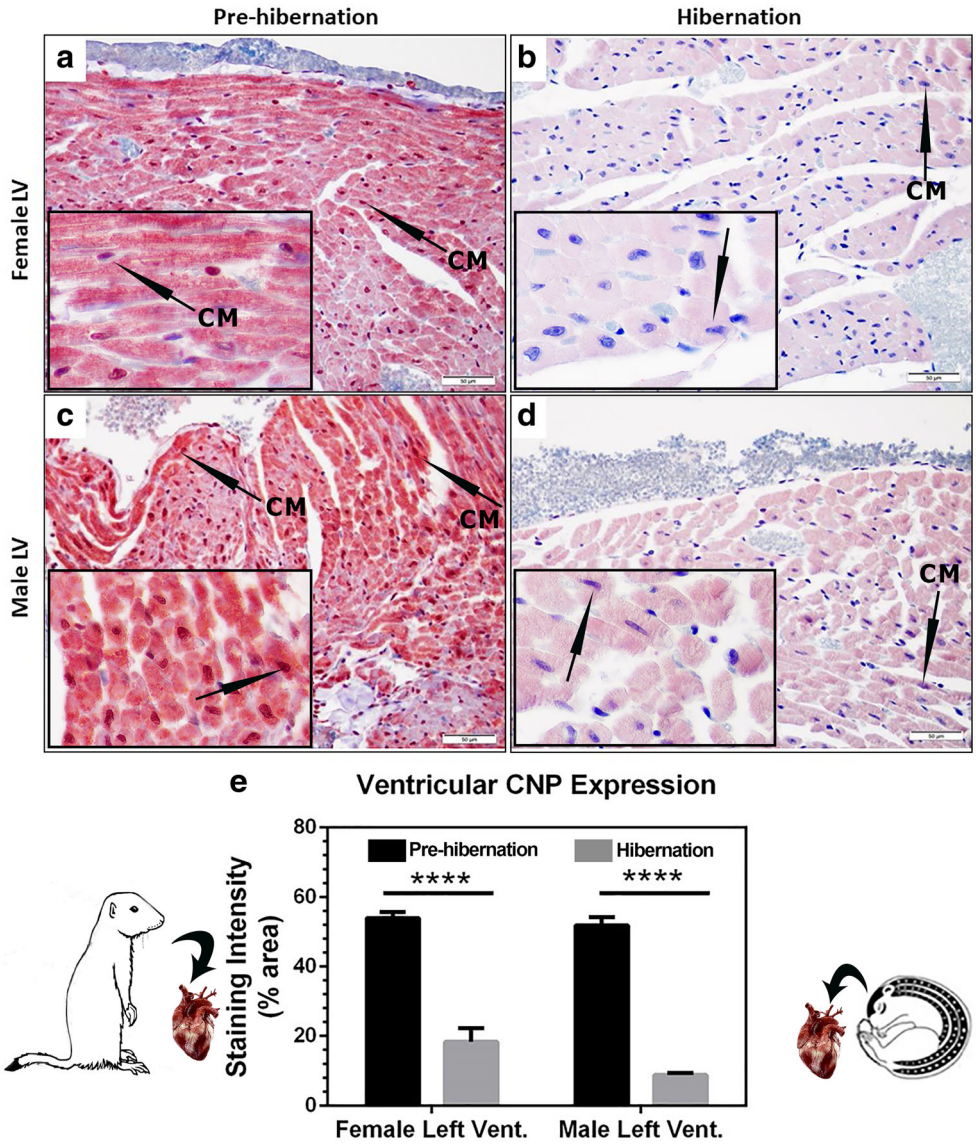


Fig. 12 The localization patterns (**a**–**d**) and staining intensity (**e**) of CNP in the left ventricles of female (**a**, **b**) and male (**c**, **d**) squirrel hearts during prehibernation and hibernation periods. **a**, Pre-hibernating female, **b**, Hibernating female, **c**, Pre-hibernating male, **d**, Hibernating male. The ventricular cardiomyocytes exhibited nuclear and cytoplasmic staining patterns for CNP (arrows) during the pre-hibernation period, while the nuclear CNP immunostaining was absent and cytoplasmic CNP staining was weak in the cardiomyocytes during the hibernation period (insets). Both immunohistochemical results and image analysis data confirmed that in the left ventricles of both sexes, CNP staining was more intense during the pre-hibernation period than during the hibernation period. **e** indicates staining intensity as the percentage according to image analysis. Data are representative of twelve images from each group. Error bar values represent SEM. For comparisons between groups, two-way ANOVA with Bonferroni's multiple comparisons was used. ***$P < 0.001$, ****$P < 0.0001$. Bars: 50 μm

known that most physiological functions are virtually halted during deep torpor (hibernation), while others continue at greatly reduced rates. For example, during torpor, heart rate is reduced from its normal 200–300 to 3–5 beats/min, minimal body temperature can decrease to as low as 1-5 °C, metabolism can be reduced to 1% of euthermic rates and oxygen consumption falls to 2% of basal levels (Tøien et al. 2001; Carey et al. 2003). Based on information in the literature (Tøien et al. 2001; Carey et al. 2003) and our evidence showing that the synthesis intensity of NPs in squirrel heart chambers during hibernation was lower than that at the pre-hibernation period, we suggest that the decrease in heart rate and oxygen consumption during hibernation may lead to decreased ANP and BNP synthesis in atrial and ventricular cardiomyocytes during hibernation.

It has been demonstrated that ANP and BNP are continuously released from the heart, but cardiac cells' stress increases their rate of release with or without a concomitant increase in synthesis (Sergeeva and Cristoffels 2013). Based on this information (Sergeeva and Cristoffels 2013), we also speculate that decreased ANP and BNP immunoreactivity in atria and ventricles during hibernation period can be the result of increase of ANP and BNP release from the atrial and ventricular cardiomyocytes in response to the cold stress occurring during the hibernation period. In order to test these or other hypotheses on the biological roles of NPs in ground squirrel cardiomyocytes during hibernation, additional experimental laboratory studies are required.

There has been no report showing CNP distribution in the heart of hibernating and/or cold-exposed animals. CNP is released mainly from vascular endothelial cells and, to a lesser extent, from cardiomyocytes (Del Ry et al. 2011; Öztop et al. 2018) and is able to induce vasorelaxation (Villar et al. 2007). It has been reported that ANP and BNP can stimulate CNP

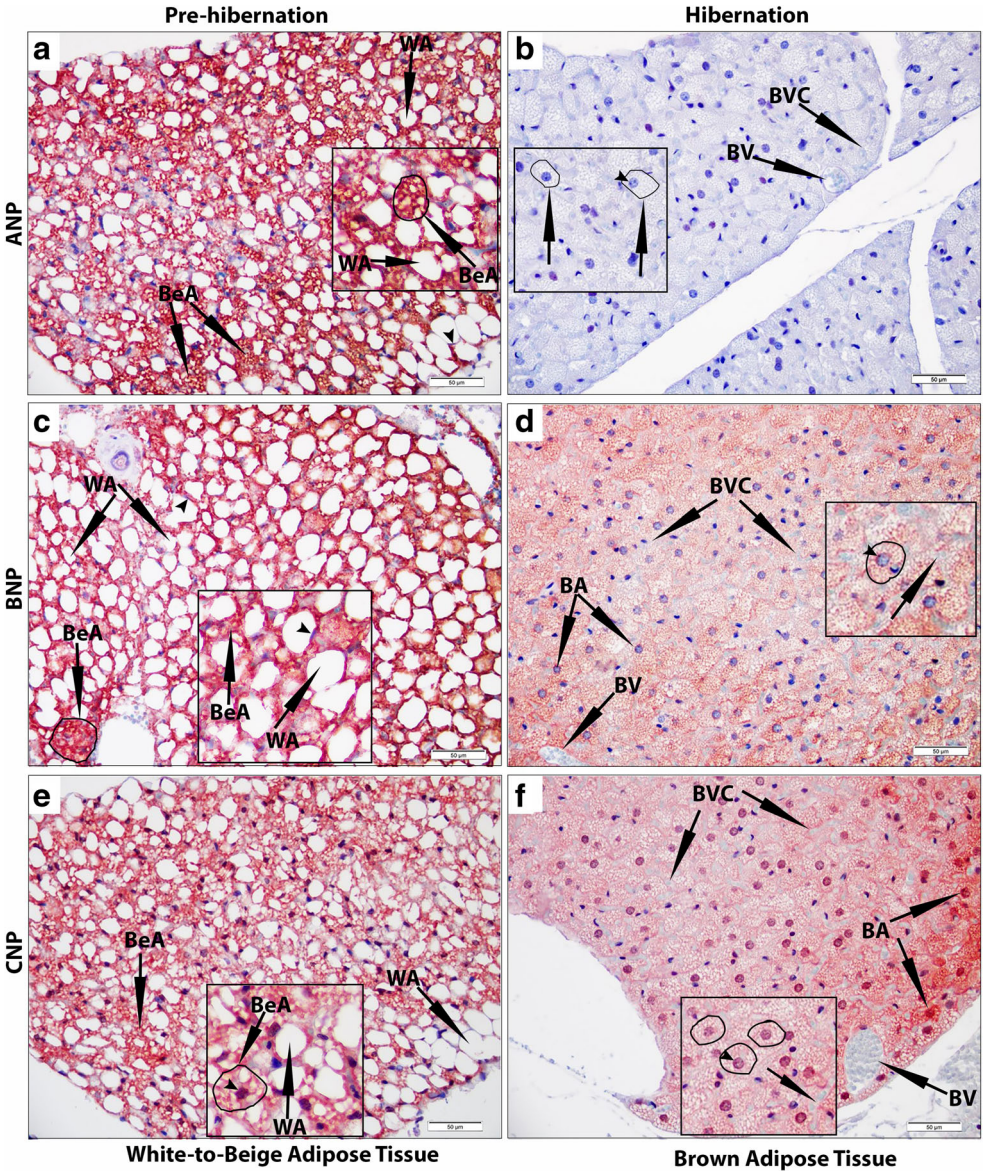


Fig. 13 ANP (**a**, **b**), BNP (**c**, **d**) and CNP (**e**, **f**) immunoreactivity in epicardial adipose tissue of ground squirrel during prehibernation and hibernation periods. Immunostainings (arrows) for all NPs were observed in both the white (WA) and beige adipocytes (BeA) (**a**, **c** and **e**) during the pre-hibernation period and in the brown adipocytes (BA) (**d** and **f**) during the hibernation except for ANP (**b**). Eccentric nucleus of a white adipocyte (WA) (**a** and **c**), and central nucleus of a beige adipocyte (BeA) (**e**) and of a brown adipocyte (BA) (**b**, **d** and **f**) are indicated by arrowheads and circles. Note that beige adipocytes are larger than brown adipocytes, as shown encircled in all. It is obvious that brown adipose tissue is characterized by rich vasculature (BV, blood vessel and BVC, blood vessel capillary) during the hibernation. Bars: 50 μm

production on endothelial cells and BNP is a much more potent stimulator of CNP secretion when compared to ANP (Nazario et al. 1995; Lumsden et al. 2010). In hibernating ground squirrels, our finding, which revealed lack of CNP nuclear immunostaining and weak cytoplamic CNP immunoreactivity in atrial and ventricular cardiomyocytes, support the opinion that the expression levels of CNP in cardiomyocytes may closely be related to those of ANP and BNP. A recent research has reported that hibernators can use selective gene/protein expression to prevent caspase activation, reversible protein phosphorylation to suppress metabolic rate and maintain ion homeostasis, and differential microRNA expression to inhibit pro-apoptotic signaling (Logan and Storey 2017). It is known that CNP exhibits several actions on cardiomyocytes such as anti-hypertrophic, pro-apoptotic, anti-proliferative, and anti-fibrotic effects (Federico 2010; Kuehnl et al. 2013). Taking into consideration all its functions, the presence of weaker cytoplasmic CNP immunoreactivity and lack of CNP nuclear immunoreactivty in the atria and ventricles of ground squirrels during the hibernation period than during the pre-hibernating period may be a reflection of inhibiting of apoptosis in the heart. However, it is difficult to interpret CNP immunostaining in the light of this information.

During deep hibernation, the lipid reserves in white adipose tissue are used as a primary source of fuel for survival. However, although brown adipose tissue is inactive, body temperature regulation is not turned off during hibernation (Cannon and Nedergaard 2004). EAT directly surrounds the heart and is in direct contact with the coronary vasculature (Talman et al. 2014). This tissue has been suggested to have a function similar to BAT and thus, be able to provide heat directly to the myocardium. Because EAT and BAT utilise glucose and lipids to generate heat and are associated with improved cardiometabolic

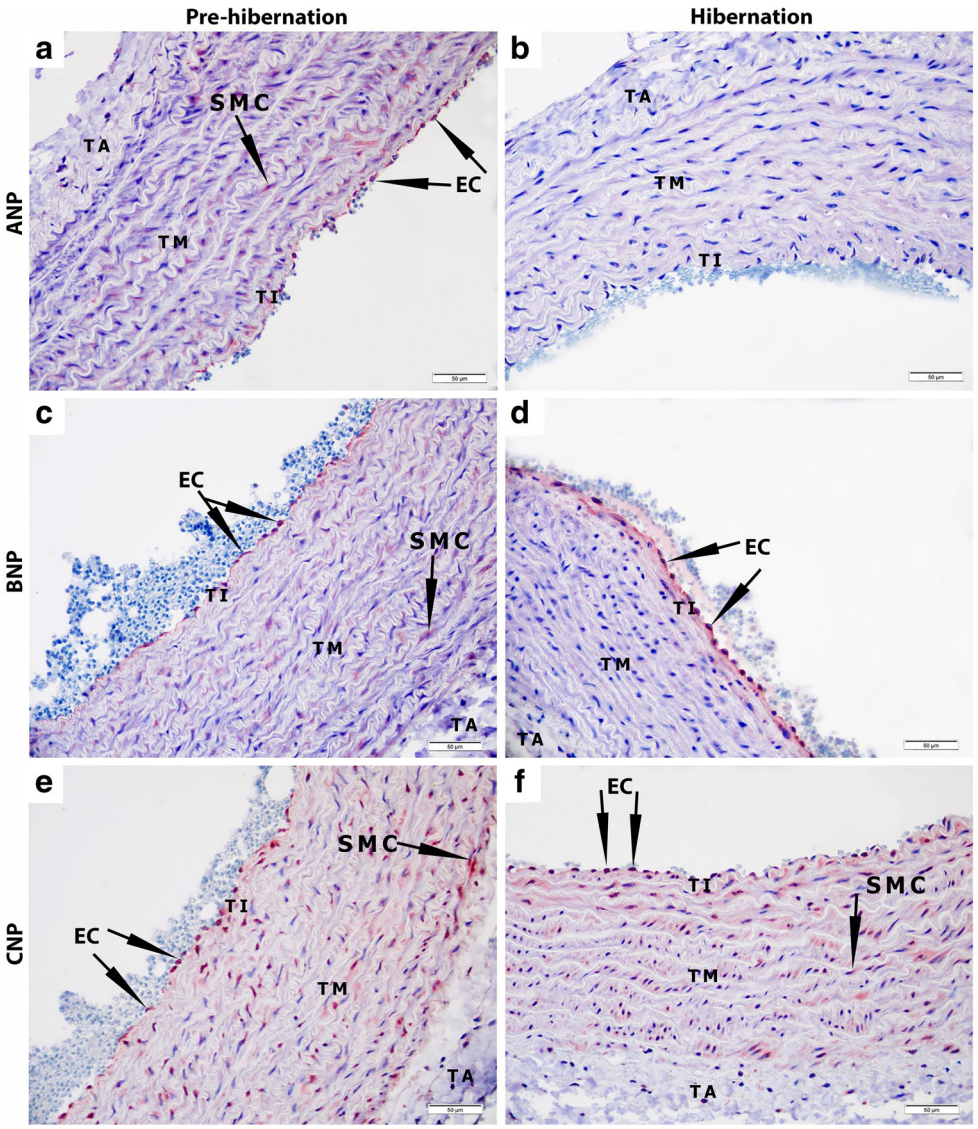


Fig. 14 ANP (**a**, **b**), BNP (**c**, **d**) and CNP (**e**, **f**) immunoreactivity in elastic artery of ground squirrel during prehibernation and hibernation periods. ANP immunostaining (arrows) was observed in the endothelial (EC) and smooth muscle cells (SMC) during the pre-hibernation period (**a**) although not being observed in these cells during the hibernation period (**b**). BNP immunostaining was evident in both types of vascular cells in blood vessel wall during the pre-hibernation period (**c**), while it was only found in the endothelial cells during the hibernation (**d**). Interestingly, CNP immunostaining was observed in these cells in both the pre-hibernation (**e**) and hibernation (**f**) periods. Note that tunica adventitia (TA) showed immunoreactivity for none of all the NPs. TI, Tunica intima; TM, Tunica media. Bars: 50 μm

health. Recent studies have suggested that an increase in the proportion of thermogenic brown or beige adipocytes in the cardiac adipose tissue reduced cardiovascular risk (Aldiss et al. 2017). Furthermore, previous studies have indicated the loss of body heat in severe heart failure (Payvar et al. 2013). Our findings, which showed that the epicardial adipose tissue consisted of brown adipocytes in the hibernation period, support the opinion that the epicardial adipose tissue may participate in protection of squirrel heart from low temperature during deep hibernation (Collins 2014).

Although NPs classically play a key role in the renal and cardiovascular systems, recent studies have indicated that natriuretic peptide receptors are expressed in adipose tissue. These studies have reported that NPs are involved in the regulation of energy metabolism, activate brown adipocytes and induce adipose tissue browning (Bordicchia et al. 2012; Collins 2014). Kimura et al. (2017) have found that the treatment with ANP exert significant thermogenic effects on brown adipose tissue and suggested that NPs keep the heart warm through the thermogenic action in the epicardial adipose tissue through browning. In the present study, we found positive immunostaining for ANP, BNP and CNP in the epicardial white and beige adipocytes of ground squirrels during pre-hibernation period. This finding could lead us to the conclusion that NPs are involved in the conversion of white into beige adipocytes to regulate the energy metabolism in the EAT. However, we observed that in hibernation period, the brown adipocytes of EAT were positive for BNP and CNP but not for ANP. This difference in the pattern of ANP immunostaining between the prehibernation and hibernation periods fuelled speculation that ANP was used for conditioning hibernation or during hibernation period. However, as yet no strong evidence exists to support or refute this notion. Therefore, further studies of experimental and naturally hibernating animals are warranted to fully delineate the thermogenic actions of NPs and NPRs in BAT, to determine their

distribution in both WAT and BAT and to explore their effects on the conversion of white to beige adipocytes or vice versa.

In summary, cardiac ANP, BNP and CNP are synthesized by, stored in, and released from atrial and ventricular cardiomyocytes of healthy Anatolian ground squirrel hearts. Their amount and localizations in atrial and ventricular cardiomyocytes are different from each other and are sex-independent. ANP and BNP, but not CNP, are expressed in a chamber-dependent manner in female and male squirrel hearts. Furthermore, cardiac NPs expression levels in hibernation period were lower than those at the pre-hibernation period. During prehibernation period, ANP, BNP and CNP are expressed in the white and beige adipocytes of EAT; while during hibernation period, BNP and CNP, but not ANP, are expressed by brown adipocytes of EAT. These data suggest that the hibernation-dependent reduction in levels of NPs, particularly ANP, in heart chambers and EAT may be associated with low heart rate and oxygen consumption during hibernation. However, further studies are needed to better delineate the roles of NPs and their receptors and their thermogenic actions in heart and EAT during hibernation.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest were disclosed.

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