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Analyses of a satiety factor NUCB2/nesfatin-1; gene expressions and modulation by different dietary components in dogs

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ABSTRACT. Nesfatin-1 is an anorexic peptide derived from a precursor, nucleobindin-2 (NUCB2), which is distributed in various organs, coexists with ghrelin in the gastric X/A-like cells and closely relates to an appetite control in rodents and humans. Nesfatin-1 may be a significant factor addressing the satiety also in veterinary medicine, however, there are few reports about nesfatin-1 in dogs. In the present study, we detected canine NUCB2/nesfatin-1 mRNA in various tissues, especially abundant in pancreas, gastrointestinal tracts, testis and cerebellum. We examined circulating nesfatin-1 concentrations and NUCB2/nesfatin-1 mRNA expressions in upper gastrointestinal tracts (gastric corpus, pyloric antrum and duodenum) in dogs fed on different types of diets. Plasma nesfatin-1 concentrations in the dogs were approximately 4 *n*g/m*l* and they did not change after feeding through the study, however, NUCB2/nesfatin-1 mRNA expressions in pyloric antrum were 1.84-fold higher in the dogs fed on a High fiber/High protein diet ($P<0.001$), 1.48-fold higher in the dogs fed on a High fat/Low protein diet ($P<0.05$) and 1.02-fold higher in the dogs fed on a Low fat/High carbohydrate diet (not significant) comparing to those on a control diet. It was concluded that High fiber/High protein and High fat/Low protein diets increased NUCB2/nesfatin-1 production in canine gastrointestinal tracts. These results may set the stage for further investigations of canine NUCB2/nesfatin-1, which may relate to satiety effects in dogs.

KEY WORDS: anorexic peptide, canine, nesfatin-1, nucleobindin-2, obesity

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Obesity is defined as excessive body fat accumulation, and the prevalence of obesity in dogs has increased in recent years [3]. Obese dogs have higher risks in hyperlipidemia, pancreatitis, arthritic disorders and shortened life-span, so it is considered to be a condition which must be treated [6, 14]. Obesity is basically caused by overfeeding and/or less exercise, so the dietary restriction is a key technique for a weight control. However, simply reduced energy feeding causes begging behaviors to animals, and the development of reducing diets with enough satiety effects is an important challenge. However, it is difficult to assess the feeling of satiety in animals, because it is a subjective sense which can not be determined by routine techniques. Recently, it has been revealed that many peptides are involved in the eating behavior in rodents, humans and dogs. For example, leptin is a representative adipokine that is produced by adipocytes and suppresses appetite. In contrast, ghrelin is a peptide produced in the stomach and increases appetite. We have investigated the functions and clinical usefulness of these peptides in cats and dogs, and discovered that leptin is available as an obese marker in dogs and ghrelin functions as a feeding stimulant in cats and dogs [12].

Nesfatin-1 is an 82-amino acid peptide derived from the precursor, NEFA/nucleobindin-2 (NUCB2), in humans and rodents [19]. Centrally, PVN-NTS-Melanocortin (MC) 3/4 receptors function as the appetite control center [7, 15, 18, 34]. Nesfatin-1 stimulates afferent vagal nerve cells locally and increases the pro-opiomelanocortin (POMC) mRNA expression in NTS through the afferent nerve system. Alpha-melanocyte-stimulating hormone which forms by processing of POMC inhibits appetite via MC 3/4 receptors [17, 26]. Nesfatin-1 has been detected in appetite-control hypothalamic nuclei and in many peripheral tissues, such as stomach, adipose tissue, pancreas, pituitary gland and testis of the rat and goldfish [8, 11, 27, 29, 35]. In particular, Stengel *et al.* [27] have reported that NUCB2 mRNA expression in gastric mucosa was 10 times of that in brain in rats. Additionally, nesfatin-1 suppresses food consumption when it was administered to rats centrally or peripherally [18, 26]. It has been hypothesized that peripheral nesfatin-1 acts via the blood-brain barrier [20, 23] or the afferent vagal nerves [1, 25, 36], however, the detail pathways underlying its action are unknown at present.

NUCB2/nesfatin-1 is located in the gastric X/A-like cells coexisting with ghrelin [27], and the NUCB2 mRNA and nesfatin-1 protein are affected by fasting/refeeding cycles in rodent [24]. Moreover, Gonkowski *et al.* [10] have detected nesfatin-1 -like immunoreactive cells in the mucosal layer of the canine digestive tracts, and Watanabe *et al.* [31] have reported that nesfatin-1 inhibited gastric contractions in the

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fasted state when administrated to dogs. In this way, the relationship between nesfatin-1 and gastrointestinal tracts is focused also in veterinary science. However, there are few reports about nesfatin-1 in dogs.

In the present study, we demonstrated following experiments for the purpose of better understanding of canine NUCB2/nesfatin-1, i.e. 1) we cloned cDNA of canine NUCB2/nesfatin-1 and revealed the tissue distribution, especially in the upper gastrointestinal tracts, 2) we investigated the change of plasma nesfatin-1 concentrations before and after feeding of different types of diets, and 3) we investigated the change of mRNA expressions of NUCB2/nesfatin-1 in the stomach and duodenum of the dogs fed on different types of diets chronically, which may relate to possible satiety effects. Focus is on the gene expression of nesfatin-1 in the gastrointestinal system, because they are directly contacting organs to dietary components.

MATERIALS AND METHODS

Sample preparation: Canine tissue samples collected and kept frozen for other research were used for RNA extraction in this study. Total RNAs of the 14 canine tissues (cerebrum, cerebellum, esophagus, stomach, pancreas, small intestine, liver, heart, spleen, kidney, bladder, skeletal muscle, testis and adipose tissue) were extracted by homogenizing each sample (50–150 mg) in TRI Reagent (Sigma, St. Louis, MO, U.S.A.). For more detailed analysis of NUCB2/nesfatin-1 expressions in canine upper-gastrointestinal tracts, 4 kinds of tissues (gastric fundus, gastric corpus, pyloric antrum and duodenum) were sampled using an endoscope from a healthy dog which were used in the following study. Total RNA was extracted using RNeasy Plus Mini Kit (QIAGEN, Tokyo, Japan).

Cloning and sequencing of canine NUCB2/nesfatin-1: Genome sequence information of canine NUCB2/nesfatin-1 was obtained from the GenBank (accession, XM534078). The designed primer sequences for cloning and sequencing of canine NUCB2/nesfatin-1 were as follows: NUCB2/nesfatin-1 Forward, 5′- atgaggtggaggatcatctt-3′; Reverse, 5′- aagagcatggtggtctatgc-3′. Total RNA extracted from a stomach sample was reverse-transcribed into cDNA using TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Otsu, Japan) at the following conditions, 42°C for 30 min and 99°C for 5 min. PCR amplification was performed using 50 μl reactions containing 5 μl of 10X PCR buffer, 4 μl of dNTPs, 2 μl of magnesium chloride, 1 μl of each primer, 0.25 μl of LA Taq DNA polymerase (TaKaRa), 2 μl of template cDNA and 34.75 μl of distilled water. The PCR protocol consisted of an initial denaturing cycle at 96°C for 2 min followed by a 35 cycle amplification step (96°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec) with a final extension cycle at 72°C for 2 min. The PCR products were electrophoresed in 2% agarose gel to confirm the expected size and subcloned into pT-7 blue vectors (Novagen, Madison, WI, U.S.A.) using the Ligation Mix (DNA Ligation Kit; TaKaRa). The ligated plasmid was transformed into NovaBlue *E. coli* cells (Novagen) and sequenced using an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Quantitative real-time PCR analysis of canine NUCB2/nesfatin-1 mRNA: For the quantitative analyses, total RNA samples of the 14 tissues and 4 upper gastrointestinal tracts were reverse-transcribed into cDNA using a QuantiTect Rev. Transcription Kit (QIAGEN). We demonstrated quantitative real-time PCR (qRT-PCR) to compare mRNA expression levels among the canine tissues. The primer sequences were as follows, NUCB2/nesfatin-1 Forward, 5′- caagtgattgatgtgctggaa-3′; Reverse, 5′- gccacttgttgtcttttcagttc-3′; beta-actin Forward, 5′-gccaaccgtgagaagatgact-3′; and Reverse, 5′-cccagagtccatgacaataccag-3′. The qRT-PCR reactions were carried out in triplicate with a commercial kit (Perfect Real Time SYBR Premix Ex Taq II) using an Applied Biosystems 7300 Real Time PCR Sequence Detection System (Applied Biosystems). The protocol was as follows, 94°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Following amplification, a melting curve analysis program was performed to verify the authenticity of the amplified products by their specific melting temperatures (Applied Biosystems). The resulting products were subjected to nucleotide sequencing to confirm their specificity. PCR amplification was carried out in 20 μl solutions containing 1 μl of template cDNA, 0.8 μl of each specific primer (10 μM), 10 μl of SYBR Premix Ex Taq, 0.4 μl of ROX reference dye II and 7.0 μl of distilled water. After qRT-PCR amplification, absolute quantification was performed according to the method of Whelan *et al.* [32], by establishing a linear amplification curve from 10-fold serial dilutions of cloned and sequenced plasmid DNA containing target PCR products. The expression level of the beta-actin gene as a housekeeping gene was quantified by the same method, and the expression levels of the target genes were rectified by the expression levels of the beta-actin gene.

Feeding and sampling protocol: Four adult beagle dogs (1 castrated male and 3 spayed females, 6.5–11.8 kg body weights and 4–7 years old) were maintained in the laboratory for research purposes and served as healthy controls. All dogs were fed on a commercial diet (Select Skin Care as maintenance diet, Royal Canin Japon, Tokyo, Japan) twice a day (8 am and 8 pm), and the energy intake was set at $1/2 \times 1.6 \times \mathrm{RER}$ ($\mathrm{BW}^{0.75} \times 70$) for each feeding period (RER, resting energy requirement; BW, body weight) for the dogs [4]. In order to investigate short-term effects of different nutrient composition on nesfatin-1 gene expressions of upper gastrointestinal tracts, the 4 healthy dogs were fed on the same diet for 7 days followed by diet rotations (Fig. 1 and See Appendix: Supplementary Table 1). The prescription diets used for the rotations were as follows, Select Skin Care dry diet as Control diet, Weight Control dry diet as High fiber/High protein diet, Renal dry diet as High fat/Low protein diet and Gastro Intestinal Low Fat dry diet as Low fat/High carbohydrate diet (Royal Canin Japon, See Appendix: Supplementary Table 2).

Three dogs (dogs 1, 3 and 4, See Appendix: Supplementary Table 2) were used for the circulating nesfatin-1 assay. On the seventh day of feeding, blood samples were collected

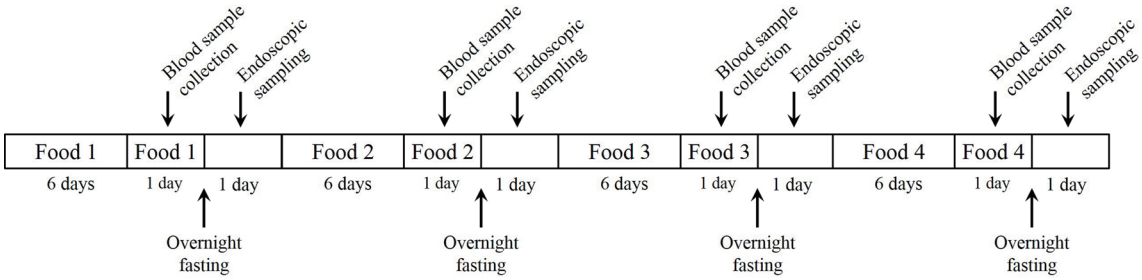

Fig. 1. Feeding rotations of the dogs. The dogs underwent diet regimen rotations, with each regimen for 7 days. The orders of the diets are shown in Supplementary Table 1. Blood was always collected on the seventh day of each diet regimen. Upper gastrointestinal tract samples were collected on the last day of each diet regimen by endoscopy from the anesthetized dogs.

from the dogs prior and 30, 60, 120 and 180 min postprandial of the diets. They were collected into chilled tubes containing EDTA-2Na and 500 KIU aprotinin (Merck Millipore, Darmstadt, Germany), and centrifuged at 1,000 g for 10 min at 4°C to obtain plasma. Plasma samples were used to determine nesfatin-1 concentration, which was measured using a commercial Human Nesfatin-1 EIA Competition ELISA Kit (LifeSpan BioSciences, Inc., Seattle, WA, U.S.A.) according to the manufacturer's protocol (the inter-assay CV and intra-assay CV were <12% and <10%, respectively).

Four dogs (dogs 1, 2, 3 and 4, See Appendix: Supplementary Table 2) were used for the NUCB2/nesfatin-1 mRNA expression assay. On the last day of each interval period, the dogs underwent overnight fasting. The dogs were sedated by intravenous administration of 0.25 mg/kg droperidol (Droleptan; Daiichi Sankyo Co., Ltd., Tokyo, Japan) and anesthetized by intravenous administration of 7 mg/kg propofol (Rapinovet; Intervet/Schering-Plough Animal Health Corp., Ltd., Tokyo, Japan). Anesthesia was maintained by inhalation of isoflurance (Escain; Merck Ltd., Tokyo, Japan) and oxygen. Stomach and small intestine samples were collected every week midmorning using an endoscope from each anesthetized animal under minimal stress conditions according to guidelines of the Nippon Veterinary and Life Science University. The tissue samples were immediately transferred into RNAlater solution (Sigma) and stores at −80°C until measurement of NUCB2/nesfatin-1 mRNA expression by qRT-PCR. Approval for the work was given by the Nippon Veterinary and Life Science University Animal Research Committee.

Statistical analyses: Data are presented as the mean ± SEM. Statistical analyses were demonstrated using two-way repeated measures ANOVA for comparison of plasma nesfatin-1 and one-way repeated measures ANOVA and Bonferroni's multiple comparison test for comparison of nesfatin-1 mRNA expressions, using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.A.).

RESULTS

cDNA cloning of canine NUCB2/nesfatin-1: Open reading frame of the canine nesfatin-1 consisted of 243 nucleotides, which encodes 81 amino acid protein sequence (registered as accession AB622125). The deduced canine NUCB2/nesfatin-1 protein sequence displayed a high identity to bovine (97%), human (92%), murine (86%) and rat (84%) NUCB2/nesfatin-1 sequences. Additionally, bovine, human and rodent nesfatin-1 proteins consist of 82 amino acids, although canine nesfatin-1 consists of 81 amino acids. Alignment analysis revealed that the 12th amino acid had been lost from the N-terminus in the dog (See Appendix: Supplementary Fig. 1).

Tissue distribution of NUCB2/nesfatin-1 mRNA: NUCB2/nesfatin-1 was expressed in 10 of 14 tissues tested in the dog, and the order of the highest to the lowest was as follows, pan creas>bladder>stomach>cerebellum>esophagus>small intest ine>spleen>testis>kidney>cerebrum (Fig. 2a). The NUCB2/nesfatin-1 mRNA expressions of cerebellum, stomach, pancreas and bladder were higher (>400 copies/*n*g cDNA), and those of cerebrum, esophagus, small intestine, spleen, kidney and testis were middle (100–400 copies/*n*g cDNA). On the other hand, liver, heart, skeletal muscle and adipose tissue had poor NUCB2/nesfatin-1 mRNA expressions (<100 copies/*n*g cDNA). In NUCB2/nesfatin-1 expression of upper gastrointestinal tracts, the order of the highest to the lowest was as follows, pyloric antrum>gastric fundus>gastric corpus>duodenum (Fig. 2b). Most rich NUCB2/nesfatin-1 mRNA expression in pyloric antrum was 567 copies/*n*g cDNA.

Influence of the different dietary components on canine plasma nesfatin-1: The results of the plasma nesfatin-1 assay of the 3 healthy dogs fed on different types of diets are shown in Fig. 3. Basal plasma nesfatin-1 concentrations did not change after 1-week feeding of different types of diets statistically, however, those after High fiber/High protein and High fat/Low protein diets tended to be higher than others. Plasma nesfatin-1 concentrations did not show significant changes from prior to 180 min after the dogs were fed.

Influence of the different dietary components on canine NUCB2/nesfatin-1: The results of the NUCB2/nesfatin-1 mRNA expressions of upper gastrointestinal tracts (gastric corpus, pyloric antrum and duodenum) examined in the 4 healthy dogs fed on different types of diets are shown in Fig. 4. In gastric corpus, nesfatin-1 mRNA expression was 1.46-fold higher in the dogs fed on a High fiber/High protein diet, 1.10-fold higher in the dogs fed on a High fat/

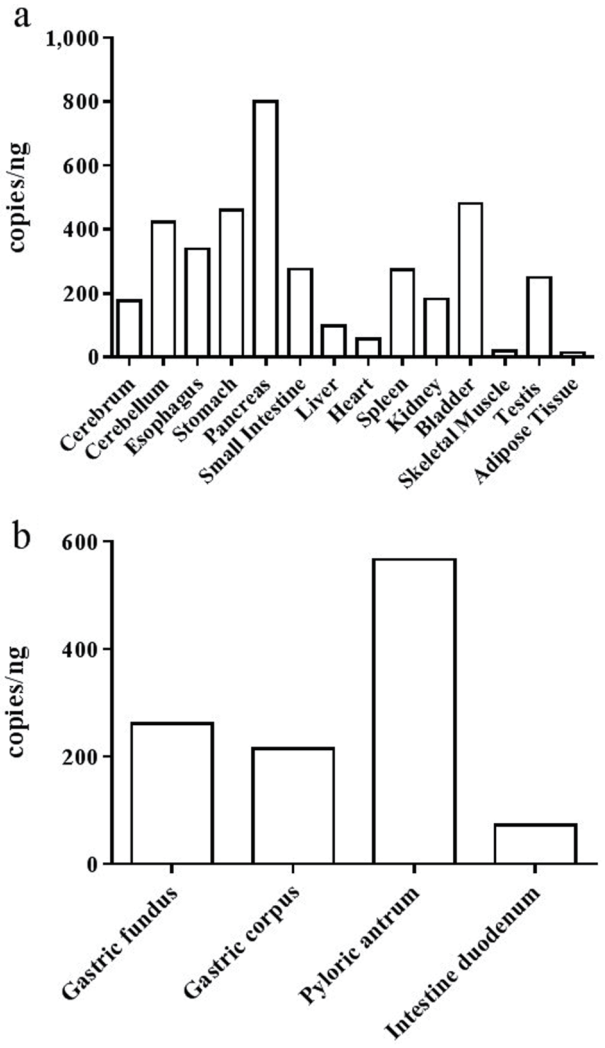

Fig. 2. Values are expressed as copies per 1 *n*g of cDNA. (a) NUCB2/nesfatin-1 mRNA expression levels in various canine tissues as determined by qRT-PCR. (b) NUCB2/nesfatin-1 mRNA expression levels in endoscopically-obtained stomach (gastric corpus, gastric fundus and pyloric antrum) and duodenum samples as determined by qRT-PCR.

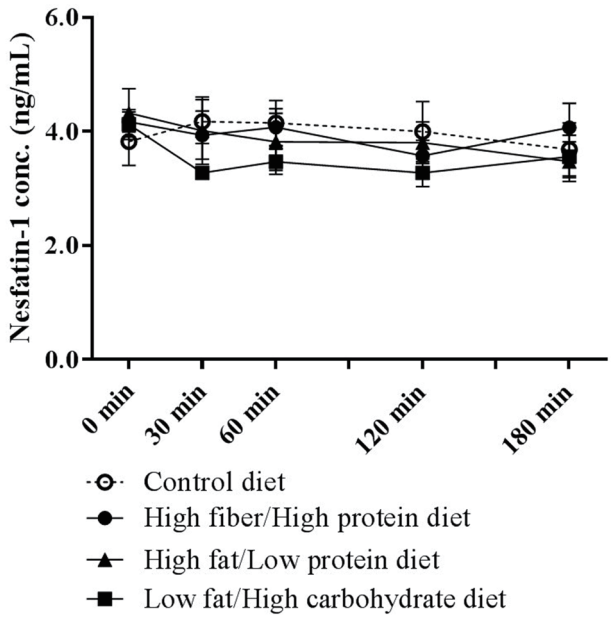

Fig. 3. Mean temporal changes in postprandial plasma nesfatin-1 concentrations under different types of diets in healthy dogs. Values are expressed as means ± SEM (n=3).

Low protein diet and 0.83-fold higher in the dogs fed on a Low fat/High carbohydrate diet diets comparing to those on a control diet, but the differences were not statistically significant. In pyloric antrum, the nesfatin-1 mRNA expression was 1.84-fold higher in the dogs fed on a High fiber/High protein diet ($P<0.001$), 1.48-fold higher in the dogs fed on a High fat/Low protein diet ($P<0.05$) and 1.02-fold higher in the dogs fed on a Low fat/High carbohydrate diet (not significant) comparing to those on a control diet. Moreover, the difference in High fiber/High protein diet and High fat/Low protein diet was statistically significant comparing to those on a Low fat/High carbohydrate diet, too ($P<0.001$ and $P<0.05$, respectively). In duodenum, nesfatin-1 mRNA expression was 1.09-fold higher in the dogs fed on a High fiber/High protein diet, 1.17-fold higher in the dogs fed on a High fat/Low protein diet and 0.83-fold higher in the dogs fed on a Low fat/High carbohydrate diet comparing to those on a control diet, but the differences were not statistically significant.

DISCUSSION

The cloned cDNA of canine NUCB2/nesfatin-1 was highly homologous to those of other species [19]. In the current study, we revealed that canine NUCB2/nesfatin-1 mRNA is abundant in pancreas, gastrointestinal tracts, testis and cerebellum. In a previous paper, it has been reported that NUCB2/nesfatin-1 was much expressed in the cerebellum, stomach (especially pyloric antrum) and pancreas in rodents [35]. Moreover, García *et al.* [9] have reported the expression of NUCB2/nesfatin-1 in Leydig cells of testis, which is under the control of developmental, nutritional and gonadotropic cues. The current results are basically identical to these reports in other species. However, these studies aimed at NUCB2/nesfatin-1 in limited types of tissues, and analyses in whole body have not ever been performed. Therefore, there are no data about other organs that we compared. In particular, we detected the highest expressions of NUCB2/nesfatin-1 in bladder, spleen and kidney, although their physiological implications are not clear.

In the present study, we measured plasma nesfatin-1 concentrations and quantified the NUCB2/nesfatin-1 mRNA expressions in the gastrointestinal tracts when the dogs were fed on different types of nutrient for 7 days. Plasma concentrations of nesfatin-1 in the dogs were approximately

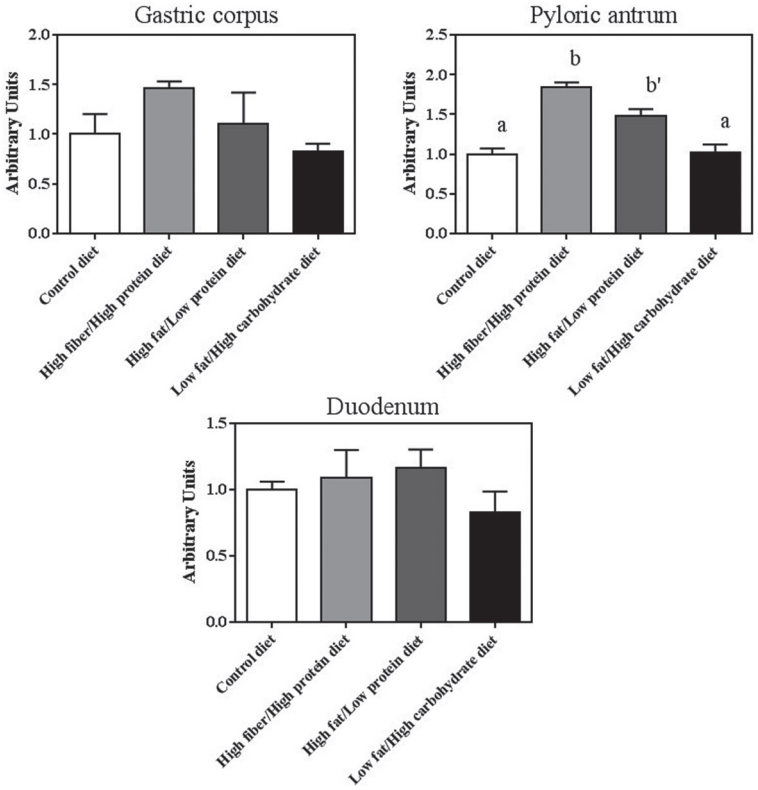

Fig. 4. NUCB2/nesfatin-1 mRNA expressions in upper gastrointestinal tracts of the dogs fed on different types of diets are indicated. The values were normalized by the expression levels of beta-actin gene. Each value of mRNA expression was denoted as the relative value to the control diet and expressed as mean ± SEM (n=4). Different alphabetic letters indicate statistically different, a vs. b, $P<0.001$; a vs. b', $P<0.05$, Bonferroni's Multiple Comparison Test.

4 *n*g/m*l*, and they did not change after feeding, harmonizing the results by Watanabe *et al.* [31]. In addition, Tsuchiya *et al.* [30] have reported that nesfatin-1 concentrations did not change significantly in humans by meal tests during 0–240 min. These results do not harmonize the hypothesis that circulating nesfatin-1 increases postprandially and acts on brains through the blood-brain barrier to cause satiety effects.

On the other hand, we detected the significant change of NUCB2/nesfatin-1 mRNA expressions, i.e. High fiber/High protein diet and High fat/Low protein diet increased NUCB2/nesfatin-1 mRNA expressions in pyloric antrum significantly. Those in other regions of gastrointestinal tracts (gastric corpus and duodenum) did not show significant change, but tended to be similar to that in pyloric antrum. It is possible that locally produced nesfatin-1 stimulates vagal nerves to cause the satiety effects by acting on brain through the afferent nerve system. However, it is no more than a hypothesis at this moment, and further studies are necessary to prove it. Ramanjaneya *et al.* [24] have reported that the mice fed on a high fat diet for 12 or 20 weeks showed increased nesfatin-1 production in adipose tissues. In the present study, we revealed that NUCB2/nesfatin-1 mRNA expressions are increased in gastrointestinal tracts after feeding of High fat (/Low protein) diet. This may support the relationship between high fat diet and NUCB2/nesfation-1 production in dogs. A number of studies have showed that fiber-rich foods can increase the sense of satiety and decrease short-term food intake in humans [5, 16, 22, 33], and it is also reported that fiber causes a satiety effect by gastric distension in companion animals [21]. In the present study, we detected the highest NUCB2/nesfatin-1 mRNA expressions in the dogs fed on High fiber (/High protein) diet. Considering the current results, it is possible that increased nesfatin-1 is another factor that may relate to satiety too. Collectively, High fat/Low protein and High fiber/High protein diets increase NUCB2/nesfatin-1 production in canine stomach, and it may relate to satiety effects in dogs. It is reported that contraction of the stomach at the fasted state is controlled by administrated nesfatin-1 in dogs [31], but there are no reports indicating that administered nesfatin-1 influences food consumption in dogs like rodent studies.

Limitations of the current study are as follows. First, we used the commercial diets which were different in more than 2 kinds of dietary components. Ideally, we should use diets which are different in only one nutrient, but when we change the amount of one nutrient of a food, keeping the metabolizable energy constant, we have to change the amount of other nutrients too, so it is difficult to design the desirable experimental protocol. Second, there are no data which

demonstrate the function of canine nesfatin-1, so the satiety effects of nesfatin-1 remain unclear in dogs. It is difficult to evaluate the sense of satiety in dogs, but there are some reports describing the satiety effects in dogs objectively. Jewell and Toll [13] and Bosch *et al.* [2] have evaluated the effects of dietary fiber on satiety in dogs, in which, voluntary food intake of an additional meal after consumption of the morning meal was found to be lower in the dogs fed on high-fiber diets. Therefore, it will be a possible approach to investigate the relationship between nesfatin-1 levels and satiety effects of dietary components in dogs using the same methods for the next step. Third, influences of adipose nesfatin-1 are not investigated. We analyzed NUCB2/nesfatin-1 in upper gastrointestinal tracts since they are organs that contact dietary components, however, Stengel *et al.* [28] have reported that nesfatin-1 produced by adipose tissues influences feeding and other metabolic actions. Although NUCB2/nesfatin-1 mRNA expression in canine adipose tissue was poor in the current result, further studies are necessary to prove the role of adipose nesfatin-1 in dogs.

In conclusion, the current study demonstrated that canine NUCB2/nasfatin-1 was very similar to those of other species in molecular structure and tissue distribution. Plasma nesfatin-1 concentrations in the dogs were approximately 4 *n*g/m*l*, and they did not change after feeding. On the other hand, High fiber/High protein and High fat/Low protein diets increased gastric NUCB2/nesfatin-1 at transcriptional levels. These results may set the stage for further investigations of canine NUCB2/nesfatin-1, which may relate to satiety effects in dogs.

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