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Pathology

# The immunohistochemical detection of peroxiredoxin 1 and 2 in canine spontaneous vascular endothelial tumors

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**ABSTRACT.** Peroxiredoxin (PRDX) is an antioxidant enzyme family with six isoforms (PRDX1–6). The main function of PRDXs is to decrease cellular oxidative stress by reducing reactive oxygen species, such as hydrogen peroxide, to $H_2O$. Recently, it has been reported that PRDXs are overexpressed in various malignant tumors in humans, and are involved in the development, proliferation, and metastasis of tumors. However, studies on the expression of PRDXs in tumors of animals are limited. Therefore, in the present study, we immunohistochemically investigated the expression of PRDX1 and 2 in spontaneous canine hemangiosarcoma (HSA) and hemangioma (HA), as well as in selected normal tissue and granulation tissue, including newly formed blood vessels. Although there were some exceptions, immunolocalization of PRDX1 and 2 in normal canine tissues was similar to those in humans, rats, or mice. In granulation tissue, angiogenic endothelial cells were strongly positive for PRDX1 and 2, whereas quiescent endothelial cells in mature vessels were negative. Both PRDX1 and 2 were significantly highly expressed in HSA compared to HA. There were no significant differences in the expression of PRDX1 and 2 among the subtypes and primary sites of HSA. These results suggest that PRDX1 and 2 may be involved in the angiogenic phenotypes of endothelial cells in granulation tissue as well as in the behavior in the malignant endothelial tumors.

**KEYWORDS:** dog, hemangiosarcoma, immunohistochemistry, peroxiredoxin

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Hemangiosarcoma (HSA) is a malignant neoplasm derived from vascular endothelial cells and occurs in various animal species [30]. Dogs are known to exhibit a high incidence of HSA. HSA in dogs can develop in various tissues. The common sites of canine HSA are the spleen, right atrium and auricle, and subcutaneous tissues [34]. Dogs that develop HSA in abdominal organs, such as the liver and spleen often show no clinical symptoms until the tumor ruptures, causing blood loss. Thus, early diagnosis of HSA occurring in the viscera is difficult [34]. The 6-months survival rate of dogs with HSA is less than 50% owing to the low efficacy of treatment, which include surgical excision, chemotherapy, and radiotherapy [9]. Similar to canine HSA, the 5-years survival rate of patients with human angiosarcoma (AS) is less than 30% [38]; thus, both human AS and canine HSA are malignant tumors with poor prognosis. Human AS accounts for only 1% of all human malignant tumors, making research difficult. However, canine HSA occurs 5–20 times more frequently than human AS, accounting for approximately 7% of canine malignancies [17, 40]. The histopathological features of canine HSA are similar to those of human AS [40]. Furthermore, both human AS and canine HSA exhibit certain mutations in several genes, such as *TP53*, *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α*, and *low-density lipoprotein receptor-related protein 1 B* [11, 28]. Thus, canine HSA can provide insight into human AS and is valuable in the search for therapeutic targets.

Peroxiredoxins (PRDXs) are a family of antioxidant enzymes expressed in various organisms. PRDXs protect cells from oxidative stress by reducing hydrogen peroxide ($H_2O_2$), a reactive oxygen species (ROS) [29]. In mammals, the PRDX family is composed of six isoforms (PRDX1–6). Furthermore, PRDXs are highly conserved in almost all organisms, suggesting their importance as a system for protecting biological functions from ROS [29, 41]. Recent studies have shown that PRDX expression in various cancers in humans, such as lung, pancreas, and liver cancers, varies from that in normal tissues [18, 22, 29, 36, 37].

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Changes in PRDX expression have been reported to alter tumor dynamics, including development, proliferation, apoptosis, and metastasis [10, 23, 24, 39, 44]. For instance, PRDX1 promotes tumor metastasis and angiogenesis in colorectal cancer, [23] and PRDX2 promotes the proliferation and metastasis of non-small cell lung cancer [4]. There is much discussion as to whether changes in the genes or protein expression of PRDXs are associated with tumorigenesis. It has been suggested that PRDXs act as tumor promoters or suppressors in different tumors, but details remain unclear.

It has been shown that PRDX6 is overexpressed in canine HSA in comparison to hemangioma (HA) and that the apoptosis of HSA cell lines derived from dogs was induced by knockdown of PRDX6 [1]. Information on PRDX in canine tumors is gradually increasing, but there are few reports on the expression of PRDXs in tumors of animals. Therefore, it is necessary to investigate PRDX expression in domestic animals. In the present study, we assessed expression of PRDX1 and 2 in spontaneous HA and HSA in dogs using immunohistochemistry.

## MATERIALS AND METHODS

### *Samples*

All canine granulation tissue, HA, or HSA samples were collected between 2012 and 2021 at the Laboratory of Veterinary Pathology, Gifu University, Japan. In the present study, 24 granulation tissue, 26 HA, and 54 HSA samples from spleen or skin were used in statistical analyses. Other HSAs were obtained from kidney (n=4), liver (n=2), bone (n=2), adrenal gland (n=1), mesentery (n=1), and abdominal cavity (n=1). All HAs were observed in skin (n=26). Information of dogs with HA and HSA were summarized in Supplementary Tables 1 and 2. All tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded samples were sectioned into 4-µm. The sections were then dewaxed, rehydrated, and stained with hematoxylin and eosin. HSA samples which occurred in spleen or skin were divided into three histopathological subtypes: conventional (n=31), Kaposiform (n=9), and epithelioid (n=14) according to the Surgical Pathology of Tumors of Domestic Animals, Vol. 3 [33].

### *Validation of anti-human PRDX1 and 2 antibodies against canine protein by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting*

To validate cross-reactivity of anti-human PRDX1 or 2 antibodies used in the present study to canine PRDX1 or 2 proteins by western blotting, HeLa which expressed PRDX1 and 2 [9, 16] and Re12 which was derived from canine HSA [27] were selected. SDS-PAGE and western blotting was performed according to previous report [8]. Briefly, protein lysates were prepared, and electrophoresed with SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Cytiva, Tokyo, Japan). After blocking for avoiding non-specific binding of antibodies, the membranes were incubated for 60 min at room temperature (RT, 20–25°C) with the following primary antibodies: rabbit polyclonal antibody to human PRDX1 (1:5,000, ab228780; Abcam, Cambridge, UK), or rabbit polyclonal antibody to human PRDX2 (1:5,000, ab109367; Abcam). Rabbit polyclonal antibody to human β-actin (1:1,000, #4967, Cell Signaling Technology, Danvers, MA, USA) was used as a loading control. The proteins were visualized using a horse radish peroxidase-conjugated anti-rabbit IgG antibody (1:2,000, #7074, Cell Signaling Technology) and enhanced chemiluminescence by Immobilon® Forte Western HRP Substrate (Merck Millipore, Burlington, MA, USA). Finally, the signals were detected using a C-Digit Blot Scanner (LI-COR, Lincoln, NB, USA).

### *Immunohistochemistry*

All canine granulation tissue and HA and HSA samples were used for immunohistochemical (IHC) staining. HSA samples were also immunostained with mouse monoclonal antibody against CD31 (ready-to-use, JC70A; Dako, Carpinteria, CA, USA) to differentiate them from other mesenchymal tumors. All paraffin-embedded samples were sectioned into 4-µm. The sections were dewaxed, rehydrated and antigen retrieved in 10% ImmunoActive solution (pH 6.0) (Matsunami Glass Ind., Osaka, Japan) for 30 min at 121°C. Tissue sections were then blocked to neutralize endogenous peroxidase activity by soaking in 0.3% $H_2O_2$ in methanol for 20 min at RT. Their sections were incubated with protein block serum-free reagent (Dako) for 30 min at RT to block non-specific antibody binding. After blocking, the sections were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal antibody to PRDX1 (1:500, ab228780; Abcam), rabbit polyclonal antibody to PRDX2 (1:1,000, ab109367; Abcam), and mouse monoclonal antibody against CD31. Sections were then incubated with the following secondary antibodies: Histofine® Simple Stain MAX PO (R) (Nichirei Biosciences Inc., Tokyo, Japan) or Histofine® Simple Stain MAX PO (M) (Nichirei Biosciences Inc.) for 30 min at RT. The sections were then visualized by 3,3′-diaminobenzidine as a chromogen (Liquid DAB + Substrate Chromogen System, Dako). After counterstaining with hematoxylin, the sections were dehydrated and mounted. Various normal tissues near the tumor, including normal endothelial cells and granulation tissues, were observed, and the staining properties of PRDX1 and 2 were qualitatively assessed. Furthermore, all HA and HSA samples stained immunohistochemically with PRDX1 and 2 were scored semi-quantitatively based on positive cell ratio and staining intensity (400 × magnification) according to Lu *et al.* (2020) [24]. The positive cell ratio was measured as follows: 1, ≤25% positive cells; 2, 25–50% positive cells; 3, 50–75% positive cells; and 4, >75% positive cells. The staining intensity were assessed according to the following measures, with macrophages used as internal positive controls for PRDX1 and some erythrocytes for PRDX2, and neutrophils were internal negative control for them: 0, negative staining; 1, weaker staining than positive controls; 2, comparable staining with positive controls; 3, stronger staining than positive controls. The IHC score was calculated by multiplying the score of the positive cell ratio and staining intensity together and averaging. To confirm reproducibility, the observation and scoring of immunohistochemistry in HA and HSA samples were repeated three times by two persons and double blind.

*Statistical analysis*

HSA samples occurred in spleen or skin were used in statistical analyses. The difference in mean IHC scores between canine HA and HSA was analyzed using the Wilcoxon rank-sum test. The comparisons of mean IHC scores in subtypes or primary locations of HSA were analyzed using the Steel-Dwass test. IHC scores were expressed as mean values with standard deviation (SD). Statistical significance was set at $P<0.05$. All analyses were performed using JMP Pro 16.0.0. software (JMP, Tokyo, Japan).

## RESULTS

*Validation of anti-human PRDX1 and 2 antibodies against canine samples*

Similar to HeLa, distinct single bands were detected in lysate from Re12 cells by western blotting using anti-human PRDX1 or 2 antibodies, and molecular weight of the band was approximately 22 kDa [9, 16] (Fig. 1). β-actin as a loading control was detected at 45 kDa in both HeLa and Re12. Therefore, anti-human PRDX1 or 2 antibodies were able to detect canine PRDX1 or 2, respectively.

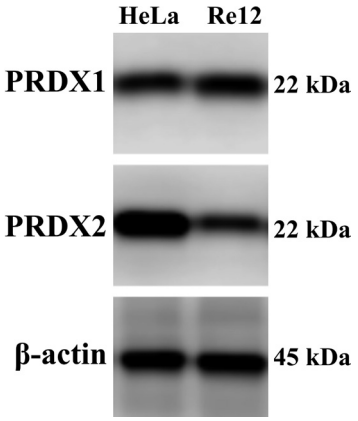


**Fig. 1.** The images of western blotting. Distinct single bands were detected in lysate from HeLa or Re12 by western blotting using anti-human peroxiredoxin (PRDX) 1 or 2 antibodies. Molecular weight of the bands was approximately 22 kDa. β-actin as a loading control was detected at approximately 45 kDa in both HeLa and Re12.

*Expression of PRDX1 and 2 in normal tissues adjacent to HA and HSA*

The expression of PRDX1 was observed in the nucleus and/or cytoplasm of various cells (Figs. 2, 3, and Table 1). In skin, the nuclei of squamous cells located in the basal and spinous layers were strongly positive for PRDX1 and 2, and the cytoplasm was weakly positive. However, the squamous cells in the granular layer were negative for them (Figs. 2A and 3A). In hair follicles, the nuclei of internal and external root sheath cells were negative to positive for PRDX1 and 2, and a few cells of the hair papilla were weakly positive (Figs. 2B and 3B). In sebaceous glands, both immature and mature glandular epithelial cells were strongly positive for PRDX1 and 2 (Figs. 2B and 3B). In apocrine glands, PRDX1 was expressed in glandular epithelial cells but not in almost all myoepithelial cells (Fig. 2C). PRDX2 was expressed strongly in glandular epithelial cells and negatively or weakly expressed in myoepithelial cells (Fig. 3C). Furthermore, PRDX1 was expressed mainly in the cytoplasm of the macrophages (Fig. 2D). However, PRDX2 was negative to weakly positive in them (Fig. 3D). In capillary vessels of the dermis and subcutaneous tissue, nuclei and cytoplasm of endothelial cells were negative for PRDX1 and 2 (Figs. 2E and 3E). Moreover, fibroblasts were negative to positive for PRDX1, and negative to weakly positive for PRDX2 (Figs. 2F and 3F). Striated cutaneous muscle cells were negative for PRDX1 and 2 (Figs. 2G and 3G). In the spleen, both of the nucleus and cytoplasm of neutrophils were negative for PRDX1 and 2 (Figs. 2H and 3H). The cytoplasm of lymphocytes and erythroid cells, including erythroblasts, was positive for PRDX1 (Fig. 2I and 2J). PRDX2 showed negative in lymphocytes (Fig. 3I), and strongly positive in cytoplasm of erythroid cells (Fig. 3J). Endothelial cells of the splenic sinus were negative to weakly positive for PRDX1, but negative for PRDX2 (Figs. 2K and 3K). Neutrophiles in the normal tissues were consistently negative for PRDX1 and 2.

In the present study, normal fibroblasts and macrophages were used as positive controls, while normal endothelial cells, striated muscle, nuclei of erythroblasts, and neutrophils were negative controls for PRDX1. Apocrine glands and erythrocytes were seen as positive controls, and normal endothelial cells, striated muscle, lymphocytes, and neutrophils were negative controls for PRDX2.

*Expression of the newly formed vascular vessels of canine granulation tissue for PRDX1 and 2*

Most proliferating endothelial cells in newly formed vascular vessels of granulation tissue samples were strongly labeled by PRDX1 and 2 with both nuclear and cytoplasmic expression observed (Fig. 4A and 4B). In contrast, quiescent endothelial cells in mature vessels of fibrous connective tissue continuous with granulation tissue had no or weak expression of PRDX1 and 2 (Fig. 4C and 4D). Neutrophils were negative to weakly positive for PRDX1 and 2, while macrophages were consistently strongly positive for them. Fibroblast staining varied from negative to positive.

*Expression and scores of PRDX1 and 2 in canine HA and HSA*

While each internal positive control in HA, macrophages or some erythrocytes were positive for PRDX1 or 2, almost all HA tumor cells were negative or very weak for PRDX1 and 2 (Fig. 5A and 5B). The average and median of IHC score for PRDX1 staining in 26 HA samples were 3.32 (SD ± 3.41) and that for PRDX2 was 1.83, and 1.04 (SD ± 1.48) and 0.67, respectively. However, neoplastic endothelial cells of HSA exhibited strong nuclear and cytoplasmic positivity for PRDX1 and 2 (Fig. 5C and 5D). The average and median of IHC scores for PRDX1 staining in 54 HSA samples were 7.69 (SD ± 3.25) and 8.00, and 7.59 (SD ± 3.98) and 9.50 for PRDX2 staining, respectively. The IHC score for PRDX1 and 2 of HSA were significantly higher than that of HA ($P<0.0001$) (summarized as box-and-whisker plots in Fig. 5E and 5F).

*Expression of PRDX1 and 2 on histopathological subtype of HSA*

In the histopathological subtypes of HSAs, the IHC scores of PRDX1 and 2 of HSA are summarized as a box-and-whisker plot (Fig. 6). The average and median of IHC scores for PRDX1 staining were 8.00 (SD ± 3.33) and 9.33 in conventional type, 7.89

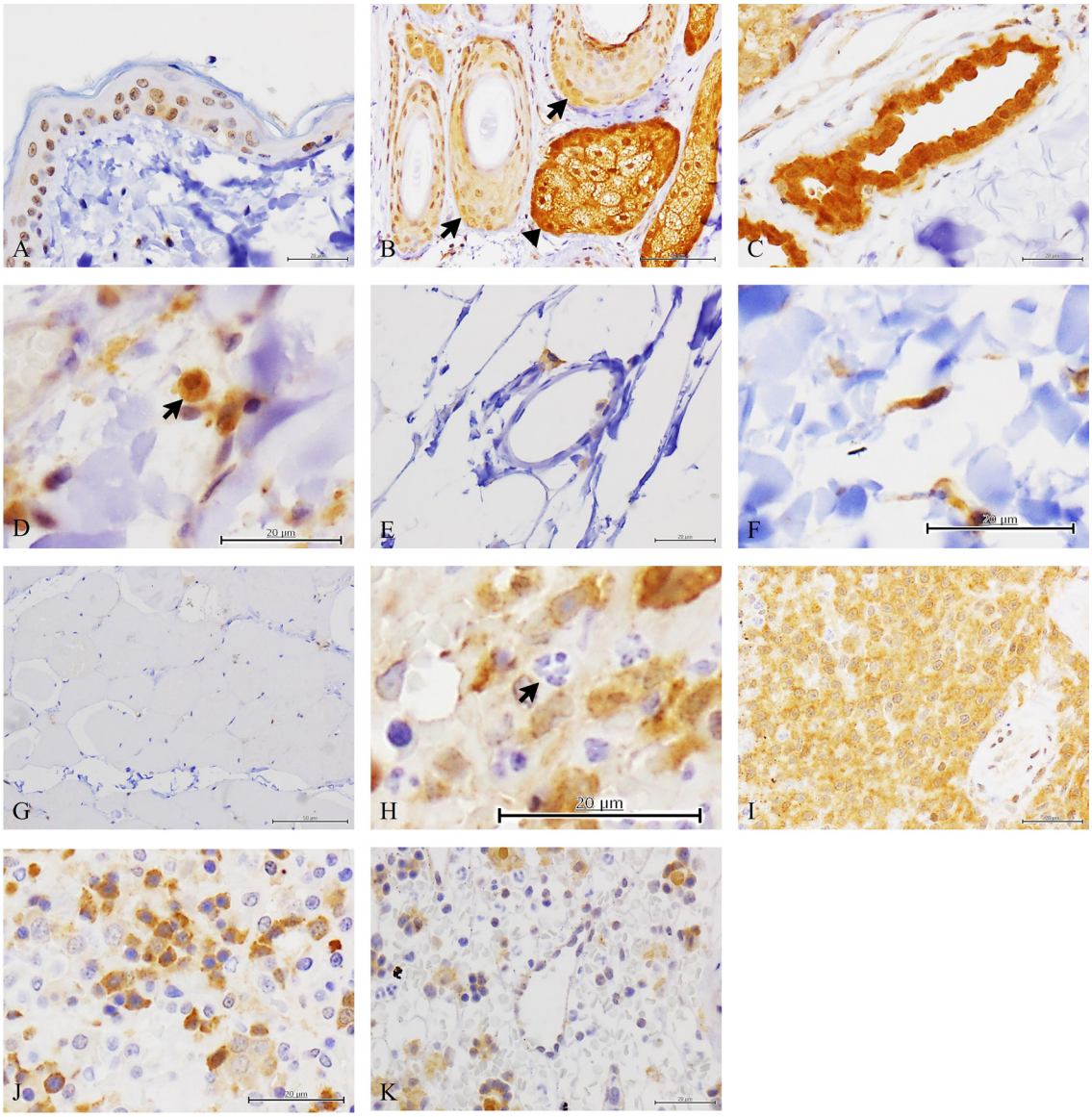


**Fig. 2.** Staining properties of peroxiredoxin (PRDX) 1 in normal canine tissues. (**A**) Squamous cells in the epidermis. The nuclei of squamous cells located in the basal and spinous layers were strongly positive for PRDX1 and the cytoplasm was weakly positive. However, squamous cells in the granular layer were negative for PRDX1. Bar, 20 µm. (**B**) Squamous cells of hair follicles (arrows) and sebaceous glands (arrowhead). The nuclei of squamous cells were positive for PRDX1 and the cytoplasm was weakly positive. Almost all sebaceous gland cells were positive for PRDX1 expression. Bar, 50 µm. (**C**) Apocrine sweat glands. Glandular epithelial cells were positive for PRDX1, but myoepithelial cells surrounding the glands were negative. Bar, 20 µm. (**D**) Macrophages in the dermis. The nucleus and cytoplasm of macrophages were labeled with PRDX1 (arrow). Bar, 20 µm. (**E**) Vascular vessels in the dermis. Almost all the endothelial cells in the vascular vessels were negative for PRDX1. Bar, 20 µm. (**F**) Fibroblasts in the dermis. Fibroblasts were negative to positive for PRDX1. Bar, 20 µm. (**G**) Striated cutaneous muscle. The nucleus and cytoplasm of striated muscle were negative for PRDX1 expression. Bar, 50 µm. (**H**) Neutrophil in the spleen. Both of the nucleus and cytoplasm of neutrophils were negative for PRDX1 (arrow). Bar, 20 µm. (**I**) Lymphocytes in the lymphoid follicles of the spleen. The cytoplasm of the lymphocytes was positive for PRDX1. Bar, 20 µm. (**J**) Erythroblasts in the spleen. Erythroblasts were positive for PRDX1 expression. Bar, 20 µm. (**K**) Endothelial cells of splenic sinusoids. The nuclei of the endothelial cells of the splenic sinusoid were negative to weakly positive for PRDX1. Bar, 20 µm.

(SD ± 3.79) and 9.33 in Kaposiform type, and 6.86 (SD ± 2.91) and 7.33 in epithelioid type, respectively (Fig. 6A). For PRDX2, they were 7.38 (SD ± 4.18) and 9.00 in conventional type, 8.96 (SD ± 3.18) and 10.67 in Kaposiform type, and 7.19 (SD ± 4.21) and 9.00 in epithelioid type, respectively (Fig. 6B). There was no significant difference in PRDX1 and 2 expressions among HSA subtypes.

**Table 1.** The staining properties of peroxiredoxin (PRDX) 1 and 2 in canine normal tissues

| Organ/tissue, cell | PRDX1 | | PRDX2 | |
|---|---|---|---|---|
| | nucleus | cytoplasm | nucleus | cytoplasm |
| Skin | | | | |
| Squamous cell | | | | |
| Basal layer | ++ | ± | ++ | ± |
| Spinous layer | ++ | ± | ++ | ± |
| Granular layer | - | - | - | - |
| Hair follicle | | | | |
| Internal root sheath cell | + | ± | -~± | ± |
| External root sheath cell | + | ± | -~± | ± |
| Hair papilla | -~± | -~± | -~± | -~± |
| Sebaceous gland | | | | |
| Immature glandular epithelial cell | ++ | ++ | ++ | ++ |
| Mature glandular epithelial cell | ++ | ++ | ++ | ++ |
| Apocrine gland | | | | |
| Glandular epithelial cell | ++ | ++ | ++ | ++ |
| Myoepithelial cell | - | - | -~± | -~± |
| Macrophage | + | + | -~± | -~± |
| Endothelial cell | - | - | - | - |
| Fibroblast | -~± | -~± | -~± | -~± |
| Striated muscle | - | - | - | - |
| Spleen | | | | |
| Neutrophil | - | - | - | - |
| Lymphocyte | -~± | + | - | - |
| Erythroblast | - | + | ± | ++ |
| Erythrocyte | No data | + | No data | + |
| Splenic sinus | -~± | -~± | - | - |

-, negative; ±, weakly positive; +, positive; ++, strongly positive.

*Expression of PRDX1 and 2 on the primary sites of HSA*

IHC scores of PRDX1 and 2 from the primary sites of HSA are summarized as a box-and-whisker plot (Fig. 7). The average and median of IHC score for PRDX1 staining in 27 splenic HSA samples were 8.48 (SD ± 3.04) and 9.33, and 7.47 (SD ± 4.13) and 9.00 for PRDX2 staining, respectively. That of 27 HSA samples from skin were 6.89 (SD ± 3.38) and 8.00 for PRDX1, and 7.72 (SD ± 3.97) and 10.0 for PRDX2, respectively. There was no significant difference in PRDX1 and 2 expressions in HSA between spleen and skin (Fig. 7A and 7B). Then, the average IHC scores for PRDX1 and 2 staining in four HSA samples from the kidney were 5.83 and 9.25, two from the liver were 8.83 and 5.67, and two from the bone were 8.33 and 6.83, respectively. In addition, those scores in HSA samples from the adrenal gland, mesentery, and abdominal cavity were 4.67 and 8.00, 7.00 and 10.0, and 4.67 and 12.0, respectively. These IHC scores of HSA located other than spleen or skin had no significant differences in those of spleen or skin HSA.

## DISCUSSION

PRDXs are antioxidant enzymes present in almost all organisms, and their amino acid sequences are highly conserved not only in mammals but also in eukaryotes. Thus, PRDXs perform essential functions in eukaryotic cells [29]. Previously, we demonstrated that PRDX6 overexpression protects against apoptosis in canine HSA [1]. PRDX6 is a unique member of the peroxiredoxin family and has peroxidase and acidic calcium-independent phospholipase A2 activities [2]. In contrast, there is no information on the involvement between PRDX1 or 2, which are other types of PRDXs, and canine tumors. Thus, we focused on the expression of PRDX1 and 2, which are typical 2-Cys PRDXs [19]. In humans and dogs, the amino acid sequence of PRDX1 or 2 is highly homologous. In addition, owing to their functional importance, it is expected that the localization of PRDX is also common in mammals; however, there is no systemic research on the immunolocalization of PRDXs in humans and animals, including dogs, except for some organs and tissues in humans, rats, or mice [6, 12–14, 20, 21, 25, 26, 31, 43, 45, 46]. Therefore, in the present study, we attempted to investigate localization of canine tissues. At first, we validated cross-reactivities of anti-human PRDX1 or 2 antibodies against canine sample by western blotting. In western blotting using anti-human PRDX1 or 2 antibodies, an obvious single band, which was approximately 22 kDa, was detected in a lysate from canine HSA cell named Re12 cells, as the same of HeLa [9, 16]. Thus, anti-human PRDX1 or 2 antibodies were able to detect the molecule corresponding to human PRDX1 or 2 in the canine cells. Then, we performed immunostaining of canine tissues sampled during tumor resection. In the skin, PRDX1 and 2 were positive in various epithelial cells, such as squamous cells and secretory epithelial cells of the apocrine glands. These results

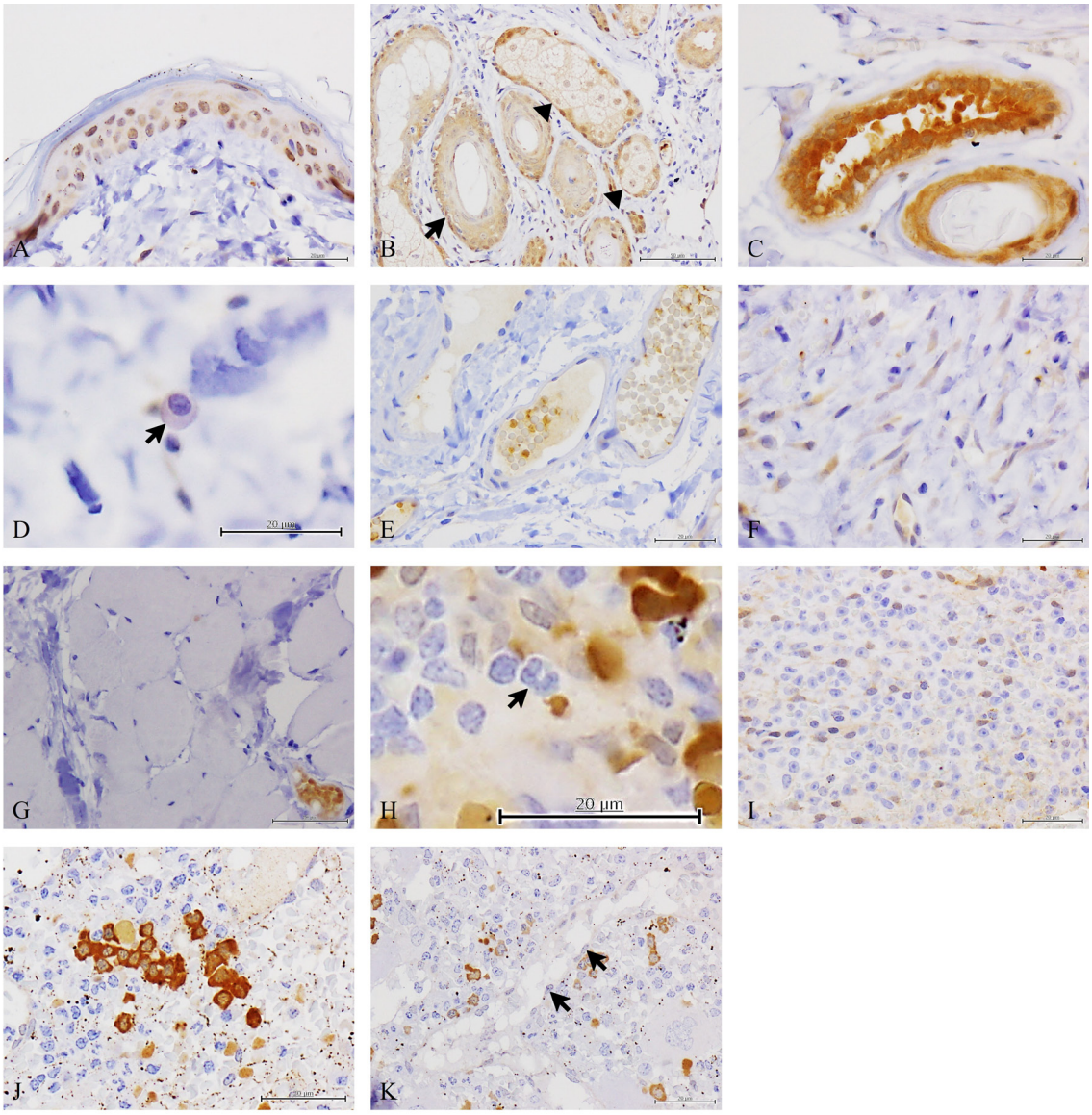


**Fig. 3.** Staining properties of peroxiredoxin (PRDX) 2 in normal canine tissues. (**A**) Squamous cells in the epidermis. The nuclei of squamous cells located in the basal and spinous layers were strongly positive for PRDX2 and the cytoplasm was weakly positive; however, squamous cells in the granular layer were negative for PRDX2. Bar, 20 µm. (**B**) Squamous cells of hair follicles and sebaceous glands. Nuclei of squamous cells were negative to positive for PRDX2 and the cytoplasm was weakly positive (arrow). Immature and mature sebaceous gland cells were positive for PRDX2 (arrowheads). Bar, 50 µm. (**C**) Apocrine sweat glands. The glandular epithelial cells were positive for PRDX2 expression. Myoepithelial cells surrounding the glands were negative to weakly positive for PRDX2 expression. Bar, 20 µm. (**D**) Macrophages in the dermis. Macrophages were negative to weakly positive for PRDX2 (arrow). Bar, 20 µm. (**E**) Microvessels in the dermis. Almost all endothelial cells in microvessels were negative for PRDX2. Bar, 20 µm. (**F**) Fibroblasts in the dermis. Fibroblasts were negative to weakly positive for PRDX2 although some erythrocytes were strongly positive for PRDX2. Bar, 20 µm. (**G**) Striated cutaneous muscle. The nucleus and cytoplasm of striated muscles were negative for PRDX2 expression. Bar, 50 µm. (**H**) Neutrophil in the spleen. The nucleus and cytoplasm of neutrophils were negative for PRDX2 (arrow). Bar, 20 µm. (**I**) Lymphocytes in lymphoid follicles of the spleen. Almost all lymphocytes were negative for PRDX2. Bar, 20 µm. (**J**) Erythroblasts in the spleen. The cytoplasm of these cells was found to be strongly positive for PRDX2. Bar, 20 µm. (**K**) Endothelial cells of splenic sinusoids. Endothelial cells of the splenic sinusoid were negative for PRDX2. Bar, 20 µm.

are in line with those reported for rat skin [21]. Similar to humans and rats, the cytoplasm of macrophages is consistently positive for PRDX1 and that of erythrocytes are positive for PRDX2 [20, 25, 26, 31]. Based on the limited results of the present study, although there were some exceptions, localization of PRDX1 and 2 might be common among mammalian species, suggesting that the expression of PRDX1 and 2 may be universal and have analogous functions in various organisms [5].

The expression of PRDX1 and 2 were clearly detected in angiogenic endothelial cells of newly formed vasculature in granulation tissues using immunohistochemistry. Quiescent endothelial cells of vessels in fibrous connective tissue continuous with

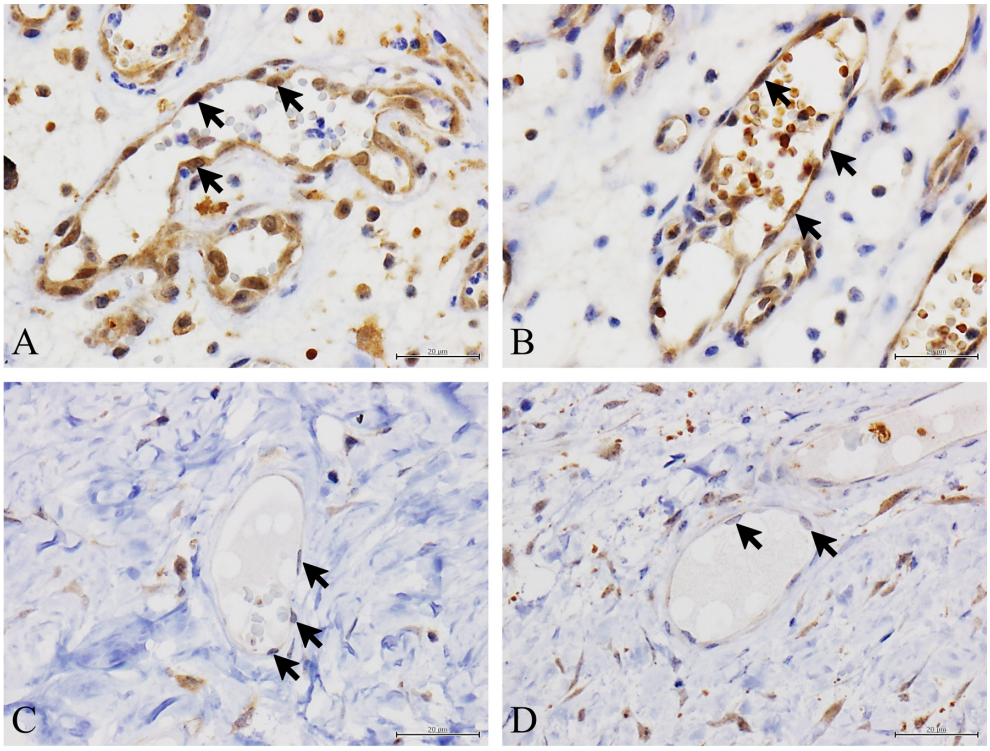


**Fig. 4.** The staining properties of peroxiredoxin (PRDX) 1 and 2 in the newly formed vascular vessels of canine granulation tissues. (**A**) The nucleus and cytoplasm of endothelial cells in angiogenic region were positive for PRDX1 (arrows). Bar, 50 µm. (**B**) The nucleus and cytoplasm of endothelial cells in angiogenic region were positive for PRDX2 (arrows). Bar, 50 µm. (**C**) The nucleus and cytoplasm of endothelial cells in resting region were negative for PRDX1 (arrows). Bar, 50 µm. (**D**) The nucleus and cytoplasm of endothelial cells in resting region were negative for PRDX2 (arrows). Bar, 50 µm.

granulation tissue were negative to slightly positive for PRDX1 and 2, as seen in endothelial cells of the normal dermis or subcutis. ROS generated by neutrophils induce PRDX expression in endothelial cells [35]. In active granulation tissue, there was infiltration of inflammatory cells, including neutrophils; thus, vascular endothelial cells may express PRDXs to protect themselves from oxidative stress. Furthermore, some studies have shown that PRDX1 and 2 play an important role in angiogenesis and proliferation of endothelial cells in inflammation or neoplastic tissues via various signaling factors, including vascular endothelial growth factors (VEGFs) or hypoxia-inducible factor-1 [15, 23, 32]. In human vascular endothelial cells, PRDX2 also prevents oxidative inactivation of VEGF receptor-2, which has an oxidation-sensitive cysteine residue [15]. Previously, we reported the expression of VEGF, basic fibroblast growth factor, and their receptors in newly formed vascular endothelial cells in canine granulation tissues [42]. Thus, it is possible that the expression of PRDXs not only protects them from oxidative stress but is also involved in the angiogenic functions of endothelial cells via regulation of expression of growth factors, such as VEGF. Moreover, VEGF and its receptors are also expressed in neoplastic endothelial cells of canine HSA [42]; thus, it is possible that the relationship between VEGF, its receptor, and PRDX in canine HSA is similar to that in angiogenic endothelial cells of newly formed vascular vessels.

It has been reported that the expression of PRDXs in various cancer tissues varies when compared to the normal tissues, suggesting that changes in PRDX expression may influence development or behavior, such as survival, proliferation, and metastasis of cancer cells [29]. Recently, some studies have shown that ROS, including $H_2O_2$, play an important role in various signaling pathways as second messengers, which are redox regulators that regulate signaling pathways by oxidizing proteins [3, 29]. Furthermore, changes in PRDX expression can play an important role in the survival and proliferation of cancer cells by influencing redox regulation. In the present study, the expression of PRDX1 and 2 in HSA was significantly higher than that in HA. These results correspond to reports in humans that PRDX1 and 2 are overexpressed in pancreatic cancer [37], liver cancer [36], lung cancers [4, 18], and esophageal squamous cell carcinomas [7]. These studies have suggested that PRDX1 and 2 are expressed in malignant tumors of humans and may enhance tumor progression; regardless of the histopathological type or primary site, it is considered a universal property of neoplastic cells of canine HSA.

In conclusion, we detected that neoplastic endothelial cells in spontaneous canine HSA overexpressed PRDX1 and 2. The results of our study suggest that PRDX1 and 2 may be involved in the proliferation of angiogenic endothelial cells in granulation tissue. Furthermore, PRDX1 and 2 were expressed higher in malignant than in benign endothelial tumor cells, suggesting that PRDX1 or/and 2 may be involved in malignant behavior in the endothelial tumors. Although further research is needed, our findings suggest that they may be therapeutic targets of canine HSA.

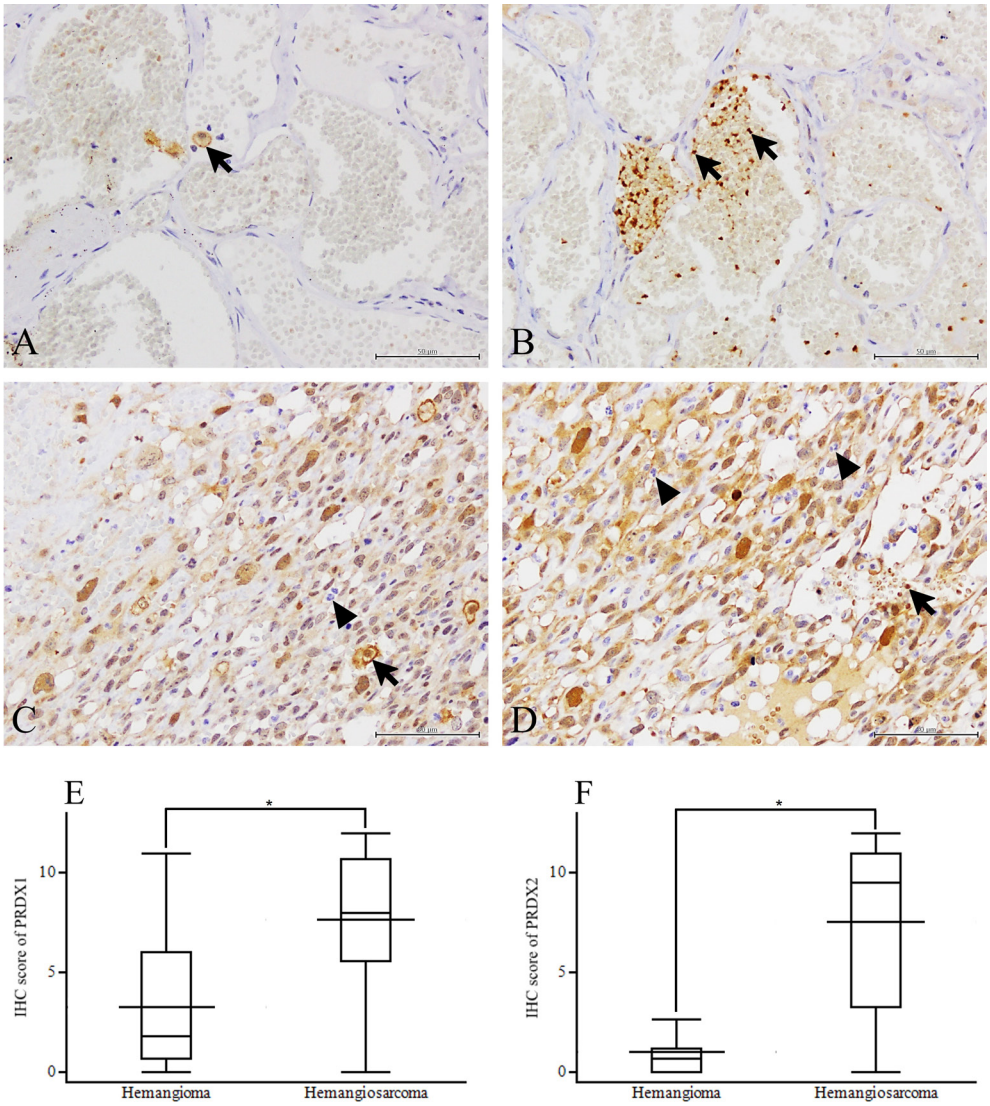


**Fig. 5.** The expression levels of peroxiredoxin (PRDX) 1 and 2 in canine hemangioma (HA) and hemangiosarcoma (HSA) tissues. (**A**) Immunohistochemical (IHC) staining image for PRDX1 expression in canine HA. Macrophages in HA tissues were positive for PRDX1 (arrow). HA cells were negative for PRDX1. IHC score of this sample is 0.00. Bar, 50 µm. (**B**) IHC staining image for PRDX2 in canine HA. Some erythrocytes were positive for PRDX2 (arrows). HA cells were negative for PRDX2. IHC score of this sample is 0.00. Bar, 50 µm. (**C**) IHC staining image for PRDX1 in canine HSA. Macrophages (arrow) and neutrophils (arrowhead) were observed as positive and negative control for PRDX1, respectively. HSA cells were positive for PRDX1. IHC score of this sample is 12.00. Bar, 50 µm. (**D**) IHC staining image for PRDX2 in canine HSA. Some erythrocytes stained positively (arrow) and neutrophils (arrowhead) were used as positive and negative control for PRDX2, respectively. HSA cells were positive for PRDX2. IHC score of this sample is 12.00. Bar, 50 µm. (**E**) The box-and-whisker plot of IHC scores of PRDX1 in canine HA and HSA. IHC score of PRDX1 in HSA was significantly higher than that in HA. Bar crossing the box-and-whisker plot expresses the sample average. (**F**) The box-and-whisker plot of IHC scores of PRDX2 in canine HA and HSA. IHC score of PRDX2 in HSA was significantly higher than that in HA. Bar crossing the box-and-whisker plot expresses the sample average. **P*<0.05.

CONFLICT OF INTEREST. The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this manuscript.

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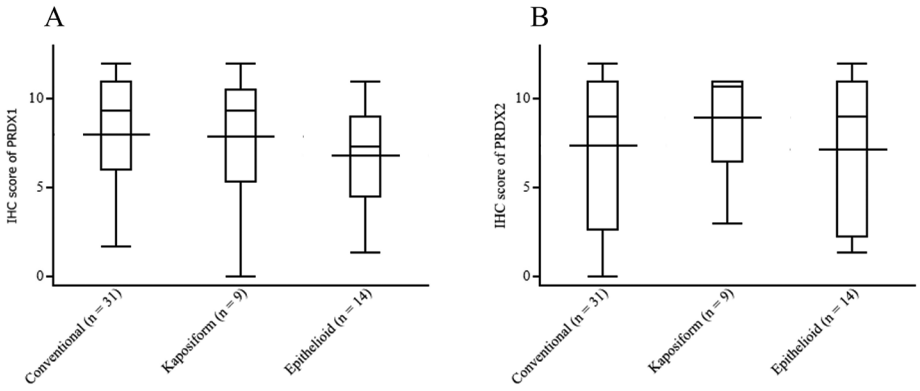


**Fig. 6.** The expression of peroxiredoxin (PRDX) 1 and 2 in histopathological subtypes of canine hemangiosarcoma (HSA). (**A**) The box-and-whisker plot of the immunohistochemical (IHC) scores of PRDX1 in each histopathological subtypes of canine HSA. There were no significant differences in the expressions of PRDX1 among three subtypes of HSA. (**B**) The box-and-whisker plot of IHC scores of PRDX2 in each histopathological subtypes of canine HSA. There were no significant differences in the expressions of PRDX2 among three subtypes. Bar crossing the box-and-whisker plot expresses the sample average.

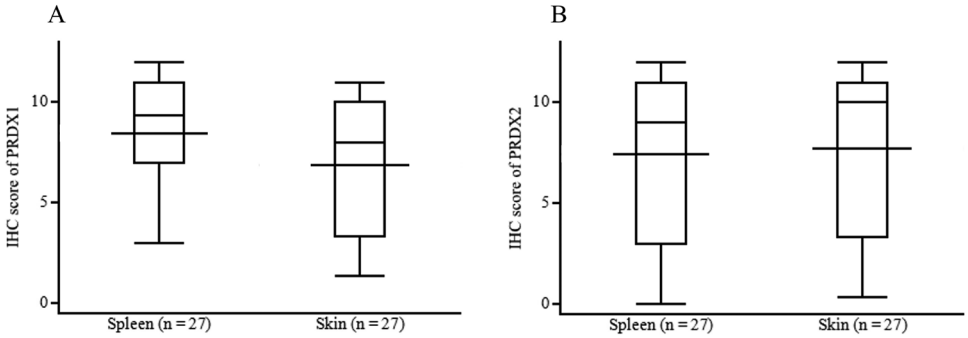


**Fig. 7.** The expression of peroxiredoxin (PRDX) 1 and 2 of canine hemangiosarcoma (HSA) between spleen (n=27) and skin (n=27). (**A**) The box-and-whisker plot of immunohistochemical (IHC) scores of PRDX1 of canine HSA in spleen and skin. There were no significant differences in the expressions of PRDX1 between spleen and skin. (**B**) The box-and-whisker plot of IHC scores of PRDX2 of canine HSA in spleen and skin. There were no significant differences in the expressions of PRDX2 between spleen and skin. Bar crossing the box-and-whisker plot expresses the sample average.

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