Studies on the Hemal Node in Japanese Black Cattle

黒毛和種牛の血リンパ節の基礎的研究

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Abstract

Combining with those secondary lymphoid organs such as normal lymph node (LN) and spleen (SP), the hemal node (HN) of bovine was investigated by morphological and immunological methods in this study.

The histology and the vasculature of HNs in Japanese black bovine were evaluated by techniques of light microscopy, scanning electron microscopy (SEM) and a combined method of vascular corrosion casting (VCC). The HNs were mainly located in three regions: subcutaneous region of the head temporal fossa, cervical part and trunk, the mesenteric region and along the large blood vessels in the thoracic, abdominal and pelvic cavities. The HN had histological structure similar as LN and SP. They were encapsulated by a capsule composed of connective collagen and the trabeculae originated from the capsule projected into the parenchyma, which could be differentiated by cortex and medulla area. The sinuses of the HN were filled with erythrocytes, and the macrophages, those lymphocytes were also found in their independent functional areas.

From the three-dimensional VCC analysis, a dense vessel network of capsule was found surrounding the cast of HN parenchyma and had no connection with the subcapsular sinus; these vessels converged and exited the HN via the hilar vein. Within the HN, many anastomoses were found between the capillary networks and the surrounding sinuses in the follicular zone and deep cortex. This may explain the origin of numerous erythrocytes in the HN sinusoids and help to understand lymphocyte migration of the HN.

The distribution and population of immunocompetent cells in bovine HN, mesenteric lymph node (MLN) and SP were analyzed comparatively by immunohistochemistry (IHC) and flow cytometry (FACS). Many $CD8^+$ cells, $CD172a^+$ cells and $TCR1\text{-}N24^+$ ($\gamma\delta$ T) cells were found in the lymphatic cord along the sinus of the HN and the splenic red pulp of SP. A few $CD8^+$ cells and $\gamma\delta$ T cells were distributed diffusely in the paracortex and medullary cord of the MLN. $CD4^+$

cells in HN and MLN were mainly present in the paracortex, and in the SP, most $CD4^+$ cells were distributed in the periarteriolar lymphoid sheath (PALS). Many germinal centers which contained $CD21^+$ cells were recognized in the lymphatic regions such as the cortex of HN and MLN, and the white pulp of the SP in these lymphoid organs. MHC class II^+ cells were mainly distributed in the lymphoid follicles, the paracortex and the sinusoid regions of HN and MLN, and in the SP, those positive cells mainly distributed in the red pulp and white pulp. The populations of $CD8^+$ cells and γδ T cells in the HNs and the SP were higher than those of the MLNs and peripheral blood mononuclear cells (PBMC). In particular, the population of γδ T cells in the HN was significantly higher than that of MLN. In addition, the populations of $CD21^+$ cells and MHC class II^+ cells in the HNs and the MLNs were significantly higher than those of the SP. The results suggest that the HN has an important role in both cellular and humoral immunity as well as the LN and the SP in bovine.

The frequency of high endothelial venule (HEV) and their regional differentiations in HN were analyzed comparing to the MLN and SP by IHC stain. In addition, population of L-selectin and other CD markers double positive cells were analyzed by FACS analysis. The HEV was found in all MLNs but absent in SP. The frequency of HEV $marker^+$ HN ($MECA\text{-}79^+$ HN) was different among the localizations, and the value of the subcutaneous region (61 %) was significantly higher than those values along aorta (33 %) and the mesenteric region (17 %). The HEV in MLN was distributed mainly in the paracortical area, but in HN, the positive signals were mainly found in the perifollicular area, a few positive signals were also located in the paracortical and interfollicular area. In HEV $marker^+$ HNs, the populations of $CD4^+$ cells, $CD4^+$ $L\text{-}selectin^+$ cells and $CD8^+$ $L\text{-}selectin^+$ cells were significantly higher than those of HEV marker negative HNs ($MECA\text{-}79^-$HN). The data suggest lymphocyte homing mechanism exist in HN, which concerns about functional differences among the localizations on immunological characteristics.

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Abbreviations

CAM	cell adhesion molecule
FACS	flow cytometry
GC	germinal center
HE	hematoxylin-eosin staining
HEV	high endothelial venule
HN	hemal node
HLN	hemolymph node
IHC	immunohistochemistry
M	medulla
MLN	mesenteric lymph node
MZ	marginal zone
LN	lymph node
PALS	periarteriolar lymphoid sheath
PBMC	peripheral blood mononuclear cells
PNAD	peripheral node addressin
RP	red pulp
SP	spleen
SEM	Scanning electron microscopy
T_{reg}	regulatory T cell
TRI	masson's trichrome staining
VCC	vascular corrosion cast

Chapter 1

General Introduction

1.1 Background

Hemal nodes (HN) are independent secondary lymphoid organs found in various mammals such as humans, rats and ruminants and also in some birds (Lewis, 1904; Turner, 1969; Gargiulo et al., 1987; Abu-Hijleh and Scothorne, 1996; Cerutti and Guerrero, 2001; Zidan and Pabst, 2004; Zidan and Pabst, 2010). But they are usually ignored by most immunologists, and the term hemolymph node (HLN) is often erroneously used instead of HN (Andreasen and Gottlieb, 1946; Turner, 1969; Nopajaroonsri et al., 1974; Kazeem et al., 1982; Ezeasor and Singh, 1988; Abu-Hijleh and Scothorne, 1996; Landsverk and Press, 1998). Different with those HLNs which possess both blood sinuses and lymphatic sinuses, HN only have blood sinuses and receive blood via small blood vessels (Weller, 1938; Singh, 1959; Bacha and Bacha, 2000; Zidan and Pabst, 2004; Casteleyn et al., 2008; Bozkurt and Kabak, 2010; Zidan and Pabst, 2010). HNs are mainly located in the thoracic, abdominal cavities along the blood circulation of different animals, with oval or round shaped and the colors varying from pink to dark red, their size, number and histological characteristics vary considerably (Getty, 1975; Ezeasor and Singh, 1988; Dellmann and Eurell, 1998; Zidan and Pabst, 2004).

The latest official reports about HNs was given on the website of the Department of Natural Resources, of the State of Michigan in the USA in 2002, which reported concern for hunters regarding HNs in deer. And in the previous studies, HNs have been described in many species such as rats (Andreasen and Gottlieb, 1946; Turner, 1969; Turner, 1971a; Turner, 1971b; Hogg et al., 1982; Abu-Hijleh and Scothorne, 1996), sheep (Al-Bagdadi et al., 1986; Gargiulo et al., 1987; Thorp et al., 1991), bovine (Winqvist, 1954; Ceccarelli et al., 1986; Cerutti et al., 1998; Bassan et al.,

1999; Cerutti and Guerrero, 2001; Casteleyn et al., 2008; Cerutti and Guerrero, 2008), goats (Ezeasor and Singh, 1988; Ezeasor et al., 1989; Ezeasor and Singh, 1990; Ozaydin et al., 2012), piglets (Akaydin and Kabak, 2006), dromedary camel (Zidan and Pabst, 2004), buffalo (Singh, 1959; Zidan and Pabst, 2010), roe deer (Bozkurt and Kabak, 2010), and the presence of HNs in humans has also been discussed comparing with those HLNs but not common in the literature and the time is too long (Warthin, 1901; Lewis, 1904; Lederer, 1923). From one recent report, neither HN nor HLN is found in the proximity of human splenic vessels (Bogacz et al., 2000).

In all animals studied previously, the HNs have been found to have a general structure like normal lymph node (LN) and spleen (SP), with some species variations, but the fine structure of blood supply is still questioned (Turner, 1969; Turner, 1971a; Cerutti et al., 1998; Dellmann and Eurell, 1998; Bozkurt and Kabak, 2010; Zidan and Pabst, 2010).

By contrast with their morphological findings, the development and function of HNs has not been fully elucidated. The development mechanisms of HNs remain unknown and the reports about their growth and compensatory modifications are very rare (Warthin, 1901; McCarthy, 1903; Lewis, 1904; Bassan et al., 1999). We can't exclude the possibility of some local environmental factor causing the temporary transformation of HNs, but HNs are persistently found close to large blood vessels in the abdomen and thorax, this makes it hard to believe that HNs are formed from LNs or SP as a result of some general growth factors, and their existence as a secondary lymphoid organ in mammals especially in ruminants is currently uncontroversial (Turner, 1969; Cerutti and Guerrero, 2001; Zidan and Pabst, 2004; Zidan and Pabst, 2010).

Those possible new functions include erythrophagocytosis, erythropoiesis, platelet formation and most recently, the immunological functions have become the hot points in the researches of HNs (Turner, 1971b; Cecarelli et al., 1986; Ezeasor et al., 1989; Thorp et al., 1991; Cerutti and Guerrero, 2001; Zidan and Pabst, 2004; Casteleyn et al., 2008; Cerutti and Guerrero, 2008). In certain diseases which affected the

animals such as sheep and bovine, HNs were found increased in number and grossly enlarged in size, and they usually had prominent histological lymphocyte follicular hyperplasia, as evidence of stimulation of the lymphoid system and antibodies production, were considered to have immunological roles in their function (Weller, 1938; Wallnerova and Mims, 1970; Snider III, 2003). However, only a few studies on immune related functions in HNs have been applied, and in further research their exact roles need more concerns to clarify.

1.2 Aim of this Study

The present study was focus on the structural features and the immune functions of HNs center on their controversial points, such as the cause of erythrocytes in sinuses, the possible roles in immunology and in addition, their associated mechanisms.

Therefore, the present work was undertaken in Japanese black bovine with the following objectives:

1) To characterize the gross anatomy and morphological structure of the HNs, their blood supply was observed by scanning electron microscopy (Chapter 2)

2) To investigate the immunological roles of the HNs, the distributions and populations of immunocompetent cells in the HNs, LNs and SP were studied comparatively (Chapter 3)

3) To investigate the lymphocyte homing in the HNs, the distribution of high endothelial venules (HEV) and their associated lymphocytes subsets were analyzed (Chapter 4)

Chapter 2

Morphology and Blood Supply of Hemal Node

Abstract

The morphology and the vasculature of hemal node (HN) in Japanese black bovine were evaluated by techniques of light microscopy, scanning electron microscopy (SEM) and a combined method of vascular corrosion casting (VCC). The HNs were mainly located in fat of three parts, includes subcutaneous region of the head temporal fossa, cervical part and trunk, the mesenteric region and along the large blood vessels in the thoracic, abdominal and pelvic cavities. They were encapsulated by a capsule composed of connective collagen and strengthened by reticular fibres and smooth muscle cells, the trabeculae originated from the capsule projected into the parenchyma, which could be differentiated by cortex and medulla area. The sinusoid pathway in the HN was characterized by subcapsular sinuses, which were continuous with the trabecular sinuses and tubular sinuses over the parenchyma, and these sinuses finally entered into the medullary sinuses which connected with the hilar veins. The sinuses of the HN were filled with erythrocytes, and the macrophages, those lymphocytes were also found in their independent functional areas.

From the three-dimensional VCC analysis, a dense vessel network of capsule was found surrounding the cast of HN parenchyma and had no connection with the subcapsular sinus; these vessels converged and exited the HN via the hilar vein. Within the HN, many anastomoses were found between the capillary networks and the surrounding sinuses in the follicular zone and deep cortex.

The HN had histological structure similar as normal lymph node (LN) and spleen (SP). In this study, direct communications between cortical capillaries and subcapsular sinuses were identified. This may explain the origin of numerous erythrocytes in the HN sinusoids and help to understand lymphocyte migration of the

HN.

2.1 Introduction

Hemal node (HN) appears to be furnished with a lymphoid structure, in which sinuses filled with erythrocytes are present in place of the lymph sinuses. HNs are independent lymphatic organs that exist in various mammalian species and also in some birds (Lewis, 1904; Turner, 1969; Gargiulo et al., 1987; Abu-Hijleh and Scothorne, 1996; Cerutti and Guerrero, 2001; Zidan and Pabst, 2004; Zidan and Pabst, 2010). Gibbes described the presence of HNs in humans for the first time, and then they were named by Robertson (Gibbes, 1884; Robertson, 1890). Since then, contradictory views have been held about their general biological significance by investigating their structure and possible functions (Warthin, 1901; Lewis, 1904; Lederer, 1923; Turner, 1969; Gargiulo et al., 1987; Cerutti and Guerrero, 2001; Zidan and Pabst, 2010).

In recent years, some new concepts have been proposed for the function of HNs including erythrophagocytosis, erythropoiesis, platelet formation and immune cell activation (Turner, 1971b; Cecarelli et al., 1986; Ezeasor et al., 1989; Thorp et al., 1991; Cerutti and Guerrero, 2001; Zidan and Pabst, 2004; Casteleyn et al, 2008; Cerutti and Guerrero, 2008). However, the blood supply of HNs, how and from where the abundant of erythrocytes in their sinuses come from have not been completely clarified and always part of those arguments (Singh, 1959; Turner, 1969; Hogg et al., 1982; Gargiulo et al., 1987; Ezeasor and Singh, 1990; Zidan and Pabst, 2004; Cerutti and Guerrero, 2008). An understanding of the HN vascular system is essential, because lymphoid tissues in HNs play an important role in the possible immune function, and the routes from which the lymphocytes and erythrocytes enter into the HNs are necessary to investigate the lymphocytes homing and erythrocytes circulation.

The technique of vascular corrosion casting (VCC) combined with scanning electron microscopy (SEM) can provide information on the three-dimensional

structure of the HN and make it possible to study the HN vasculature in detail. Since previous studies of the HN vasculature were performed in rats (Turner, 1969), we undertook a study of the HN to define its vascular architecture using a combination of VCC and SEM, and other morphological techniques, to help discuss the blood supply of HN especially the possible originations of the numerous erythrocytes, and provide basic theory for the lymphocyte circulation.

2.2 Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki. Japanese Black bovines (1-24 months old, n=80) and two foetuses (foetal age approximately 5 months and 9 months) were obtained from local farms in Miyazaki Prefecture. The animals were killed by electric shock following the combination of xylazine (0.2mg/kg) and pentobarbital (15mg/kg) intravenous injection.

2.2.1 Light Microscopy

Following dissection procedures, the HNs were examined carefully, 200 HNs were selected from the bovines, and from the fetuses, totally 3 HNs were found and removed. All HNs were immediately fixed in phosphate-buffered 10% formaldehyde solution for at least 48 hours, and finally they were embedded in paraffin. The sections (4 μm) were prepared according to routine procedures. Hematoxylin-eosin Staining (HE) method was applied to investigate the general structure of the nodes, while Masson's Trichrome Staining (TRI) method was used to differentiate the collagen fibres and smooth muscle cells.

2.2.2 Scanning Electron Microscopy

For observation with scanning electron microscope, HNs in the abdominal and pelvic cavities were perfused with warm heparinized saline through the abdominal artery supplying them for 15 minutes. After the perfusion, 55 HNs were removed

from the calves and cut into small pieces with a razor blade. The specimens were dehydrated with alcohol and immersed in amyl acetate, after which they were dried according to the critical point method (Critical Point Dryer HCP-2; Hitachi Co., Ltd., Tokyo, Japan) (Anderson, 1951). The dried specimens were coated with gold (Ion Sputter E-1030; Hitachi, Co., Ltd., Tokyo, Japan) and observed by scanning electron microscopes (SEM-4100, Hitachi, Co., Ltd., Tokyo, Japan; JSM-6390, JEOL Datum Ltd., Tokyo, Japan). To investigate the blood sinuses, the spleen was also flushed and taken for comparation.

2.2.3 Vascular Corrosion Casting

For observation with vascular corrosion casting, 30 HNs along the cervical artery of 10 calves were collected with surrounding tissue. The HNs were cannulated with a fine needle after isolation of the ascending arteriole under a stereomicroscope. The HNs were injected according to the previous reports (Castenholz and Castenholz, 1996; Okada et al., 2002) (Fig2.1). Briefly, after flushing with warm heparinized saline, 2-3 ml resin (Mercox II Kit, Ladd Research, Williston, VT, USA) was injected into the HN slowly using a disposable syringe. For polymerization of the resin, the HN with some surrounding tissue was immersed in hot water (60°C) for at least 15 min immediately after resin injection. All tissue surrounding the resin cast was removed by digestion in 1% NaOH solution for 24 hr. Then, after washing in hot tap water (50-60°C) for at least 10 min, the specimen was immersed in 1% sodium hypochlorite solution for another 24 hr, followed by washing in hot tap water. These digestion steps were repeated over several days, and when all tissue was removed, the cast was rinsed several times in distilled water and air dried. The cast was then mounted on an SEM stub, sputter coated with gold (Ion Sputter E-1030; Hitachi, Co., Ltd., Tokyo, Japan), and then observed under scanning electron microscopes (SEM-4100, Hitachi, Co., Ltd., Tokyo, Japan; JSM-6390, JEOL Datum Ltd., Tokyo, Japan). Except in the cases of incomplete injection of two HNs and one broken cast because of misoperation, all casts of HNs showed consistent basic vasculature.

2.3 Results

2.3.1 Gross Anatomy and General Histology

The HNs in Japanese Black bovine were in oval or round shape with colors varying from pink to dark red, they were located mainly along the big vessels of the thoracic, abdominal and pelvic cavities (Fig. 2. 2a and 2b), and in the mesenteric region (Fig. 2. 2c and 2d). In addition, the HNs also could be found in the axillary region and the subcutaneous region of the head temporal fossa, cervical area and trunk, which located along with the blood vessels (Fig. 2. 2e and 2f).

All HNs were embedded in adipose tissue, sometimes they grew up with LNs nearby, and those HNs had communication only with blood vessels (Fig. 2. 2b and 2c; Fig. 2. 3a and 3b). Unlike those of the normal LNs, the capsules of HNs varied in thickness of a wide range, and they contained many small vessels, but typical afferent or efferent lymphatic vessels were not observed in the capsule (Fig. 2. 3c-3h).

The most developed lymphoid regions in HNs consisted of three main regions: the follicular region in which many germinal centers were located; the paracortex which consisted of the lymphatic region and located between cortex and medulla; and the sinusoid region which consisted of the lymphatic cord and sinus (Fig. 2. 3c-3h). In HNs, most of the lymphoid follicles within the cortex were secondary lymphoid follicles. The lymphoid follicles and lymphatic cords in the HNs contained many lymphocytes, they were strongly supported by reticular fibres and cells (Fig. 2. 3d, 3f and 3h; Fig. 2. 4a and 4b).

The parenchyma of HNs was encapsulated by connective collagen tissues and smooth muscle cells, and those smooth muscle cells were usually observed in the trabeculae and inner connective tissue (Fig. 2. 4a and 4b). The trabeculae originated from the capsule and projected into the parenchyma, which helped form and support the independent areas such as cortex and medulla (Fig. 2. 4c-4f).

The supcapsular sinus was just beneath the capsule and was filled with numerous erythrocytes and lined with endothelial cells (Fig. 2. 5a and 5b). Collagenous trabeculae and reticular fibers were usually found extended through the lumen of the

sinuses (Fig. 2. 5c and 5d). Trabecular sinuses surrounded the trabeculae, at the interior part of the nodes, and were continuous with the subcapsular sinus (Fig. 2. 4a-4d; Fig. 2. 5c-5g). Those trabecular sinuses extended into medullary sinuses and finally left the node from the hilus (Fig. 2. 5g and 5h).

No significant differences were seen in the basic structure of the HNs between the individual animals or between the nodes from different regions, but the lymphoid tissues of HNs had many variations (Fig. 2. 6a-6d).

In the case of the nodes from two bovine foetuses , the foetal HNs were found only in the foetus of 9 months foetal age. They located along the big vessels of pelvic cavities, totally 3 in number, pink or red in color. The appearances of foetal HNs and their unusual, interesting microstructures were noted because it might attract the interests of other researchers. In foetal HNs, the capsule was very thin and there were no trabeculae in the parenchyma of the nodes, the blood vessels within the capsule were observed and there were no lymphatic vessels had connection with those nodes (Fig. 2. 7a and 7c). The subcapsular sinus was present in foetal HNs, but it's hard to find erythrocytes in subcapsular sinus and some other interior sinuses, and no trabecular sinuses were observed as trabeculae were not present in the foetal HNs (Fig. 2. 7a-7d). However, there were still many blood sinuses in the interior nodes and the erythrocytes were found numerous within those sinuses (Fig. 2. 7b and 7d). In the parenchyma, a cortex and medulla could not be differentiated, the lymphoid tissues were composed of lymphoid cord, which had dispersed lymphocytes and some free erythrocytes, and many capillaries could be observed (Fig. 2. 7e). In addition, there was no primary or secondary lymphoid follicle within the nodes, and many sinuses could usually be found with few erythrocytes (Fig. 2. 7a, 7c and 7f).

2.3.2 Morphological Features under Scanning Electron Microscope

The structural components of the HN, the capsule, trabeculae, lymphoid follicles, medullary cords, blood sinuses and vessels were clearly observed under the low magnification of SEM (Fig. 2. 8a and 8b).

The HN capsule showed that its cut surface were densely piled layers consisted of dense fibrous connective tissue including collagen and reticular fibers (Fig. 2. 8c and 8d). The inner surface of the capsule was attached to the reticular cells by their feet traversing the subcapsular sinus, and some erythrocytes were usually found in the capillaries of capsule (Fig. 2. 8d). Trabeculae of variable thickness extended from the capsule into the sinuses, the blood vessels could also be observed within them (Fig. 2. 8e and 8f). When a trabeculae occurs in the sinus, a continuous reticular structure was usually formed and supported the sinusoid pathway. Its reticular fibers stretched to reach the wall of the sinus and connected with it forming a dense network (Fig. 2. 8e and 8f).

The subcapsular sinuses running beneath the capsule of the node and extended with the trabecular sinuses which connected with the medullary sinuses, they contained a very dense network of reticular cells (Fig. 2. 9a and 9b). Sometimes, in place of trabeculaes, blood vessels were found running through the sinuses of cortex and medulla, or only some reticular fibres existed (Fig. 2. 9c and 9d). In addition, in some portions of HNs especially in the medullary cord, the sinuses contained few reticular cells in their lumen were usually observed, they appeared like a hollow vessel, and somewhat similar as the splenic sinuses in SP (Fig. 2. 9e and 9f).

Except for erythrocytes, the macrophages, reticular cells and lymphocytes were observed in the subcapsular sinus and interior blood sinuses of HNs (Fig. 2. 10a-10c). In the cortex, there were few macrophages but many small round cells which may correspond to lymphocytes and plasma cells (Fig. 2. 10c and 10d). In the sinuses, reticulum cells were polygonal or stellate in form and aggregated with each other (Fig. 2. 10e). A group of cells including lymphocytes, erythrocytes and so on were usually seen either in the flat, oblique or side view, all the round type cell in the sinuses were attached to the reticular cells by the processes of the latter, especially the macrophages, they touched the reticular tissue and were usually located in the meshes of the reticulum (Fig. 2. 10c-10f).

2.3.3 Three-Dimensional Structure of Blood Supply in HN

Corrosion casts injected from the cervical artery clearly showed the vascular supply of HNs (Fig. 2. 11a-11f). The cast of HN parenchyma was surrounded by a fine network of capsule vessels with various diameters and branching patterns (Fig. 2. 11b-11f; Fig. 2. 12a-12c). In capsule, the arterioles ran along with the venules, partly interweaving with each other, and a branched venous network converged to leave the HN via the hilar vein (Fig. 2. 12a-12d). Unlike normal lymph nodes in which the hilus usually showed a concave appearance, the position of HN hilus could only be identified by the supplying vessels, where it had a relatively smooth, flattened or convex surface (Fig. 2. 12e and 12f). The vessels of the capsule entered into the parenchyma of HN along with the trabeculae processes, these vessels finally connected with the medullary sinuses but never drained into the subcapsular sinuses (Fig. 2. 13a-13h). Other vessel branches of HN penetrated into the parenchyma and formed dense capillary networks in the follicular zone and deep cortex (Fig. 2. 14a-14c). There were usually two or more arterioles supplying the lymph follicles of the HN. The capillaries of the lymph follicles formed dense plexuses, and most of them opened into adjacent subcapsular sinuses (Fig. 2. 14b and 14c). The connections of capillaries and subcapsular sinuses in the follicular zone were observed (Fig. 2. 14c and 14d), and in deep cortex, many capillaries also drained into surrounding tubular sinuses (Fig. 2. 14e). Many small, delicate sinuses in the deep cortex were found between the tubular sinuses and venules, and these venules jointed the medullary vein (Fig. 2. 14f).

The subcapsular sinuses of the HN showed a convex-shaped layer, which penetrated the parenchyma of the HN as trabecular sinuses either to join the medullary sinuses or to become continuous with a system of tubular sinuses in the cortex. Ultimately, all sinuses converged into the medullary veins (Fig. 2. 13c and 13d; Fig. 2. 15d and 15e). The cast layer of subcapsular sinuses outlined the shape and dimension of the HN; many dome-like protrusions indicated the position of the lymph follicles (Fig. 2. 15a-15c). The surfaces of the medullary veins in the cast had

distinctive endothelial impressions, which were different from the surfaces of the arteries. These medullary veins formed the venous tributaries of the hilar vein and finally exited the HN (Fig. 2. 15e and 15f).

The main structure of the vascular system in the HN of bovine cervical region is shown schematically in Fig. 2. 16.

2.4 Discussion

The HNs were found in each examined bovine and one foetus. They were found between the abdominal aorta and caudal vein, as well as in the thoracic cavity, subcutaneous region and mesenteric region, as reported previously in other ruminants (Ezeasor and Singh, 1988; Abu-Hijleh and Scothorne, 1996; Cerutti,1998; Snider et al., 2003; Zidan and Pabst, 2004; Bozkurt and Kabak, 2010; Zidan and Pabst, 2010). Even though the size and number of HNs were variable and some researchers thought they may just pathological changes that transmitted from normal lymph nodes (McCarthy, 1903; Vaida et al., 1974), the HNs had been considered as the permanent secondary lymphoid organ based on the previous and our findings.

From micro-morphological observation, the HNs of bovine consisted of a cavernous capsule of connective tissue and smooth muscles enclosing many blood sinuses, which were tightly packed with erythrocytes. The blood sinuses were partitioned by lymphoid cords rich in various blood cells, this shared a structure similar to that of spleen and lymph nodes, somewhat like an "accessory spleen." The morphological alterations in HNs in splenectomized cattle showed that HLNs presented hypertrophy and hyperplasia in lymphatic tissue due to the splenectomy along with the absence of any modifications in the lymph nodes (Bassan et al., 1999). Their conclusion was that HNs could be structures capable of performing a supplementary role in spleen-like functions in young splenectomized cattle. However, an opposite opinion was advanced by researchers who confirmed that radical splenectomies in Wistar rats failed to demonstrate any compensatory or regressive changes (Konstanty et al., 2000).

The capsule and trabeculae of bovine HNs were supported by smoothe nuscle cells and reticular fibres, similar to that reported for the HNs of many ruminants (Gargiulo et al., 1987; Ezeasor and Singh, 1988; Cerutti et al., 1998; Zidan and Pabst, 2004; Casteleyn et al., 2008; Bozkurt and Kabak, 2010). As shown in the SEM results, the reticular network extended throughout the HNs forming the structural backbone, the large number of free blood cells seemed to collect in the subcapsular sinus by flowing through the reticular meshwork supported by the particular alignment of some of the reticular cells in channel-like sinusoid passageways (Gargiulo et al., 1987).

There were various literature reports with different results on the presence of afferent and efferent lymphatic vessels in HNs. Afferent and efferent lymphatic vessels were present in the HNs of goats (Ezeasor and Singh, 1988; Ezeasor and Singh, 1990) and camels (Zidan and Pabst, 2004), but not in those of sheep (Thorp et al., 1991), buffalo calves (Singh, 1959), oxen (Constantinescu, 1988) and roe deer (Bozkurt and Kabak, 2010). Our results showed that the HNs of bovine lacked lymphatic vessels, similar to those of sheep, buffalo calves and roe deer.

The lymphoid tissue of bovine HNs contained numerous lymphocytes and reticular cells, and macrophages could also be found touching the reticulum, distributed in the subcapsular sinuses and medulla, which indicated that bovine HNs were involved in the phagocytosis of old or defective blood cells from the circulation, and also the possible immunological functions (Winqvist, 1954; Cecarelli et al., 1986; Ezeasor and Singh, 1988; Zidan and Pabst, 2004). This study also showed a few primary and many secondary follicles in the bovine HNs, and the presence of secondary follicles was indicative of the role of the HNs in antibody production (Cecarelli et al., 1986). Based on the previous findings, the primary immunological response to extrinsic antigens was located in the areas from the marginal to the intermediate lymph node sinuses (Fujino et al., 1996; Sakita et al., 1997). Given that the lymphocytes and macrophages coming into intimate contact as an expression of special interaction, some immune functions might be performed by similar cells in bovine HNs (Casteleyn et al., 2008).

The existence of the blood sinuses in the HNs of bovine foetus of 9 months old suggested that the development of the HNs in bovine occurred early since the fetal stage. The lymphoid tissue, which mostly resembled lymphocyte infiltration, was loosely structured in the foetus, as reported previously (Winqvist, 1954; Ezeasor and Singh, 1988; Bozkurt and Kabak, 2010). It was reported that lymphatic infiltrations existed in the HNs of young animals (Dellmann and Eurell, 1998; Akaydin and Kabak, 2006), and that lymphoid follicles developed with age (Yoon et al., 1999).

In our study, the three-dimensional view of the HN from the bovine cervical region was clearly demonstrated by the corrosion cast. The vascular models were analyzed both by inspection of the outer vasculature and by sectioning the cast to show the architecture of the inner vasculature. The parenchyma of the HN was surrounded by a dense vessel network of the capsule. Unlike the regular arrangement of arterioles and venules in this network, the capsular vessels in the cast of rat HN was bundle-like and had large interspaces (Castenholz and Castenholz, 1996). The numerous vessels in this network may well be associated with the thickness of the bovine HN capsule. These vessels increased the surface area of the HN, and this might act as a reservoir for erythrocytes; contraction of the capsule can expel these erythrocytes into the circulation when needed. The presence of venules from the capsule suggests blood leaves the bovine HN through a series of venules that eventually converge and exit the HN via the hilar vein. As in the spleen, they might originate from blood sinuses and reach the capsule via the adjacent trabeculae (Cesta, 2006).

The mechanism by which erythrocytes enter the node is still controversial. The earlier opinion was that erythrocytes entered the rat HN by reflux via lymphaticovenous communications, but this was discounted based on the presence of venous valves and the pattern of erythrocyte distribution (Job, 1918; Andreasen and Gottlieb, 1946). Some studies showed that erythrocytes entered the rat HN via afferent lymphatics, and this argued strongly against any direct communication between blood vessels and sinuses (Drinker and Field, 1931; Hogg et al., 1982; Kazeem, 1982). However, those findings were refuted by studies that failed to find

afferent lymphatics and found no erythrocytes in the few afferent lymph streams (Turner, 1969; Nopajaroonsri, 1974). In our previous histological study, we could find neither afferent nor efferent lymphatics in bovine HNs, and this denied the possibility that erythrocytes came from the lymphatic system. The HNs had been described as a hemopoietic organ that could produce erythrocytes within the node (Jordan, 1926), and evidences of erythropoiesis were also shown (Cerutti and Guerrero, 2008), but no erythrocyte precursors were seen in any of the nodes examined by another researcher (Turner, 1969). No evidence of direct communication between the vascular and sinus systems was obtained from cast studies of the rat HN (Castenholz and Castenholz, 1996). However, our study showed opposite results, because many anastomoses were found in the cortical capillaries opening into the blood sinusoids in the follicular zone and deep cortex of the bovine HN. Therefore, this route is probably one of the main sources of erythrocytes in the HN sinuses. In addition, within the normal lymph node, the deep cortex unit is a functional entity that allows contact and activation of lymphocytes by antigens or cytokines (Okada et al., 2002), so the capillary networks associated with the adjacent sinuses in the deep cortex of the bovine HN may suggest a similar immunological role in facilitating immunological interactions.

In the casts of bovine HNs from the cervical region, the presence of sinusoids could be taken as evidence of another pathway for blood flow. The subcapsular sinus of the HN was shown to be always engorged with numerous erythrocytes and had a variable size (Okada et al., 2002). In our research, there were many sieve openings in the cast of this sinus, and these perforations may due to large reticular processes and numerous small trabeculae (Castenholz and Castenholz, 1996; Zidan and Pabst, 2010). From the casts, the subcapsular sinus penetrated into the parenchyma along with the trabecular sinuses and partly connected with the tubular sinuses, and these tubular sinuses seemed comparable to the lymph labyrinths described by He (He, 1985), which were located between the cortical capillaries and medullary sinuses and thought to be closely associated with the lymphocyte recirculation in the lymph node.

The tubular sinuses developed well in the cortex, and this might be important for erythrophagocytosis and lymphocyte transport in HNs (Heath, 1987).

According to the classification of Weller (Weller, 1938), the nodes we examined in the bovine is HNs, as they possess abundant blood sinusoids, without any afferent or efferent lymphatics, similar to that in buffalo and sheep (Dellman and Brown, 1976; Zidan and Pabst, 2010). The data obtained in the morphological study and lymphoid tissue observation may contribute to future research of HNs, but there were still some problems remain.

For example, first, the HNs of bovine, with their numerous blood sinuses filled with blood and the direct contact between immune cells and the blood, may have a similar hematological and immunological function to the spleen, such as defense against the blood-borne infection and the clearance of damaged blood cells. The similar histological appearances of the hemal node, lymph node and spleen point to the need for comparative structural and functional studies of these basic lymphoid organs in order to help define evolutionary steps in immunology.

Second, the vascular study identified the anastomoses between cortical capillaries and sinusoids in the bovine HN from the cervical region for the first time, but the classification of capillaries in the HN and the migration mechanisms for erythrocytes and lymphocytes remain unclear and will need to be resolved with other methods in further studies.

FIGURE CAPTIONS

Figure 2. 1

Schematic diagram of process in vascular corrosion casting (VCC) technology. After injection, the specimens were prepared following resin polymerization, tissue digestion and air drying, and were finally observed under scanning electron microscopy (SEM).

Figure 2. 2

Appearances of bovine hemal nodes (HNs) in the gross anatomy. The number and size of HNs (arrows in a-f) varied among different anatomical regions and different calves. The HNs were mainly located along the big vessels (a and b), in the mesenteric region (c and d) and in the subcutaneous region of scapular and trunk (e and f). A: aorta, V: vein, arrow: HNs, arrow head: branches of vessels.

Figure 2. 3

Histological characteristics of bovine HNs in different regions. The HNs usually growing near the lymph nodes (LNs) (a and b). The histological structure of HNs along the aorta (c and d), in the mesenteric region (e and f) and from the subcutaneous region (g and h) showed that HNs were surrounded by connective tissue capsules, and the parenchyma consisted of the sinus, cortex, paracortex, medulla and hilus. The sinus was filled with numerous erythrocytes, and many germinal centers were located in the cortex. C: capsule, S: sinus, CX: cortex, PC: paracortex, M: medulla, H: hilus, asterisk: germinal center, arrow: LNs, arrow head: HNs. Bar=1 mm. HE stain.

Figure 2. 4

The capsule and associated collagenous tissue of bovine HNs. The HNs were supported by numerous trabeculae (a and b), which were originated from the capsule and projected into the parenchyma (c and d). The collagenous tissue and smooth muscle cells were usually observed in the trabeculae and inner connective tissue (e

and f). C: capsule, CX: cortex, T: trabeculae, TS: trabecular sinus, M: medulla, H: hilus, asterisk: germinal center, arrow: capsular vessels, arrow head: collagenous tissue and smooth muscle cells. Bar=1 mm (a-d), Bar=400 μm (e), Bar=200 μm (f). TRI stain.

Figure 2. 5

The sinuses, trabeculae and hilus of bovine HNs. The subcapsular sinus was usually filled with erythrocytes and supported by reticular fibres, but sometimes only a few erythrocytes could be observed (a and b). The trabecular sinuses extended into the medullary sinuses and the vessels could also be found within the trabeculae (c-f), the blood finally left the HNs from the hilus (g and h). C: capsule, S: subcapsular sinus, CX: cortex, T: trabeculae, TS: trabecular sinus, M: medulla, A: arteriole, V: venule, H: hilus, arrowhead: reticular fibres, arrow: valve, asterisk: germinal center. Bar=1 mm (d and g), Bar=400 μm (c and h), Bar=200 μm (a and b, e and f). HE stain (a and b, e-g), TRI stain (d and h).

Figure 2. 6

The variations of lymphoid tissues in bovine HNs. The size and number of germinal centers were usually different among different HNs (a-d), and sometimes the lymphoid tissues were loose, which seemed like lymphocyte infiltration (c and d). CX: cortex, M: medulla, asterisk: germinal center, arrow head: interior sinuses with a few erythrocytes. Bar=400 μm. HE stain.

Figure 2. 7

Histological characteristics of bovine foetal HNs. The foetal HNs had loose lymphoid tissue (a-f). The erythrocytes could hardly be found in the subcapsular sinuses (a and c), and the erythrocytes also had different distribution in the interior sinuses (b and d, e and f). Some capillaries were found existed in the medullary cords (e). S: subcapsular sinuse, IS: interior sinuses, MC: medullary cord, arrow

head: interior sinuses with few erythrocytes, arrow: capillary vessels. Bar=1 mm (a and c), Bar=200 μm (b, d and f), Bar=100 μm (e). HE stain.

Figure 2. 8

The capsule and trabeculae of bovine HNs under the observation of scanning electron microscopy (SEM). The basic structure were clearly shown and the big venules were observed in the hilar area (a and b). The capsule consisted of collagenous tissue and many capillaries could usually be found within them (c and d). The trabecular sinuses were observed to have network formed by trabeculae and reticular fibres, and in trabeculae, capillaries could be found (e and f). C: capsule, S: subcapsular sinus, CX: cortex, T: trabeculae, TS: trabecular sinus, M: medulla, H: hilus area, arrow head: venules, arrow: collagenous tissue. Bar: a, 800 μm; b, 800 μm; c, 120 μm; d, 4.50 μm; e, 72.0 μm; f, 180 μm.

Figure 2. 9

The sinuses in the bovine HNs under SEM. The blood sinuses were numerous in HN which contained dense network of reticular cells (a and b), in place of trabeculae, sometimes there were only small vessels existed in the sinuses (c and d). The splenic sinus-like structure was observed in HNs (e), which seemed like those sinuses in spleen (f). The wall of the splenic sinus-like structure was formed by similar endothelial cells that touching the blood cells in the lumen (e and f). MS: medullary sinus, RT: reticular fibres, IS: interior sinuses, V: venules, H: hilus area, asterisk: splenic sinus-like structure. Bar: a, 514 μm; b, 12.0 μm; c, 225 μm; d, 72.0 μm; e, 65.0 μm; f, 75.0 μm.

Figure 2.10

The cells in the sinus and pulp of the bovine HNs under SEM. Many cells includes erythrocytes, lymphocytes, plasma cells, reticular cells and macrophages were observed in the sinuses (a-c) and pulp (d). Most of them were found to have

communications with reticular cells in the sinuses (e) and pulp (f). RT: reticular fibres, IS: interior sinuses, R: erythrocyte, L: lymphocyte, P: plasma cell, asterisk: macrophage, arrow head: degraded cells. Bar: a, 20.0 μm; b, 16.4 μm; c, 6.00 μm; d, 10.0 μm; e, 6.00 μm; f, 3.00 μm.

Figure 2. 11

Full view of corrosion casts of bovine HNs under lower magnification of SEM. After digestion, corrosion casts injected from the cervical artery clearly showed the vascular supply of HNs (a), and other HNs were also observed under SEM (b-f). The capsular vessels networks embrace the HN parenchyma (b-d) and the hilus had a relatively flattened surface from where the supply artery and vein could be found (e and f). CV: capsular vessels network, A: artery, V: vein, H: hilus, arrow: the position of injection, arrow head: HNs after digestion. Bar: b, 720 μm; c, 1.64 mm; d, 1.64 mm; e, 3 mm; f, 3 mm.

Figure 2. 12

Corrosion casts of capsule vessels and hilar surfaces in bovine HNs under SEM. The arterioles in capsule ran along with the venules and they had various diameters (a-d). The position of the hilus had convex surface and the position could only be identified by the supplying vessels (e and f). CV: capsular vessels network, A: artery, V: vein, H: hilus, asterisk: valves, arrow: the groove-like imprints of artery, arrow head: the oval endothelial imprints of vein. Bar: a, 2 mm; b, 1 mm; c, 200 μm; d, 72.0 μm; e, 1.80 mm; f, 1.80 mm.

Figure 2. 13

Corrosion casts of capsule vessels and blood sinuses in bovine HNs under SEM. The vessels of the capsule entered into the parenchyma along with the trabecular sinuses (a and b, e and f), which were continuous with the tubular sinuses and then medullary sinuses (c and d, g and h). CV: capsular vessels network, S: subcapsular

sinus, TS: trabecular sinus, MS: medullary sinus, TV: trabecular vessels, arrow head: the position capsular vessels entered into the parenchyma, arrow: tubular sinuses. Bar: a, 200 μm; b, 250 μm; c, 400 μm; d, 2 mm; e, 400 μm; f, 100 μm; g, 400μm; h, 250μm.

Figure 2. 14

Corrosion casts of cortical capillaries in bovine HNs under SEM. The capillaries in the cortex formed dense plexuses (a, c and e), and there were at least two capillaries supported the lymphoid follicle (b and c). The connections between capillaries and sinuses were observed in the cortex (c and d) and paracortex (e and f). C: capillary, LF: lymphoid follicles, S: subcapsular sinuses, arrow head: the connections of capillaries and sinuses, asterisk: delicate sinuses in the deep cortex. Bar: a, 200 μm; b, 200 μm; c, 120 μm; d, 60 μm; e, 100 μm; f, 72.0 μm.

Figure 2. 15

Corrosion casts of subcapsular sinuses and medullary area in bovine HNs under SEM. The subcapsular sinuses showed a layer which coved the parenchyma of HN, and also indicated the position of lymphoid follicles (a-c). The blood sinuses converged in the medullary area and left the nodes from the hilar area (d-f). TS: trabecular sinus, LF: lymphoid follicle, M: medullary area, H: hilar area, A: artery, V: vein, asterisk: branches of big vessels, arrow head: the big branches of subcapsular sinuses, arrow: the different imprints of artery and vein. Bar: a, 360 μm; b, 400 μm; c, 400 μm; d, 600 μm; e, 400 μm; f, 164 μm.

Figure 2. 16

Schematic diagram of the vascular system in HNs of bovine cervical region. The artery (A) reaches the medulla of the HN through the hilus (H) and gives off many arteriole branches (AB), which finally form capillary networks (C) in the follicular zone and deep cortex (P). These capillaries connect with the subcapsular sinuses (S)

and delicate sinuses, separately. The sinuses merge with the tubular sinuses (TS) and then the medullary sinuses (MS), which finally collect into the hilar vein (V) and leave the HN in combination with some venous branches formed by the capsular vessel network (CV). CA: capsule, TV: trabecular venule. LF: lymphoid follicle. H: hilus.

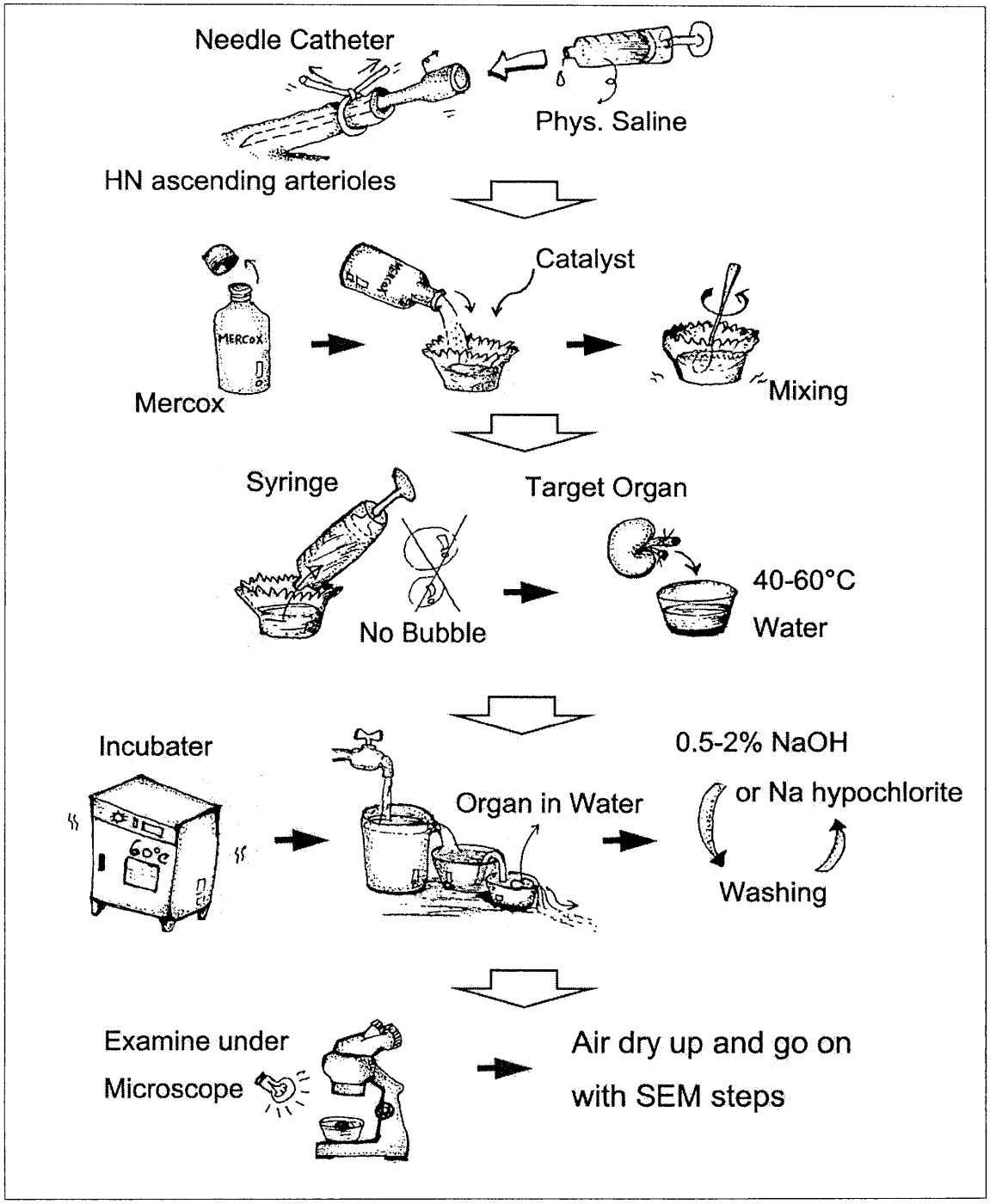

Fig. 2. 1 Schematic diagram of vascular corrosion casting (VCC) technological process.

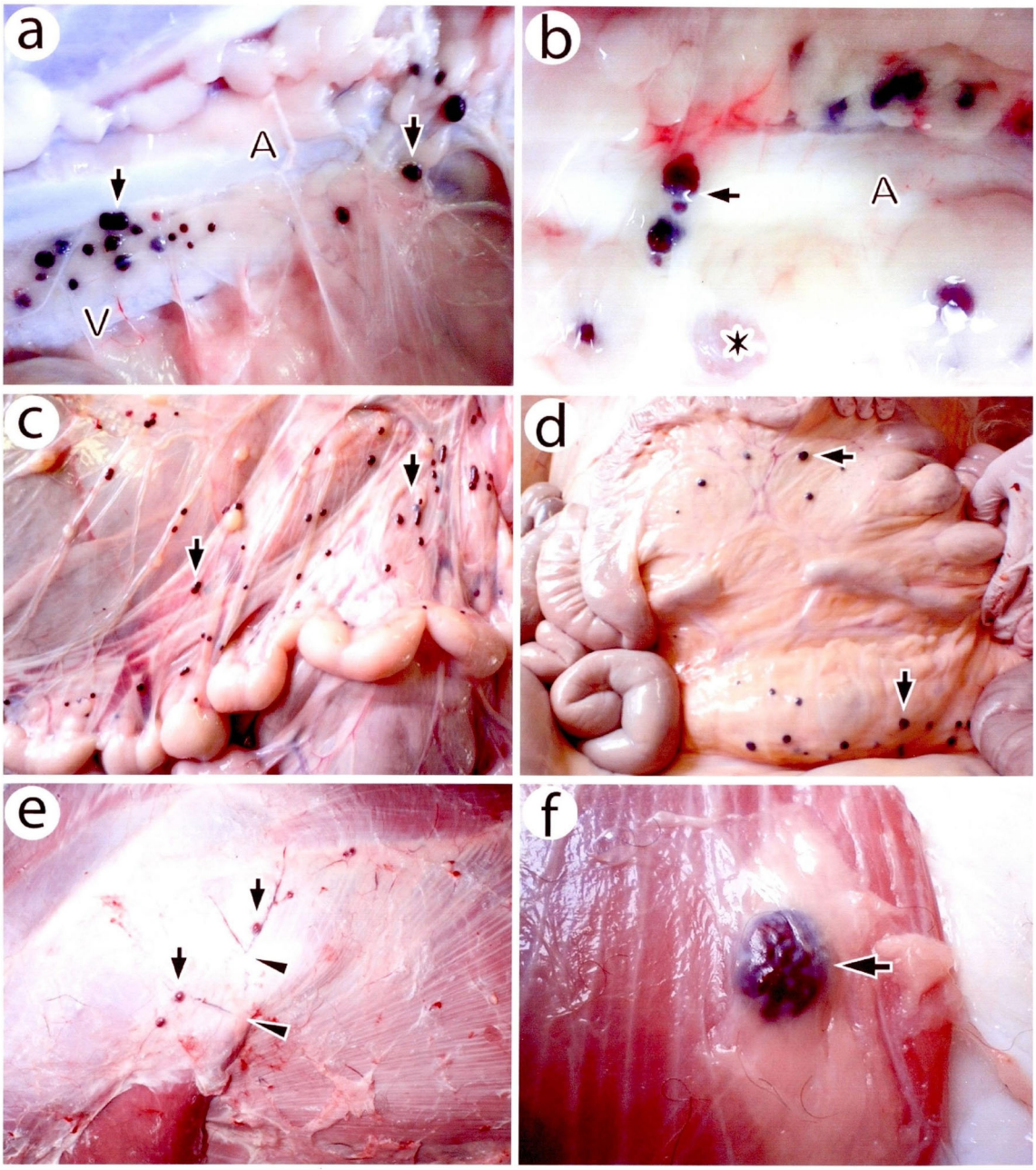

Fig. 2. 2 Appearances of bovine hemal nodes in the gross anatomy.

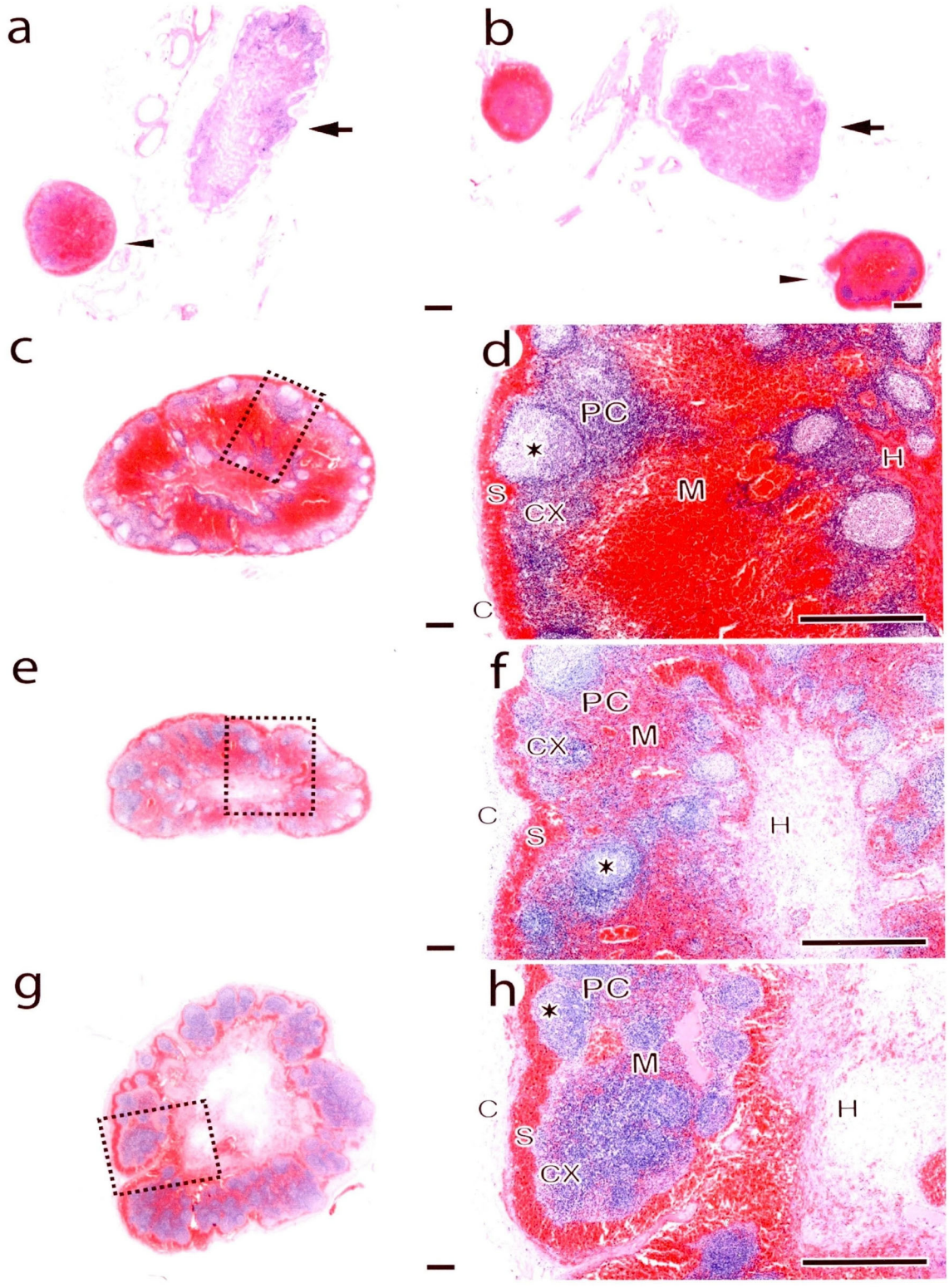

Fig. 2. 3 Histological characteristics of bovine hemal nodes in different regions.

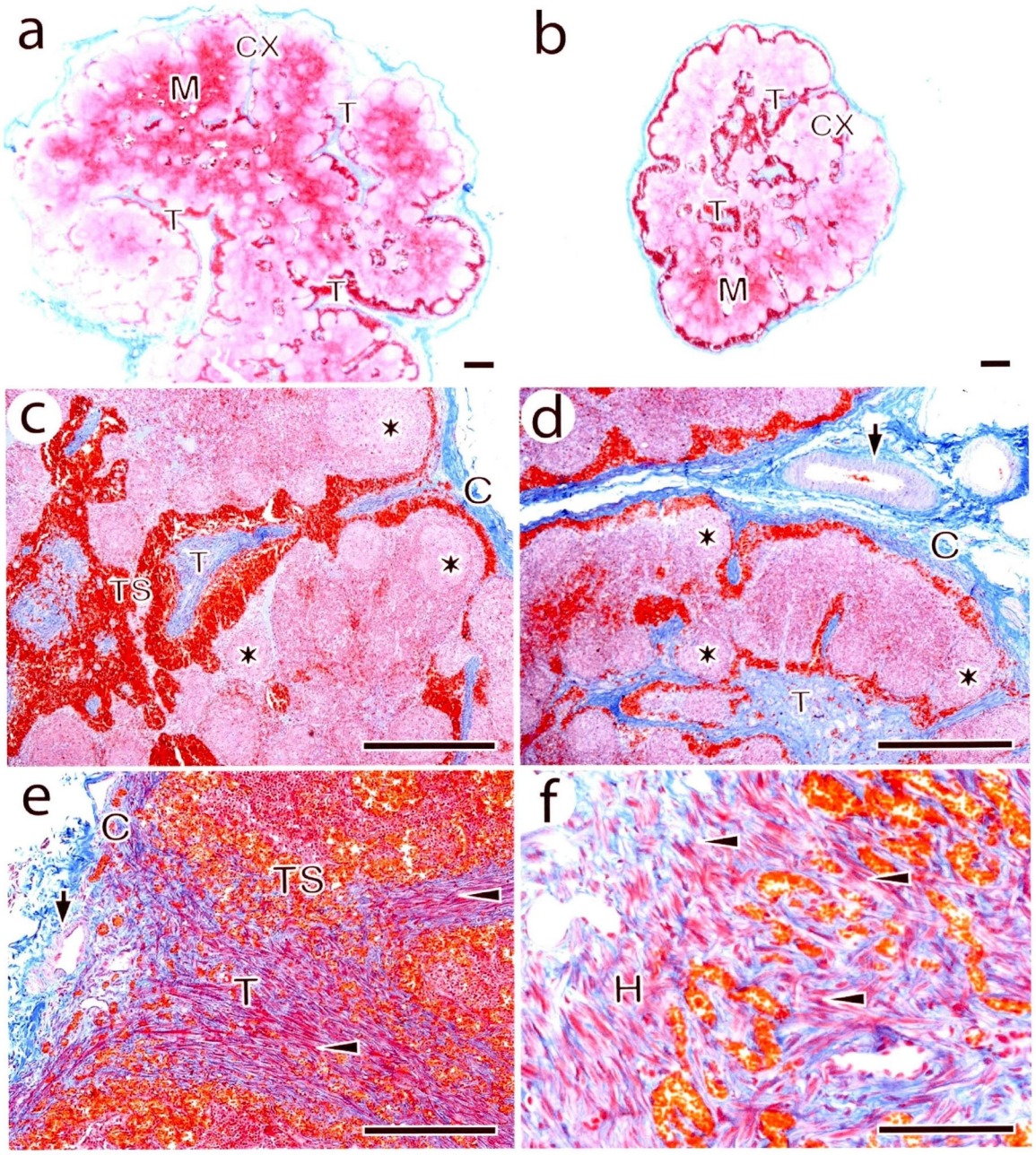

Fig. 2. 4 The capsule and associated collagenous tissue of bovine hemal nodes.

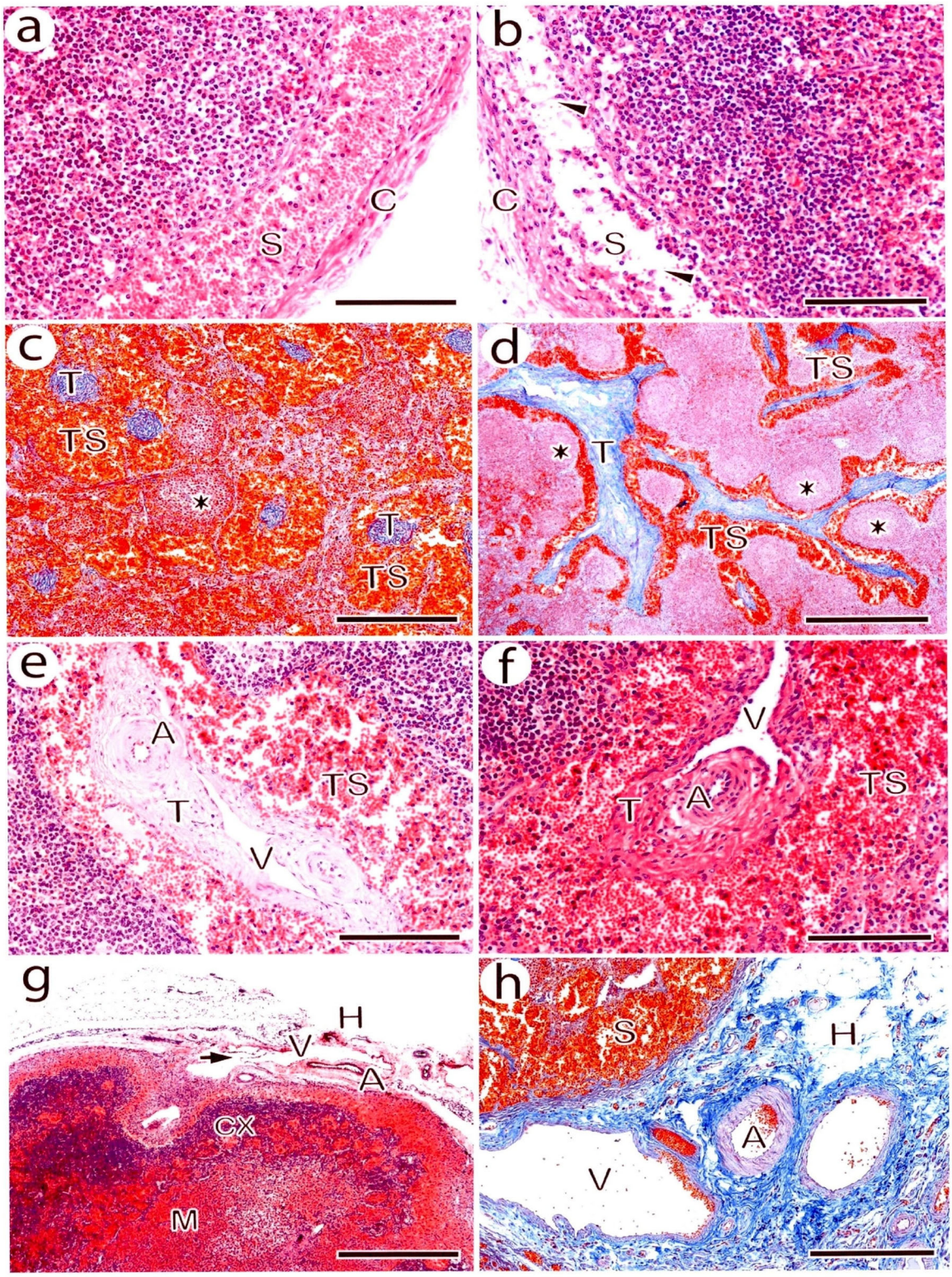

Fig. 2. 5 The sinuses, trabeculae and hilus of bovine hemal nodes.

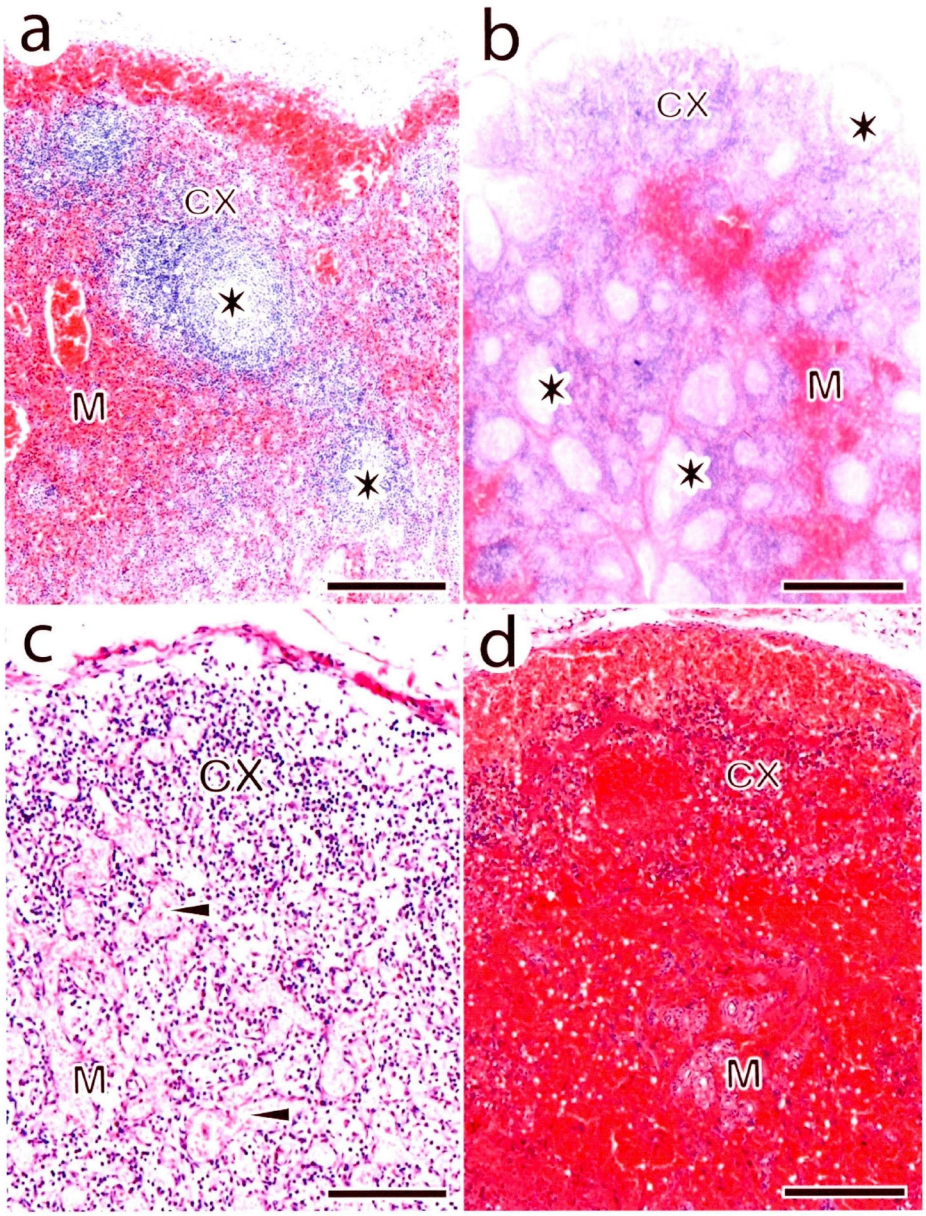

Fig. 2. 6 The variations of lymphoid tissues in bovine hemal nodes.

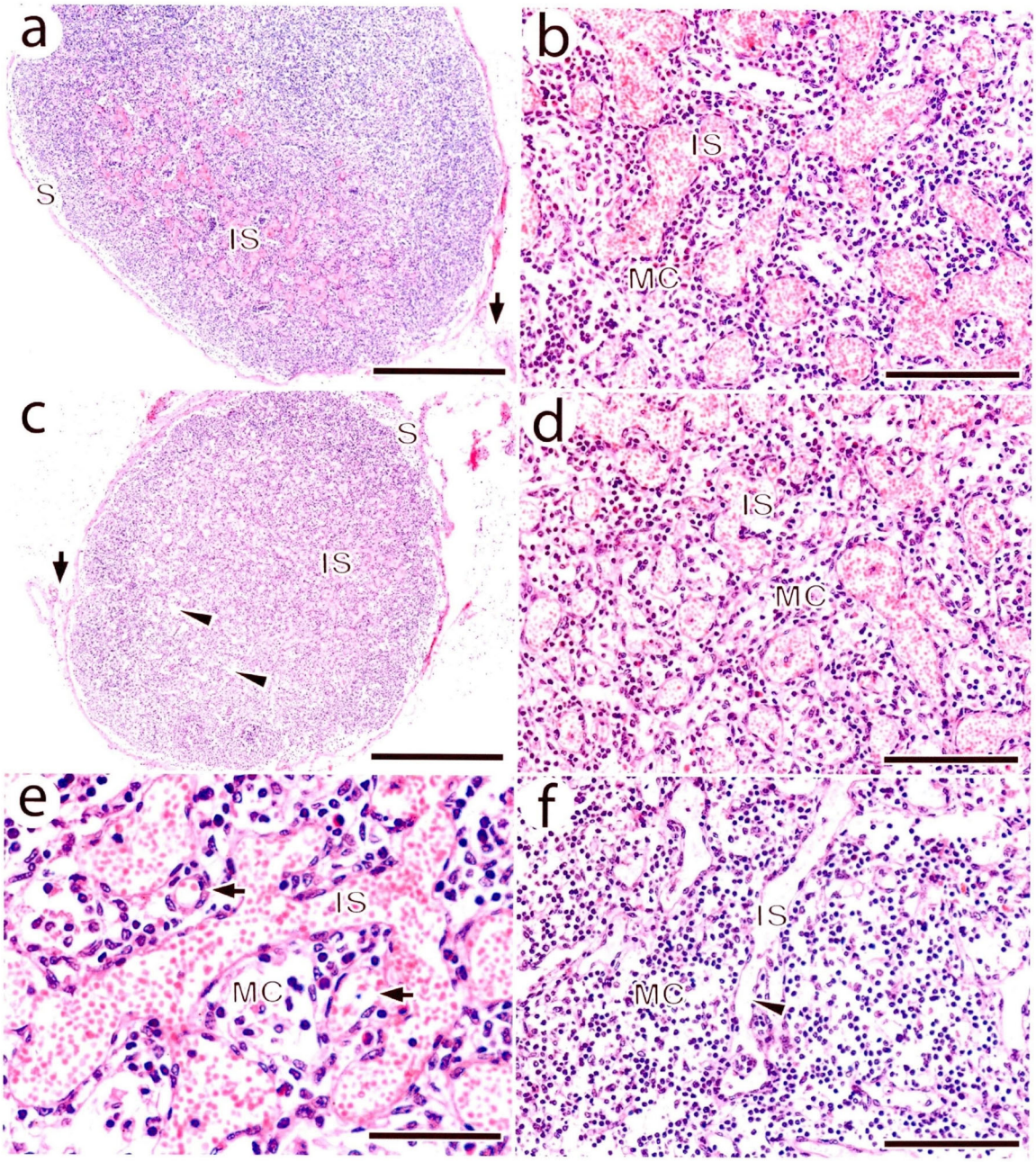

Fig. 2. 7 Histological characteristics of bovine foetal hemal nodes.

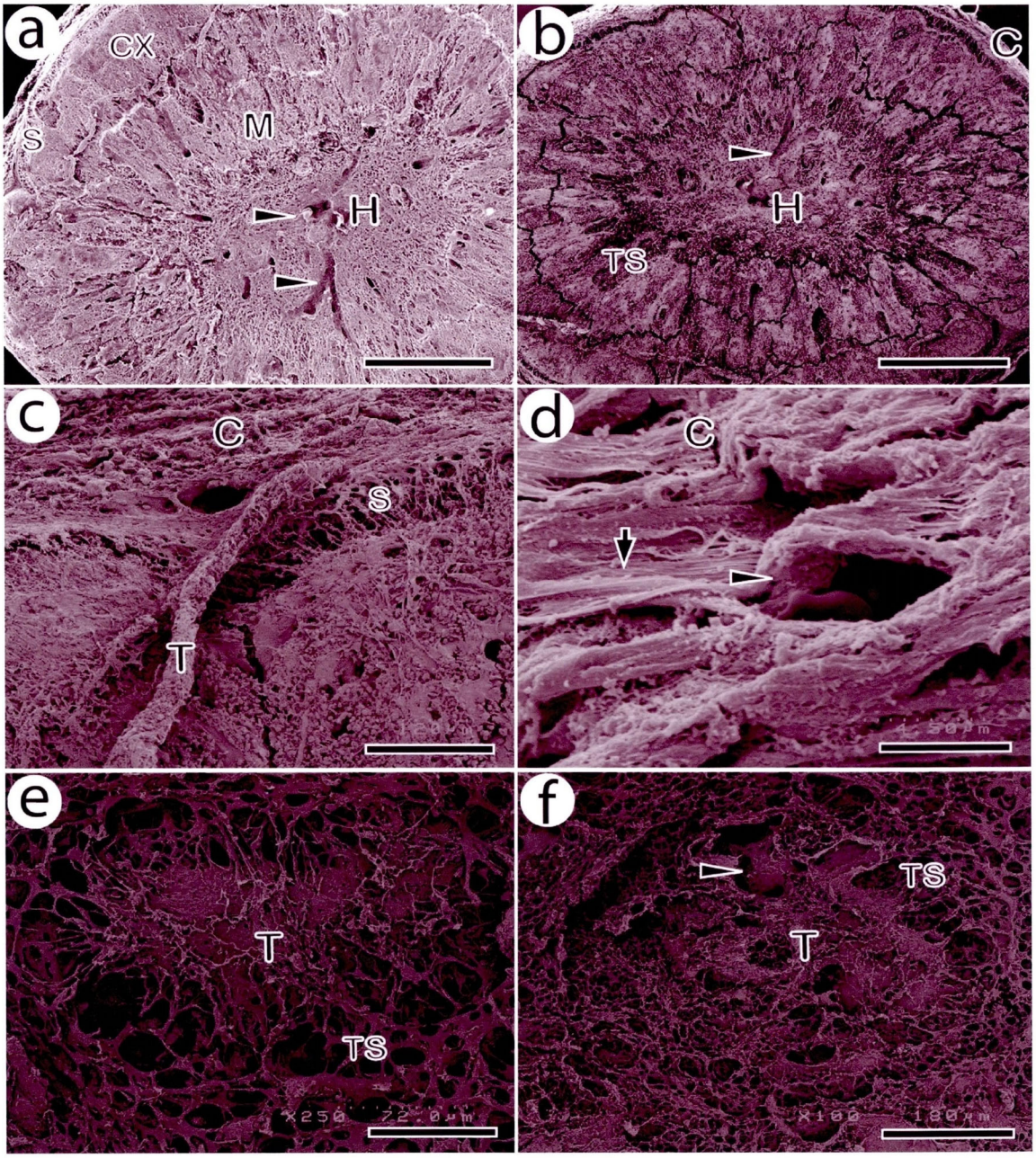

Fig. 2. 8 The capsule and trabeculaes of bovine hemal nodes under the observation of scanning electron microscopy (SEM).

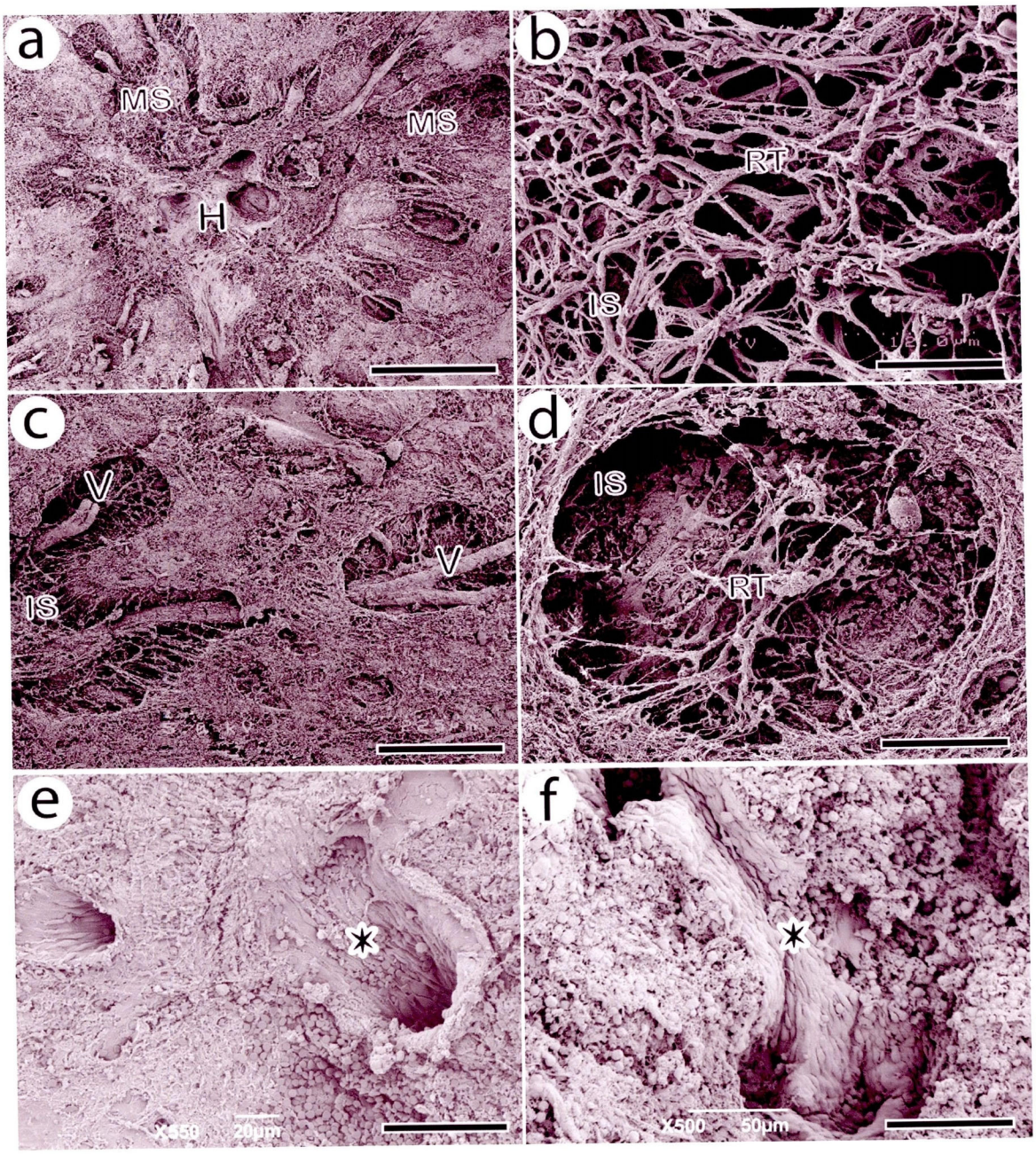

Fig. 2. 9 The sinuses in the bovine hemal node under SEM.

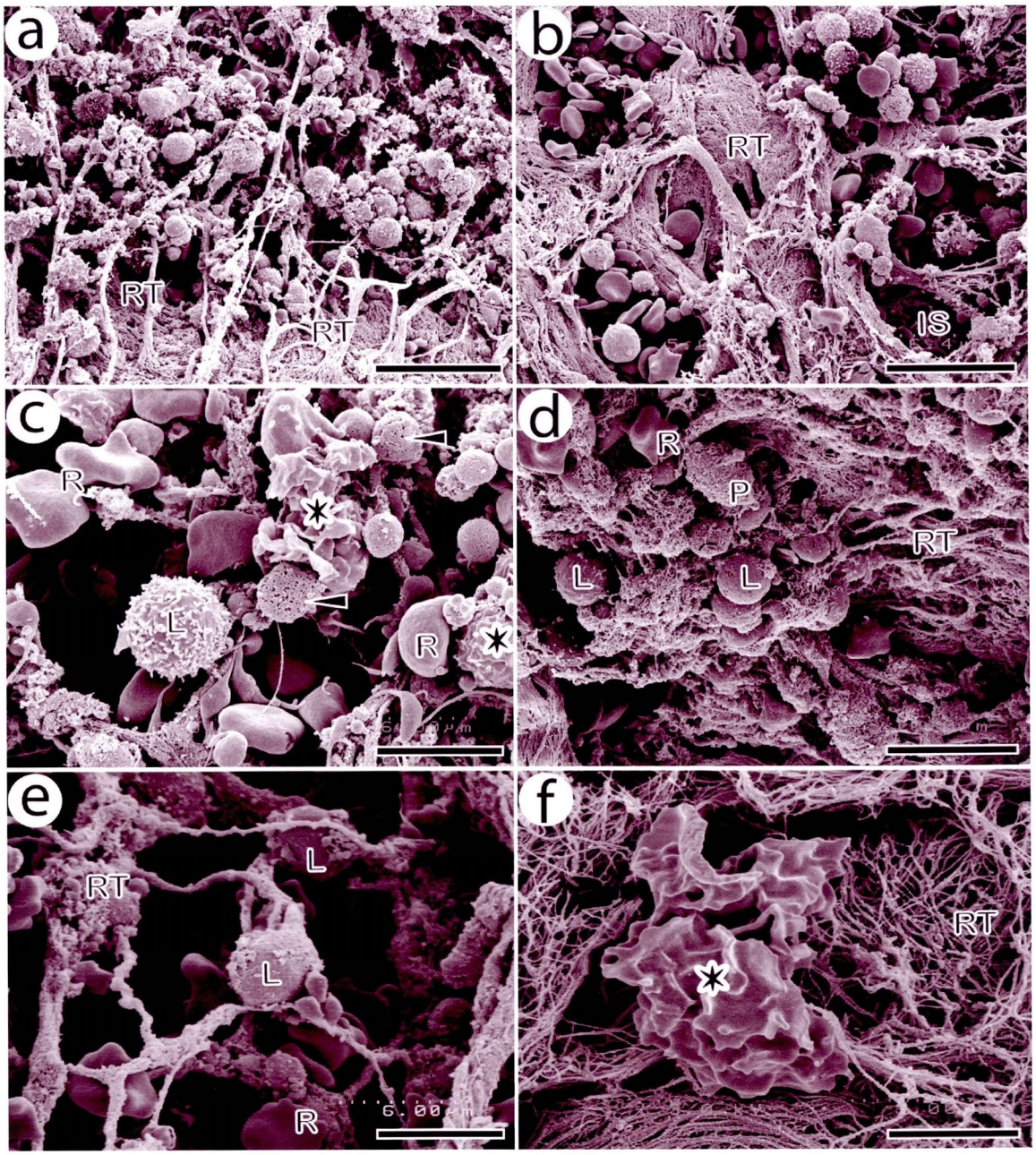

Fig. 2. 10 The cells in the sinus and pulp of bovine hemal nodes under SEM.

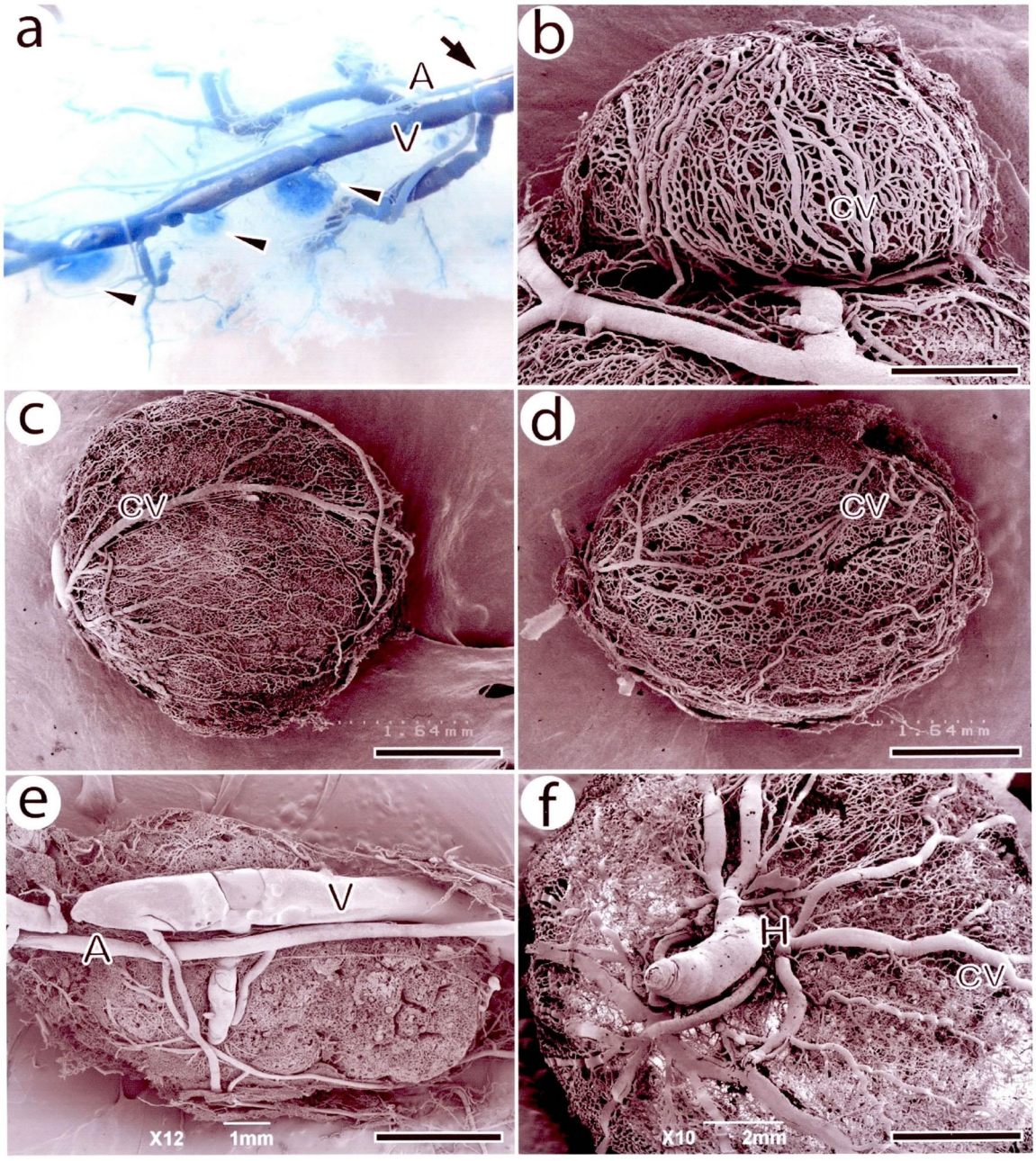

Fig. 2. 11 Full view of corrosion casts of bovine hemal nodes under lower magnification of SEM.

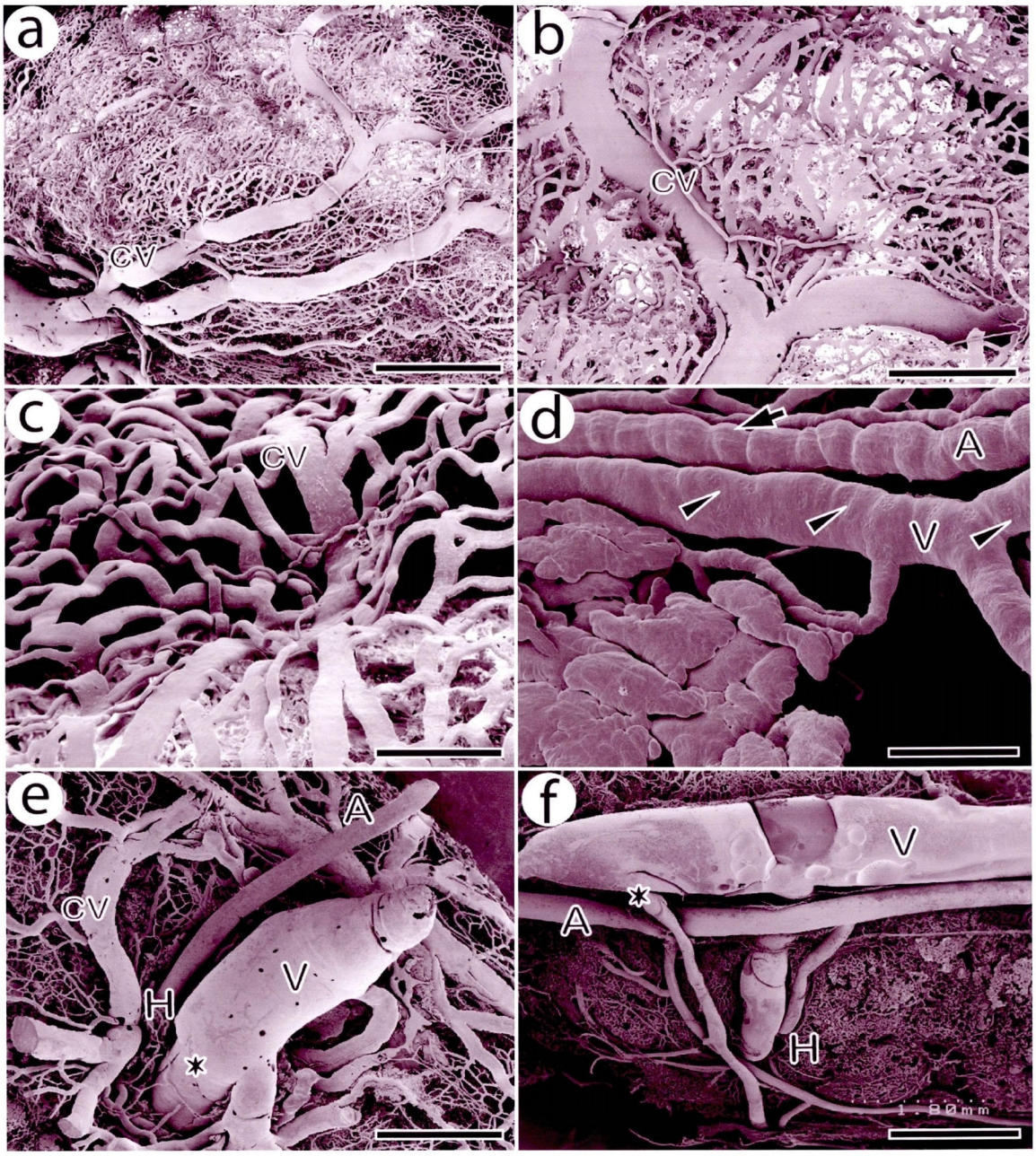

Fig. 2. 12 Corrosion casts of capsule vessels and hilar surfaces in bovine hemal nodes under SEM.

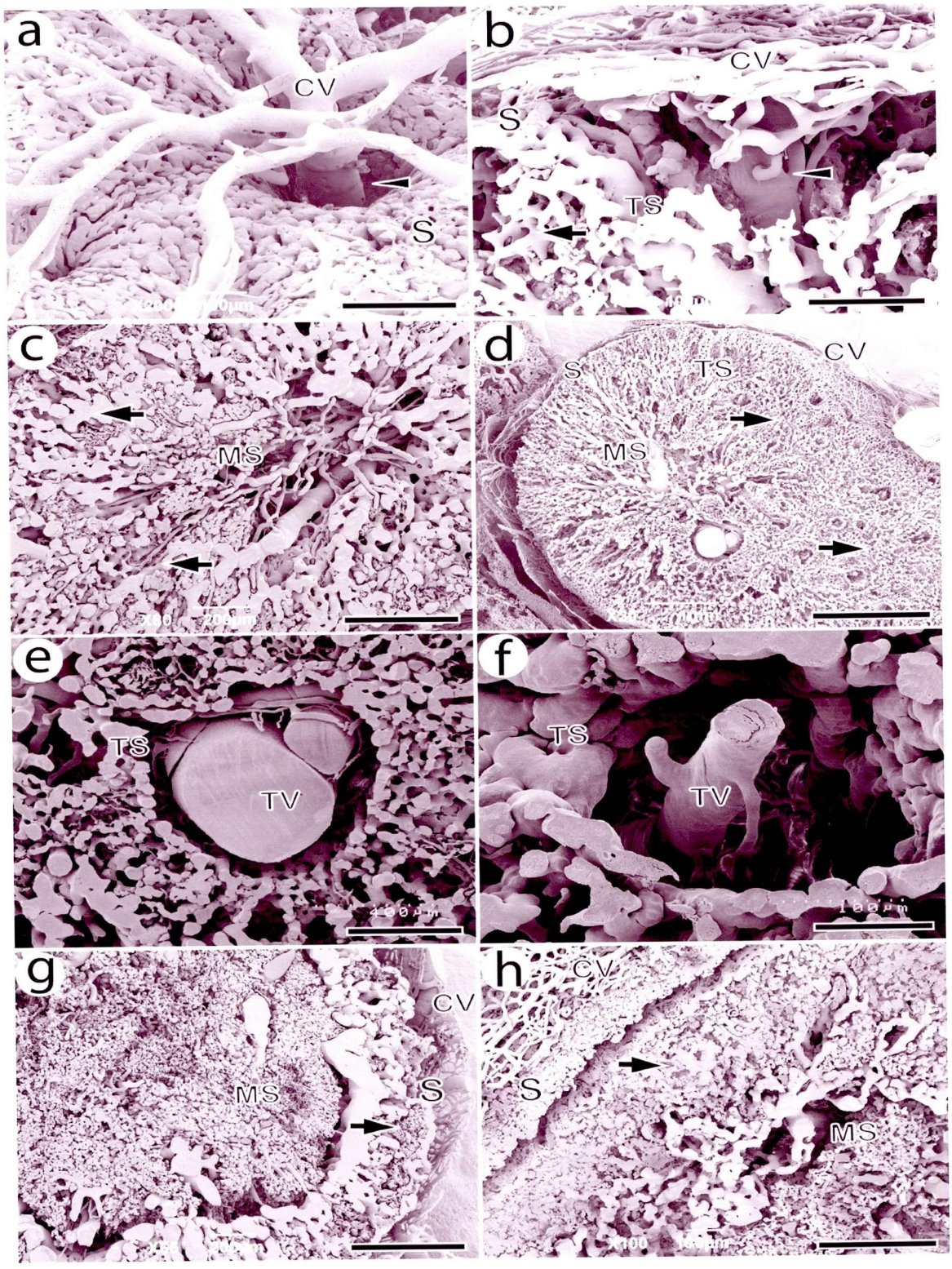

Fig. 2. 13 Corrosion casts of capsule vessels and blood sinuses in bovine hemal nodes.

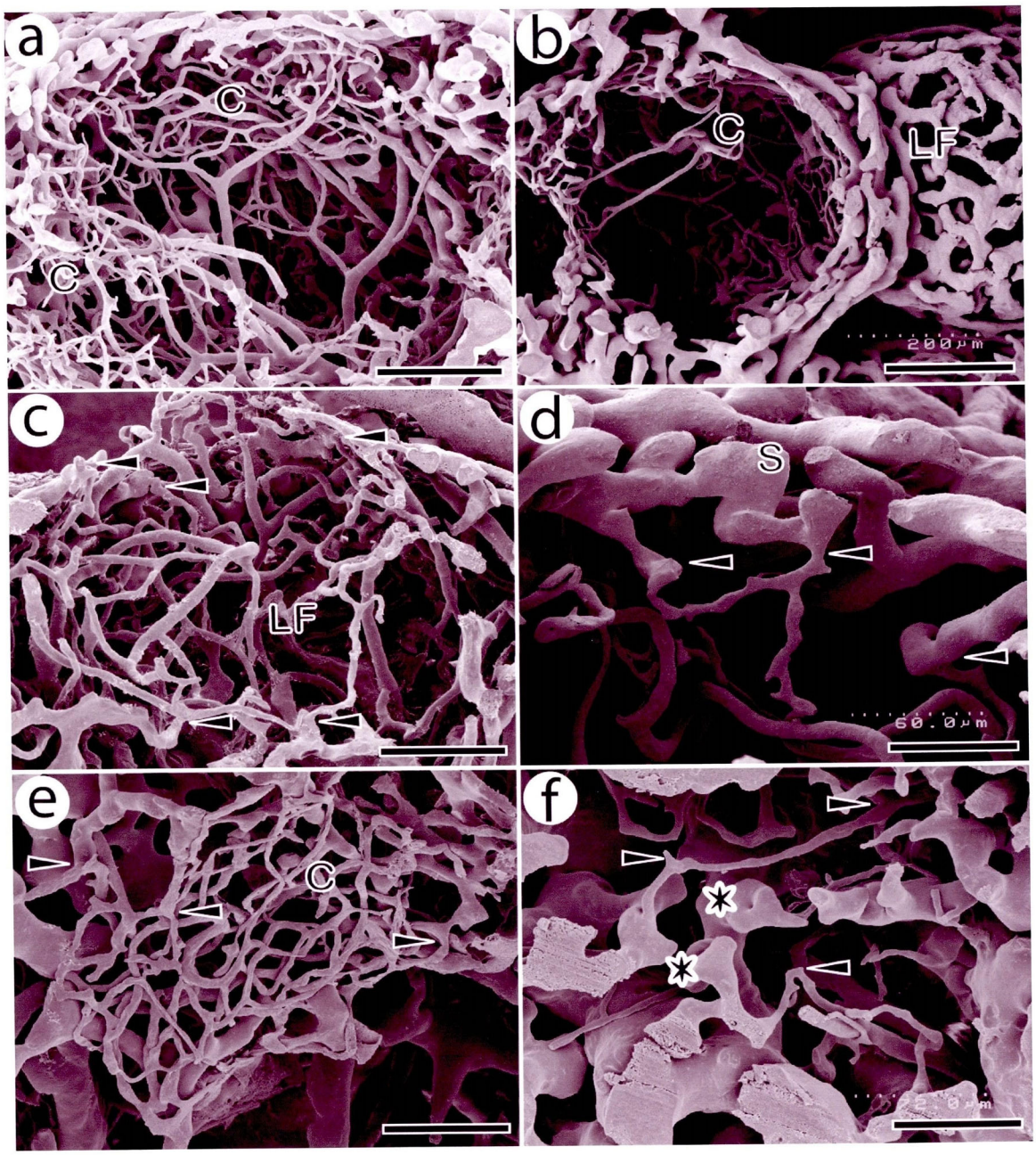

Fig. 2. 14 Corrosion casts of cortical capillaries in bovine hemal nodes under SEM.

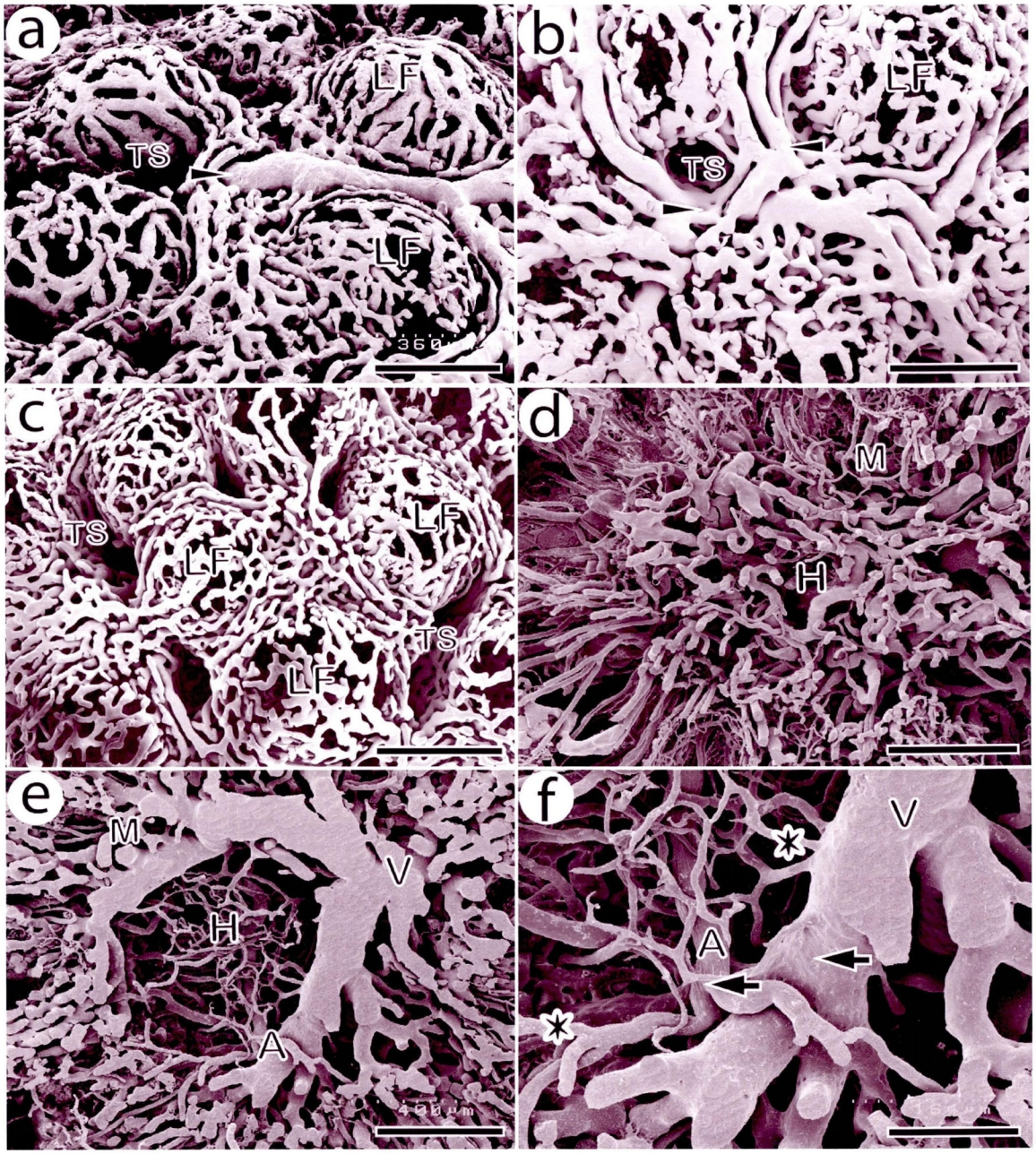

Fig. 2. 15 Corrosion casts of subcapsular sinuses and medulla in bovine hemal nodes under SEM.

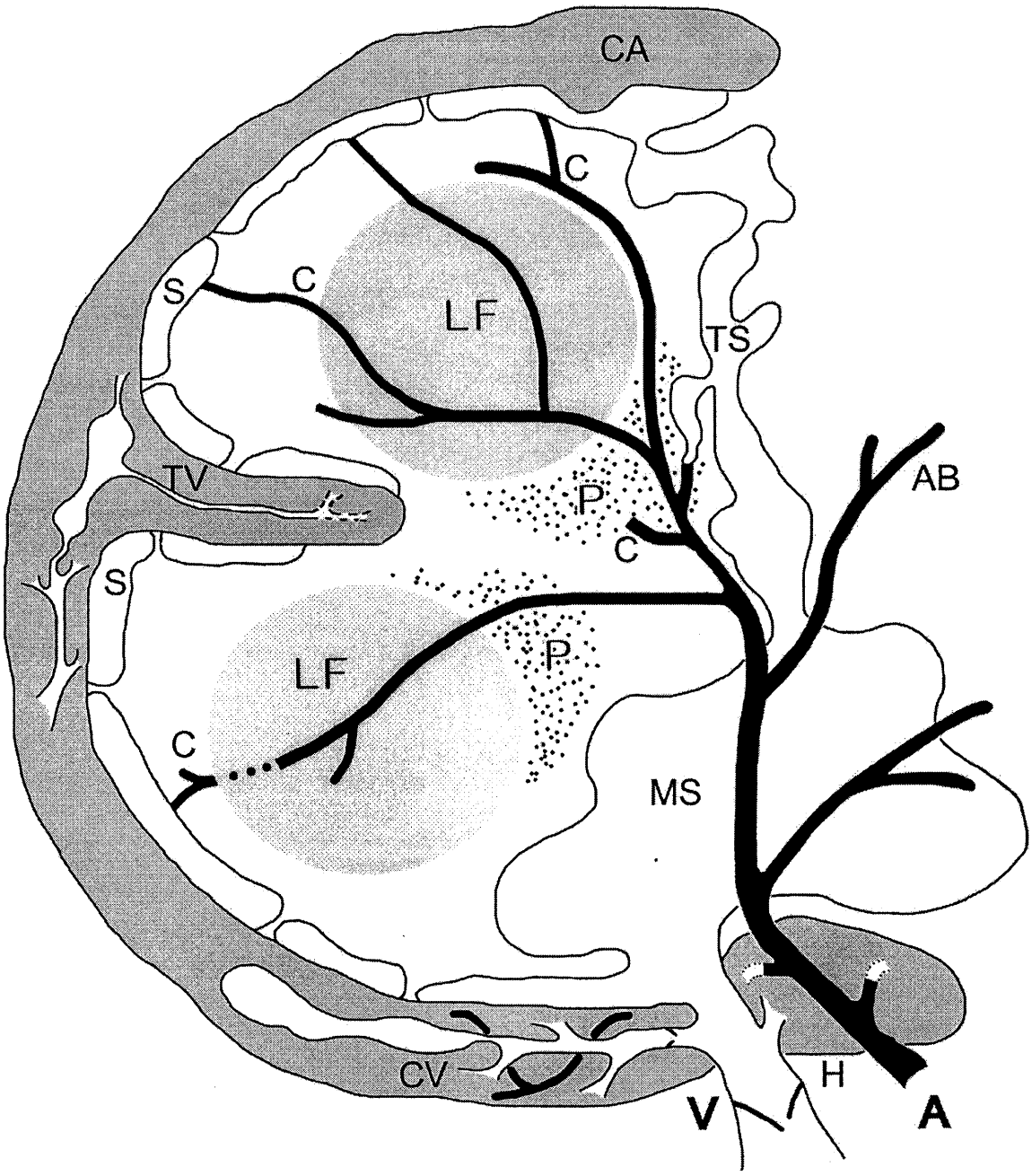

Fig. 2. 16 Schematic diagram of the vascular system in hemal node of bovine cervical region.

Chapter 3

Comparative Studies on the Distribution and Population of Immunocompetent Cells in Bovine HN, MLN and SP

Abstract

The distribution and population of immunocompetent cells in bovine hemal node (HN), mesenteric lymph node (MLN) and spleen (SP) were analyzed comparatively by immunohistochemistry and flow cytometry. Many $CD8^+$ cells, $CD172a^+$ cells and TCR1-$N24^+$ ($\gamma\delta$ T) cells were found in the lymphatic cord along the sinus of the HN and the splenic red pulp of SP. A few $CD8^+$ cells and $\gamma\delta$ T cells were distributed diffusely in the paracortex and medullary cord of the MLN. $CD4^+$ cells in HN and MLN were mainly present in the paracortex, and in the SP, most $CD4^+$ cells were distributed in the periarteriolar lymphoid sheath (PALS). Many germinal centers which contained $CD21^+$ cells were recognized in the lymphatic regions such as the cortex of HN and MLN, and the white pulp of the SP in these lymphoid organs. MHC class II^+ cells were mainly distributed in the lymphoid follicles, the paracortex and the sinusoid regions of HN and MLN, and in the SP, those positive cells mainly distributed in the red pulp and white pulp. The populations of $CD8^+$ cells and $\gamma\delta$ T cells in the HNs and the SP were higher than those of the MLNs and peripheral blood mononuclear cells (PBMC). In particular, the population of $\gamma\delta$ T cells in the HN was significantly higher than that of MLN. In addition, the populations of $CD21^+$ cells and MHC class II^+ cells in the HNs and the MLNs were significantly higher than those of the SP. The results suggest that the HN has an important role in both cellular and humoral immunity as well as the lymph node and the SP in bovine.

3.1 Introduction

The hemal node (HN) is a hematopoietic and secondary lymphoid organ that is found in some mammals such as humans, rats and ruminants (Turner, 1969; Gargiulo et al., 1987; Abu-Hijleh and Scothorne, 1996; Cerutti and Guerrero, 2001; Zidan and Pabst, 2004; Zidan and Pabst, 2010). In ruminants, HNs are located in the subcutaneous region of the head and trunk, the mesenteric region and along the large blood vessels such as the aorta in the thorax and abdomen; however, the distribution and number of HNs in these regions seem to be diverse (Vincent and Harrison, 1897; Bacha and Bacha, 2000; Snider III et al., 2003; Casteleyn et al., 2008). The histological structure of HNs resembles that of lymph nodes and consists of a lymphoid region, in which the cortex and paracortex are recognized, and a sinusoid region, in which the lymphatic cord and sinus are formed (Turner, 1971b; Cerutti et al., 1998; Casteleyn et al., 2008). The HN sinus is filled with numerous erythrocytes, lacks lymphatic vessels and only connects with small blood vessels (Turner, 1969; Sakita, 1997; Bacha and Bacha, 2000; Zidan, 2010). In addition, the HN structure also resembles that of the spleen, which consists of white pulp, in which the germinal center is formed, and red pulp, in which the splenic sinus is filled with numerous erythrocytes (Sakita, 1997; Zidan, 2010). The antibody-producing ability and immunological competence of rat HN after antigen challenge have been described (Turner, 1971b; Sakita, 1997). In certain diseases which affected the animals such as sheep and bovine, HNs were found increased in number and grossly enlarged in size, and they usually had prominent histological lymphoid follicular hyperplasia, as evidence of stimulation of the lymphoid system and antibodies production, were considered to have immunological roles in their function (Weller, 1938; Wallnerova and Mims, 1970; Snider III, 2003).

However, only a few studies on immune related functions in HNs have been applied, the immunological role of the bovine HN has not yet been determined. In this study, we analyzed the immunological function of bovine HNs by comparing the distribution and population of immunocompetent cells in the HNs of calves, with those of the mesenteric lymph nodes (MLNs) and spleen (SP).

3.2 Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki. Animals and collection of specimens: Japanese black calves (5-8 months old, n=10) were obtained from local farms in Miyazaki Prefecture. The animals were killed by electric shock following the combination of xylazine (0.2mg/kg) and pentobarbital (15mg/kg) intravenous injection. The HNs, MLNs and spleens were collected for immunohistochemistry (IHC) and flow cytometry (FACS). The specimens for IHC were mounted in OCT embedding compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) on Cryomold (Sakura Finetek Japan Co., Ltd.), frozen on dry ice, and then stored at -80°C until used. The specimens for FACS were transported on ice and then treated as described below.

For histological analysis, HNs were fixed with 10% buffered formalin. The samples were passed through ethanol and toluene and embedded in paraffin. The samples were cut into 4-μm thick sections and stained with hematoxylin-eosin (HE) stain.

Monoclonal antibodies (mAbs) used in IHC and FACS analysis: the mAbs specific for calf CD4 (IL-A11), CD8 (CACT80c), CD21 (GB25A), CD172a (DH59B), TCR1-N24: δ chain specific (GB21A) and MHC class II (TH14B) were purchased from VMRD Inc. (Pullman, WA, U.S.A.). A 1:500 dilution of each mAb was used for IHC and FACS.

3.2.1 Immunohistochemistry

Cryostat sections were stained with the mAbs by using the indirect immunoperoxidase technique described by Yasuda et al (Yasuda, 2002). In brief, the sections (7-10 μm thick) were air-dried on slides and fixed with ice-cold acetone for 10 min. To block any nonspecific binding, the sections were rehydrated in PBS and incubated with 10% normal horse serum in PBS for 30 min. The sections were stained with mAbs for 60 min and washed three times with PBS. They were then

incubated with a secondary antibody absorbed with acetone powder from calves HN, MLN and spleen. After incubation with the secondary antibody, endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min followed by incubation with ABC complex (Vector Lab., Burlingame, CA, U.S.A.) for 15 min. After the sections were rinsed three times in PBS, the reactions were made visible with metal-enhanced diaminobenzidine (DAB, Pierce, Rockford, IL, U.S.A.). All immunohistochemical staining was performed at room temperature in a moist chamber. Control staining, in which the primary antibody was replaced with PBS, was performed simultaneously.

3.2.2 Flow Cytometry

Cell suspensions were obtained from HNs, MLNs and spleens by teasing the minced organs through nylon mesh for removing the debris. The cell suspensions from each lymphoid organ and peripheral blood mononuclear cells (PBMC) were further purified by density gradient centrifugation with Ficoll-Paque (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England). For immunofluorescence, the cells were suspended in PBS supplemented with 0.5% bovine serum albumin and 0.05% sodium azide. Viable cells were incubated with mAbs at 4°C for 30 min. After washing, a second incubation was conducted with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (H+L) F $(ab)_2$ (Silenus Lab., Boronia, Victoria, Australia) at 4°C for 30 min. Relative immunofluorescence intensities were determined by flow cytometry with a FACS Canto^{TM}II system (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

3.2.3 Statistical Analysis

The statistical analysis consisted of an analysis of variance (ANOVA) and multiple comparison tests performed with Origin Pro 8.0 software (OriginLab CO., Northampton, MA, U.S.A.). *P*-values less than 0.05 were regarded as statistically significant.

3.3 Results

3.3.1 Distribution of HNs and Their General Histological Structure

The HNs in Japanese black bovine were located mainly in the mesenteric region and along the aorta of the thoracic, abdominal and pelvic cavities (Figs. 3. 1a and 1c). In addition, HNs also could be found in the axillary region and the subcutaneous of the scapular region and abdominal region, which located along with the blood vessels (Figs. 3. 1e). The HNs were encapsulated by connective tissues, in which many small blood vessels were found, and the sinus was filled with numerous erythrocytes. There were many variations of lymphoid regions in HNs. The most developed lymphoid regions in HNs consisted of three main regions: the follicular region in which many germinal centers were located; the paracortex which consisted of the lymphatic region and located between cortex and medulla; and the sinusoid region which consisted of the lymphatic cord and sinus (Figs. 3. 1b, 1d and 1f).

3.3.2 Distribution of Immunocompetent Cells in HNs, MLNs and SP

In HNs, $CD21^+$ cells were distributed in the lymphoid follicles, especially in the germinal center (GC), and a few positive cells were also located in the medulla (M) (top left figure in Fig. 3. 2). The distribution of $CD21^+$ cells in MLNs was similar to those of HNs (middle left figure in Fig. 3. 2). But, many $CD21^+$ cells were also found in the medulla of MLNs. In the spleen, many $CD21^+$ cells were located in the GC and red pulp (RP) (bottom left figure in Fig. 3. 2). $CD4^+$ cells in HN and MLN were mainly present in the paracortex, and a few positive cells were found in the GC and M (top right and middle right figures in Fig. 3. 2). In the spleen, many $CD4^+$ cells were distributed in the periarteriolar lymphoid sheath (PALS), and a few positive cells were also present in the GC, the marginal zone (MZ) and the RP (bottom right figure in Fig. 3. 2).

In HNs, many $CD8^+$ cells were found in the paracortex, and a few positive cells were found in M (top left figure in Fig. 3. 3). In MLN, $CD8^+$ cells were infiltrated diffusely in the paracortex and M (middle left figure in Fig. 3. 3). In the spleen,

many $CD8^+$ cells were located in RP, and a few positive cells were found in PALS and MZ (bottom left figure in Fig. 3. 3). Many TCR1-$N24^+$ ($\gamma\delta$ T) cells in HNs were distributed in the lymphatic cord along the sinus of M (top right figure in Fig. 3. 3). A few $\gamma\delta$ T cells were scattered in the paracortex and M of MLN (middle right figure in Fig. 3. 3). In the spleen, many $\gamma\delta$ T cells were found in RP, and very few positive cells were found in MZ (bottom right figure in Fig. 3. 3).

$CD172a^+$ cells, which were expressed on the macrophages and granulocytes, were distributed in the lymphatic cord along the sinus and M in HN (top left figure in Fig. 3. 4). In MLN, the distribution of $CD172a^+$ cells was very similar to those of HN (middle left figure in Fig. 3. 4). In the spleen, many positive cells were also distributed in RP, and a few positive cells were recognized in PALS and MZ (bottom left figure in Fig. 3. 3). MHC class II^+ cells were mainly distributed in the lymphatic follicles, the paracortex and the sinusoid regions of HN (top right figure in Fig. 3. 4). In MLN, the distribution of positive cells was very similar to those of HN (middle right figure in Fig. 3. 4). In spleen, many positive cells were distributed in RP and the white pulp (bottom right figure in Fig. 3. 4).

No positive staining was found in the control slides (data not shown). The staining intensities of the immunocompetent cells in the different locations of those secondary lymphoid organs were summarized (Table. 3. 1).

3.3.3 Population of Immunocompetent Cells in PBMC, HN, MLN and SP

The populations of those immunocompetent cells from PBMC, HN, MLN and SP were shown with mean ± SD (Table. 3. 2), and as shown in Fig. 3. 5, the populations of $CD21^+$ cells and MHC class II^+ cells in HN and MLN were significantly higher than those of the spleen and PBMC ($P<0.05$); but these vales for HN was similar to those of MLN.

In addition, the populations of $CD8^+$ cells, $\gamma\delta$ T cells and $CD172a^+$ cells in the spleen were significantly higher than those in HN, MLN and PBMC ($P<0.05$); but, these values of $CD8^+$ cells and $\gamma\delta$ T cells for HN were higher than those for MLN.

In particular, the population of γδ T cells in HN was significantly higher than that of MLN ($P<0.05$). The population of $CD4^+$ cells was highest in PBMC, but there was no significant difference among these lymphoid tissues.

3.4 Discussion

The spleen is an immunoreactive lymphoid organ situated in the blood circulation, which provides an efficient immunologic defense against invading antigens, and the lymph nodes are located in the lymph circulation, which evokes immunologic responses to a limited region of the body (Sakita et al., 1997). The histological analysis showed that the arrangement of the cells in HN resembled the normal MLN, thus the direct contact between lymphatic tissue and blood was more reminiscent of the SP. The distribution and population of immunocompetent cells in these lymphoid organs has been well established (Wilson, et al., 1996; Caro et al., 1998; Wu et al., 1999; Sopp and Howard, 2001; Faldyna et al., 2005; Kampen et al., 2006). However, the distribution and population of immunocompetent cells in HNs have not been sufficiently investigated to understand the exact role of HN in the immune system. Here, we compared the distribution and population of immunocompetent cells in bovine HN with those of MLN and spleen to analyze the immunological function of HN.

From the IHC analysis, there were many germinal centers in the cortex of HN, which consists of mostly $CD21^+$ cells and a few $CD4^+$ cells. In addition, the population of $CD21^+$ cells in HN was higher than that of other lymphoid organs. The germinal center is thought to be associated with the development B-cell memory and is the site of somatic hypermutation, selection and differentiation during immune responses (Liu et al., 1989; Leanderson et al., 1992; Grandien et al., 1994). Therefore, we speculate that the HN is also induction sites of antigen-specific B-cell responses as well as other lymphoid organs like MLN and spleen.

The $CD172a^+$ cells were found in the lymphatic cord along the sinus of HN and MLN. In addition, the population of $CD172a^+$ cells in HN was also very similar to

that of MLN. The lymphatic cord along the sinus is thought to be a major antigen-trapping site, in which many antigen-presenting cells such as macrophages and dendritic cells are distributed in the cord to englobe the external antigen from lymphatic and blood vessels (Galeotti et al., 1993; Goldsby et al., 2002). MHC class II^+ cells were distributed in most areas of the HN as well as the spleen. It has been reported that MHC class II is expressed by antigen-presenting cells including dendritic cells, macrophages, B ells and some activated T cells (Baldwin et al., 1986; Van Kampen and Mallard, 1997; Banchereau et al., 2000; Janeway Jr et al., 2001; Cheng et al., 2008). In addition, the population of MHC class II^+ cells in HN was higher than that of other lymphoid organs, indicating that HN may have a potential ability in humoral immune reactions.

The distribution and population of $CD4^+$ cells in HN is quite similar to those of other lymphoid organs. $CD4^+$ cells in MLNs and the spleen play important roles in the regulation of cellular immunity (Bennett et al., 1997; Ossendorp et al., 2000). Therefore, $CD4^+$ cells in HN might have similar functions as those in the MLNs and spleen.

The distributions of $CD8^+$ cells and γδ T cells in HN are very similar to those of the spleen, but not those of MLNs. In particular, many $CD8^+$ cells and γδ T cells were found in the lymphatic cord along the sinus of HN and the splenic cord in RP, in which external antigen trapping is thought to occur (Goldsby et al., 2002; Cerutti and Guerrero, 2008). The different distributions of $CD8^+$ cells and γδ T cells between HN and MLN could be explained by the different expression of chemokines and their receptors (Blumerman et al., 2007). However, the migration mechanisms of the γδ T cells in HN require further study. In addition, young cattle have high levels of γδ T cells, which are found in blood circulation and can respond to autologous molecules and recognize non-peptide antigens (Hein and Mackay, 1991; Tanaka et al., 1995; Pollock and Welsh, 2002; Martino et al., 2005; Toka et al., 2011). It has also been reported that γδ T cells can act as antigen-presenting cells; but, the induction sites for professional antigen-presenting γδ T cells have not been fully determined in vivo

(Collins et al., 1998; Brandes et al., 2005; Wu et al., 2009; Toka et al., 2011). We speculate that γδ T cells, which are distributed in the lymphatic cord along the sinus of HN, have a potential for antigen presenting cell (manuscript in preparation). Meanwhile, the high population of γδ T cells in HN, reached about two times higher than that in MLN, may suggest the different mechanism of this lymphocyte subset in lymphocyte homing in HN, and more immune related characteristics should be promoted in the continued study on this hypothesis.

In addition, there are two types of nodes form: hemal nodes containing blood-filled sinusoids, which are peculiar to ruminants, and hemolymph nodes containing both blood and lymph sinusoids, which occur in other animal (Singh, 1959; Constantinescu et al., 1988; Cerutti and Guerrero, 2008). However, it has been described that the ruminant hemal nodes also have both blood vessel and lymphatics (Zidan and Pabst, 2004; Zidan and Pabst, 2010). Therefore, the functional and morphological differences between two types of ruminant hemal nodes should be addressed in near future.

Taken together, these results suggest that the HN has the potential to be an antigen-capturing site and the induction site of antigen-specific humoral and cellular immunity. However, further studies are needed to determine if HNs provide local immunity like MLNs, systemic immunity like the spleen, or both local and systemic immunity.

FIGURE CAPTIONS

Figure 3. 1

Gross anatomical and histological observations of HNs in the mesenteric region (a and b), the region along the aorta (c and d) and the subcutaneous of the scapular region (e and f). The number and size of HNs varied among different anatomical regions and different calves. HNs were surrounded by connective tissue capsules, and the parenchyma was clearly shown. The sinus was filled with numerous erythrocytes, and many germinal centers were located in the cortex. C: capsule, S: subcapsular sinus, CX: cortex, PC: paracortex, M: medulla, H: hilus, asterisk: germinal center, Arrow: HNs, Arrowhead: blood vessels. Bar= 1 mm.

Figure 3. 2

Distribution of $CD21^+$ and $CD4^+$ cells in HN (top row), MLN (middle row) and spleen (bottom row). Each mAb is shown in the same column, and the dotted square indicates the details of the same area. $CD21^+$ cells were mainly distributed in GCs of these lymphoid organs and RP of spleen (left column). $CD4^+$ cells were found in the paracortex of HN and MLN and the PALS of spleen (right column). M: medulla, PC: paracortex, asterisk: germinal center, arrow: periarterial lymphatic sheath, arrowhead: marginal zone. Bar=1 mm.

Figure 3. 3

Distribution of $CD8^+$ and TCR1-$N24^+$ cells in HN (top row), MLN (middle row) and spleen (bottom row). Each mAb is shown in the same column, and the dotted square indicates the details of the same area. In HN, $CD8^+$ cells were distributed in the paracortex and distributed diffusedly in the lymphatic cord along the sinus (top figure of left column). In MLN, $CD8^+$ cells were found diffusedly in the paracortex and medulla (middle figure of left column). In the spleen, many $CD8^+$ cells were found in RP (bottom figure of left column). TCR1-$N24^+$ ($\gamma\delta$ T) cells of HN were mainly distributed in the lymphatic cord along the sinus (top figure of right column).

In MLN, γδ T cells were scattered in the paracortex and medulla (middle figure of right column). In spleen, many γδ T cells were found in the RP (bottom figure of right column). M: medulla, PC: paracortex, asterisk: germinal center, arrow: periarterial lymphatic sheath, arrowhead: marginal zone. Bar=1 mm.

Figure 3. 4

Distribution of $CD172a^+$ and MHC class II^+ cells in HN (top row), MLN (middle row) and spleen (bottom row). Each mAb is shown in the same column, and the dotted square indicates the details of the same area. Many $CD172a^+$ cells were found in the lymphatic cord along the sinus of HN and RP of spleen (left column). MHC class II^+ cells were found in most of the lymphatic cord and sinusoid region in three organs (right column). M: medulla, PC: paracortex, asterisk: germinal center, arrow: periarterial lymphatic sheath, arrowhead: marginal zone. Bar=1 mm.

Figure 3. 5

Analysis graphics of the populations of immunocompetent cells in PBMC, HN, MLN and spleen. The data show mean ± SD. The exponents $^{a\text{-}d}$ reflect significant differences ($P<0.05$) as compared to PBMC ^{a}; HN ^{b}; MLN ^{c}; or spleen ^{d}.

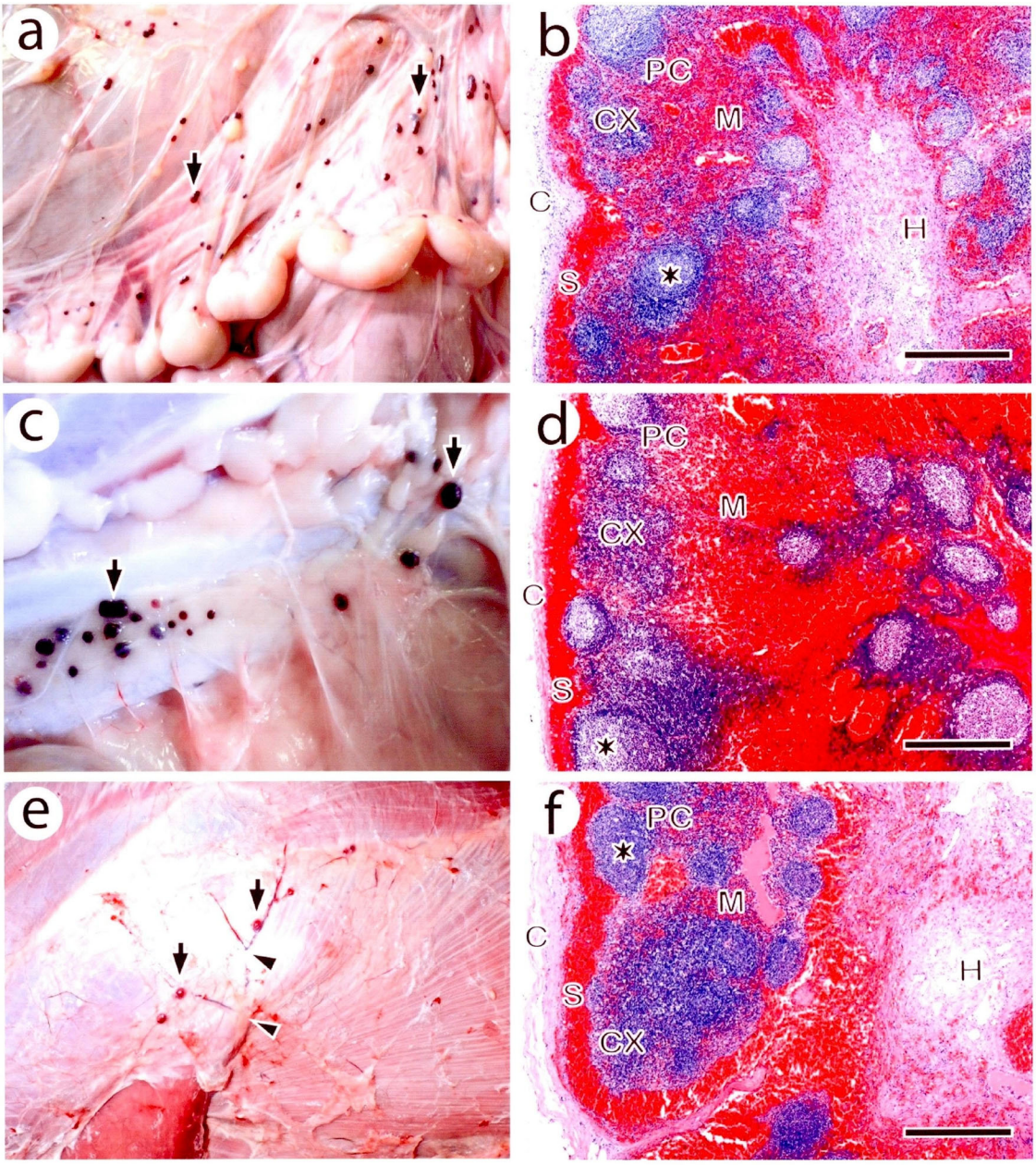

Fig. 3. 1 Gross anatomical and histological observations of HNs in different regions.

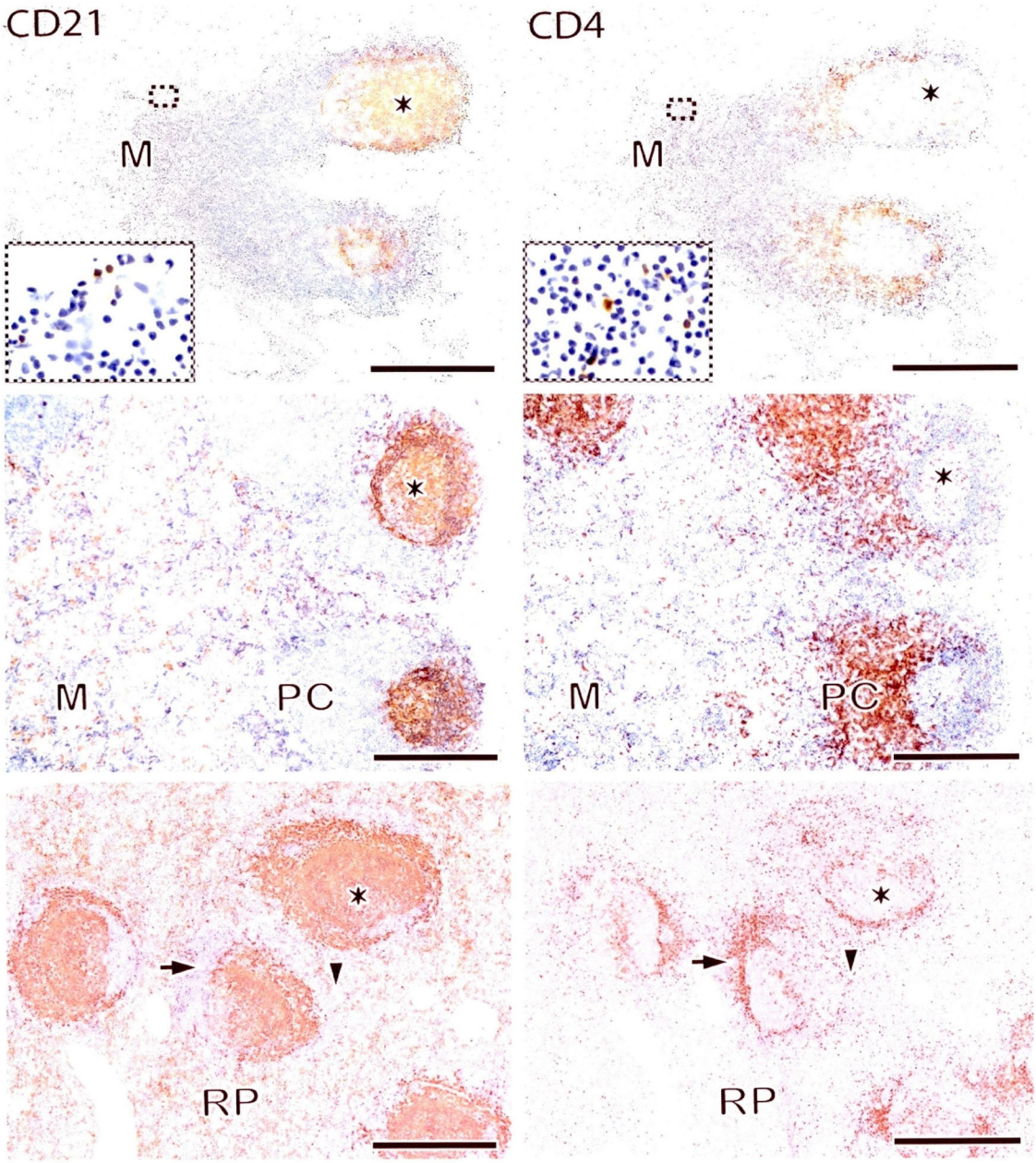

Fig. 3. 2 Distribution of $CD21^+$ and $CD4^+$ cells in HN (top row), MLN (middle row) and spleen (bottom row).

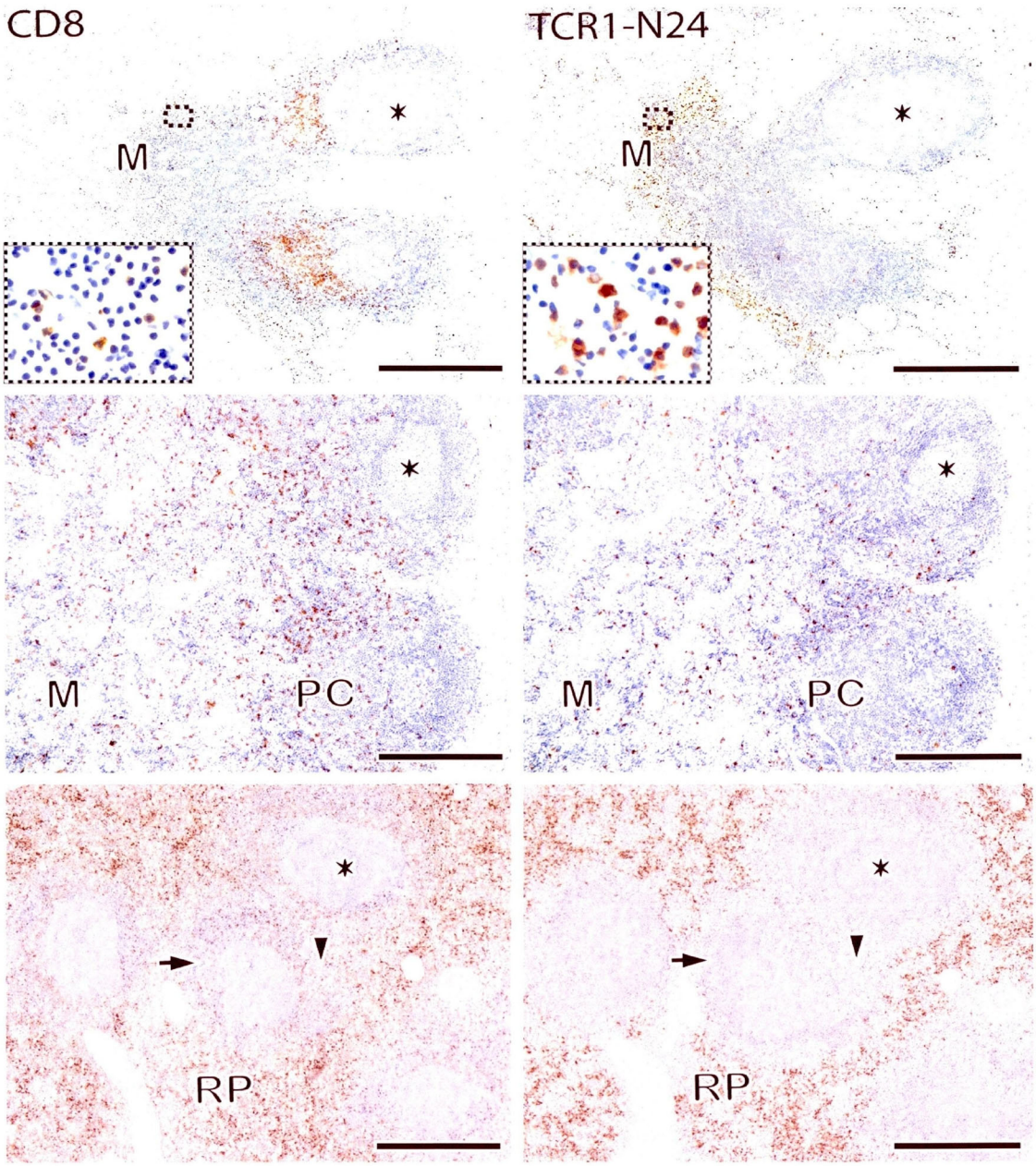

Fig. 3. 3 Distribution of $CD8^+$ and $TCR1\text{-}N24^+$ cells in HN (top row), MLN (middle row) and spleen (bottom row).

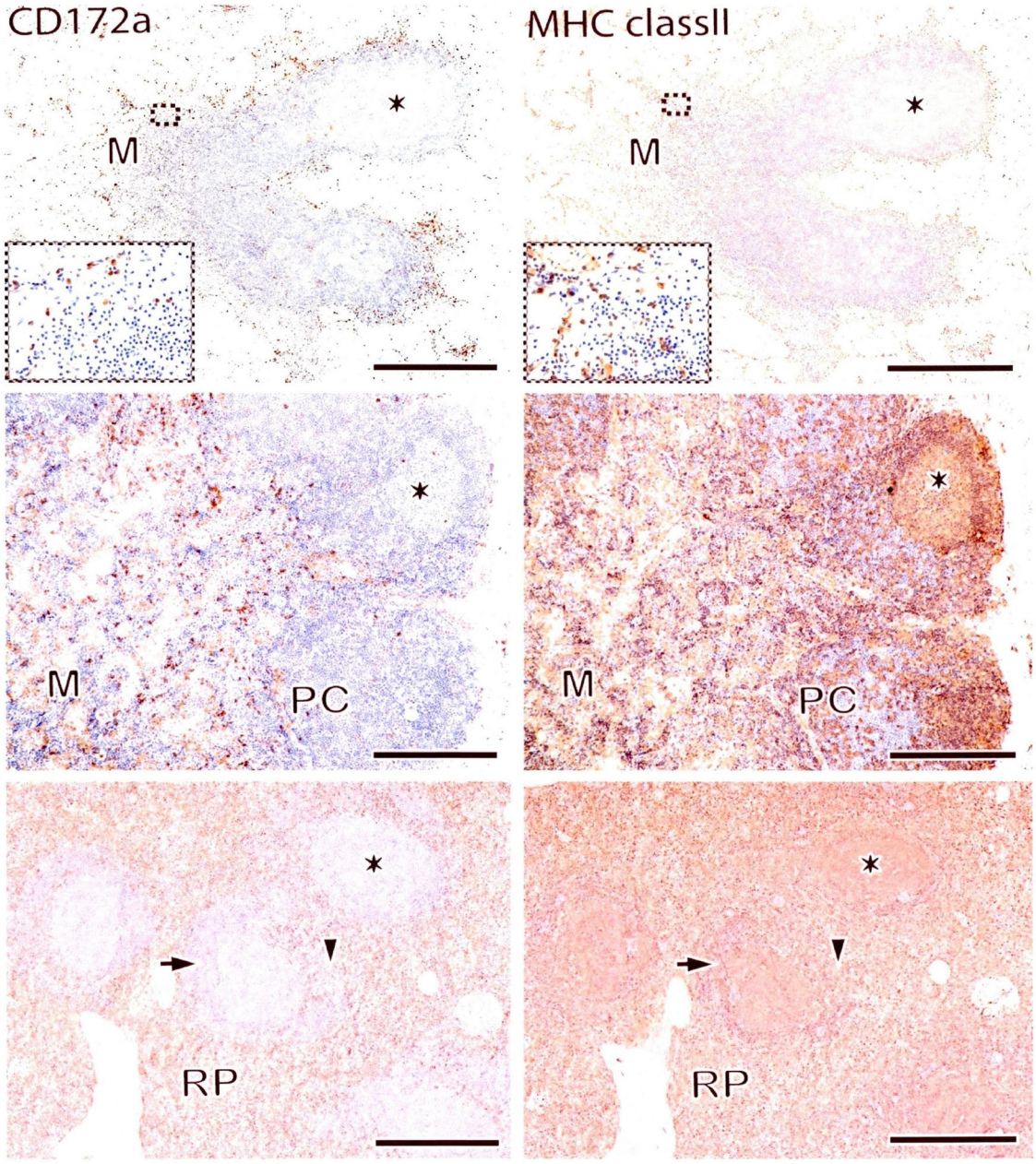

Fig. 3. 4 Distribution of CD172a^{+} and MHC class II^{+} cells in HN (top row), MLN (middle row) and spleen (bottom row).

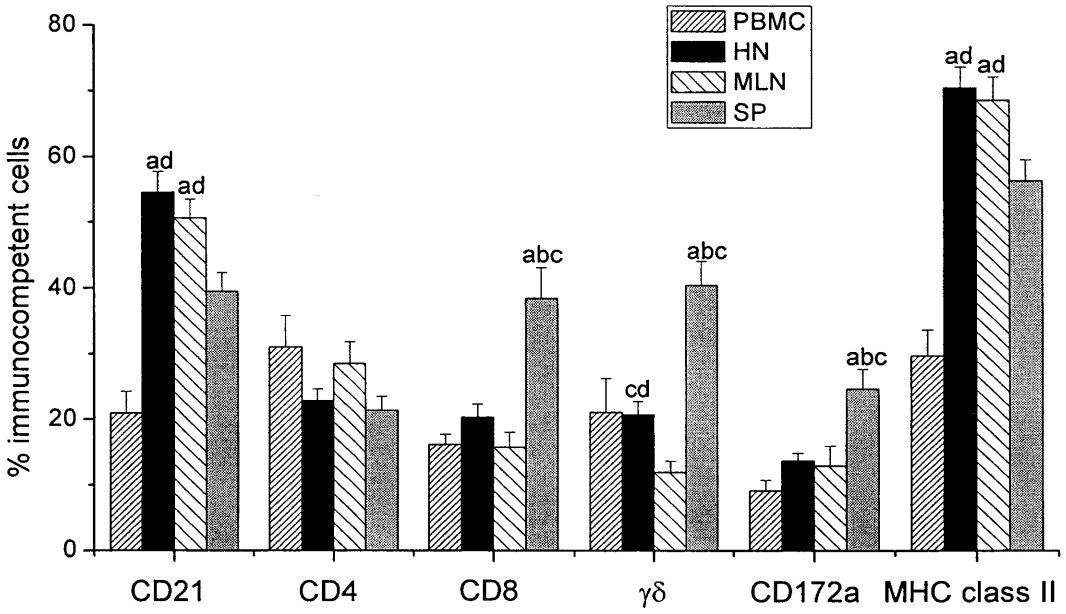

Fig. 3. 5 Analysis graphics of the populations of immunocompetent cells in PBMC, HN, MLN and spleen. The data show mean ± SD. The exponents [a-d] reflect significant differences ($P<0.05$) as compared to PBMC[a]; HN[b]; MLN[c]; or spleen[d].

Table 3. 1. The distributions and staining density of immuno-competent cells in HN, MLN and SP (n=28).

organs	areas	Monoclonal antibodies used in immunohistology		
		CD21	CD4	CD8
HN	PC	--	+++	+++
	GC	+++	+	--
	M	+	+	+
	LS	--	--	--
MLN	PC	--	+++	++
	GC	+++	+	--
	M	++	+	+
	LS	--	--	--
SP	PALS	--	+++	++
	GC	+++	+	--
	RP	++	--	+++
	MZ	--	+	+

Table 3. 1 Continued.

organs	areas	Monoclonal antibodies used in immunohistology		
		TCR1–N24	CD172a	MHC class II
HN	PC	--	--	++
	GC	--	--	+++
	M	+++	++	+++
	LS	+	++	++
MLN	PC	+	--	++
	GC	--	--	+++
	M	+	++	+++
	LS	+	++	++
SP	PALS	++	+	+++
	GC	--	--	+++
	RP	+++	+++	++
	MZ	+	+	++

-- , negative; + , slight; ++ , moderate; +++ , strong staining; PC , paracortex; GC , germinal center; M , medulla; LS , lymphoid cord along the sinus; PALS , periarteriolar lymphoid sheath; RP, red pulp; MZ, mantle zone.

Table 3. 2. Percentages with mean ± SD of the various immuno-competent cells populations as determined by flow cytometry (n=10).

mAbs	Populations of different immunocompetent cells (%)			
	PBMC	HN	MLN	SP
CD21	20.93±3.30	54.51±3.18	50.58±2.88	39.50±2.80
CD4	30.99±4.75	22.85±1.78	28.48±3.31	21.32±2.13
CD8	16.16±1.53	20.34±1.97	15.75±2.28	38.42±4.65
TCR1-N24	21.04±5.23	20.68±2.02	11.92±1.72	40.40±3.64
CD172a	9.07±1.63	13.65±1.19	12.88±2.99	24.59±3.03
MHC Class II	29.62±3.98	70.44±3.22	68.57±3.56	56.23±3.24

mAbs , monoclonal antibodies; PBMC , peripheral blood; HN , hemal node; MLN , mesenteric lymph node; SP , spleen.

Chapter 4

Preliminary Analysis on High Endothelial Venules of Bovine HNs and Associated Subpopulation of T Lymphocytes

Abstract

The frequency of high endothelial venule (HEV) and their regional differentiations in hemal node (HN) were analyzed comparing to the mesenteric lymph node (MLN) and spleen (SP) by immunohistochemistry (IHC). In addition, population of L-selectin and other CD markers double positive cells were analyzed by flow cytometry (FACS). The HEV was found in all MLNs but absent in SP. The frequency of HEV marker$^+$ HN (MECA-79$^+$ HN) was different among the localizations, and the value of the subcutaneous region (61 %) was significantly higher than those values along aorta (33 %) and the mesenteric region (17 %). The HEV in MLN was distributed mainly in the paracortical area. In HN, the positive signals were mainly found in the perifollicular area, a few positive signals were also located in the paracortical and interfollicular area. In HEV marker$^+$ HNs, the populations of CD4$^+$ cells, L-selectin$^+$ CD4$^+$ cells and L-selectin$^+$ CD8$^+$ cells were significantly higher than those of HEV marker negative HNs. The data suggest lymphocyte homing mechanism exist in HN, which concerns about functional differences among the localizations on immunological characteristics.

4.1 Introduction

Primary immune responses are initiated in the lymph node and other secondary lymphoid tissues (Ohtani et al., 2003). In particular, the hemal node (HN) that is endowed only with blood vessels has already been accepted as an independent secondary lymphoid organ, in which the lymph sinuses are replaced by blood sinuses (Bacha and Bacha, 2000; Zidan and Pabst, 2010). By gross anatomy, the HNs can be found in mammals such as human and rats, especially in ruminants (Max Lederer,

1923; Turner, 1969; Snider III et al., 2003). Although the distribution and number of HNs seem to be diverse among species, but in ruminants, they mainly located in the subcutaneous region of the head, neck and trunk, the mesenteric region and along the aorta in the thorax and abdomen (Cerutti and Guerrero, 2001; Snider III et al., 2003), which was also supported by our previous study in bovine (Zhang et al., 2012).

The population and distribution of immunocompetent cells in HNs and their possible immunological functions has been proposed in our previous studies (Chapter 3), but exactly how and from where recirculating T lymphocytes migrate into the HN system remains to be fully illustrated (Cerutti and Guerrero, 2001; Casteleyn et al., 2008; Zhang et al., 2012). From current knowledge, the first critical step of lymphocyte migration from circulation into lymphoid tissue is the adhesion of lymphocytes to vascular endothelium, and in secondary lymphoid organs like peripheral lymph nodes this occurs in high endothelial venules (HEVs), which have specialized endothelium for multistep interactions with adhesion molecules on the surface of lymphocytes (homing receptors) (Girard and Springer, 1995; Miyasaka and Tanaka, 2004). From previous studies, cell adhesion molecules (CAM) L-selectin, has been identified as a peripheral lymphocyte homing receptor in cattle, which can bind to peripheral node addressin (PNAD) that is present at high levels of HEVs and identified by the MECA-79 monoclonal antibody (the HEV marker currently available, which stains HEVs in various secondary lymphoid organs of many species) (Bosworth et al., 1993; Girard and Springer, 1995; Miyasaka and Tanaka, 2004; Drayton et al., 2006). The aim of this study is to analyze the variation of the lymphoid structure and functional difference of bovine HNs.

4.2 Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki. Japanese black bovine (male, 12; female, 16) were obtained from local farms in Miyazaki Prefecture, aged from 3 to 15 months. The animals were killed by electric shock following the combination of xylazine

(0.2mg/kg) and pentobarbital (15mg/kg) intravenous injection. The HNs were collected separately from subcutaneous region of the neck and trunk, mesenteric region and the area along the abdominal aorta, HNs were numbered and half of each was fixed with 10% buffered formalin for at least three days together with mesenteric lymph node (MLN) and spleen (SP), another half of numbered HNs were collected for flow cytometry (FACS) immediately.

Monoclonal antibody (mAb): mAbs specific for calf CD4 (IL-A11), CD8 (CACT80c), TCR1-N24: δ chain specific (GB21A) and L-selectin (DUI-29) were purchased from VMRD Inc. (Pullman, WA, U.S.A.). Labeling Kits-NH2 including Allophycocyanin (APC), Fluorescein (FITC) and R-Phycoerythrin (PE) were purchased from Dojindo Molecular Technologies Inc. (Minato-ku, Tokyo, JAPAN). A 1:250 dilution of each labeled mAb was used for two-color analysis by FACS. In addition, mAb MECA-79 (sc-19602) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.), and a 1:200 dilution was used for immunohistochemistry (IHC) analysis.

4.2.1 Histological Analysis and Immunohistochemistry

Specimens were passed through ethanol and toluene and embedded in paraffin. They were cut into 4μm thick continuous sections and went on with hematoxylin-eosin (HE) stain for HN histology. For IHC analysis, continuous sections of HN, MLN and SP were rehydrated through xylol and ethanol, endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min, and incubated with normal 10% rabbit serum for 20 min. The sections were stained with mAb MECA-79 for 30 min and washed three times with PBS, after incubation with secondary antibody and washed three times with PBS, the process was followed by incubation with ABC complex (Vector Lab., Burlingame, CA, U.S.A.) for 30 min. After the sections were rinsed three times in PBS, the reactions were made visible with metal-enhanced diaminobenzidine (DAB, Pierce, Rockford, IL, U.S.A.). All immunohistochemical staining was performed at room temperature in a moist

chamber. Control staining, in which the primary antibody was replaced with PBS, was performed simultaneously.

4.2.2 Flow Cytometry

Cell suspensions were obtained from other half of each numbered HN by teasing the minced organs through nylon mesh for removing the debris. The cell suspensions of HNs were further purified by density gradient centrifugation with Ficoll-Paque (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England). For immunofluorescence, the cells were suspended in PBS supplemented with 0.5% bovine serum albumin and 0.05% sodium azide. Viable cells were incubated with mixed labeled mAbs (different mAbs conjugated with APC, FITC and PE, separately) at 4°C for 30 min. After washing three times by PBS, relative immunofluorescence intensities were determined by multi-parametric flow cytometry with a FACSCanto^{TM}II system (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

4.2.3 Statistical Analysis

The statistical analysis consisted of an analysis of variance (ANOVA) and multiple comparison tests performed with Origin Pro 8.0 software (OriginLab CO., Northampton, MA, U.S.A.). *P*-values less than 0.05 were regarded as statistically significant.

4.3 Results

4.3.1 Histology of HNs and the Frequency of HEV Positive Bovine HNs

The HNs were encapsulated by connective tissues and the sinuses were filled with numerous erythrocytes. The most developed lymphoid tissue in HNs consisted of three main regions: the follicular region in which many germinal centers were located; the paracortex which consisted of the lymphatic region and located between cortex and medulla; and the sinusoid region which consisted of the lymphatic cord and sinus (Fig. 4. 1a).

The frequency of HEV marker$^+$ HNs was summarized by three main regions (Table. 4. 1). Compared with those from other two regions, the HEV marker$^+$ HNs of subcutaneous region showed the highest percentage reaching 61 %, followed with 33 % and 17 % which showed the frequency in the area along the aorta and the mesenteric region, respectively. Different with HN, MLN was always a HEV marker$^+$ lymphoid organ, when SP was exactly HEV marker negative.

4.3.2 Distribution of HEVs in Bovine Lymphoid Organs

In HEV marker$^+$ HNs, the HEVs could be found numerous in the perifollicular area, which immediately beneath the subcapsular sinus, and a few positive signals also distributed in the paracortical and interfollicular area (Fig. 4. 1b-d). There are many variations on number and positive staining intensity of HEVs in HNs, especially in those HEV marker$^+$ HNs from the region along the aorta, the HEVs were usually found mainly present in the perifollicular zone, with weak positive signals (Fig. 4. 1e). No positive signal was found in the control slides (Fig. 4. 1f).

As shown in the study, the immunohistological examination revealed that MECA-79 stained HEVs in all MLNs (Fig. 4. 2a-c). The HEVs of MLN distributed mainly in the paracortical area, with a few present in the interfollicular area (Fig. 4. 2a-c), but HEVs were absent in SP (Fig. 4. 2d).

No positive staining was found in the control slides (data not shown). The staining intensities of the HEV marker$^+$ cells in the different locations of those secondary lymphoid organs were summarized (Table. 4. 2).

4.3.3 The Subpopulations of Lymphocytes in HNs

L-selectin$^+$ and T cell subpopulations of HNs associated with the existence of HEV marker MECA-79 was shown (Fig. 4. 3, Fig. 4. 4 and Table. 4. 3), and the significant differences were also summarized (Fig. 4. 5). In HEV marker$^+$ HN, the populations of CD4$^+$ cells, CD4$^+$ L-selectin$^+$ cells and CD8$^+$ L-selectin$^+$ cells were significantly higher than those of HEV marker negative HN ($P<0.05$). In addition, upon the

populations of L-selectin$^+$ cells, CD8$^+$ cells, TCR1-N24$^+$ cells (γδ T), γδ T $^+$ L-selectin$^+$ cells and CD8$^+$ γδ T cells, there was no significant difference between the HEV marker$^+$ HN and HEV marker negative HN; but the population of γδ T $^+$ L-selectin$^+$ cells was higher than both CD4$^+$ L-selectin$^+$ cells and CD8$^+$ L-selectin$^+$ cells in HEV marker$^+$ HN and HEV marker negative HN. The populations of CD4$^+$ CD8$^+$ cells and CD4$^+$ γδ T cells were lower than 1% so they weren't included in the analysis.

4.4 Discussion

The conventional HN is usually thought to be a lymphoid organ that functions on the blood filtration and may take part in the immune system, but the answers to the controversies about the immunological role and the mechanism of lymphocyte homing mode by which blood reaches the inside of the node still remain unclear (Turner, 1969; Cerutti and Guerrero, 2008; Zidan and Pabst, 2010). Some studies showed that blood cells possibly entering the HNs of rats via some capillary vessels, which sent branches leading directly into the sinusoids, through an opening guarded by two bulging endothelial cells, but that were not conclusively demonstrated in their study (Turner, 1969). Some researcher claimed that blood cells entered the HNs by diapedesis through the walls of HEVs and small intranodal blood vessels (Nopajaroonsri et al., 1974), but their hypothesis was denied by others (Abu-Hijle and Scothorne, 1996). Our study revealed that in bovine HNs, there were HEV marker$^+$ HN and HEV marker negative HN existed, which indicated that the pathway of blood entering was multiple and there may be a selective mechanism in HNs, it operated to direct entering of blood cells into the HNs and affected the erythrocytes distribution and lymphocyte migration.

The population of HEV marker$^+$ HN was highest in subcutaneous region, and this may be relevant to the nearby cutaneous inflammation. It is generally considered that most leukocyte migration to cutaneous sites is regulated by the expression of E- and/or P-selectin on dermal venules (Kupper and Fuhlbrigge, 2004), but L-selectin had

also been demonstrated an important role in mediating cutaneous inflammation (Tedder et al., 1995; Tang et al., 1997; Steeber et al., 1999; Nagaoka et al., 2000; Shimada et al., 2003). The multiple adhesion pathways can support leukocyte migration into cutaneous sites of inflammation, and this may affect the lymphocyte trafficking in skin-drained HNs nearby.

L-selectin$^+$ cells were similar in their populations between HNs of HEV marker$^+$ and HEV marker negative, but for CD4$^+$ cells and CD4$^+$ L-selectin$^+$ cells, the populations had significant differences between HNs defined by HEV marker, this may imply L-selectin was essential in lymphocyte trafficking in HNs, but more important, there may other cell adhesion molecules like integrins act in this progress, especially in HEV marker$^+$ HNs (Steeber et al., 1998). In addition, L-selectin function is required for normal regulatory T cell (T_{reg}) migration, regulatory T cells, typically identified as having a CD4$^+$ CD25$^+$ Foxp3$^+$ phenotype, are involved in regulating a broad array of immune functions such as T cell homeostasis (Grailer et al., 2009). Like conventional CD4$^+$ T cells, the majority of T_{reg} cells express L-selectin (Huehn et al., 2004) and utilize its function for migration and maintenance of normal tissue distribution (Venturi et al., 2007). By contrast, T_{reg} cells have twogold higher L-selectin mRNA levels and have a higher rate of cell-surface L-selectin turnover than conventional CD4$^+$ T cells (Venturi et al., 2007). In HNs defined by MECA-79, there may some differences on the distribution and population of T_{reg} cells, which continue to influence the population of L-selectin.

The cellular immunity of bovine HNs had been suggested in previous studies (Snider III, 2003; Zhang et al., 2012). The population of CD8$^+$ L-selectin$^+$ cells in HEV marker$^+$ HNs had significant difference with those in HEV marker negative HNs, which indicated that the coactions of L-selectin and HEV were essential for a part CD8$^+$ cells trafficking into HNs, which may suggest a high cellular immune function in those HNs with HEVs.

For the population of γδ T cells and γδ T$^+$ L-selectin$^+$ cells, there was no significant difference in two types of HNs defined by MECA-79. It had been reported that the

expression of L-selectin alone did not predict a LN homing capacity (Walcheck and Jutila, 1994), they found that bovine γδ T cells accumulated along the vascular wall of venules that support lymphocyte extravasation into LN (MECA-79$^+$ venules) in vivo, and those γδ T cells did not appreciably extravasate from the blood into the parenchyma of LNs. From our previous studies, the population of γδ T cells in the HN was significantly higher than that of MLN, and they were distributed near the paracortex, but in this study, the MECA-79$^+$ venules was also located in the perifollicular area and interfollicular area except for the paracortical area, the pathways by which γδ T cells migrated into the HNs may be different with those in LN, furthermore, besides L-selectin, there may be other vascular of lymphocyte adhesion molecular exist in the migration of γδ T cells, which needs to be identified in further studies.

In this study, , there was no significant difference on the CD8$^+$ γδ T cells between the HEV marker$^+$ HNs and HEV marker negative HNs. In primary γδ T cells, most CD8$^+$ γδ T cells were found to express L-selectin and the $\alpha_4\beta_7$ integrin (Wilson et al., 1999; Wilson et al., 2002) and many adhesion moleculars and chemokines were found to play roles in the migration of CD8$^+$ γδ T cells (Zabel et al., 1999; Pan et al., 2000; Wang et al., 2000), this may also support the hypothesis that the migration mechanism of lymphocytes exist in HNs, which concerns about functional differences among the localizations on immunological characteristics.

In conclusion, the HNs of different types defined by MECA-79 were investigated on their HEV distribution and associated T lymphocyte subsets population. The existence of lymphocyte homing mechanism was suggested and the immunological characteristics in HNs including HEV neogenesis and development, the adhesion molecular and related chemokines on the lymphocyte trafficking and their independent pathways should be discussed in further studies.

FIGURE CAPTIONS

Figure 4. 1

HN histology and distribution of high endothelial venule (HEV) in HNs. The histology of HN was clearly identified (a). In MECA-79$^+$ HNs, the HEVs could be found numerous in the perifollicular area, and a few positive signals also distributed in the paracortical and interfollicular area (b-d, the figure b and d indicated the details of upper and lower dotted square in figure c, separately). In some HNs, the HEVs were usually found mainly present in the perifollicular zone, with weak positive signals (e), and no positive signal was found in the control slides (f). C: capsule, S: subcapsular sinus, CX: cortex, PX: paracortex, IF: interfollicular area, M: medulla, asterisk: germinal center, arrow: MECA-79 positive signals, arrow head: HEVs in perifollicular area. Bar= 250μm.

Figure 4. 2

Distribution of high endothelial venule (HEV) in LN and SP. In LNs, the HEVs could be found numerous in the paracortical area (a-c, the figure b indicated the details of dotted square in figure a). In SP, no positive signal was found (d). C: capsule, CX: cortex, PX: paracortex, RP: red pulp, asterisk: germinal center. Bar= 250μm.

Figure 4. 3

Two color flow cytometry analysis of CD8$^+$ L-selectin$^+$ cells (a) and CD4$^+$ L-selectin$^+$ cells (b) in HNs defined by MECA-79. The right upper quadrant represents cells that are positive for both mAbs (PE-L-selectin, FITC-CD8, APC-CD4), while the single positive cells are present in the left upper and right lower quadrants.

Figure 4. 4

Two color flow cytometry analysis of γδ T L-selectin$^+$ cells (a) and CD8$^+$ γδ T cells

(b) in HNs defined by MECA-79. The right upper quadrant represents cells that are positive for both mAbs (PE-L-selectin, FITC-CD8, APC-γδ T), while the single positive cells are present in the left upper and right lower quadrants.

Figure 4. 5

Analysis graphics of The populations of immunocompetent cells in HNs defined by MECA-79. The data show mean ± SD. The asterisks showed the significant difference between different HNs ($P<0.05$)

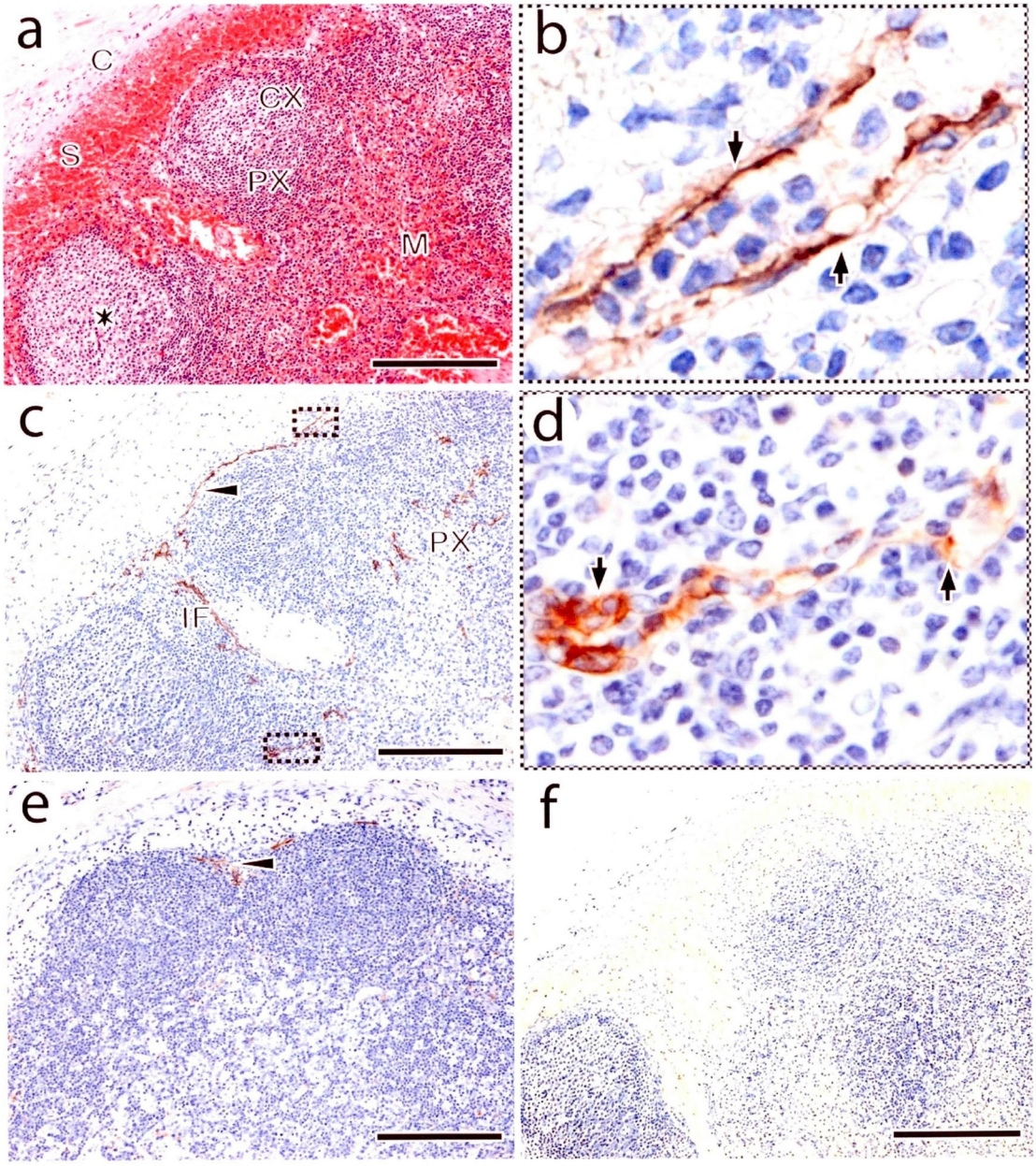

Fig. 4. 1 HN histology and distribution of high endothelial venule (HEV) in HNs.

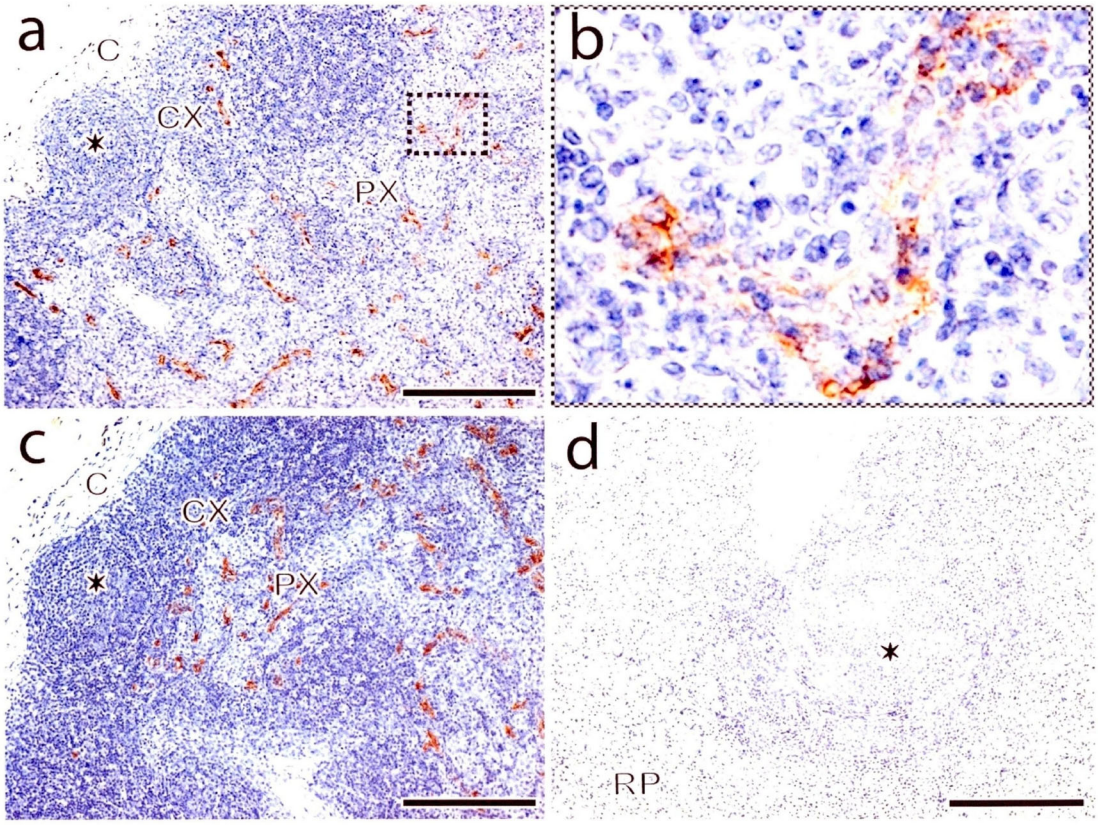

Fig. 4. 2 Distribution of high endothelial venule (HEV) in LN and SP.

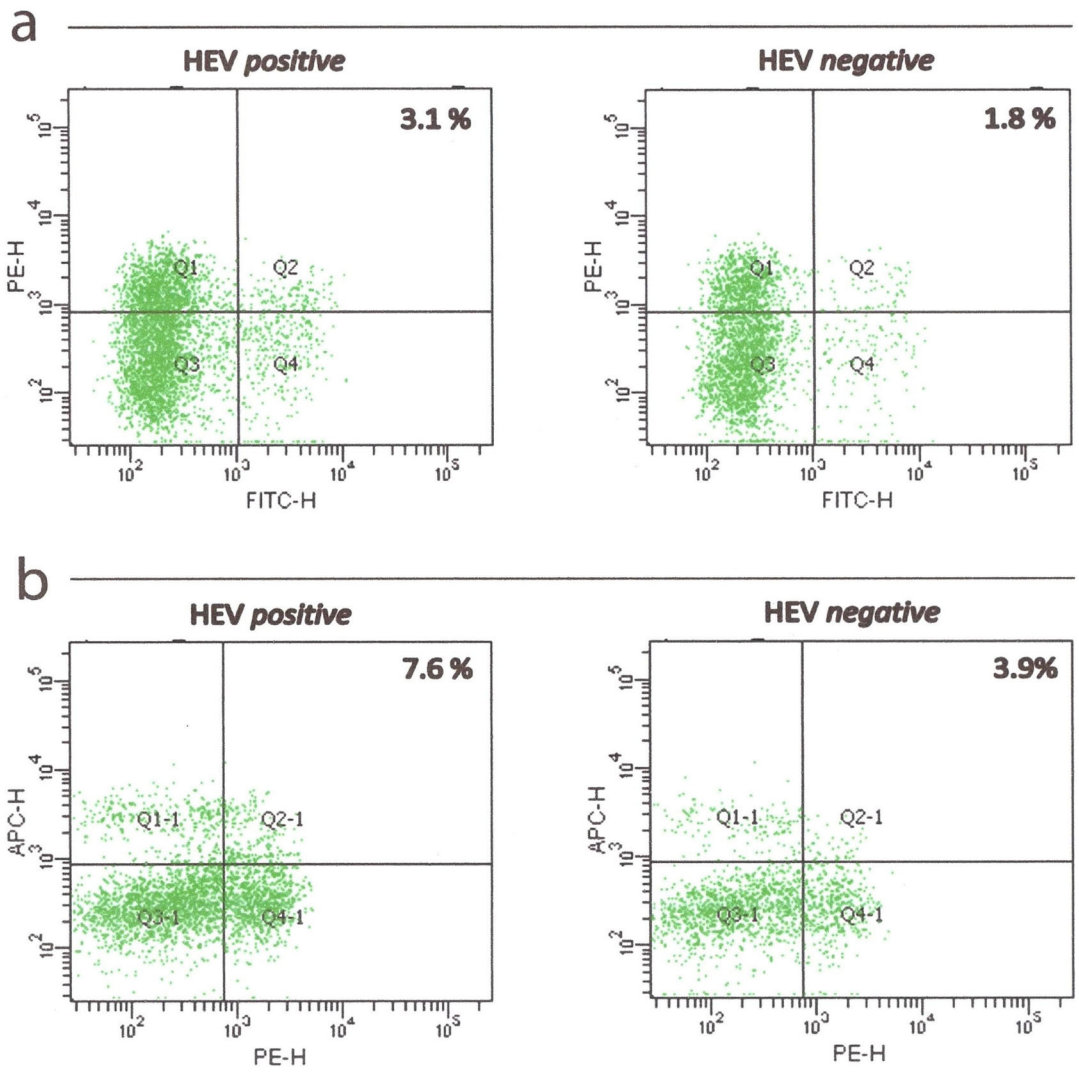

Fig. 4. 3 Two color flow cytometry analysis of $CD8^+$ L-selectin$^+$ cells (a) and $CD4^+$ L-selectin$^+$ cells (b) in different HNs defined by MECA-79.

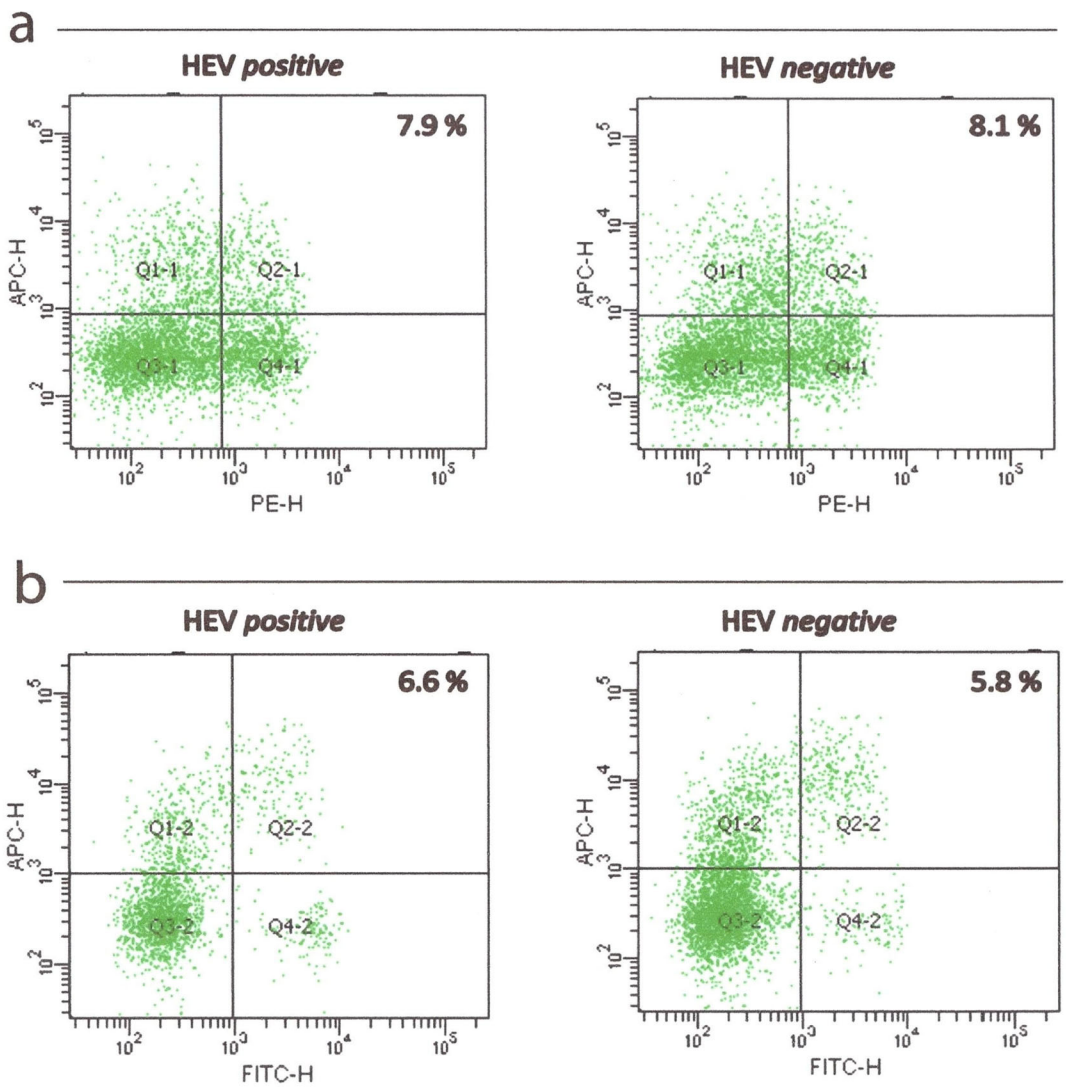

Fig. 4. 4 Two color flow cytometry analysis of γδ T L-selectin^{+} cells (a) and CD8^{+} γδ T cells (b) in different HNs defined by MECA-79.

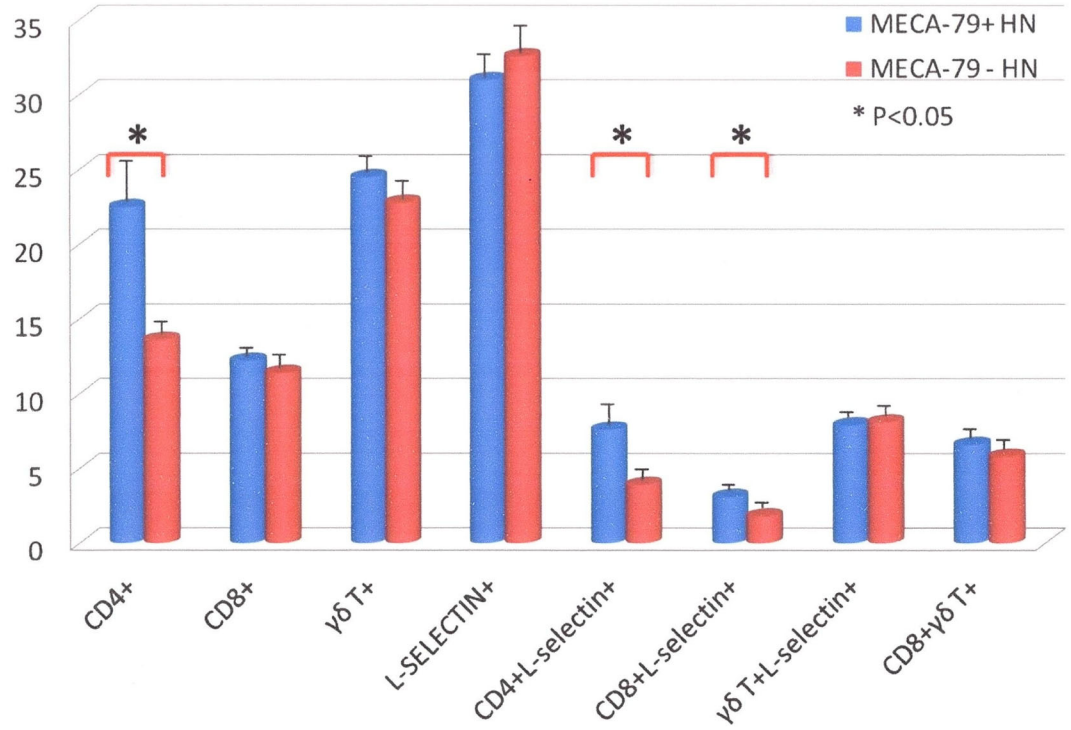

Fig. 4. 5 Analysis graphics of The populations of immuno-competent cells in different HNs defined by MECA-79.

Table 4. 1. The percentages of HEV-positive HNs in different regions of bovine (n=28).

Number	HNs				MLNs	SP
	Subcutaneous	Aorta	Mesenteric	Total		
Specimens	77	81	87	245	28	28
Positive	47	27	15	89	28	0
Percentage (%)	61	33	17	36	100	0

HNs , hemal nodes; MLNs , mesenteric lymph nodes; SP , spleen.

Table 4. 2. The distributions of MECA-79^{+} venules in HN, MLN and SP.

organs	Areas		
	PEF	PX	INF
HN	+++	++	++
MLN	--	+++	+
sp	--	--	--

-- , negative; + , slight; ++ , moderate; +++ , strong staining; PEF , perifollicular area; PX , paracortical area; INF , interfollicular area.

Table.4.3 Percentages with mean ± SD of the various immuno-competent cells populations as determined by flow cytometry in different HNs defined by MECA-79 (n=245).

mAbs	Populations of different immunocompetent cells (%)	
	MECA-79^{+} HN	MECA-79^{-} HN
L-selectin	31.0±1.7	32.6±2
CD4	22.5±3	13.6±1
CD8	12.2±0.7	11.4±0.9
TCR1-N24(γδ T)	24.5±1.2	22.8±1.7
CD4 L-selectin	7.6±1.5	3.9±0.8
CD8 L-selectin	3.1±0.4	1.8±0.2
γδ T L-selectin	7.9±0.4	8.1±0.7
CD8 γδ T	6.6±0.6	5.8±0.7

mAbs , monoclonal antibodies; HN , hemal node.

Chapter 5

General Conclusion

The hemal nodes (HNs) were found between the abdominal aorta and caudal vein as well as in the thoracic cavity, subcutaneous region and mesenteric region, as reported previously in other ruminants (Ezeasor and Singh, 1988; Abu-Hijleh and Scothorne, 1996; Cerutti,1998; Snider et al., 2003; Zidan and Pabst, 2004; Bozkurt and Kabak, 2010; Zidan and Pabst, 2010). The morphological alterations in HNs in splenectomized cattle showed that HLNs presented hypertrophy and hyperplasia in lymphatic tissue due to the splenectomy along with the absence of any modifications in the lymph nodes, which indicated that HNs could be structures capable of performing a supplementary role in spleen-like functions in young splenectomized cattle (Bassan et al., 1999). However, an opposite opinion was advanced by researchers who confirmed that radical splenectomies in Wistar rats failed to demonstrate any compensatory or regressive changes (Konstanty et al., 2000). The histological analysis showed that the arrangement of the cells in HN resembled the normal MLN, thus the direct contact between lymphatic tissue and blood was more reminiscent of the SP, somewhat like an "accessory spleen."

The capsule and trabeculae of bovine HNs were supported by smoothe nuscle cells and reticular fibres, similar to that reported for the HNs of many ruminants (Gargiulo et al., 1987; Ezeasor and Singh, 1988; Cerutti et al., 1998; Zidan and Pabst, 2004; Casteleyn et al., 2008; Bozkurt and Kabak, 2010). There were various literature reports with different results on the presence of afferent and efferent lymphatic vessels in HNs. They were present in the HNs of goats (Ezeasor and Singh, 1988; Ezeasor and Singh, 1990) and camels (Zidan and Pabst, 2004), but not in those of sheep (Thorp et al., 1991), buffalo calves (Singh, 1959), oxen (Constantinescu, 1988) and roe deer (Bozkurt and Kabak, 2010). Our results showed that the HNs of bovine

lacked lymphatic vessels, similar to those of sheep, buffalo calves and roe deer.

The lymphoid tissue of bovine HNs contained numerous lymphocytes and reticular cells, and macrophages could also be found touching the reticulum, distributed in the subcapsular sinuses and medulla, which indicated that bovine HNs were involved in the phagocytosis of old or defective blood cells from the circulation, and also the possible immunological functions (Winqvist, 1954; Cecarelli et al., 1986; Ezeasor and Singh, 1988; Zidan and Pabst, 2004). This study also showed a few primary and many secondary follicles in the bovine HNs, and the presence of secondary follicles was indicative of the role of the HNs in antibody production (Cecarelli et al., 1986). Based on the previous findings, the primary immunological response to extrinsic antigens was located in the areas from the marginal to the intermediate lymph node sinuses (Fujino et al., 1996; Sakita et al., 1997). Given that the lymphocytes and macrophages coming into intimate contact as an expression of special interaction, some immune functions might be performed by similar cells in bovine HNs (Casteleyn et al., 2008). And this was investigated by analyzing the distribution and population of immunocompetent cells in bovine HN with those of MLN and spleen.

From the IHC analysis, there were many germinal centers in the cortex of HN, which consists of mostly $CD21^+$ cells and a few $CD4^+$ cells. In addition, the population of $CD21^+$ cells in HN was higher than that of other lymphoid organs. The germinal center is thought to be associated with the development B-cell memory and is the site of somatic hypermutation, selection and differentiation during immune responses (Liu et al., 1989; Leanderson et al., 1992; Grandien et al., 1994). Therefore, we speculate that the HN is also induction sites of antigen-specific B-cell responses as well as other lymphoid organs like MLN and spleen.

The $CD172a^+$ cells were found in the lymphatic cord along the sinus of HN and MLN. In addition, the population of $CD172a^+$ cells in HN was also very similar to that of MLN. The lymphatic cord along the sinus is thought to be a major antigen-trapping site, in which many antigen-presenting cells such as macrophages and dendritic cells are distributed in the cord to englobe the external antigen from

lymphatic and blood vessels (Galeotti et al., 1993; Goldsby et al., 2002). MHC class II^+ cells were distributed in most areas of the HN as well as the spleen. It has been reported that MHC class II is expressed by antigen-presenting cells including dendritic cells, macrophages, B ells and some activated T cells (Baldwin et al., 1986; Van Kampen and Mallard, 1997; Banchereau et al., 2000; Janeway Jr et al., 2001; Cheng et al., 2008). In addition, the population of MHC class II^+ cells in HN was higher than that of other lymphoid organs, indicating that HN may have a potential ability in humoral immune reactions.

The distribution and population of $CD4^+$ cells in HN is quite similar to those of other lymphoid organs. $CD4^+$ cells in MLNs and the spleen play important roles in the regulation of cellular immunity (Bennett et al., 1997; Ossendorp et al., 2000). Therefore, $CD4^+$ cells in HN might have similar functions as those in the MLNs and spleen.

The distributions of $CD8^+$ cells and γδ T cells in HN are very similar to those of the spleen, but not those of MLNs. In particular, many $CD8^+$ cells and γδ T cells were found in the lymphatic cord along the sinus of HN and the splenic cord in RP, in which external antigen trapping is thought to occur (Goldsby et al., 2002; Cerutti and Guerrero, 2008). The different distributions of $CD8^+$ cells and γδ T cells between HN and MLN could be explained by the different expression of chemokines and their receptors (Blumerman et al., 2007). However, the migration mechanisms of the γδ T cells in HN require further study. In addition, young cattle have high levels of γδ T cells, which are found in blood circulation and can respond to autologous molecules and recognize non-peptide antigens (Hein and Mackay, 1991; Tanaka et al., 1995; Pollock and Welsh, 2002; Martino et al., 2005; Toka et al., 2011). It has also been reported that γδ T cells can act as antigen-presenting cells; but, the induction sites for professional antigen-presenting γδ T cells have not been fully determined in vivo (Collins et al., 1998; Brandes et al., 2005; Wu et al., 2009; Toka et al., 2011). We speculate that γδ T cells, which are distributed in the lymphatic cord along the sinus of HN, have a potential for antigen presenting cell (manuscript in preparation).

Meanwhile, the high population of γδ T cells in HN, reached about two times higher than that in MLN, even in HNs of MECA-79+ and MECA-79- venules, the population of γδ T cells had no significant difference, this may suggest the different mechanism of this lymphocyte subset in lymphocyte homing in HN, and more immune related characteristics should be promoted in the continued study on this hypothesis.

In our study, the HN casts were investigated by VCC method to show the architecture of the inner vasculature. Unlike the capsular vessels in the cast of rat HN which was bundle-like and had large interspaces (Castenholz and Castenholz, 1996), regular arrangement of arterioles and venules in HN capsular vessels network was observed. These vessels increased the surface area of the HN, and this might act as a reservoir for erythrocytes; contraction of the capsule can expel these erythrocytes into the circulation when needed.

The mechanism by which erythrocytes enter the node is still controversial. The earlier opinion was that erythrocytes entered the rat HN by reflux via lymphaticovenous communications, but this was discounted based on the presence of venous valves and the pattern of erythrocyte distribution (Job, 1918; Andreasen and Gottlieb, 1946). Some studies showed that erythrocytes entered the rat HN via afferent lymphatics, and this argued strongly against any direct communication between blood vessels and sinuses (Drinker and Field, 1931; Hogg et al., 1982; Kazeem, 1982). However, those findings were refuted by studies that failed to find afferent lymphatics and found no erythrocytes in the few afferent lymph streams (Turner, 1969; Nopajaroonsri, 1974). In our previous histological study, we could find neither afferent nor efferent lymphatics in bovine HNs, and this denied the possibility that erythrocytes came from the lymphatic system. The HNs had been described as a hemopoietic organ that could produce erythrocytes within the node (Jordan, 1926), and evidences of erythropoiesis were also shown (Cerutti and Guerrero, 2008), but no erythrocyte precursors were seen in any of the nodes examined by another researcher (Turner, 1969). No evidence of direct

communication between the vascular and sinus systems was obtained from cast studies of the rat HN (Castenholz and Castenholz, 1996). However, our study showed opposite results, because many anastomoses were found in the cortical capillaries opening into the blood sinusoids in the follicular zone and deep cortex of the bovine HN. Therefore, this route is probably one of the main sources of erythrocytes in the HN sinuses. In addition, within the normal lymph node, the deep cortex unit is a functional entity that allows contact and activation of lymphocytes by antigens or cytokines (Okada et al., 2002), so the capillary networks associated with the adjacent sinuses in the deep cortex of the bovine HN may suggest a similar immunological role in facilitating immunological interactions. The distribution of MECA-79$^+$ venules in HNs was similar as those capillaries observed in VCC studies opening into the sinuses, this also support the immunological interactions and imply the immune function of HNs.

Some studies showed that blood cells possibly entering the HNs of rats via some capillary vessels, which sent branches leading directly into the sinusoids, through an opening guarded by two bulging endothelial cells, but that were not conclusively demonstrated in their study (Turner, 1969). Some researcher claimed that blood cells entered the HNs by diapedesis through the walls of HEVs and small intranodal blood vessels (Nopajaroonsri et al., 1974), but their hypothesis was denied by others (Abu-Hijle and Scothorne, 1996). Our study revealed that in bovine HNs, there were HEV marker$^+$ HN and HEV marker negative HN existed, which indicated that the pathway of blood entering was multiple and there may be a selective mechanism in HNs, it operated to direct entering of blood cells into the HNs and affected the erythrocytes distribution and lymphocyte migration.

In the casts of bovine HNs from the cervical region, the presence of sinusoids could be taken as evidence of another pathway for blood flow. The subcapsular sinus of the HN was shown to be always engorged with numerous erythrocytes and had a variable size (Okada et al., 2002). In our research, there were many sieve openings in the cast of this sinus, and these perforations may due to large reticular processes

and numerous small trabeculae (Castenholz and Castenholz, 1996; Zidan and Pabst, 2010). From the casts, the subcapsular sinus penetrated into the parenchyma along with the trabecular sinuses and partly connected with the tubular sinuses, and these tubular sinuses seemed comparable to the lymph labyrinths described by He (He, 1985), which were located between the cortical capillaries and medullary sinuses and thought to be closely associated with the lymphocyte recirculation in the lymph node. The tubular sinuses developed well in the cortex, and this might be important for erythrophagocytosis and lymphocyte transport in HNs (Heath, 1987).

The population of HEV marker$^+$ HN was highest in subcutaneous region, and this may be relevant to the nearby cutaneous inflammation. It is generally considered that most leukocyte migration to cutaneous sites is regulated by the expression of E- and/or P-selectin on dermal venules (Kupper and Fuhlbrigge, 2004), but L-selectin had also been demonstrated an important role in mediating cutaneous inflammation (Tedder et al., 1995; Tang et al., 1997; Steeber et al., 1999; Nagaoka et al., 2000; Shimada et al., 2003). The multiple adhesion pathways can support leukocyte migration into cutaneous sites of inflammation, and this may affect the lymphocyte trafficking in skin-drained HNs nearby.

L-selectin$^+$ cells were similar in their populations between HNs of HEV marker$^+$ and HEV marker negative, but for CD4$^+$ cells and CD4$^+$ L-selectin$^+$ cells, the populations had significant differences between different HNs defined by HEV marker MECA-79, this may imply L-selectin was essential in lymphocyte trafficking in HNs, but more important, there may other cell adhesion molecules like integrins act in this progress, especially in HEV marker$^+$ HNs (Steeber et al., 1998). The cellular immunity of bovine HNs had been suggested in previous studies (Snider III, 2003; Zhang et al., 2012). The population of CD8$^+$ L-selectin$^+$ cells in HEV marker$^+$ HNs had significant difference with those in HEV marker negative HNs, which indicated that the coactions of L-selectin and HEV were essential for a part CD8$^+$ cells trafficking into HNs, which may suggest a high cellular immune function in those HNs with HEVs.

For the population of γδ T cells and γδ T^+ L-selectin$^+$ cells, there was no significant difference in two types of HNs defined by MECA-79. It had been reported that the expression of L-selectin alone did not predict a LN homing capacity (Walcheck and Jutila, 1994), they found that bovine γδ T cells accumulated along the vascular wall of venules that support lymphocyte extravasation into LN (MECA-79$^+$ venules) in vivo, and those γδ T cells did not appreciably extravasate from the blood into the parenchyma of LNs. From our previous studies, the population of γδ T cells in the HN was significantly higher than that of MLN, and they were distributed near the paracortex, but in this study, the MECA-79$^+$ venules was also located in the perifollicular area and interfollicular area except for the paracortical area, the pathways by which γδ T cells migrated into the HNs may be different with those in LN, furthermore, besides L-selectin, there may be other vascular of lymphocyte adhesion molecular exist in the migration of γδ T cells, which needs to be identified in further studies.

In this study, , there was no significant difference on the CD8$^+$γδ T cells between the HEV marker$^+$ HNs and HEV marker negative HNs. In primary γδ T cells, most CD8$^+$γδ T cells were found to express L-selectin and the $\alpha_4\beta_7$ integrin (Wilson et al., 1999; Wilson et al., 2002) and many adhesion moleculars and chemokines were found to play roles in the migration of CD8$^+$γδ T cells (Zabel et al., 1999; Pan et al., 2000; Wang et al., 2000), this may also support the hypothesis that the migration mechanism of lymphocytes exist in HNs.

According to the classification of Weller (Weller, 1938), the nodes we examined in the bovine is HNs, as they possess abundant blood sinusoids, without any afferent or efferent lymphatics, similar to that in buffalo and sheep (Dellman and Brown, 1976; Zidan and Pabst, 2010).

Taken together:

1) This study found the anastomoses between the capillaries and sinuses, which were taken as the pathways of some erythrocytes in HN.

2) The HN has the potential to be an antigen-capturing site and the induction site of antigen-specific humoral and cellular immunity.

3) The existence of lymphocyte homing mechanism was suggested by the HEVs in HNs, and the perifollicular area was a new site which may concern about the lymphocytes trafficking in the HNs and affect their lymphoid tissue formation.

However, further studies are needed to determine if HNs provide local immunity like MLNs, systemic immunity like the spleen, or both local and systemic immunity, and the immunological characteristics in HNs including HEV neogenesis and development, the adhesion molecular and related chemokines on the lymphocyte trafficking and their independent pathways should be discussed in further studies.

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