Angiogenesis of Buffalo Choroid Plexuses: Structural and Immunocytochemical Study

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KEY WORDS Ang-2; VEGFR-3; CD133; SEM; TEM

ABSTRACT Mammalian choroid plexuses (CPs) are vascularized structures involved in numerous exchange processes that supply nutrients and hormones to the brain, and that remove deleterious compounds and metabolites from the brain. Studies in the adult Mediterranean buffalo have investigated the morphology of CPs using histochemical and immunohistochemical techniques. To date, however, there have been no studies conducted on ruminants regarding this removal process which serves to repair functional vascular damage in the CPs. Each of these vascular repair processes is a very complex and none of these has not yet been completely understood. Then, the aim of the present study is to investigate the morphological processes during angiogenesis in the CPs of healthy adult buffaloes, utilizing transmission electron microscopy (TEM), scanning electron microscopy (SEM), and immunogold-labeling SEM analysis (biomarkers: angiopoietin-2 [Ang-2], vascular endothelial growth factor receptor-3 [VEGFR-3], and CD133). At TEM, the inner surface of the blood capillaries sometimes showed pillar-like cells, which in contact with endothelial cells formed prominences, which in turn formed neo-blood capillaries. With immunogold-labeling SEM analysis, the CP blood capillaries showed Ang-2 and VEGF-3, respectively, in positive particles and spheroid formations. In addition, the external surface of the blood capillaries showed spheroid formations that originated from the neo-vascular capillaries whose terminals formed a capillary network, positive to CD133. On the basis of these results, the following hypothesis can be made, namely, that these CPs are vascular structures which play a fundamental role in maintaining brain homeostasis and self-repairing of functional vascular damage, independently of the presence of rete mirabile in this species. *Microsc. Res. Tech. 75:1104–1112, 2012.*

The mammalian choroid plexuses (CPs) are highly vascularized structures that protrude in the two lateral ventricles, in the third ventricle and in the fourth ventricle of the brain ventricular system. The CPs consist of tufts of blood vessels (arterioles, capillaries, and venules) covered by a single continuous layer of epithelium cells. The main function of the CPs is to secrete the cerebrospinal fluid that fills the ventricular system, and then circulates around the brain and in the spinal cord, and is being reabsorbed into the blood circulation system. Furthermore, owing to the localization of the CPs between the blood compartment and the cerebrospinal fluid compartment, the CPs are involved in numerous exchange processes that supply nutrients and hormones to the brain, and remove the deleterious compounds and metabolites from the brain. Emerich et al. (2005) has shown that alterations in the anatomy and physiology of the CPs are linked to aging and several central nervous system diseases and that a fundamental role is played by the blood vessels of the CPs in bringing about these alterations.

Recent studies have indicated that the maintenance of the CP vascular system requires constant remodeling and dynamic adaptation of the blood vessels in response to the functional needs of the brain. This maintenance can be performed by various processes:

1. Angiogenesis, or the formation of neo-blood vessels by sprouting from preexisting vessels, which occurs principally in response to ischemia (Carmeliet, 2005);
2. Vasculogenesis, or the de novo differentiation of mature endothelial cells from endothelial progenitor cells (EPCs) which may derive either from bone marrow or from precursors in specific regions of the blood vessel wall (Coultas et al., 2005);
3. Arteriogenesis or the process of increasing the size of the lumen of pre-existing arterioles by modeling and growth to form collateral arteries (Fischer et al., 2006).

The above studies were all conducted on animals which did not have a rete mirabile (*Rete mirabile epidurale rostrale*), that is, on nonruminants. The rete mirabile is an arterial system found in many domestic ruminants (cattle, sheep, goat, etc.) composed of branches from one or more arteries that then converge in a single artery. In the buffalo, the rete mirabile is a mass of arterial network lying at the base of the brain on either side of the sella turcica, and extends in the cavernous sinus from the sphenooccipital junction to the foramen orbitorotundum (Bamell et al., 1975). This

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Received 18 October 2011; accepted in revised form 14 February 2012
DOI 10.1002/jemt.22037
Published online 21 March 2012 in Wiley Online Library (wileyonlinelibrary.com).

arterial network constitutes the main source of arterial blood to the brain of buffalo and regulates blood temperature principally as the blood passes through the arterial meshwork of the rete in close association with the cavernous sinus, thereby protecting the brain (Baker and Hayward, 1968; Jessen, 2001). Previous studies have investigated the morphology and microvasculature of CPs in ruminants (Scala et al., 1994), and more specifically in the Mediterranean buffalo, using ultrastructure, histochemical, and immunocytochemical techniques (Pavone et al., 2007; Scala et al., 2007, 2011; Tafuri et al., 2009). To date, however, there have been no studies conducted on ruminants regarding the above-mentioned maintenance processes which serve to repair functional vascular damage in the CPs. Each of these vascular repair processes is very complex, and none of these has yet been completely understood. The aim of the present study is to investigate the morphological processes during angiogenesis in the CPs of healthy adult buffaloes utilizing transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In addition, it is used in immunogold-labeling SEM analysis to study the morphofunctional processes during angiogenesis to mark the immunocytochemical characteristics of the blood vessels in CPs utilizing the following biomarkers: Angiopoietin-2 (Ang-2), vascular endothelial growth factor receptor-3 (VEGFR-3), and CD133.

MATERIALS AND METHODS

Animals

Thirty-six heads were collected immediately after slaughter from adult buffaloes (*Bubalus bubalis*). From buffalo brains, CPs (lateral ventricles, III and IV) were collected and processed in a different way depending on the type of microscopy to be used.

TEM

Six heads were perfused through the maxillary artery with 0.1 M cacodylate buffer, pH 7.2, and then fixed with a mixture of this buffer and 2% glutaraldehyde. After 1 h, brains were removed, and the CPs were collected. CPs were cut into minute fragments, immersed in glutaraldehyde for 1 h, rinsed in cacodylate buffer, postfixed with 2% OsO_4 for 2 h, dehydrated, and embedded in an Embed of Embed-812 resin (Electron Microscopy Supplies, Fort Washington, PA). All embedded specimens were sliced into thin sections using an ultramicrotome (Ultratome IV-LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate (Ultrastain-LKB), examined under a transmission electron microscope (Philips EM 201, Eindhoven, The Netherlands) at 40 kV, and photographed.

SEM-Vascular Corrosion Cast and Intact Tissue Technique

SEM analysis was performed on CP samples obtained by two distinct procedures: (1) vascular corrosion cast technique and (2) intact tissue technique.

1. In the case of vascular corrosion cast technique, six heads were perfused through the maxillary artery with a 0.9% physiological saline for cleaning of the vascular system. They were then injected whit a low-viscosity, colored methylmethacrylate mixture, to obtain a cast of CPs (Ohtani et al., 1982). The brains were macerated in 30% KOH solution for 1–2 weeks, changing the solution every 4–5 days. Upon complete corrosion, the casts were rinsed with bidistilled water, and dried in desiccators. CP casts from lateral, III, and IV ventricles were separated, mounted on stubs (diameter, 25 mm), and coated with gold using a sputter coater (SC500, BioRad, Hemel Hempstead, United Kingdom). All gold-coated samples were examined, and photographed under a scanning electron microscope (LEO 435 VP, Leica, Cambridge, United Kingdom) at 10 kV.
2. In the case of intact tissue technique, six heads were perfused through the maxillary artery with 0.1 M phosphate-buffered saline (PBS), pH 7.3, and then fixed with Karnovsky's solution (4% paraformaldehyde, 2.5% glutaraldehyde). After 12 h, the brains were removed, and the collected CPs were cut into small fragments (length, 0.5 cm). All fragments were immersed in a solution of 4.5% glucose in phosphate buffer, pH 7.3, for 24–48 h, dehydrated in ethyl alcohol, and dried to the critical point (CPD 030, Balzers, Liechtenstein). The specimens were mounted on stubs (diameter, 12.5 mm), coated by gold and examined under a LEO 435 VP microscope at 20 kV, and photographed.

Immunogold-Labeling SEM Analysis

For the immunogold-labeling SEM analysis, CPs were immediately removed from the brain of 18 buffaloes. Six heads were used for every antibody and their CPs were immersed in PBS for 1 h. Samples were incubated for 2 h in a solution containing normal goat serum (X 0907, Dako Italia s.p.a., Milano, Italy) diluted 1:10 in PBS, and then in the primary rabbit polyclonal antibodies: Ang-2, VEGFR-3, and CD133.

1. Ang-2, (sc-20718, Santa Cruz Biotechnology) is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to 171–240 amino acids of Ang-2 of the human origin, diluted 1:1,500 in PBS, overnight at 4°C;
2. VEGFR-3 (sc-20734, Santa Cruz Biotechnology) is an affinity-purified rabbit polyclonal antibody raised against epitope corresponding to 8–240 amino acids mapping to the N-terminus of the human vascular endothelial growth factor receptor 3, diluted 1:1,500 in PBS, overnight at 4°C;
3. CD133 (sc-30220, Santa Cruz Biotechnology) is a rabbit polyclonal antibody raised against to 508–792 amino acids mapping within an extracellular domain of human diluted 1:1,500 in PBS, overnight at 4°C.

After washing in PBS, all samples were incubated with gold-conjugated goat anti-rabbit IgG (E.M. GAR10, Agar Scientific, Stansted, United Kingdom) diluted 1:200 in PBS, for 1 h at room temperature. The secondary antibody was conjugated with gold particles of different sizes, namely 5 and 15 nm. After washings in PBS, samples were fixed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing $CaCl_2$, pH 7.2, for 30 min. After the fixation step and washings with dis-

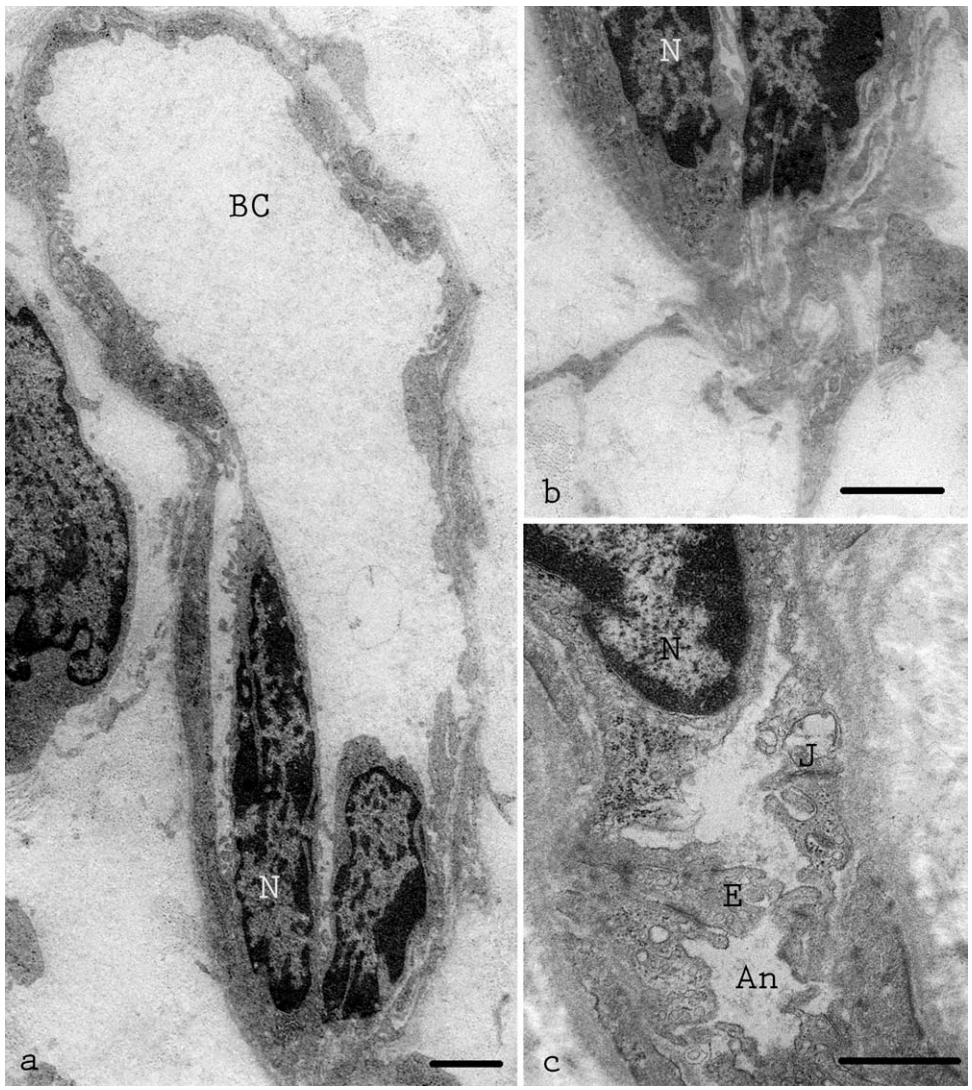

Fig. 1. TEM buffalo choroid plexus. **a**: Transverse section of the blood capillary. **b**: Beginning of the vascular neo-formation (angiogenesis). **c**: Angiogenesis. An, Angiogenesis. BC, Blood capillary. E, Cytoplasmic extension. J, Cytoplasmic junction. N, Nucleus of the EPCs. Scale bar, 1 μm.

tilled water, samples were subjected to silver enhancement process (British BioCell International, Cardiff, Wales, United Kingdom). The enhancement process enables the use of antibodies conjugated to smaller (1–5 nm) gold particles, preserving the advantage of faster penetration and higher labeling efficiency (Owen et al., 2002). Next, samples were dehydrated through an ethanol series, and dried to the critical point. The specimens, mounted on stubs, were examined under a LEO 435 VP scanning electron microscope at variable pressure (80–120 Pa) in the backscattered electron mode, which allows detecting gold particles associated to cells even if they are intracellular located (Richards et al., 2001). Samples were not been coated by gold, so that SEM observed only the conjugated gold deriving from immunocytochemical reaction, and photographed. No immunoreactivity was observed in CP samples treated with PBS substituting the primary antibody (negative control).

RESULTS

TEM

The inner surface of the blood capillaries sometimes showed pillar-like cells in contact with the endothelial cells. These pillar-like cells presented many cytoplasmic processes on their surfaces, and had a high nuclear–cytoplasmic ratio. Their nuclear chromatin was highly electron-dense, and located around the periphery of the nucleus. Their cytoplasm formed a thin strip that surrounded an elongated and irregular nucleus, and its basal portion was adjacent to the endothelial cells and showed the profiles of many free ribosomes.

In addition, the contact between the pillar-like cells and the endothelial cells formed prominences in the blood capillary wall. The endothelial cells in these prominences formed neo-blood capillaries, which had many extensions of the cellular surface, and junctions between the cytoplasmic extensions of endothelial cells

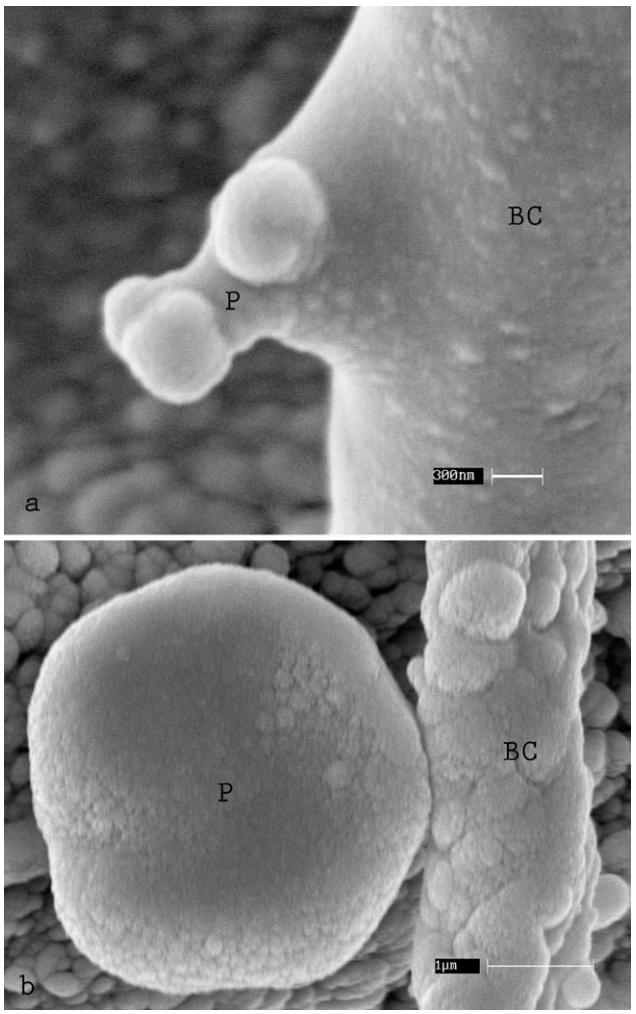

Fig. 2. SEM-intact tissue of the buffalo choroid plexus. The samples were subjected to ultrasonic treatment of 15′. **a**: Beginning of the vascular neo-formation (prominence). **b**: Spheroid form of the advanced prominence. BC, Blood capillary. P, Vascular prominence of the blood capillary.

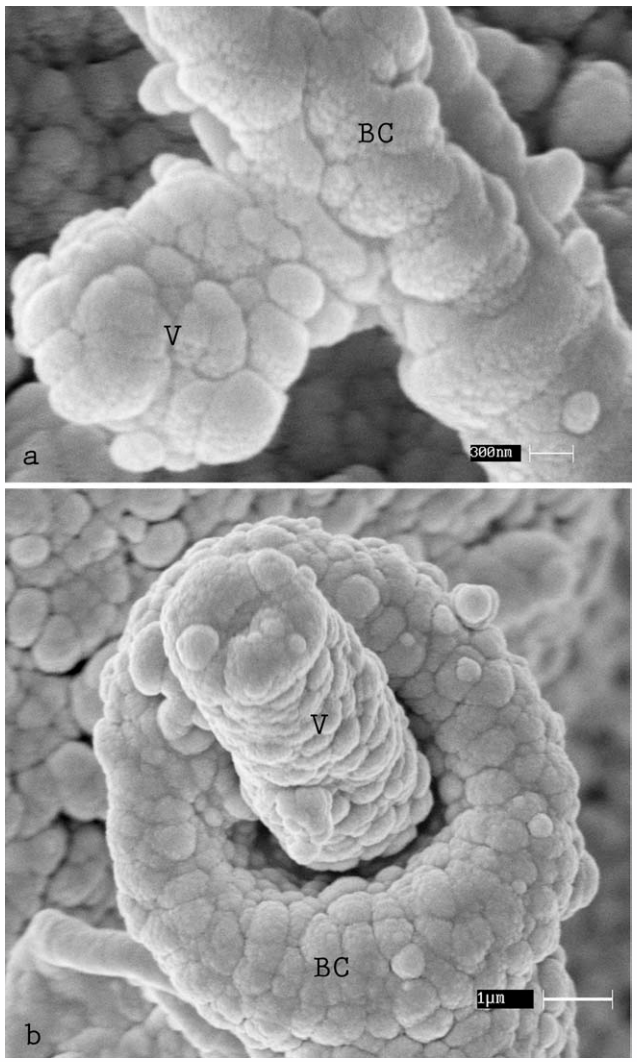

Fig. 3. SEM-intact tissue of the buffalo choroid plexus. The samples were subjected to ultrasonic treatment of 15′. **a**: Beginning of the blood capillary branch. **b**: Extension of the blood capillary. BC, Blood capillary. V, Neo-blood vessel.

(Fig. 1c). The formation of neo-blood capillaries from these prominences is the evidence of angiogenesis taking place.

SEM-Intact Tissue Technique

The scanning electron micrographs of the CP samples that were subjected to ultrasonic treatment (15 min) showed the presence of small spherical prominences (approx. diameter, 500–800 nm) on the external surface of the blood capillaries. When these prominences expand, they produce spheroid prominences on the surface of the blood capillary, which, in turn, become neo-blood capillaries (Fig. 2). The sequence of the pictures in Figures 3a and b shows a morphological expression of angiogenesis which may be termed either "nonsprouting" angiogenesis, or "intussusceptions" angiogenesis.

SEM-Vascular Corrosion Cast

The surface of the vascular casts of the CPs presented details of two stages of the morphological change in blood capillaries during the formation of the neo-blood capillaries:

1. The beginning of angiogenesis from pre-existent blood capillaries;
2. The advanced angiogenesis in the pre-existent blood capillaries, and in neo-blood capillaries (Fig. 4).

Immunogold-Labeling SEM Analysis

1. The external wall of the CP blood capillaries showed Ang-2-positive particles uniformly distributed (Fig. 5a).
2. The external wall of the CP blood capillaries showed VEGFR-3-positive spheroid formations (approx. diameter, 8–15 μm) (Fig. 5b).

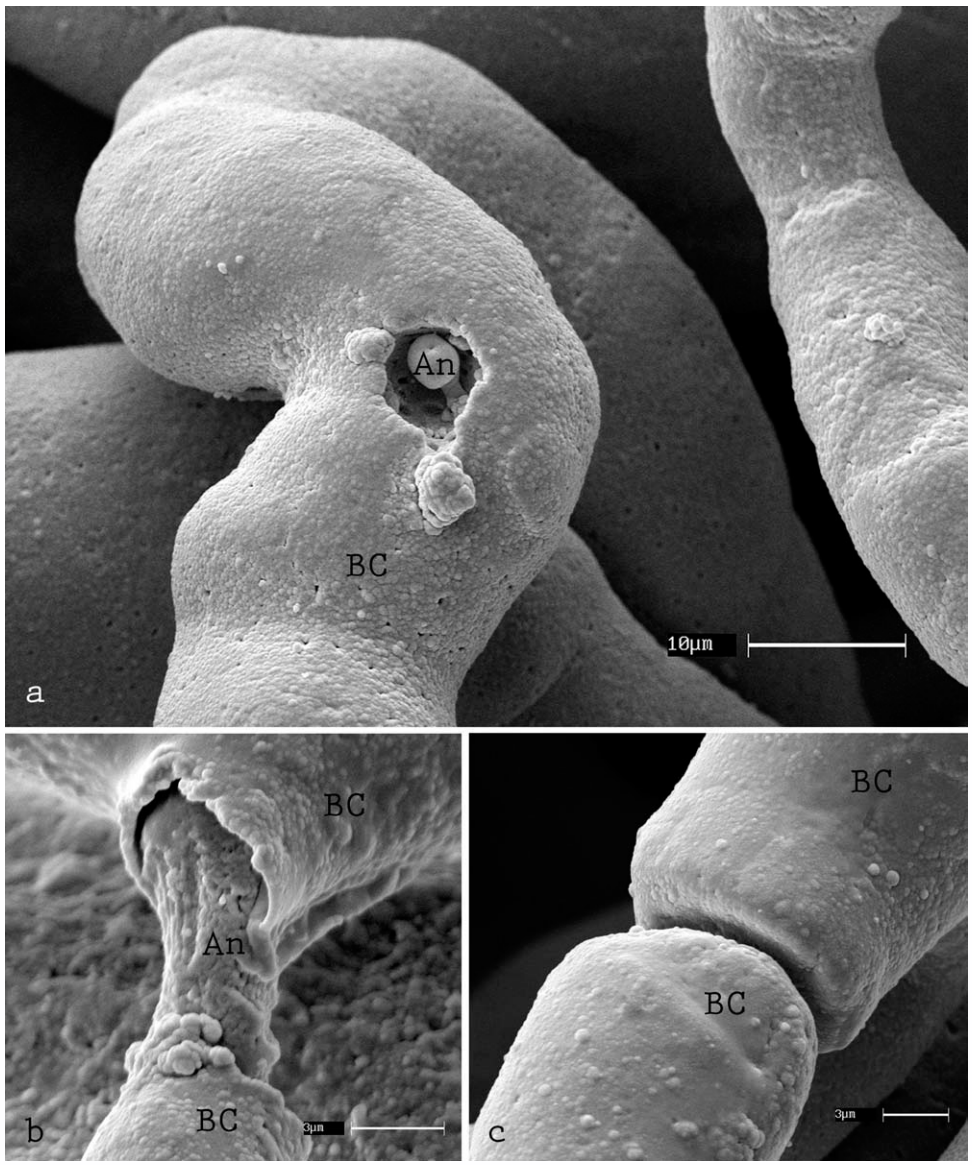

Fig. 4. SEM-vascular corrosion cast of the buffalo choroid plexus. **a**: Beginning of the angiogenesis. **b**: Hooking of the neo-blood vessel to blood capillary. **c**: Hooking between two new blood capillaries. An, Angiogenesis. BC, Blood capillary.

3. The inner surface of the CP blood capillaries showed a single CD133-immunopositive ovoid cellular formation adhering to the capillary wall. This formation had an elongated small cavity which extended outside of the blood capillary (Fig. 6a). Some cells of the formation emitted long cytoplasmic extensions that embraced a neo-capillary bed (Fig. 6b). These extensions were not far from the blood capillary, and they had many cellular extensions, which hooked to the blood capillary (Figs. 7a and b). In addition, these cellular extensions were also evident on the capillary extremities of the CP samples observed at SEM-intact tissue and at immunogold SEM-labeling analysis (Figs. 8a and 8b). A vast area of the external surface of the blood capillaries showed many spheroid formations (approx. diameter, 30–40 μm) which were hooked to the blood capillaries by short peduncles (Figs. 9a and b). These formations originated from the neo-vascular capillaries that ended in forming a capillary network, which was positive to CD133 (Fig. 10).

DISCUSSION

On the basis of the observations made in the present study of angiogenesis in the CPs of buffaloes, the following hypothesis can be made: buffalo CPs are vascular structures which play a fundamental role in maintaining brain homeostasis as the blood capillaries of these CPs have the capacity to ameliorate vital functions, and/or the self-repairing of functional vascular

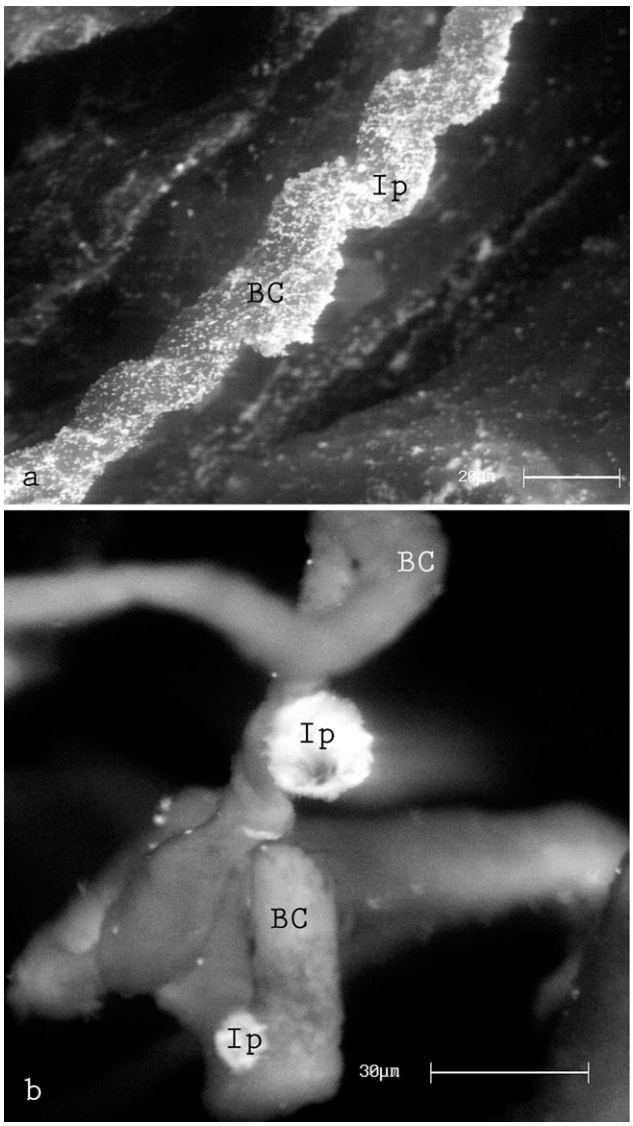

Fig. 5. Immunogold-labeling SEM analysis of the buffalo choroid plexus. **a**: Expression of Ang-2. **b**: Expression of VEFGR-3. BC, Blood capillary. Ip, Immunopositivity.

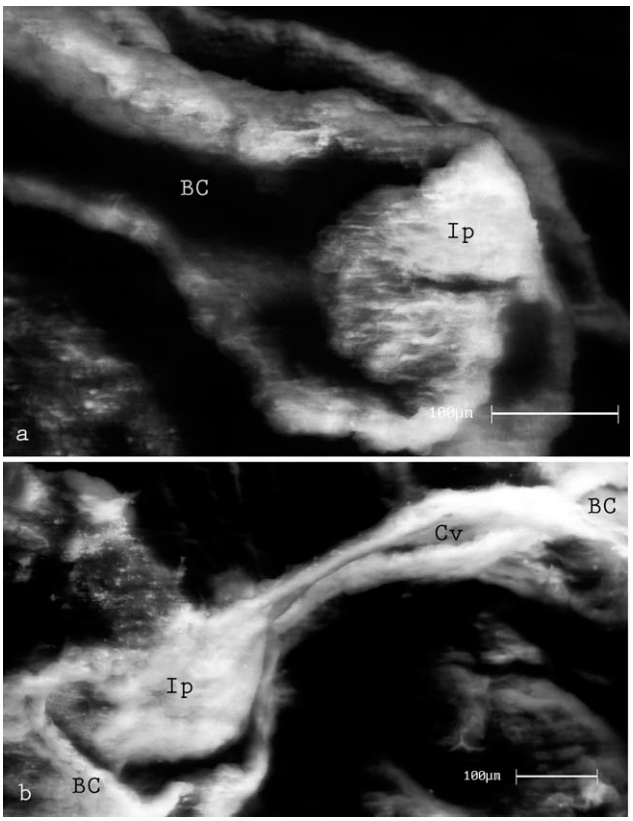

Fig. 6. Immunogold-labeling SEM analysis of the buffalo choroid plexus. Expression of CD133. **a**: Ovoid positive formation, adherent to inner wall of the blood capillary. **b**: Extension of positive formation directed to blood capillary with evident a small cavity. BC, Blood capillary. Cv, Vascular cavity of the neo-blood vessel. Ip, Immunopositivity.

damage. This capacity is independent of the presence of the rete mirabile (Lluch et al., 1985). This hypothesis is consistent with the following results found in the previous studies of angiogenesis in humans and animals:

1. The pillar-like cells that adhere to the endothelial cells of the CP blood capillaries may be the immature EPCs, observed by Rae et al. (2011) which derive from the hematopoietic lineage displaying endothelial-like cell properties. More specifically, these pillar-like cells may be the putative true EPCs of the endothelial lineage presumably derived from circulating precursor in vivo. Previous studies (Assmus et al., 2002; Kocher et al., 2001; Walter et al., 2002) in animals and humans have suggested that the capacity of EPCs to ameliorate the functioning of ischemic organs possibly takes place by means of both the induction of angiogenesis in areas where oxygen supply is reduced, and the stimulation of the re-endothelialization of injured vessels. As a consequence, it can be tentatively affirmed that the angiogenesis capacity of the blood vessels serves to maintain tissue homeostasis in response to perturbations. In fact, EPCs have been proposed as a new form of cell therapy for damaged organs (Becher et al., 2010).
2. The angiogenesis capacity of the blood vessels of the buffalo CP has been demonstrated by our results obtained with immunogold-labeling in SEM of Ang-2, VEGFR-3, and CD133.

The presence of positive particles to Ang-2 in the blood capillaries of the CPs demonstrates the presence of a protein growth factor which promotes angiogenesis. Other recent studies have also observed the presence of Ang-2 at sites where angiogenesis takes place. Maisonpierre et al. (1997) observed the presence of Ang-2 in adult tissue at, and only at, sites where vascular remodeling takes place. A more recent study by Zadeh et al. (2010) identified Ang-2 as a marker of tumor cell invasion in high-grade astrocytomas, and an

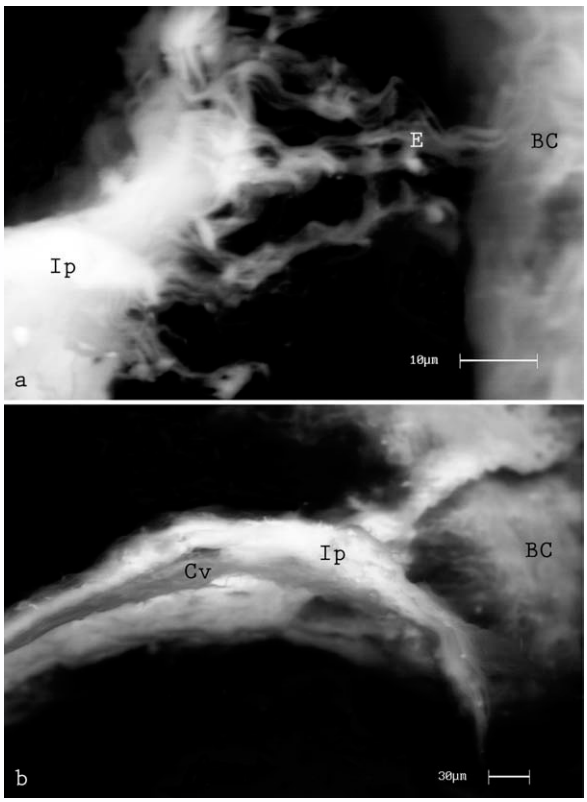

Fig. 7. Immunogold-labeling SEM analysis of the buffalo choroid plexus. Expression of CD133. **a**: Cytoplasmic extension of the angiogenesis. **b**: Hooking of the cytoplasmic extension of the angiogenesis to blood capillary. BC, Blood capillary. Cv, Vascular cavity of the neoblood vessel. E, Cytoplasmic extension. Ip, Immunopositivity.

earlier study by Srirajaskanthan et al. (2009) both confirm the role of Ang-2 in both physiological and pathological angiogenesis.

Our results regarding the intense immunoreactivity anti-VEGFR-3 expressed in the blood vessels of the buffalo CPs demonstrate that this receptor plays a role in adult blood vessel angiogenesis. These results are consistent with those of the previous studies (Tammela et al., 2005a; Witmer et al., 2001).

Our results regarding the presence of the CD133 immunopositive ovoid cellular formations in the CPs demonstrate the presence of pluripotent stems cells in the EPCs. As a consequence, it may be affirmed that the cells that compose the EPCs are stem cells. Yamahara and Itoh (2009) have shown that the EPCs are similar to stem cells in that they too are found in many different types of tissues as well as in the general blood circulation. In addition, these researchers have found that EPCs are endowed with the ability to actively find and adhere to damaged endothelial surfaces, and to differentiate into endothelial cells to reconstitute vascular integrity. This reconstitution process is especially important for the repair of damaged blood vessels of the CPs. In animal models of ischemia, Arbab et al. (2006) observed that EPCs were embedded into the sides of active angiogenesis.

3. The present study has shown that vascular angiogenesis in buffalo CPs is a nonsprouting, or intussusceptions type of angiogenesis (IA). This type of angiogenesis consists in the insertion of transcapillary tissue pillars into the vascular lumen, with the subsequent growth of these pillars in the vascular lumen resulting in a division of blood vessels (Djonov et al., 2000; Rossi-Schneider et al., 2010). This morphofunctional process can be clearly visual-

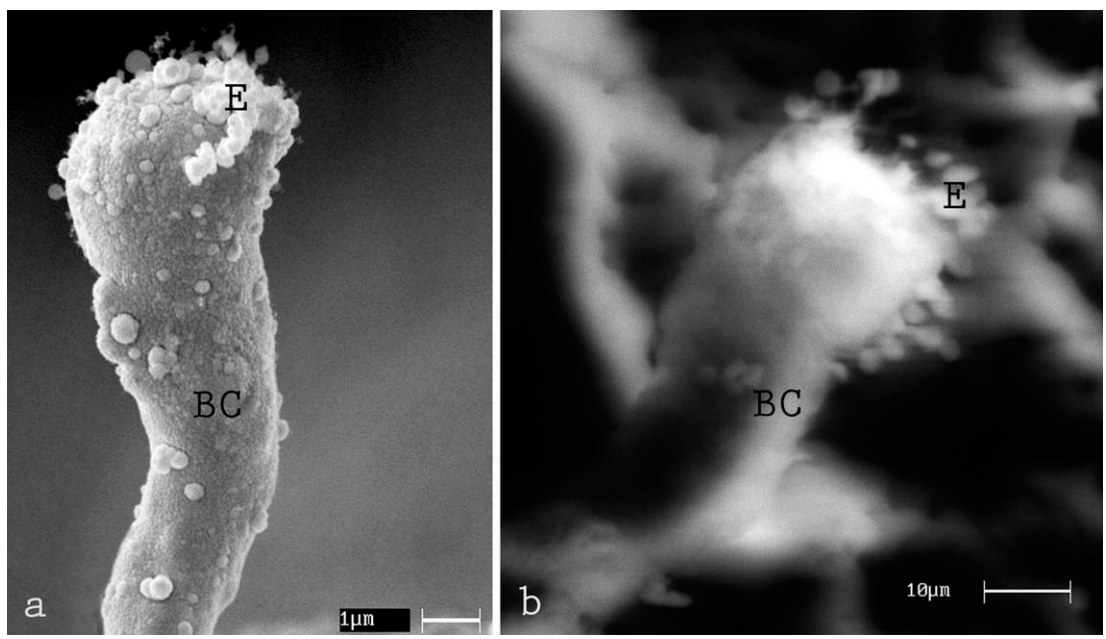

Fig. 8. SEM blood capillary of the buffalo choroid plexus. **a**: SEM-intact tissue blood capillary. **b**: Immunogold-labeling SEM analysis of the blood capillary. BC, Blood capillary. E, Cytoplasmic extension.

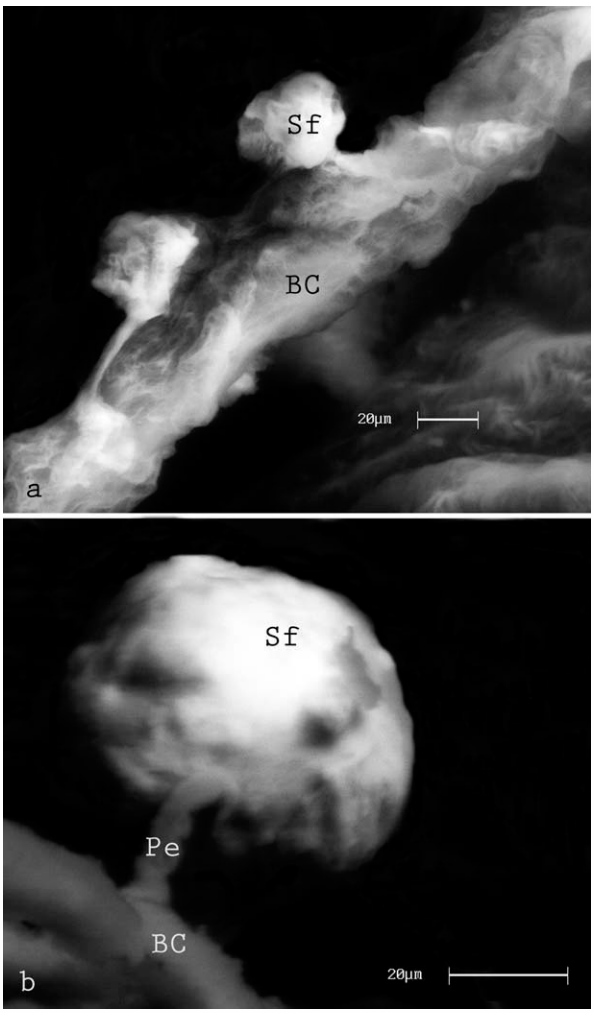

Fig. 9. Immunogold-labeling SEM analysis of the buffalo choroid plexus. **a**: Spheroid formations on the external surface of the blood capillary. **b**: Particular of the spheroid formation. BC, Blood capillary. Pe, Peduncle. Sf, Spheroid formation.

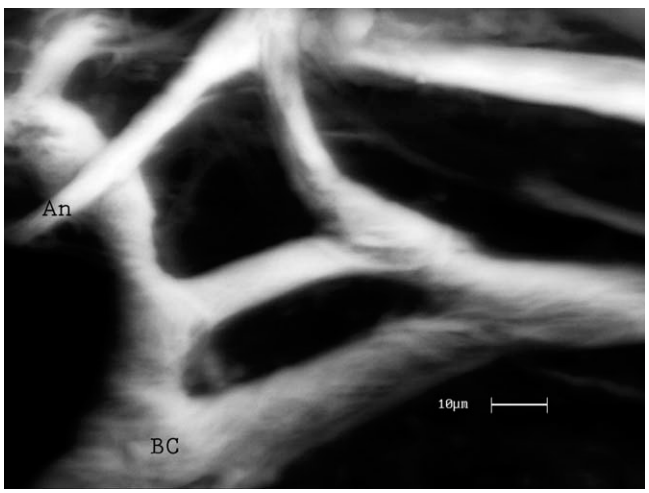

Fig. 10. Immunogold-labeling SEM analysis of the buffalo choroid plexus. Network of the neo-blood capillary immunopositive to CD133. An, Angiogenesis. BC, Blood capillary.

ized by TEM, SEM-vascular corrosion cast, and intact tissue techniques, and by immunogold-labeling SEM analysis.

Further confirmation of the above hypothesis, given the omnipresent nature of stem cells, would involve the finding of EPCs in organs other than the one studied here.

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