# Original Article Effect of mixed endothelin receptor antagonist bosentan on brain damage after experimental cardiac arrest

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**Abstract:** We investigated the effect of dual endothelin receptor (ETR) antagonist-bosentan on post ischemic ultrastructural abnormalities and endothelin binding sites in the brain after 10 min cardiac arrest in rats. After seven days, CA1 sector of the hippocampus and cortical neurons in bosentan-treated animals exhibited less severe morphological abnormalities compared with non-treated rats. Although the number of damaged neurons was not substantially modified after bosentan application, cell organelles were well preserved. The beneficial effect of bosentan on neuronal cells was accompanied by a reduced swelling of astrocytes and the intensification of angiogenesis, with endothelial cells (EC) hypertrophy and the presence of young capillaries. Centrioles in dividing endothelial cells were also seen. Bosentan treatment resulted in lowered levels of <sup>125</sup>I-endothelin-1 maximum density of receptors ( $B_{max}$ ) within the brain. The results indicate that bosentan, through endothelin receptor modulation, may act towards salvage of organelles in neurons, while ameliorating astrocytic and vascular changes in the vulnerable brain regions after cardiac arrest.

Keywords: Cardiac arrest, endothelin receptor antagonist, electron microscopy, neurons, astrocytes, microvessels

#### Introduction

Cardiac arrest (CA) produces global cerebral ischemia due to a complete cessation of blood supply into the brain, which can however be restored with resuscitation [1]. While the global ischemia produces well known pattern of cell death observed under light microscope, the underlying mode of cell death (apoptotic or necrotic) remains debatable [2]. However under the electron microscope, numerous subtle changes have been revealed post ischemia, what poses a question as to whether ultrastructural assessment is necessary for in depth insight on cell injury and evaluation of brain protective interventions [3].

Cell organelle stress can be induced by endothelins after brain ischemia, for their vasoconstrictive and proinflammatory actions [4, 5]. However, it is unknown how endothelin system in the brain contributes to organelle damage in neurons after cerebral ischemia. Therefore, we wanted to conduct electron microscopic investigations of the brain in order to determine the impact of endothelin antagonism on neuronal organelle damage after cardiac arrest. We also evaluated its effects on microvascular and astrocytic brain compartments post ischemia. We carried out experimental cardiac arrest with or without administering mixed ETA and ETB endothelin receptor antagonist bosentan in rats [6], while hypothesizing that the blockade of the receptors would largely ameliorate organelle damage detected under electron microscope. To verify the interaction of endothelin antagonist with endothelin receptors we investigated the impact of bosentan on endothelin binding sites in the brain.

#### Materials and methods

#### Experimental design

The experiments were performed on female Wistar rats with body weight range between 180 and 250 g, divided into untreated groups and the groups treated daily with bosentan. They were sacrificed without CA, or at 3 min, 10 min, 1 h, 24 hrs and 7 days after CA for endothelin binding studies (n=5). Seven days after cardiac arrest the rats were sacrificed for EM studies (n=4).

The animals were housed at 22-24°C with free access to food and water. Cardiac arrest was carried out according to previously published protocols [7, 8]. Fasting was administered 12 hrs prior to surgery. Experiments involving laboratory animals complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institute of Health Publications no. 85-23, revised 2010) and were carried out upon the consent issued by the Polish Local Ethic Council for Medical Research.

# Surgical procedures

The induction of cardiocirculatory arrest in anesthetized rats was conducted according to previously published protocols [1]. In order to induce cardiac arrest a hook-like device was inserted into the chest in the right parasternal line at the third intercostal space and pulled up from the level of vertebral column in order to compress the heart vessel bundle onto the ribcage [1, 7]. The compression was maintained for 3.5 min. on average, until isoelectric line was reached. Then the cardiorespiratory arrest was continued for a total duration of 10 minutes. Rats in the sham operation group received the intubation and insertion of hook device into thoracic cavity, without causing heart bundle occlusion. Then external heart compressions were started at a rate 300/min, along with the mechanical ventilation of rats. until they regained spontaneous heart rate and breathing. Electrocardiogram from I, II, III, aVL, aVR, and aVF leads was monitored with ECG apparatus Ascard A4 (Aspel) for continuous evaluation of heart function during the resuscitation and recovery period [9]. Arterial blood pressure from the femoral artery catheter was continuously monitored by means of pressure transducer (UFI) and BP monitor (50115 Stoelting).

# Endothelin antagonist treatment

Dual endothelin receptor antagonist bosentan was generously provided by Dr. Martine Clozel from Actelion Pharmaceutical (Switzerland). Bosentan, suspended in 5% Arabic gum/PBS with the use of Polytron homogenizer was administered by gavage 2 hrs before cardiac arrest and once daily for a total of 7 days [7]. The administered dose was 100 mg/kg b.w., as recommended by the manufacturer.

# Electron microscopy procedures

On the 7<sup>th</sup> day of the experiment, the frontal cerebral cortex and the hippocampus were collected for electron microscopic studies. All animals were anesthetized using Nembutal (80 mg/kg b.w., i.p.) and perfused by the ascending aorta, initially applying 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer pH 7.4 (PBS), and afterwards perfusing with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Following the perfusion, brains were collected from skulls and immersed for 2 h in the same fixative agent. The specimens were post-fixed in  $1\% (w/v) OsO_{1}$ solution in deionized water, dehydrated in an ethanol gradient, and encased in the epoxy resin (Epon 812). Ultrathin (60 nm) sections were prepared as described earlier [10]. Material was examined in a transmission electron microscope (JEM-1200EX, Jeol, Japan).

In order to perform the analysis of each selected feature of ultrastructural injury, 15-20 random electron micrographs were taken per hippocampus or cerebral cortex, from all animals in untreated and treated groups. The number of morphological structures with incorrect ultrastructural features was counted and expressed per total number of these structures contained in analyzed photographs. Percentage of structures exhibiting features of injury was classed as follows: ++++,  $\geq 50\%$ ; +++, < 50% and  $\geq 25\%$ ; ++, <25% and ≥10%; +, <10% and >0%; -, none. We evaluated the following components: neuron, neurotubules, neuronal mitochondria, Golgi apparatus, nucleus, nuclear envelope; neuropil, astrocyte, perivascular space, endothelial cells, interendothelial junctions, and microvessels. In total one thousand electron micrographs were analyzed in all groups at the magnification of ×2500-×7500.

# Preparation of membrane fractions

The anesthetized animals were euthanized, skulls were opened and fragments of the hippocampus (H), cortex (C) and medulla (M) were dissected out, immediately weighted, snap fro-



**Figure 1.** Neurons of the hippocampal CA1 region after cardiac arrest. A. Fragments of neurons with moderate fragmentation of neurotubules and swollen mitochondria with remnants of cristae. B. Total loss of cristae is seen in mitochondria. C. Segmental distention of cisterns within the Golgi apparatus. D. Obliterated structure of nuclear envelope with invaginations and numerous nuclear pores (arrows). E. Electron lucidity (rarefaction) of neuronal structure. F. Swelling of the neuropil elements.

zen in liquid nitrogen and stored at -80°C until receptor binding assays in membrane fractions. To obtain membrane fractions, brain samples were homogenized with Polytron homogenizer in 10 ml of cold Tris buffer and centrifugated at 40 000×g for 20 min. This step was repeated twice. The final pellet was resuspended in 10 ml of buffer [11].

#### Endothelin receptor binding assay

The competitive receptor binding experiments were run on 0.5 ml membrane suspensions

and with I-125-endothelin-1 diluted with unlabeled compounds (Peninsula) to the concentrations from 0.1 to 300 nM. For each brain structure, the total counts, unspecific binding, the total binding and the binding in 6 sequential dilutions were measured. After 2 h incubation at room temperature using Cell Harvester apparatus (Brandel), individual samples were filtered through GF/C glass filters (Whatman), and after cold buffer rinsing the filters were placed in tubes for radioactivity measurements. The radioactivity of I-125 was measured by means of gamma scintillation counter 1470 Wizard



**Figure 2.** Astroglial and microvascular changes of the CA1 sector after cardiac arrest. A. Astrocyte (a) with swollen cytoplasm, adjacent to neuron. B. Mild swelling of perivascular astrocytic processes. C. Altered ultrastructure of the intercellular junction and swelling of the perivascular region. D. Increased number of pinocytic vesicles (arrows) in microvascular endothelial cells. E. The presence of microvilli (arrows) on endothelial cells and perivascular bundles of gliofibrils (gf). F. Lucent perivascular space partly occupied by an astrocyte showing the accumulation of gliofibrils.

(Wallac). Values of  $B_{max}$  and  $K_{D}$  were individually calculated for each brain structure-H, C and M by nonlinear regression using the computer PRISM program from Graph Pad (San Diego, CA). The results were calculated per mg of wet tissue [11].

#### Data analysis

Data were expressed as means  $\pm$  SEM. Statistical analysis of differences between experimental groups was performed by means of one-way ANOVA followed by Tukey's test. A probability value of *P*<0.05 was considered statistically significant.

#### Results

Ultrastructural alterations in the CA1 sector of the hippocampus after cardiac arrest

In the hippocampal CA1 sector of untreated rats the neuronal compartment exhibited several major alterations. Seven days after cardiac arrest, disrupted neurotubules could be frequently seen. Only occasionally well preserved neurotubules were present (**Figure 1A**). In CA1 neurons, damage to mitochondria comprised the loss of mitochondrial cristae (**Figure 1B**). Conspicuously present was the swelling of Golgi zone structures (**Figure 1C**). Neuronal nuclei

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**Figure 3.** CA1 neurons and neuropil after cardiac arrest in bosentan-treated rats. A. Normal shape of neurotubules, however corrugated nucleus is present in the neuron. B. Preserved mitochondria (m) in neuronal cell. C. The Golgi apparatus in neurons with minute distention. D. Invagination (arrow) of the nuclear envelope. E. Neuron showing sparse mitochondrial alterations and the enlargement of Golgi apparatus cisterns. F. Neuropil without signs of swelling.

exhibited numerous nuclear pores and invaginations of the nuclear envelope (Figure 1D). Electron lucent neurons in this area exhibited rarefaction of their structure, invaginations of nuclei as well as the altered morphology of mitochondria and Golgi apparatus (Figure 1E). Marked swelling was seen in the brain neuropil (Figure 1F). Also astrocytes with electronlucent, swollen cytoplasm were frequently seen in the vicinity of neurons (Figure 2A). Perivascular regions of microcapillaries were occupied by swollen astrocytic processes (Figure 2B). Endothelial cells (EC) of microcapillaries displayed several abnormalities of their ultrastructure. Widened interendothelial junctions showed blurred contours (**Figure 2C**). Endothelial cells exhibited numerous pinocytic vesicles (**Figure 2D**) and microvilli (**Figure 2E**). Gliofibrils were abundantly deposited in astrocytic processes surrounding capillaries in this brain region (**Figure 2E**, **2F**).

The effect of bosentan on ultrastructural alterations in CA1 sector of the hippocampus after cardiac arrest

In bosentan-treated rats, neurons of CA1 showed lessened disintegration of neurotubules



**Figure 4.** Astroglial and vascular elements in the CA1 after cardiac arrest in bosentan-treated rats. A. Astrocyte exhibiting condensed cytoplasm with gliofibrillar bundles. B. Absence of perivascular swelling. C. Intercellular junction (arrow) shows normal structure. D. Moderate number of pinocytic vesicles (arrow) in endothelial cells. E. Smooth surface of endothelial cells with absence of projections. F. Morphological features of the immature capillary vessel.

(Figure 3A) and the mitochondria were better preserved (Figure 3B). The morphology of Golgi apparatus showed smaller distention (Figure 3C). However, the invaginations of nuclear envelope were frequently seen although delaminating within nucleolemma was present only occasionally (Figure 3D). Neurons showed sparse irregularities of mitochondrial shape and a mild segmental distention of the Golgi apparatus cisterns (Figure 3E). The neuropil was dense, without signs of swelling and occasionally contained cells with dark, condensed structure (Figure 3F). Astrocytes frequently displayed condensed cytoplasm with well-preserved organelles (Figure 4A). Similarly, cellular processes surrounding vascular capillaries were not swollen (**Figure 4B**). Mirocapillaries in this group included properly formed interendothelial junctions (**Figure 4C**), the limited number of pinocytic vesicles localized in endothelial cells, (**Figure 4D**) and the absence of microvilli in vascular profiles (**Figure 4E**). However, the capillaries with features of immature vessels were frequently seen in the hippocampus (**Figure 4F**).

# The effect of cardiac arrest on cerebral cortex ultrastructure

The cortical neurons 7 days after cardiac arrest also exhibited fragmentation of neurotubules.

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**Figure 5.** Neurons and neuropil of the cerebral cortex after cardiac arrest. A. Cortical neuron with fragmented neurotubules (arrow). B. Neuronal mitochondria (m) showing depletion of cristae and bizarre morphology. C. Neuron presenting with distentions of cisterns within the Golgi apparatus (ga). D. Delamination of nuclear envelope (arrows). E. Neuron with morphological features of necrosis. F. Swelling of the neuronal and astroglial processes in the neuropil.

Compartmentalization of reticular cisterns, loose aggregates of ribosomes and detached polysomes were present as well (Figure 5A). Mitochondria were often swollen, with a depletion of cristae and irregularities of their shape (Figure 5B). Similar to what was found in CA1, dilated Golgi apparatus cisterns were often seen in cortical neurons (Figure 5C). Delaminating of the nuclear envelope was present. The increased abundance of nuclear pores could be also observed (Figure 5D). In subsets of neurons, nuclei with peripheral condensation of the chromatin, lyzosomes and small electron dense granules (lipofuscin) were noted (Figure 5E). The neuropil exhibited swollen processes, occasionally with large numbers of presynaptic vesicles (Figure 5F). Astrocytes were often mildly swollen, although their organelles showed normal appearance (Figure 6A). Microcapillary vessels were surrounded by a rim of swollen processes (Figure 6B). Interendothelial junctions showed several abnormalities, including elongation of the junction, widening of the cleft and blurring of its structure (Figure 6C). Numerous pinocytic vesicles were seen within endothelial cells (Figure 6D). Most of vascular profiles in the cortex showed microvilli on the surface of endothelial cells as well as tight junction abnormalities (Figure 6E, 6F).



**Figure 6.** Astroglial and vascular elements of the cerebral cortex after cardiac arrest. A. Mildly swollen astrocyte (a). B. Microcapillary vessel (v) with ultrastructural signs of perivascular swelling. C. Elongated and widened tight junction cleft between endothelial cells. D. Endothelial cells with increased numbers of pinocytic vesicles (arrows). E. Presence of microvilli (arrows) on endothelial cells of microvessels. F. Microvessel shows microvilli and widening of interendothelial junctions (arrow).

### The effect of bosentan on ultrastructural alterations in rat cerebral cortex after cardiac arrest

In bosentan-treated rats, the long neurotubules of cortical neurons were better preserved as compared to untreated animals after cardiac arrest (**Figure 7A**). Mitochondria of cortical neurons showed normal appearance (**Figure 7B**). The Golgi apparatus was less extended and revealed nearly normal ultrastructure. In addition, signs of vesicle transport to trans-Golgi network were visible (Figure 7C). Invaginations of nuclei were often seen, although not accompanied by delaminations of nuclear envelope. Nuclear pores were less numerous than in the group without treatment (Figure 7D). Neurons showed a reduced ribosomal detachment from endoplasmic reticulum and reduced distention of cis-Golgi stacks (Figure 7E). The neuropil appeared dense, with no signs of swelling (Figure 7F). Normal astrocytes in the cerebral cortex of treated rats contained preserved mitochondria and, occasionally, a mildly dis-



**Figure 7.** Neurons and the neuropil in the cerebral cortex after cardiac arrest in bosentan-treated group. A. Improved preservation of microtubules (arrow) in the cortical neuron. (original mag. ×6000). B. Mitochondria (m) in neurons display a regular shape and preserved cristae. C. Golgi apparatus (ga) and signs of vesicle transport to trans Golgi network in the neuron. D. Nucleus with invagination (arrow) of the nuclear envelope. E. Neuron showing nuclear invaginations and well preserved organelles. F. Fragment of the neuropil without morphological features of swelling.

tended Golgi apparatus (Figure 8A). The structures of perivascular region showed electron dark appearance (Figure 8B). As compared to the untreated group, interendothelial junctions were less elongated and possessed well defined morphology, with narrow clefts (Figure 8C). Only a modest abundance of pinocytic vesicles was found in microvascular endothelial cells after treatment with bosentan (Figure 8D). Single projections were occasionally seen on the endothelial surface (Figure 8E). However, numerous microcapillaries contained hypertrophic endothelial cells. Centrioles in the cytoplasm of endothelial cells could be found. Vascular profiles exhibited the shape of newly formed vascular lumen (**Figure 8F**).

#### Analysis of ultrastructural features in the hippocampus and cerebral cortex

Semiquantitative assessment of ultrastructural alterations revealed that the number of dark or dead neurons in CA1 and cerebral cortex did not differ substantially between bosentantreated and untreated groups (**Table 1**) after cardiac arrest. In neurons of CA1 and cerebral cortex, bosentan reduced abnormalities of



**Figure 8.** Astroglial cells and microvessels in the cerebral cortex after cardiac arrest in the bosentan-treated group. A. Normal appearance of the astrocyte. B. Perimicrovascular space without features of swelling. C. Interendothelial junction shows a tight cleft and correct structure. D. Sparsely seen pinocytic vesicles. E. Sparse microvilli on the endothelial surface. F. Microvessel with characteristics of a young capillary. Hypertrophic endothelial cell with centriole (c) in the cytoplasm.

neurotubules, mitochondria and Golgi apparatus. Nuclear envelope delaminations were reduced with the treatment as well.

In the brain microvascular compartment, bosentan reduced abnormalities within interendothelial junctions, decreased the abundance of pinocytic vesicles and the number of microvilli after cardiac arrest. Bosentan also reduced the perivascular swelling and the number of swollen astrocytes. However, it enhanced invaginations of the nuclear envelope. In the group treated with bosentan, the number of vascular profiles showing features of angiogenesis was greater as compared to the untreated rats.

Bosentan effect on endothelin binding sites in the brain post cardiac arrest

**Figures 9** and **10** show the alterations of maximum density ( $B_{max}$ ) and equilibrium dissociation constant ( $K_D$ ) of ET-1 binding sites in the hippocampus (H), cerebral cortex (C) and medulla (M), preischemically and during recovery from 10-min cardiac arrest in the animals untreated and treated with bosentan.

Table 1. Semiquantitative assessment of ultrast	ructural features
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	CA1 un-	CA1 w/	Cortex	Cortex w/
	treated	bosentan	untreated	bosentan
Disrupted neurotubules	++++	+++	++++	+++
Mitochondrial abnormalities	++++	++	++++	++
Golgi apparatus distention	++++	++	++++	++
Nuclear invaginations	++++	++++	++	++++
Nuclear envelope delamination	++++	+	++++	++
Multiple nuclear pores	++++	+++	+++	++
Dark/necrotic neurons	+++	+++	+++	+++
Neuropil swelling/degeneration	++++	++	++++	+++
Swollen astrocytes	+++	+	++++	++
Perivascular swelling	++++	++	++++	++
Disturbed EC junctions	++++	+++	++++	++
Multiple pinocytic vesicles	++++	+++	++++	+++
Abundant microvilli	++++	+++	++++	+++
Angiogenic features	+	+++	+	+++



**Figure 9.** Regional changes of the maximum density of receptors ( $B_{max}$ ) for endothelin-1 receptors in the rat brain after cardiac arrest. \*P<0.05 versus prearrest; #P<0.05 versus untreated with bosentan.

In untreated animals,  $B_{max}$  of endothelin receptors in the hippocampus, cerebral cortex and medulla decreased until 24 hrs after cardiac arrest (CA) as compared to pre-arrest levels (**Figure 9**). The lowest values in the hippocampus were noted after 1 h of reperfusion, while in the cortex after 24 hrs-13.3% and 31% of pre-arrest levels, respectively. Also at 1 h, 14.9% of pre-arrest level was noted in the medulla. One week after CA in untreated animals, the maximal density values reached 82.6% of pre-arrest level in the hippocampus, 74.7%

in the cerebral cortex, and 99.5% in the medulla. In the group with bosentan, hippocampus B<sub>max</sub> was lower as compared to untreated rats-prior to cardiac arrest, then 3 minutes and 7 days after ischemia. In the cerebral cortex,  $B_{max}$  in the group with bosentan treatment was lower than in the untreated at prearrest and 10 minutes after the arrest. Endothelin receptor activity, as determined by the  $B_{max}$  to  $K_{p}$ ratio was reduced with the treatment, in the cerebral cortex and hippocampus at 7 days. In the medulla, lower B<sub>max</sub> values with bosentan were noted prior to cardiac arrest and at 3 minutes, 10 minutes, and 7 days.

In the untreated group, K of endothelin receptors in the hippocampus decreased at 1 h and 24 h after cardiac arrest, by 34.06 and 28.31% respectively, while in the cerebral cortex only 3 min after cardiac arrest (by 37%), and later corresponded to pre-arrest level. In the medulla, K<sub>n</sub> increased already after 3 min and 10 min of reperfusion, by 49 and 64.7 %, respectively, followed by normalization at 1 h and 24 h, and resurgence (58.7%)

increase compared to pre-arrest) at 7 days (Figure 10). In the bosentan-treated group  $K_{\rm D}$  values during the acute post-resuscitation period were maintained within pre-arrest levels although increased at 24 h in the cerebral cortex and after one week in all examined structures.

#### Discussion

This present study provides several major observations. Cardiac arrest induced cerebral



**Figure 10.** Changes of the equilibrium dissociation constant ( $K_D$ ) for endothelin-1 receptors in the rat brain after cardiac arrest. \*P<0.05 versus prearrest; #P<0.05 versus untreated with bosentan.

ischemia that injured neuronal organelles as determined in the brain on day 7 after ischemia. Bosentan preserved organelles in the cells surviving ischemic injury. Bosentan also reduced swelling of astrocytes and their perivascular processes. Bosentan treatment ameliorated vascular injury after ischemia and enhanced the process of angiogenesis. Furthermore, bosentan administration resulted in the alterations in  $B_{max}$  and  $K_{D}$  of endothelin binding sites in the brain that evolved in a different direction than after the ischemia alone. Bosentan reduced B<sub>max</sub> in all structures prearrest, while in the hippocampus and the medulla, at 7 days after cardiac arrest. In parallel, K<sub>p</sub> increased with bosentan in all structures at 24 hrs, while in the cortex and medulla at 7 days, pointing towards a reduced affinity of endothelin towards its receptors with the treatment. Observed effects can be ascribed to the mixed blockade of ETA and ETB with bosentan, while endothelin receptor antagonism has been implicated as carrying a therapeutic potential in stroke [12].

In this present study, the organelle alterations, observed under electron microscope in the cerebral cortex and CA1, show similarities to those previously reported after experimental cardiac arrest, although there are also differences.

Similar to the earlier authors' findings, neuronal shrinkage and neuronal chromatin condensation was observed in the CA1 sector of the hippocampus and cerebral cortex on day 7. Also, mitochondrial degeneration was pronounced on day 7 in this present and in earlier reports. Mitochondrial alterations in CA1 neurons included flattening and depletion of mitochondrial cristae as well as swelling of the entire organellum. Progressive swelling of the mitochondria was seen over several days after cardiac arrest [13]. The swollen astrocytes were found especially in the vicinity of injured neurons. Alterations in astrocytic ultrastructure, including swollen

cytoplasm and chromatin abnormalities, were reported in a porcine model of cardiac arrest [14]. However, after cardiac arrest, the mitochondrial structure in neurons showed normal morphology in examined brain regions [14]. Also, in contrast with our findings, previous observations pointed out that the formation of nuclear pore complexes in neurons was much reduced by day 7 after brain ischemia [13]. Likewise the fragmentation of neurotubules was not reported [13]. Moreover, the swelling of the Golgi zone structures was conspicuous in this study while 10 min cardiac arrest in male Wistar rats did not cause prominent changes in theses organelles [13].

Bosentan has exerted a profound effect on cytoskeleton disarrangements. However, the mechanism of preservation of neurotubules with endothelin antagonist appears quite unclear. Studies have shown that endothelin-1 is capable of F-actin filament cytoskeleton disruption [15], however, endothelin-1 may have no effect on tubulin expression [16]. Several other questions warrant further investigation e.g. whether endothelin blockade reduces the activity of calcium depended enzymes that degrade cytoskeleton proteins.

Neuronal mitochondria also appeared to be better preserved with bosentan treatment. Studies have shown, that ET-1 can promote oxidative stress in mitochondria via ROS formation [17]. Interestingly, antagonism of ETA receptors increases the activity of respiratory chain components [18]. Bosentan combined with phosphodiesterase-5 inhibition, improved mitochondrial capacity of the right ventricle in a rat model of arterial hypertension [19]. In addition, the reduced cristae loss observed in our study can also be involved in the mechanism of bosentan treatment. The respiratory chain function is, among other factors, determined by the integrity and morphology of mitochondrial cristae [20]. Stabilization of cristae shape may play a role in the inhibition of apoptosis. Thus bosentan, through amelioration of mitochondrial function in surviving cells, may stabilize their respiration.

Ischemia-induced Golgi apparatus alterations were also attenuated in the treatment group. Golgi apparatus was less distended, and the vesicular transport between the Golgi and endoplasmic reticulum appeared well preserved. Of note, others have reported that endothelin receptors can be found on the Golgi apparatus [21, 22]. The reduced cistern enlargement with bosentan may be deemed as an improvement of the Golgi complex morphology [23]. Reduced size of Golgi may also indicate a reduced neuronal activity and thereby a diminished metabolic demand. The Golgi apparatus processes multiple lysosomal, plasmalemmal, and secretory proteins in the cell. In neurons it controls the axonal flow and vesicle trafficking within presynaptic terminals [24]. These functions could be ameliorated with bosentan treatment.

Interestingly, nuclear invaginations were much increased with the treatment. Bosentan might interact with filaments involved in formation of nuclear invaginations such as actin and vimentin, thus reducing cell smoothness. The increased number of grooves and invaginations, by increasing surface area of nucleus, might facilitate nucleocytoplasmic transport or Ca2+ release from nuclear envelope into nucleoplasm [25]. In the bosentan treated group, less abundant nuclear pores were also present as compared to untreated conditions. Within cell nucleus, endothelin receptors are localized in close proximity to the nuclear pore forming proteins [26]. The suppressive effect of bosentan on nuclear pores may suggest a stimulating role of endothelins in nuclear pore formation. Also the reduction of nuclear envelope delamination was found with the treatment, what speaks in favor of protective action of bosentan towards lamins and alleviation of nuclear skeleton derangements. Such action might prevent tearing of nuclear lamina and protect from the envelope breakdown. Indeed, other authors have shown that bosentan reverted tacrolimusinduced decrease in the expression of lamins within vascular tissues [27].

These results differ from the impact of some other neuroprotective agents for cardiac arrestinduced global brain ischemia, examined at the ultrastructural level. PBN, for instance, increased mitochondrial swelling in CA1, and increased ER swelling in CA3, while, similarly to bosentan, it tended to reduce nuclear pore formation in all examined structures [13]. The inhibition of MMP9 with SB-3CT in cerebral tissues after cardiac arrest, reduced the overall extent of morphological injury, however ultrastructural alterations in surviving cells remained equivalent to those in untreated control after cardiac arrest in rats [28].

Although the number of damaged neurons was not substantially modified with bosentan treatment in the present study, the neuropil swelling/degradation was much reduced. The beneficial effect of bosentan on neuronal compartment was accompanied by the reduced swelling of astrocytes. Opposition to the action of endothelin-1 on astrocytes, responsible for cerebral water accumulation, might contribute to this effect [29]. Bosentan might also produce anti-inflammatory effect, as ET-1 increases mRNA levels of several inflammatory mediators in astrocytes including CCL2/MCP1 and CXCL1/CINC-1 [30].

The effect of bosentan on brain microvascular ultrastructure after cardiac arrest to our knowledge has not been previously reported. Bosentan reduced number of endothelial microvilli, associated with the risk of thrombus formation [31]. Other observed effects of bosentan may relate to a modulation of blood brain barrier (BBB) permeability. Bosentan ameliorated postischemic alterations within interendothelial junctions. The reduced number of microvascular profiles with enhanced pinocytosis and lessened perivascular swelling were also seen in the bosentan group. Reduced thrombosis and diminished microvascular constriction by swollen brain structures may work towards amelioration of cerebral microcirculation after cardiac arrest. Thus consistent with recent reports, bosentan seems to provide vascular protection in our study [32].

Furthermore, bosentan treatment shows the effect on postischemic angiogenesis. The observed characteristics of this process include the presence of hypertrophic endothelial cells, rich in organelles that form pillars (bridging) and then lumen of young capillaries. The presence of centrioles in dividing endothelial cells was also noted. Elsewhere, as in the vicinity of mature vessels, young capillary vessels were formed by immature endothelial cells surrounded by the basement membrane, that have not yet formed a lumen [33]. The number of vascular profiles showing features of angiogenesis post-ischemically was greater with bosentan treatment. However, literature data on the impact of bosentan on new vessel formation are somewhat conflicting. Bosentan reduced dysfunctional cerebrovascular angiogenesis in diabetic rats [34]. However, bosentan favored angiogenesis in the chronic limb ischemia [35] and in systemic sclerosis cases [36], while in carcinogenic tissues it reduced neovascularization [37]. This may relate to the complex role of ETR in angiogenesis, depending on underlying pathway activation and cell type involved. ET-1 stimulated angiogenesis in HUVEC, while others have found that ET-1 may actually impair this process in pulmonary artery EC [38, 39].

Studies have shown that cerebral ischemia causes significant alterations of endothelin binding sites. Upregulation of the ETB vasoconstrictive receptor system (ETB2) was seen in ischemic brains of male Wistar rats [40]. Postischemia, the density of endothelin binding sites increased in CA1 and cerebral cortex in rats [41]. Increased tissue levels of endothelins in cerebral ischemia may, through up-regulated receptors, contribute to ischemic damage at the subcellular level, e.g. ET-1 may cause endoplasmic reticulum stress [4]. Endothelin binding sites were found on the Golgi complex and on highly purified nuclei of rat liver cells, what allowed earlier authors to argue that endothelins may exert part of their biological function intracellularly [22].

While, in our study there was a decrease of B<sub>max</sub> in untreated rats shortly after ischemia, which could reflect internalization of the receptors upon agonist binding [42], or proteolysis of the receptor protein by ischemia-induced proteases, possibly as a mechanism protecting from endothelin overstimulation [43]. On 7 days post arrest, however, the level of ET binding sites was largely restored in examined structures. In the bosentan treated rats,  $B_{max}$  was decreased in the hippocampus at 3 minutes after cardiac arrest and in the cortex at 10 minutes and in all structures at 7 days. After bosentan treatment, a higher K<sub>n</sub> at 24 hrs and 7 days points towards a reduced affinity of endothelin to the receptors. Bosentan, by reducing B<sub>max</sub> (binding sites) while increasing  $K_{p}$  (reduced affinity), lowered the activity of the endothelin receptors, prior to cardiac arrest and at 1 and 7 days after.

Collectively, these findings point towards a notion that the observed salvage of neuronal organelles after cardiac arrest may be acquired via blocking endothelin receptors with bosentan. However, the link between endothelin receptor antagonism and organelle protection after ischemia requires further studies.

This present work may carry implications for the clinical management aimed at achieving neurological recovery after resuscitation. Bosentan is used in the clinical management of pulmonary arterial hypertension and idiopathic pulmonary fibrosis [44, 45]. While neuroprotective properties of bosentan so far remain debatable, here comes an indication that it may alleviate neuronal organelles damage, thereby contributing to a protection against brain injury after cardiac arrest.

# Conclusions

Mixed endothelin receptor antagonist bosentan ameliorates ultrastructural characteristics of neurons, astroglia and blood vessel in the vulnerable brain regions and may enhance cerebral angiogenesis after cardiac arrest.

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#### Disclosure of conflict of interest

#### None.

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