Inflammatory cell populations in early neuroinflammation in the core and peri-ischemic lesions of rat brain after transient focal cerebral ischemia: A morphometric study

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Background and Objective: Clinical and experimental observations emphasize the role of inflammation as a direct risk factor for stroke. To better characterize the inflammation, we have conducted a detailed histological analysis of the inflammatory cell population after transient middle cerebral artery occlusion in a rat model.

Methods: Fifteen adult Wistar male rats were divided randomly into test (n=10) and sham (n=5) groups. In the ischemic group, transient focal cerebral ischemia was induced with an intraluminal filament technique. Histologic lesions of the ischemic core and the surrounding penumbra zone were evaluated, based on a complex algorithm. Representative morphological changes in the core and the penumbra zone were compared. Immunohistochemistry was performed for leukocytes markers (CD15, CD68, CD3), leukocyte-released effectors (MMP-9 and COX-2), and FXIII (possibly involved in microglia and macrophage activation)

Results: Neuronal vacuolation and degeneration were significantly more in the core lesion, whereas cellular edema and inflammatory infiltrate were increased in the penumbra. CD68, CD3, FXIII and Cox-2 expression were significantly higher in the penumbra than in the core ($p=0.026$; $p=0.006$; $p=0.002$; and $p<0.001$).

Discussion: In the rat model of middle cerebral artery occlusion, inflammatory mechanisms, microglia/macrophage cells, and T-lymphocytes likely play an important role in the penumbra. The deterioration of neurons is less in the penumbra than in the core. Appreciation of the role of the inflammatory cells and mechanisms involved in stroke might lead to measures to inhibit the injury and save brain volume.

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ABSTRACT

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Keywords: middle cerebral artery occlusion; transient focal cerebral ischemia; inflammatory infiltrate; digital morphometry; Cox-2; FXIII

INTRODUCTION

Stroke is the second leading cause of death globally and can lead to permanent disability *(The World Health Organization updates, 2017)*. In humans, acute therapy of stroke focuses on reducing the volume of ischemia and improving the clinical outcome. The therapeutic options are limited to early recanalization by intravenous thrombolysis with the clot-busting drug, tissue plasminogen activator administered up to 4.5 hours after the onset of symptoms, or/and thrombectomy; both methods have a narrow therapeutic window *(Gronberg et al. 2013)*. The involved parenchyma in acute ischemic stroke has three components: ischemic core, penumbra, and the region named benign oligaemia, which is characterized by reduced blood flow. The penumbra is affected by hypoxemia and is at increased risk of being merged into the ischemic core. Correct estimation of size of the penumbra and minimizing its extent is critical in guiding stroke therapy. Thus, a focus of acute stroke intervention should be reperfusion of the penumbra *(Manning et al. 2014; Fuhrer, Günter & Zinke, 2017)*.

The severity of neural tissue damage after stroke is dependent on duration and intensity of the ischemia, but also on circulatory independent mechanisms, especially in the peri-infarct area. Neuronal damage is triggered by chemokines, cytokines, and matrix metalloproteinases (MMPs) resulting from exocytosis. Inflammatory cell infiltration and activation in cerebral parenchyma are additional harmful effects *(Wolinski & Glabinski, 2013)*. Recent clinical observations emphasize the role of inflammation as a direct risk factor for stroke, and suggest the benefit of anti-inflammatory therapy in reducing the incidence of stroke in animal models and humans *(Chiba & Umegaki, 2013)*.

The brain responds to ischemic injury with a complex inflammatory process, characterized by the participation of resident microglia and blood-derived macrophages, polymorphonuclear neutrophils (PMNs), and T-lymphocytes. Microglia/macrophages perform phagocytotic functions and produce neurotropic factors that are involved in neuroprotection. On the other hand, microglia have cytotoxic activities through secreted neurotoxic substances, reactive oxygen species and pro-inflammatory cytokines *(Ito et al., 2001; Desestret et al, 2013)*. Thus, the inflammatory process in stroke cannot be categorized as either exclusively beneficial or detrimental. Study of the inflammatory pathways, with their variety of cells and enzymes involved in the early stages of neuroinflammation in cerebral ischemia, is difficult to carry out in humans.

We have conducted a detailed histological analysis of the inflammatory cell population after transient middle cerebral artery occlusion (tMCAO) in a rat model. We determined the extent of the ischemic area with magnetic resonance imaging (MRI) and a complex algorithm of neurological assessment. We hypothesize that relationships between the inflammatory cell-positive area and the neuronal damage are different in the core and the penumbra zone, with possible implications for targeted therapy.

MATERIALS AND METHODS

Rat model of acute focal cerebral ischemia: unilateral transient middle cerebral artery occlusion (tMCAO) and reperfusion

The procedures used to create this experimental model were conducted according to the guidelines for care and use of animals in research (Directive 2010/63/EU of the European Parliament and the Council on the protection of animals). The study was approved by the Ethics Committee of the University of Medicine and Pharmacy Târgu-Mures (Ethical Committee approval number 17/17.03.2016).

Initially, 15 healthy adult male Wistar rats of 280 to 320 g, purchased from Laboratory Animal Facility-Center for Experimental Medicine of University of Medicine and Pharmacy Cluj-Napoca, Romania, were included in our study. Before and during experiments, the animals were group-housed (5 animals per cage) in a climate-controlled environment (temperature 21-23°C, humidity 60%, and natural circadian day-night cycle). All the animals had free access to water and standard rat chow.

The animals were assigned to two groups by a simple randomization. The first group (S, composed of 5 animals) was assigned to control conditions (Sham group); the second (I, consisting of 10 animals) was the experimental ischemic (test) group. After two weeks of accommodation, transient focal cerebral ischemia was induced in the test group by an intraluminal filament technique: obstruction of the left middle cerebral artery (MCA) for 90 minutes, followed by gentle removal of the filaments, according to the surgical technique we have described *(Oradan et al., 2017)*. All efforts were made to avoid animal suffering. Postoperative pain was minimized with optimal hydration and subcutaneous tramadol. The sham-operated group (S), used as controls, was subjected to the same anaesthesia and surgical procedures without tMCAO. The animals were monitored before, during and after the tMCAO procedure with rectal temperature recording. Body temperature was maintained with a heating pad (Doccol Corp.). Animals that died during the tMCAO procedure (in post-ischemic/reperfusion period) were excluded from further study.

Neurological deficit measurement

Behavioural tests for neurological assessment were performed at 90 min (the reperfusion time) and 24 h after reperfusion by a person blinded to the experiments. An expanded five-point scale (neuroscore) was used for evaluation of neurological deficit: 0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, inability to walk spontaneously *(Bederson et al, 1986; Woodruff et al., 2011)*. Only animals with a value equal to or greater than 2 points were used for further investigation.

Brain ischemia control and measurement of infarct volume with MRI

At 24 h after tMCAO, neural tissue damage in each animal (S and I groups) was assessed with MRI (Bruker Bio Spec 70/16 USR scanner, operated at 7 Tesla). After MRI acquisition, the ischemic region was reconstructed and evaluated with 3D Slicer (https://www.slicer.org/), an open-source software platform. Animals that had no characteristic signs of ischemia were excluded from the following morphological study. At the end-point of this experiment (24 h after tMCAO), the animals were sacrificed under deep anaesthesia with isoflurane. After vital functions and limb and corneal reflexes were absent (adequate anaesthesia confirmed by the veterinarian), the animals were perfused with 20 ml of 10% formalin through the left ventricle; the brains were removed and kept 24 h in 10% neutral buffered formalin for assessment of the morphological and molecular changes.

Morpho-histological analysis and immunohistochemistry

Twelve animals (5 sham and 7 of the ischemic group) were used for the morphological analysis. Three were excluded; two from the ischemic group (I_1 and I_4) died after ischemia/reperfusion, and one from the ischemic group (I_3) had negligible ischemic lesion on MRI examination.

Tissue processing and quantification of neural damage

The brains were processed in rostrocaudal direction, embedded in paraffin wax and cut into 4-5 µm thick sections at multiple levels. The serial sections were stained using haematoxylin and eosin (H&E) complemented with cresyl violet/Nissl (CV) staining to verify the diagnose and anatomic orientation of the ischemic lesion, and to select representative areas for analysis of the histological output in the ischemic core (characterized by the presence of dead neurons) in comparison with penumbra (containing degenerated, yet viable neurons) *(Paxinos & Watson, 1986)*. Adjacent sections to those processed by H&E were selected for immunohistochemistry. The contralateral hemisphere served as negative control.

The cellular modifications induced by acute ischemia changes were examined in sections stained with H&E and CV, particularly in the middle cerebral area. We focused on these morphological cellular and stromal changes: a) focal acute eosinophilic necrosis associated with neural-cell vacuolation; b) neuronal degeneration (pyknotic nuclei associated with vacuolation of neutrophils); c) areas of oedema; and d) the presence of inflammatory cells. For each variable, scores between 1-10 were assigned, as Randell *(Randell et al., 2016)* have recommended for quantification of brain-tissue damage. To determine the final score, we examined and quantified these variables in the core and penumbra of each sample, using a magnification of 200 x, the score indicating the degree of neural destruction.

Immunohistochemistry

One of all sections with representative neuronal injury was immunostained to establish the rate of glial cell loss (glial fibrillary acidic protein staining), and the immunological profile of inflammatory cell types involved in the focal cerebral ischemia and reperfusion was determined

(neutrophil granulocytes, macrophages and T-cells). We evaluated the core using the following leukocyte markers: anti-CD15 clone Carb-3 (Dako), anti-CD68 EKP1 ab955, anti-CD3 SP7 ab 21703 (Abcam), anti-MMP-9 ab 15W2 (Novocastra), anti COX-2 ab CX-294 (Dako) and anti-Factor XIII a, clone E980.1 (Novocastra). Dilutions were made according to the manufacturers' recommendation, and incubation of the primary antibody was performed overnight. Secondary antibodies were goat anti-mouse IgG and goat anti-rabbit IgG in combination with 3,3'-diaminobenzidine chromogen (DAB) followed by haematoxylin counterstain. As an endogenous control, we used contralateral non-damaged hemisphere tissue. For negative antibody control, normal serum was substituted for the primary antibody.

The H&E and immunostained slides were digitally scanned with Mirax Scanner and examined with the associated Panoramic Viewer 1.15.4 software (3DHISTECH Ltd., Budapest, Hungary). To determine the total tissue damage score (TTDS) for each case, five representative foci from core and penumbra were chosen, captured with a 20x objective, and saved in JPEG format. The effects of acute ischemia on the cell-modification score, and counts of inflammatory cell types, were determined with Image J 1.46 software (National Institutes of Health, Bethesda, MD USA) *(Ferreira & Rasband, 2012)*. The results were calculated individually for each case based on the arithmetic mean.

In the case of immunohistochemically stained slides, in the first phase, we selected the immunopositive cells by colorimetric segmentation followed by measurement of the positive areas in the five selected foci for each case, preceded by conversion of the image from 24-bit RGB format to 8-bit B&W (second phase), for the completely automated analysis. The result was calculated individually for each case based on the arithmetic mean. All specimens (H&E, CV and immunostained) were analysed and assessed individually by two of the authors; if necessary, discrepancies of their findings were discussed, and a consensus was reached.

Statistical Analysis

Descriptive and comparative statistics were performed with the GraphPad Prism 7.0 1 software (GraphPad Software, Inc.). Between-group comparisons (morphological and immunohistochemical scores) were performed with the non-parametric Mann-Whitney U test. Correlation analysis was performed according to Spearman. The level of statistical significance was set at $p<0.05$.

RESULTS

Measurement of neurological deficit

The neuroscore evaluation confirmed the success of the tMCAO procedure. All ischemic-group rats had a score above 2, except one (I_3) that had no neurological deficit (score 0) and no MRI signs of ischemia; this animal was excluded from subsequent morphological evaluation and immunohistochemical analysis. All S-group specimens had a neurological deficit score of 0.

Brain ischemia control measurement using MRI

After MRI acquisition, the volume of ischemia in 8 brains subjected to digital image examination was determined. The median total brain volume (TBV) of group I was 4066.5 mm^3 (min. 3726.94, max. 5264.65), associated with median ischemic volume (IV) 406.0 mm^3 (min. 10.73, max. 969.9). The median IV/TBV ratio was 0.12 (min. 0.002, max. 0.3). The specimen with a IV/TBV ratio <0.01 (min. value) was excluded from further studies (specimen I_3 with IV/TBV =0.002).

Characterization of tissue damage in the cortex: histology and immunohistochemistry

One day after stroke was induced, ischemic tissue was clearly demarcated from normal tissue (Figure 1A and 1B), involving the anatomical region irrigated by the occluded middle cerebral artery. The histological changes were characterized by eosinophilic coagulation necrosis in the ischemic core and decrease in glial-fibrillary acidic protein-positive cells (Figure 1C), along with cell vacuolisation (Figure 1D) and neuronal degeneration (Figure 1 E), oedema and inflammatory cell infiltration (Figure 1F). In all cases, the contralateral (control) hemisphere had a normal distribution of glial cells and no ischemic changes in the neural tissue. No representative morphological modifications were found in the sham group, so this group was not subjected to quantification of morphological changes.

The scores obtained from the morphological characteristics in ischemic group are presented in Table 1. The median total tissue damage score (TTDS) in the tMCAO group was 23 in the penumbra (20-27) and 24 (22-27) in the core, not a statistically significant difference (p=0.69). Scores of cell vacuolization and neuron degeneration were significantly higher in in the core (p=0.006 and p=0.003), in opposite, cellular oedema and inflammatory infiltration were more enhanced in the penumbra (p=0.009 and p<0.0001). The most impressive morphological modification in penumbra was high-grade oedema, while in the core, cell vacuolization and degenerating neurons dominated. Analyzing the correlation between the ischemic volume/total brain volume ratio (IV/TBV) and the total tissue damage score (TTDS) in the penumbra and core, we found no significance (r=-0.18, p=0.70 and r=0.36, p=0.45, respectively).

Cellular inflammatory response

In the first 24 h after tMCAO, signs of inflammatory-cell activation were present in the ischemic core and the peri-infarction area. Table 2 summarizes the immunological profile of the inflammatory cell types in the core and penumbra of the ischemic rats 24 h after reperfusion. CD15+ neutrophils and MMP-9+ cells were the most frequent inflammatory cells in the ischemic areas, but there was not significant difference between these populations in the penumbra and core (p>0.05; Table 2). After 24 h survival, CD68-positive and blood-derived macrophages were relatively few in ischemic tissue. CD 68 positive microglia/macrophage cells, (some of them expressing FXIII) and CD3 positive T-lymphocytes appeared in damaged tissue (Figure 2 A-F), and these cells were significantly more numerous in penumbra than in the core (p=0.026 for CD68,

p=0.006 for CD3, respectively; Table 2). In addition, more round FXIII-positive cells appeared in the penumbra zone (p=0.002; Table 2). COX-2 expressing cells (Figure 2 G-H), were also significantly more numerous in penumbra than in the core (p<0.001; Table 2). The positive surface scores of differently expressed markers: CD68, FXIII, CD3 and COX-2 in the core and penumbra are shown as dot-plot representation in Figure 3 A-D.

DISCUSSION

In this study on tMCAO in a rat model, we evaluated the ischemic core and the penumbra in parallel by morpho-histological and immunohistochemical examination. The inflammatory cell infiltration was more pronounced in the penumbra, and thus we hypothesised a correlation between the inflammatory cell-positive areas in the core and the neuronal damage in the penumbra, occurring within the first 24 hours after transient ischemia.

The dramatically affected brain tissue after tMCAO is the core, with severe morphological changes characterized by intense neuronal cell oedema and degeneration. Hypoxia caused by vascular occlusion leads to decreased energy production with consequent leukocyte activation, massive formation of reactive oxygen species, accumulation of intracellular calcium, and induction of cell membrane injury with neuronal death *(Traystman, 2003; Gronberg et al., 2013)*. This complex mechanism is not limited to the lesion itself, but affects also the perilesional and even remote areas. The region bordering the core, known as the ischemic penumbra, is the region that can be saved if adequate and timely treatment is administrated *(Popp et al, 2009; Fluri, Schumann & Kleinschitz, 2015)*.

Human ischemic stroke, usually resulting from middle cerebral artery occlusion, is a multifactorial disease caused by heterogeneous and complex risk factors and conditions, which makes it difficult to reproduce in an animal model. The proximal middle cerebral artery occlusion model induced by direct mechanical occlusion is the most frequently used procedure in stroke research, with high reproducibility, rare complications and focal brain damage like that occurring in human stroke *(Bacigaluppi, Comi & Hermann, 2010)*. Furthermore, the presence of a significant ischemic penumbra early after occlusion makes this technique suitable for neuroprotection studies *(Sicard & Fisher, 2009; Canazza et al., 2014)*.

To mimic all aspects of transient ischemia/reperfusion, we used a microsurgical procedure recommended by Güzel *(Güzel et al., 2014)*, with minor modifications as described *(Oradan et al., 2017)*, followed by a complex algorithm that includes neurological assessment, evaluation by MRI measurement of ischemic-tissue volume, and a histopathological and immunohistochemical examination. Our success rate of inducing an infarction by a 90-minute middle cerebral artery occlusion was 90 % (only one animal had no ischemic lesions according to the MRI examination), in accordance with another report *(Bacigaluppi, Comi & Hermann, 2010)*. With reference to the animal models, the infarction size reportedly is dependent on rat strains: the Wistar strain and

the intraluminal filament procedure, using a silicone-coated filament, gives the lowest variability *(Strom et al., 2013)*.

We found that the variability of infarction size, measured by MRI was high, but the mean infarct volume may be related to the volumes of the three t-MCAO-affected areas, as determined with stereotaxic atlas data. The histological examination confirmed the presence of ischemia, with more oedema and inflammatory cell infiltration in the penumbra than in the core. The positive cells were quantified with digital morphometry, a method that greatly improves the comparability of results.

Examining the immunophenotype of inflammatory cells, we found that CD15-positive leucocytes and MMP9-positive cells constituted most of the cellular elements that infiltrated the damaged brain tissue after 24-h survival, but without significant difference in cells in the penumbra and core. The number of positive MMP-9 cells was slightly higher than that of CD15-positive leucocytes both in the penumbra and core. The grade of neutrophil influx (the main source of free oxygen radicals), which directly destroys neurons, gains significance through the generation of post-ischemic tissue damage. The neutrophil granulocytes also produce MMP-9, thus amplifying the brain-inflammatory response and increasing the extent of the infarction area. MMP-9 probably mediates degradation of the extracellular matrix, which is followed by disruption of the blood-brain barrier, with initiation of neuronal death and erythrocyte extravasation. Regardless of the cellular origin (PMNs, microglia or macrophages), the MMP-9 positive inflammatory cells were the most common cellular elements in the ischemic area. In the core, both the neutrophils and activated microglia displayed a cellular localization of MMP-9 after ischemic stroke, the microglia presenting a shape and phenotype shift to resemble macrophage properties. Numerous pro-inflammatory agents triggered by ischemia increase the MMP expression immediately after a stroke. An increased number of MMP-9 positive cells has been described not only in the experimental models of cerebral ischemia but also in post mortem ischemic brain tissue, in association with neutrophil infiltration and activated microglia cells *(Rosell et al., 2006)*. These findings suggest a deleterious role of neutrophils and microglia in human brain injury (oedema following cerebral acute ischemia) and a potential therapeutic target in stroke *(Yang & Rosenberg, 2015; Zhang et al., 2017)*. In contrast to the significance of leukocytes, the significance of lymphocytes recruited into the brain after ischemic stroke remains uncertain. However, recent studies emphasize the role of T-lymphocytes in mediating reperfusion injury in post-ischemic brain tissue, even though the manner in which lymphocytes and neutrophils interact in the pathophysiology of stroke is unknown *(Jin, Yang & Li, 2010, Lee et al., 2015)*. In our experimental model, after one-day survival, the total T-cell influx in comparison with neutrophils was reduced. We found a significantly greater CD3-positive cell count in the penumbra than in the core. Studies on the role of T-cell subsets after stroke *(Jin, Yang & Li, 2010)* found an early peak (day 1) in helper T-cells expressing pro-inflammatory cytokines, and a later peak (day 3 to 7) with cytotoxic T-cells releasing anti-inflammatory cytokines. Stroke stimulates bone marrow production of myeloid cells, which are recruited to the brain *(Ritzel et*

al., 2015). We detected reduced numbers of CD68-positive macrophages in early infiltrates of both investigated zones, with a stronger expression in the penumbra. Due to phagocytotic activity, these cells remove cellular debris and, like neutrophils, recruit and activate lymphocytes *(Gronberg et al, 2013)*. Resident microglia cells are activated rapidly on day 1 after focal cerebral ischemia. In contrast, blood-derived macrophages are rare on day 2, as they infiltrate the damaged brain tissue 24-48 hours after focal cerebral. In their activated status in the penumbra, these cells shift from a thin, ramified morphology to a large, amoeboid structure, reflecting their activation *(Lee et al., 2014; Ritzel et al., 2015)*.

We found that part of the CD68-positive cells with macrophage and amoeboid microglia morphology express coagulation factor XIII (FXIII), with a significant difference in number of these cells between the positive surfaces in the penumbra and the core. Based on this result, we hypothesised that FXIII is involved in the activation of these cells. Recently, FXIII-A was reported to be a good marker of macrophage alternative pathway activation, but the interaction of coagulation factor FXIII with cells of the immune system has been demonstrated infrequently *(Navarrete et al., 2014)*. This relationship includes the modification of FXIII expression during monocyte differentiation and monocyte-macrophage activation, and is supported by the presence of FXIII in the cytoplasm of monocytes/macrophages with phagocytic potential. Cytoplasmic FXIII (cFXIII) is implicated in phagocytosis, and the effect of monocyte activation on cFXIII expression depends on the activation pathway: the classical pathway down-regulates cFXIII expression, while the alternative pathway up-regulates it *(Bagoly, Katona &Muszbek, 2012)*. cFXIII produced by macrophages from the penumbra and core is a substrate for neutrophil elastase released by activated PMNs, inducing a limited cleavage of cFXIII, which results in enzyme activation, followed by a much slower proteolytic inactivation *(Bagoly et al., 2008)*.

Cyclooxygenase-2 (COX-2) is an inflammatory signal-induced key enzyme in arachidonic acid metabolism, being induced in normal cell types, including blood-derived macrophages, microglia, and endothelial cells in response to inflammatory cytokines *(Patrono, 2016)*. COX-2 expression in ischemic tissue is quickly up-regulated after tMCAO together with MMP-2 and MMP-9 activity. Increased levels of COX-2 increase the level of prostaglandin E, with tissue destruction and early development of oedema *(Kim et al., 2017)*. In our study, we found significantly higher COX-2- positive surface area in the penumbra than in the core. The high COX-2-positive area in comparison with the CD68-positive surface is probably due to the appearance of numerous microglia and macrophages in the penumbra and core, activated through a dual pathway (classical and alternative), but also to CD15-positive neutrophils. PGE-2, which is the product of COX-2 activity, is involved in the early development of high-grade oedema, which in our experiments was the most representative morphological modification in the penumbra, with less in the core.

Our study has limitations. First, in the t-MCAO model, primary core damage may recover, and a secondary delayed injury evolves after a free interval of up to 12 hours. This is a long therapeutic

window that is not seen in human stroke, an important difference between human and experimental stroke pathways, as described by Hossmann *(Hossmann, 2012)*. Second, we did not perform a time-dependent characterization of the inflammatory response. Lacking the determination of M1/M2 polarization of macrophages and microglia, we could not collect data referable to the rate of the pro-inflammatory (M1)/anti-inflammatory (M2) character type. Considering that M1 and M2 macrophages and microglia can be converted into each other in their specific microenvironment, polarization of macrophages and microglia plays an important role in controlling the balance between the induction and suppression of inflammation *(Nakagawa & Chiba, 2014)*. Therefore, the role of cFXIII in macrophage activation needs further investigation.

CONCLUSION

Applying digital morphometry in an experimental rat model of cerebral ischemia, we determined that microglia/macrophage subsets and T-lymphocytes were present in the penumbra zone. We elucidated a higher grade of cellular vacuolization and neuronal degeneration in the core lesion, whereas cellular oedema, macrophage and T-cell infiltration were more intense in the penumbra. Significantly higher Cox-2 and cytoplasmic FXIII expression was present in the penumbra zone. The early inhibition of these cells and regulatory factors with pro-inflammatory properties may decrease the extent of the penumbra zone and enlarge the narrow therapeutic window in the first 24 hours of ischemic stroke.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no conflicts of interests.

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Table 1(on next page)

Results of the comparative study based on the applied score system (cell vacuolation, neural degeneration, cellular edema and inflammatory infiltration) for the core and penumbra.

Co-core. P-penumbra. Score A- cell vacuolization. Score B- neuronal degeneration. Score C-cellular oedema. Score D –inflammatory infiltration. IV-ischemic volume. TBV-total brain volume. *Mann-Whitney U test.

Ischemic Group (n=7)	Score A		Score B		Score C		Score D		Total Tissue Damage Score		IV/TBV ratio
	P	Co	P	Co	P	Co	P	Co	P	Co	
I_2	6	9	7	8	9	6	5	3	27	26	0.01
I_5	4	8	5	6	9	6	5	2	23	22	0.1
I_6	7	9	4	8	9	8	4	2	24	27	0.25
I_7	6	9	6	7	8	7	6	3	26	26	0.19
I_8	4	7	4	8	8	5	4	3	20	23	0.12
I_9	6	9	5	9	7	4	5	2	23	24	0.3
I_{10}	6	6	4	8	7	6	6	3	23	23	0.1
Median (min.-max.)	6 4-7	9 6-9	5 4-7	8 6-9	8 7-9	6 4-8	5 4-6	3 2-3	23 20-27	24 22-27	0.12 (0.01-0.3)
p*	0.006		0.003		0.009		<0.0001		0.69		

Table 2(on next page)

The immunological profile of inflammatory cell types represented by median (min-max) value positive cell surface (% of the total area) in the core and penumbra of the ischemic-rat group (n=7).

*p values calculated by Mann-Whitney U test.

Inflammatory Cell Type	% positive cells/total examined area	
	Penumbra	Core
Neutrophils (CD15+)	6.13 (5.52-7.36)	5.5 (3.92-7.7)
	p=0.274	
Macrophages (CD68+)	2.68 (1.87-2.95)	1.91 (1.78-2.03)
	p=0.026	
FXIII+	1.67 (1.34-2.26)	1.27 (0.92-1.49)
	p=0.002	
T-Cells (CD 3+)	1.48 (1.26-1.56)	1.08 (0.87-1.49)
	p=0.006	
MMP-9+	7.53 (7.32-7.83)	7.81 (7.26-8.12)
	p=0.4	
COX-2+	5.13 (4.96-5.55)	3.7 (3.57-4.18)
	p<0.001	

Figure 1

Representative morphological features of tMCAO-induced ischemia around the middle cerebral area.

(A) Clearly demarcated ischemic zone in the ipsilateral cerebral cortex and thalamus represents the penumbra, surrounding the core (red arrows), Nissl staining, 1x obj**. (B)** Core (c) and penumbra (p) interface viewed with H&E staining, 4x.obj. **(C)** Decrease of the Glial-fibrillary acidic protein-positive astrocytes in the ischemic core, DAB staining, 10x obj. **(D)** The most impressive morphological modifications in the ischemic core: focal acute necrosis associated with neural-cell vacuolation (red arrows), and **(E)** Neural degeneration associated with vacuolation of neuropil (red arrows), Nissl staining, 20x obj. **(F)** Penumbra zone with areas of edema (red arrows) and inflammatory cell infiltration (circle), Nissl staining, 10x obj.

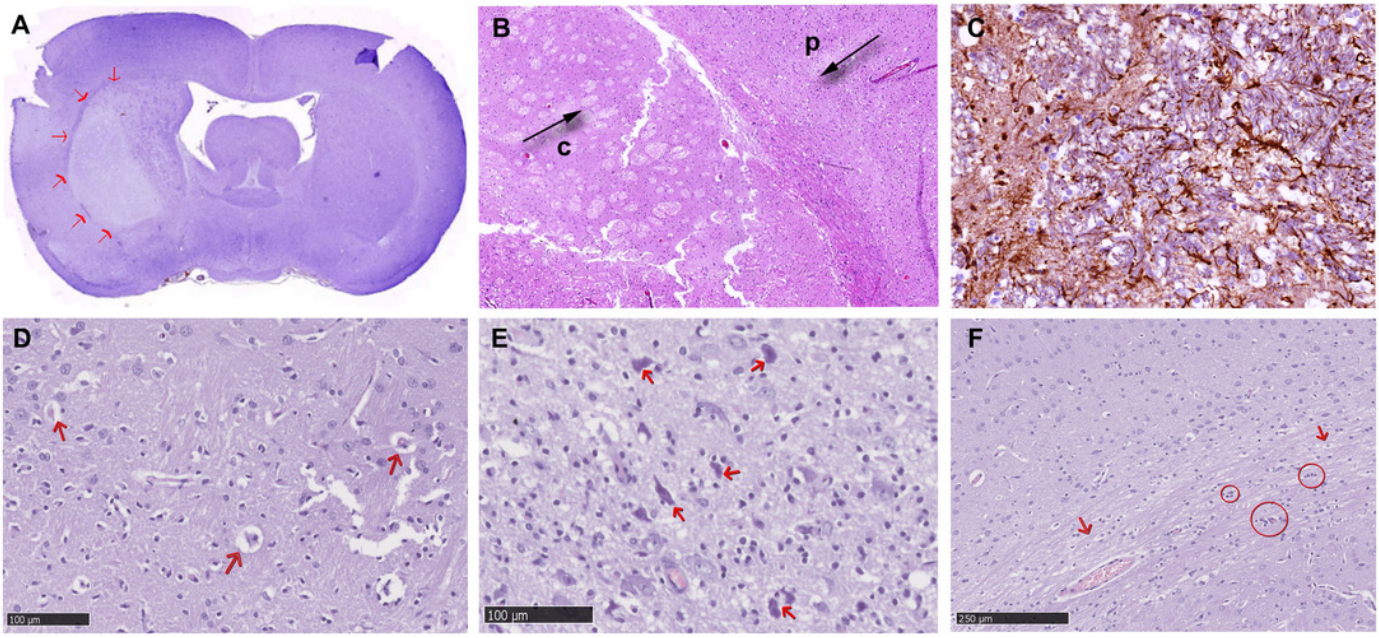


Figure 2

The immunological profile of the inflammatory cell types, with significantly higher positive surface area (calculated morphometrically by Image J) in the penumbra than in the ischemic core, after 24 h survival following tMCAO (DAB chromogen, 20x).

Fewer CD68-positive macrophages in the core **(A)** than in the penumbra **(B).** Rare, round FXIII-positive cells in the core **(C)** in comparison with the peri-ischemic area **(D),** associated with reduced influx of CD3 positive T-cells both in the ischemic core **(E)** and penumbra zone **(F).** Areas infiltrated by inflammatory elements with COX-2 immunophenotype in core **(G)** and penumbra **(H).**

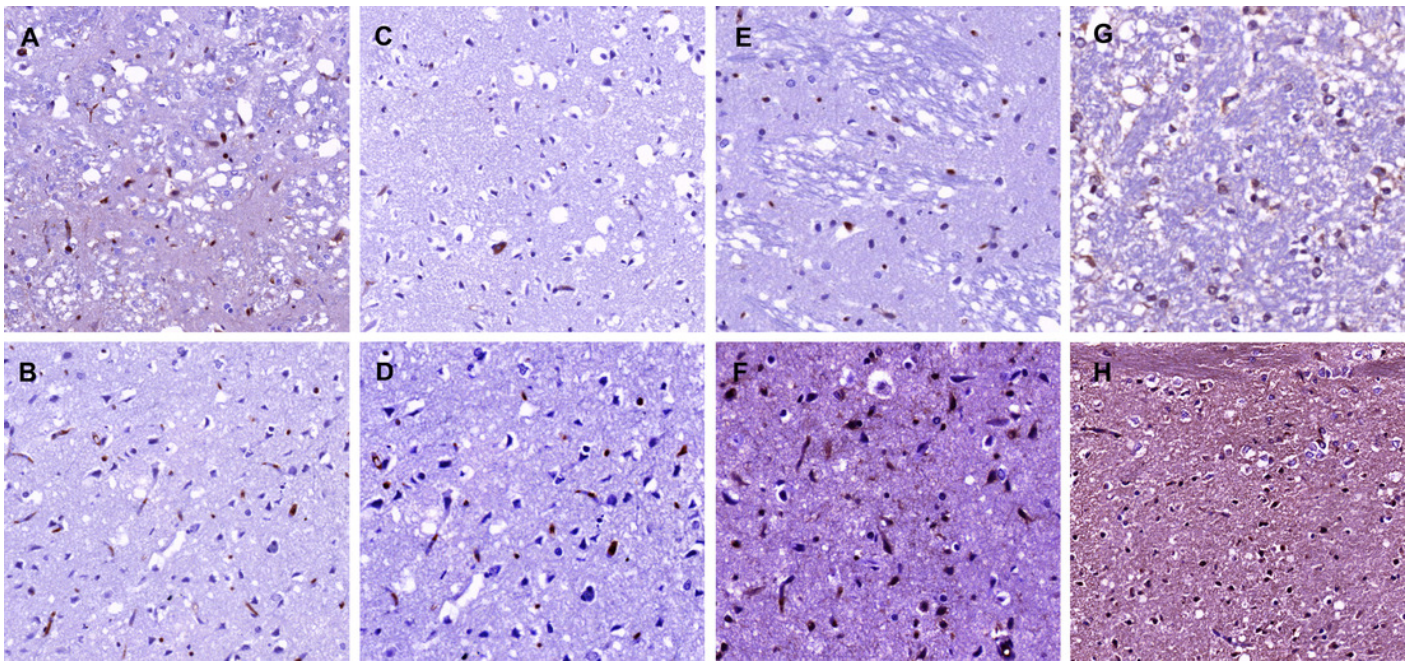
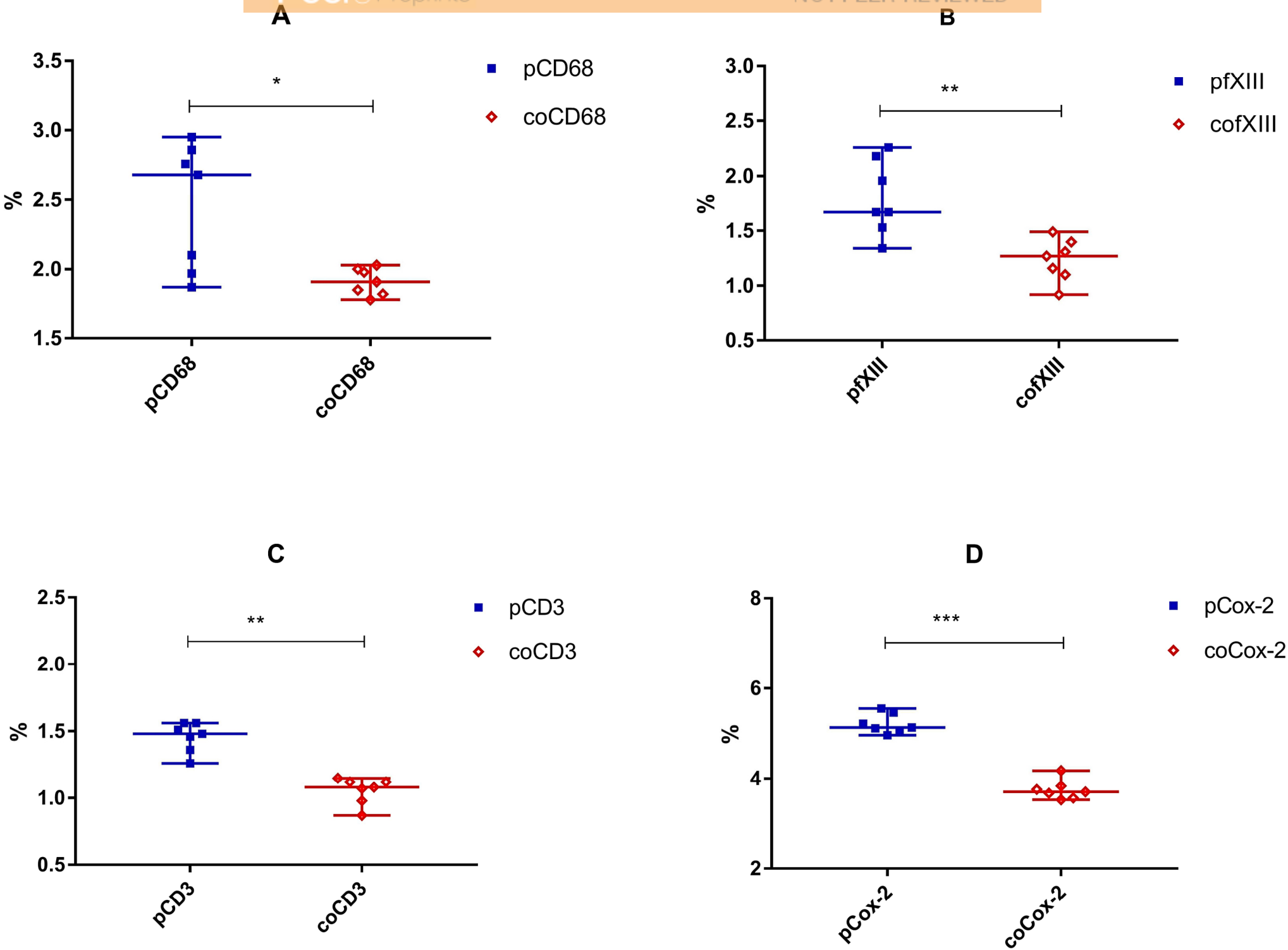


Figure 3(on next page)

Comparative dot-plot representations of significantly different inflammatory cell markers. (A) CD68, (B) FXIII, (C) CD3 and (D) Cox-2.

p – penumbra, co – core; all markers represented as % of the positive surface area, shown as median and 95% CI, * $p<0.05$, * * $p<0.01$, * ** $p<0.001$, calculated by the Mann-Whitney U test.



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