Cerebral ischemia in the aged. Limited anti-inflammatory efficacy of the indomethacin treatment

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Abstract
Ischemic stroke is a disease of aging and causes high mortality or long-term disability. Diminished neurological recovery after stroke in aged subjects is possibly associated with exaggerated non-specific inflammatory reaction. The focus of the present study was on neurobiological and behavioral differences between young and old rats modulated by indomethacin daily treatment starting at four hours after acute cerebral ischemia in animal model. Our results indicate age-independent positive consequences of non-specific inhibition of inflammation by indomethacin including increased NeuN-positive surviving neurons, reduced infarct volume and enhanced neuroprotective response of innate immune system evidenced by increased Iba1 and Anx3 immunoreactivities and moderately activated microglia in the peri-infarcted area. Quite relevantly, the efficacy of therapy with indomethacin was reduced. In the aged rats, specifically indomethacin is ineffective in inhibiting phagocytic activity, which is probably due failure of the aged brain to up-regulate the expression of several cytokines including TNFα and Cxcl4. At protein level, we observed no change of lysosomal ED1 immunoreactivity under treatment. Our study demonstrates the beneficial anti-inflammatory treatment with indomethacin. However, aging blunted the positive effect.

Keywords: aging, stroke, indomethacin, neurological recovery, microglia, reversible middle cerebral artery occlusion.

Introduction
Aged individuals experience the highest incidence of stroke, 75% of stroke victims are above 65-year-old, and recover less from stroke [1–3]. Stroke mortality is rated between 20 and 30%. Of the surviving patients, one-third improves within a week, 40% remain unchanged in their disability, and 20% deteriorate further during the first week [4]. Surviving stroke patients often have persistent symptoms like impaired motor function, sensory deficits, perceptual deficits, impaired balance, aphasia, depression, dementia and other cognitive impairments [5–7]. Most patients experience some degree of spontaneous recovery of function [8], and this can be further enhanced by rehabilitation or stroke unit care [9].

The understanding of the molecular and biochemical processes during ischemia induced brain damage have increased during recent years, but still there are limited possibilities for treatment of stroke patients. The only approved treatment in clinical practice today, is thrombolytic treatment with recombinant tissue plasminogen activator (rt-PA). However, only a fraction of the stroke patients benefit from thrombolysis since the treatment has to start within the first 3–6 hours, which has been defined as the ‘therapeutic window’ for acute stroke [10].

Although each step along the ischemic cascade offers a potential target for therapeutic intervention, and neuro-protection has shown benefit in animal studies, this has been difficult to translate to stroke patients. One reason is that animal models of stroke are conducted mostly in young adult rodents to evaluate neuropathological or behavioral outcome [11]. However, aged animals differ from young animals in physiology, pathophysiological features and behavioral outcome after a cerebral ischemic episode [12–14]. For that reason, animal models of stroke using aged animal may be more clinically relevant than current models employing young adult rats [15–19]. Thus, it is mandatory to investigate other potential therapies for acute and subacute stroke and also to discover new treatments for late intervention using aged animal models. The present study confirmed and extended current data on neurobiological and behavioral differences between young and old rats modulated by pharmacotherapy, in particular indomethacin treatment after ischemic episode.

Materials and Methods
Animals
Young (three months of age, n=45) and aged (20 months, n=50) Sprague–Dawley male rats, bred in-house were maintained on a 12-hour light/dark cycle (07.00–19.00 h) and allowed constant access to food (hard food) and water. Young and aged rats groups were divided subsequently into subgroups of equal number (n=15 each group) and assigned survival times 14 days following surgery, also one treatment group, per age (14 days). These time points were chosen based on our previous work on this model [20]. For the older rats, kept alive for two weeks, the group was larger (n=18) to compensate for the higher post-ischemic mortality rate (15%). For
comparison, the running results to experimental groups for each age were added two sham-operated control groups ($n=15$). Body weights ranged from 290 to 360 g for the young rats and from 600 to 700 g for the aged rats.

The animals were behaviorally tested (see below) and then subjected to reversible occlusion of the middle cerebral artery (MCAO) or sham surgery ($n=15$), per age group (see below) or MCAO and indomethacin treatment (see below).

**Surgery**

Blood flow through the middle cerebral artery was transiently interrupted as previously described [2, 18]. Briefly, this method begins with external reversible occlusion of the right middle cerebral artery followed by the bilateral common carotid arteries external occlusion. After 90 minutes, the MCA is released and the clamps from common carotid arteries are removed.

Overnight fasted rats were generally anaesthetized with Sevoflurane [2,2,2-trifluoro-1-(trifluoromethyl)-ethyl ether], 3% in a mixture of 70% nitrous oxide and 30% oxygen through a specially designed mask. Arterial blood pressure level were controlled and kept within physiological ranges during surgery.

After survival times 14 days, the rats were deeply anesthetized with 4% Sevoflurane in a mixture of 70% nitrous oxide and 30% oxygen through a specially designed mask and perfused with buffered saline followed or not by 4% freshly depolymerized paraformaldehyde (PFA) in 5× phosphate buffer (PB), depending on future tissue propose. In sterile condition, using Lempert rongeur (Fine Science Tools) the skull was carefully opened and the brains removed with one micro spatula. For RNA and proteins extraction, the rats were perfused with buffered saline. After removal, the brains were cut into 2 mm slices that were dipped in TTC (triphenyltetrazolium chloride) to visualize the infarct. For cryosection, the rats were behaviorally tested (see below) and then subjected to reversible occlusion of the middle cerebral artery (MCAO) or sham surgery ($n=15$), per age group (see below) or MCAO and indomethacin treatment (see below).

**Indomethacin treatment**

For studying the effect of indomethacin in rat model with focal reversible ischemic stroke, we chose 14 days survival time after middle cerebral artery occlusion episode. Two treatment groups, young and aged rats were injected intraperitoneally (i.p.) with Indocid PDA (indomethacin sodium trihydrate, powder for solution for injection) from Merck Sharp & Dohme. The powder was dissolved in 0.9% physiological saline to give a concentration of 2 mg/mL. Indomethacin (i.p.) administration began in operation day at four hours. The treatment was administrated divided in two doses at 12 hours interval and in total for 14 days.

The ethical approval of all experiments was granted by the University of Medicine and Pharmacy of Craiova, Romania.

**Bromodeoxyuridine (BrdU) administration**

To label proliferating cells, rats were injected intraperitoneally with bromodeoxyuridine (BrdU; 50 mg/kg body-weight; Sigma). For 14-day post-ischemic group (treated and untreated), rats were intraperitoneally given BrdU daily for 12 days starting from the 2nd day of post-ischemia until the 13th. Similar to 14-day group, sham-operated control group rats were daily administrated BrdU for 12 days. Rats were transcardially perfused 24 hours after the last injection and those brains after fixation were used for sections (all the rat brains used for sections derive from rats BrdU injected).

**Immunohistochemistry and immunofluorescence**

We used coronal brain sections processed for immunohistochemistry as previously described [2]. Frozen brain was sectioned at 25 μm thickness using a freezing cryotome (Cryostat CM3000 Leica, Bensheim). Briefly, the sections were blocked for two hours in 10 mM phosphate-buffered saline (PBS) containing 5% serum, 0.3% Tween 20, 0.005% hydrogen peroxide (to inactivate endogenous peroxidase) followed by overnight or 48 hours incubation at 4°C with optimum primary antibody titer in blocking solution without hydrogen peroxide. After three washes with 10 mM PBS solution containing 0.3% Tween 20, sections were then incubated overnight at 4°C with biotinylated or fluorescent-labeled secondary antibody with optimal dilution also in blocking solution with 3% serum. For the enzyme-mediated detection (colorimetric detection for light microscopy), we used substrate: peroxidase and chromogen 3,3′-diaminobenzidine (DAB) with or without nickel. Finally, the sections were mounted onto slides, air-dried for two hours at 55°C, counterstained with Methyl Green (nuclear marker) and coverslipped using a xylene based mounting medium. The specificity of the antibodies was verified by omission of the primary antibodies in immunohistochemical experiments.

In short, after incubation with blocking solutions containing 3% donkey serum/10 mMol/L PBS/0.3% Tween 20, tissue section were incubated overnight at 4°C with monoclonal antibodies recognizing one of the following: (1) a cytoplasmic determinant of brain macrophages (clone ED1, 1:400 dilution; Serotec, UK); (2) the neuronal marker, NeuN (1:1000 dilution; Millipore, Schwalbach, Germany) diluted in PBS containing 3% normal donkey serum and 0.3% Tween 20. The primary antibody was detected using the ABC (Avidin–Biotin complex) system.

The detection of BrdU-positive cells was done as described by Eriksson et al. [21], with the following modifications. Free-floating sections were pre-treated with 50% formamide, 0.3 M sodium chloride, 10 mM SSC (sodium chloride and sodium citrate solution) at 55°C for two hours, incubated in 2 M hydrochloric acid at 40°C for 40 minutes, and rinsed in 0.1 M boric acid (pH 8.5) at a room temperature for 10 minutes. After neutralization, sections were incubated in blocking solution containing 10% lamb serum, 0.3% Triton X-100, 0.2% gelatin in PBS) overnight at 4°C, then mouse monoclonal anti-BrdU antibody (1:300 dilution; Roche, Mannheim, Germany) at
4°C for 48 hours. Sections were washed with PBS, incubated with biotinylated donkey anti-mouse secondary antibody (1:400 dilution; Jackson ImmunoResearch, West Grove, PA) followed by the Avidin–Biotin complex and DAB staining were performed as described above. Fluorescence signals detection and 3D reconstructions were described previously [18]. To establish the three dimensional characteristics of double or triple-labeled cells, a sequence of confocal image spaced 0.1 μm apart through 25 μm section was taken. The resulting sequence of the image was loaded and processed into Velocity 3D analyses software (Improvision, Coventry, UK).

**Determination of the infarct volume**

To assess the volume of the infarct induced by reversible focal ischemia, every 14 coronal section was stained with NeuN, a marker of neuronal viability (mature neuron marker). Images of the stained sections were taken to cover the entire infarcted area and scanned and the total volume of infarct and ipsilateral hemisphere was estimated applying Cavaleri method. Then the ratio of V infarcted mean/V ipsilateral hemisphere was calculated, which was then expressed as percent of infarct to the total volume of the hemisphere per group (“Cavaleri method”, Howard & Reed (1998) [22]).

**Statistical analysis**

The data required for statistical analyses was parametric data and non-parametric data. Parametric data is presented as mean ± SD and nonparametric data as median (range) unless otherwise stated.

Parametric data was analyzed running a One-Way Repeated Measures Analysis of Variance (ANOVA) followed by the Dunnett’s post-hoc test (experiment-Treated vs. control-Unreated) and One-Way ANOVA followed by Tukey’s post-hoc test (all pairwise) to compare young vs. aged group.

Non-parametric data (score) was analyzed with the Kruskal–Wallis test or Repeated Measures Analysis of Variance (ANOVA) on Ranks followed by the Bonferroni’s post-hoc test (Siegel & Castellan (1988) [23]). P-values lower than 0.05 were considered statistically significant.

**Results**

**Indomethacin decreased the infarct volume in both age groups**

Indomethacin, a non-steroidal anti-inflammatory drug, is known to penetrate blood-brain barrier and decreases inflammatory reaction in brain. We found that in young rats, the indomethacin treatment greatly reduced the infarct volume from 89.6±28 mm³ (9.35±3%) to 58.4±21 mm³ ($p=0.05$).

In aged rats, the volume of cortical infarct dropped to 91±11 mm³ (9±1.8%) in treated groups as compared to 132±30 mm³ ($p=0.05$) (Figure 1A).

The most affected cortex area by stroke in untreated rats is the parietal area 1, forelimb and hindlimb area and frontal area 1 with extensiveness upper to frontal area 2 or lower to parietal area 2 in aged rats. The anatomical localization of the infarct coincides with primary and secondary somatosensory cortex with some extension to the frontal primary motor cortex. Treated animals had affected predominantly parietal area 1, the primary somatosensory cortex. The infarct length in these groups is 2.5±1.5 mm, with the mean between Bregma -0.8 mm and Bregma -1.8 mm (Figure 1B). The age difference of the infarct volume was nevertheless not significant.

![Figure 1](image-url) - Infarct volume by NeuN immunohistochemistry at low resolution (magnification 0.5×). Fourteen days of treatment with indomethacin decreased significantly the infarct volume after in both groups of aged rats with focal cerebral ischemia. UT: Untreated; T: Treated.
morphology of penumbral neurons. After treatment, neurons had an intensified NeuN immunoreactivity and showed evidence of neurites outgrowth in penumbra of both age groups. Without treatment, some few penumbral neurons exposed ischemic cell change characterized by irregularities in the plasma membrane and the nuclear membrane, and the cytoplasm generally containing many large vacuoles, predominantly in aged rats (Figure 2, A and B).

**Indomethacin diminished phagocytosis in the infarct core of aged rats**

Indomethacin is a non-steroidal anti-inflammatory drug. ED1 is an antibody, which identify lysosomal proteins of phagocytic cells.

In areas corresponding devoid of NeuN immunoreactivity, there was a high expression of the lysosomal marker ED1, which is indicative of phagocytic transformation at 14 days in both age groups. In the infarct core, there was a significant decrease in the number of ED1 positive-cells of young animals as compared to untreated animals ($p<0.01$) (Figure 3A). As opposed to young rats indomethacin treatment in aged rats did not have any effect on ED1 expression in infarct core (Figure 3B).

Morphology of most of these inflammatory cells in infarct core showed the phagocytic cells with round and ovoid shapes, with many cytoplasmatic vacuoles and round nucleus. This morphology characterized the infiltrated macrophages (blood-born monocyte) and the activated microglial cells of the amoeboid type (amoeboid microglia). Moderate microglial activation with preserved ramified morphology is observed in areas with selective neuronal loss (penumbra) in untreated rats (Figure 4A).

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**Figure 2** – *Indomethacin treatment is neuroprotective and has a beneficial effect on the infarct volume especially in the young post-stroke rats. IC: Infarct core; Pn: Penumbra; UT: Untreated; T: Treated. Magnification, 10×.*

**Figure 3** – *Indomethacin, as non-steroidal anti-inflammatory drug, decreases the ED1 immunoreactivity in young treated group in infarct core, but interestingly increases the ED1 expression in penumbra in both age groups. UT: Untreated; T: Treated.*
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Indomethacin influences proliferation of ED1-positive cells

To detect the proliferation pattern of ED1-immunoreactive cells in different age groups, we quantified the ED1-BrdU positive cells in both groups with or without treatments. There were statistically significant decreases in the number of ED1-BrdU colocalized cells in infarct core of treated young group as compared with the corresponding untreated group \((p=0.05)\) (Figure 5Ic). However, the number of ED1-BrdU colocalized cells in the infarct core of treated old group was higher as compared with the corresponding untreated group. Since BrdU is a marker of proliferating post-stroke cells, we conclude that in the peri-infarct area of old rats the indomethacin treatment stimulated significantly microglia proliferation \((p=0.045)\) (Figure 5Pi). No ED1-BrdU positive cells were detected in sham-operated animals and in the contralateral cortex of animals subjected to MCA occlusion.

Figure 4 – Immunohistochemistry of ED1+ cells and counterstaining of nuclei with Methyl Green. Indomethacin decreases the number of ED1+-activated macrophages/microglia in the infarct core in the young rats. Unexpected, this treatment modulate the hyperplasic reaction of moderate reactive microglia. Magnification, 20×, 100×.

Figure 5 – Indomethacin decreases the number of proliferating ED1-positive cells in infarct core of young animals and increases the number of proliferating microglia in the peri-infarct area in both age groups. Nuclei are shown in blue and ED1-positive cells are shown in green. Ic: Infarct core; Pi: Peri-infarct; UT: Untreated; T: Treated. Magnification, 20×.
Inflammatory reaction after cerebral ischemia has a “tripodal” mechanism: vascular, blood and brain inflammatory-immune response leading to the activation of pro-inflammatory genes and causing local and systemic inflammation [24, 25].

Indomethacin, a common non-steroidal anti-inflammatory drug is widely used for the treatment of pain and inflammation. Recent studies demonstrated its efficacy in periventricular hemorrhagia hypoxic newborn and in Alzheimer’s disease. Since aged rats show a fulminating inflammatory reaction after cerebral ischemia, we investigated the efficacy of indomethacin as possible adjuvant treatment of ischemic stroke in aging animals. In this study, we showed that indomethacin treatment is more effective in reducing infarct volume in young rats than in the aged rats.

Previous studies demonstrated that indomethacin-activated peroxisome proliferator-activated receptors γ (PPARγ) [26] and PPARγ activation is essential for angiotensin AT2 receptor-mediated neurite outgrowth [27]. Recently [28], it was shown that overexpression of angiotensin AT2 receptor predominantly in neuronal neurites protected against cerebral ischemia and induced extensive neurite outgrowth, which was correlated to improved neurological outcome.

In this study, NeuN immunostaining is highly suggestive of improved survivability of neurons after treatment. We hypothesize that surviving neurons might preserve their function since the indomethacin protected against neurological deterioration in first three days after ischemic episode. Our hypothesis is supported by results obtained after non-steroidal anti-inflammatory drug (NSAID – Cox2 inhibitor) administration in young male Sprague–Dawley rats with focal transient ischemia model [29] or with traumatic brain injury [30]. Although there are numerous studies showing that indomethacin or other NSAIDs improve age-dependent neurological and cognitive deficits in neurodegenerative diseases in animal model and patients with Alzheimer’s disease [31–34], studies on ischemic stroke in the context described above are limited. Trials on indomethacin treatment in acute stroke patients are quite a few. Nekhimenko et al. [33, 34] observed that indomethacin treatment daily for 14 days after stroke decreased the neurological deficit in patients with ischemic stroke.

Indomethacin decreased phagocytic activity in the infarct core of young animals but is not effective in aged rats

Morphologically, “activated” microglia is identified as hypertrophic with thicker and shorter processes [35]. Frequently those morphological changes are correlated with microglial hyperplasia, but the histological transformation from ramified resting microglia to the amoeboid microglia does not necessarily reflect their function in specific environment. The traditional macrophagic/microglial marker ED1 (recognizes a lysosomal membrane glycoprotein that is a rat homolog of human CD68) can be useful to assess the phagocytic activity of those cells. Similarly, Iba1, a novel cytokine-inducible EF-hand protein recognizing the ionized calcium-binding adapter molecule 1, is specifically expressed in microglia/macrophages [36] and is expected to be a key factor in membrane ruffling by facilitating cell migration and phagocytosis [37].

At day 14 after an ischemic episode, our findings indicates a strong induction of phagocytic microglia/macrophages specific antigen in areas devoid of NeuN immunostaining and are consistent with previous study in our lab [2]. Our findings also showed a reduction in the proliferation of ED1⁺ inflammatory cells (macrophagic/microglial phagocytic activity) in the infarct core of young rats.

Our results agree with the study of Hoehn et al. [38]. They have shown that indomethacin diminished microglial/monocyte phagocytic activity in male young rats following focal cerebral ischemia. Using the ratio of ED1⁺ to CD11b⁺ cells the authors showed that ED1 immunoreactivity was reduced significantly in CD11b-positive cells, in infarcted area. CD11b (Integrin Alpha M) is commonly used as a microglial/macrophagic/granulocytes marker.

However, indomethacin was not as effective in aged animals. More specifically, indomethacin did not influence ED1 immunoreactivity in infarct core of aged rats. One explanation is that elderly rats have an elevated macrophagic synthesis of Cox2. Indeed, recent studies in aging macrophages highlighted a higher ceramide concentration probably mediated by up-regulation of NFXB induced increase of Cox2 level [39, 40]. Furthermore, endogenously ceramides in post-ischemic aging brain may activate microglia [41–44]. Thus, aging could contribute to the amplification of neuroinflammatory reaction and limit the efficacy of anti-inflammatory drug.

Conclusions

In the present study, we showed that (i) indomethacin administration has neuroprotective efficacy by increasing the survivability of penumbral neurons and by decreasing the infarct size after transient focal ischemia; (ii) in aged rats, indomethacin is ineffective in inhibiting phagocytic activity; (iii) in young animals, indomethacin administration decreases phagocytic activity after ischemic stroke.

Conflict of interests

The authors declare that they have no conflict of interests.

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