Inhibition of cyclooxygenase-1 does not reduce mortality in post-ischemic stroke rats

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ARTICLE INFO

Keywords:
Cyclooxygenase-1
Ischemic stroke
Fever
Mofezolac
Mortality

ABSTRACT

Background: Ischemic stroke is one of the leading causes of mortality and morbidity. The currently available non-invasive therapeutic options are not sufficiently efficacious. Post-ischemic brain is characterized by a prominent inflammatory response. Little is known about the involvement of cyclooxygenase (COX)-1 in the pathophysiology of ischemic stroke.

Objective: This study was undertaken to examine the effects of a highly selective COX-1 inhibitor – mofezolac – on clinical outcomes and brain inflammatory markers in post-stroke rats.

Methods: Stroke was induced by subjecting rats to permanent middle cerebral artery occlusion (MCAO). Control rats underwent a sham surgery. Rats were treated with mofezolac (50 mg/kg, intraperitoneally [*ip*]) once daily for 14 days. Control animals were treated with vehicle. Body temperature (BT), neurological score (NS) and cumulative mortality were monitored at different time points. At the end of the experiment, rats were euthanized and three brain regions (hypothalamus, hippocampus and frontal cortex) were extracted. Levels of interleukin (IL)-6, prostaglandin (PG)E_2 and tumor necrosis factor (TNF)-α in these brain regions were determined by ELISA kits.

Results: BT, NS and cumulative mortality were all significantly higher in post-MCAO rats than in sham-operated rats, irrespective of the treatment given. BT, NS and mortality rate did not differ significantly between mofezolac-treated and vehicle-treated sham-operated animals. BT was significantly lower in mofezolac-treated as compared to vehicle-treated post-MCAO rats. Mofezolac did not significantly alter NS in post-MCAO rats at any time-point. Cumulative 14-day mortality was non-significantly higher in mofezolac-treated as compared to vehicle-treated post-MCAO rats (48 % *vs*. 21 %, respectively; $P = 0.184$). Mostly, IL-6 and TNF-α levels did not differ be- tween post-MCAO and sham-operated rats and were not affected by mofezolac treatment. In contrast, mofezolac significantly decreased PGE_2 levels in post-MCAO rats' brains.

Conclusion: Overall, these results suggest that chronic treatment with the selective COX-1 inhibitor mofezolac did not reduce morbidity or mortality in post-stroke rats.

1. Introduction

In 2016, the World Health Organization reported that stroke was the second cause of death and the third leading cause of disability worldwide [1]. The American Stroke Association placed stroke as the fifth cause of death in the United States [2]. Moreover, cerebrovascular disease was graded as the leading cause of burden of disease in several parts of the world [3]. These figures emphasize the extent at which

Abbreviations: BT, body temperature; COX, cyclooxygenase; FC, frontal cortex; HC, hippocampus; HT, hypothalamus; IL, interleukin; MCAO, middle cerebral artery occlusion; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α.

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stroke is fatal and debilitating.

Stroke may be sub-divided into two major types: ischemic and hemorrhagic. More than eighty percent of stroke cases are ischemic [4,5]. Due to the resulting decreased blood flow, nutrient supply to specific areas of the brain is reduced or totally abolished [6]. Immediately after the occurrence of ischemia, activation of glutamate receptors initiates an ischemic cascade contributing to the damage that occurs in the early stages of cerebral ischemia [7]. In the ischemic core, neurons suffer metabolic stress and cellular death, while surrounding the ischemic core a structurally intact region known as the ischemic penumbra is formed. Therefore, immediate medical treatment is critical for attenuating further irreversible damage in the penumbra zone [8,9]. In more progressed stages, following the occurrence of ischemia, the tissue damage continues to evolve, further aggravated by inflammation and apoptosis [9]. After ischemia onset, blood-derived leukocytes and microglia are activated within the brain [9]. In an effort to repair the damaged tissue, an inflammatory response takes place. However, in cases where this response is uncontrolled, it can lead to inflammation-induced injury [9].

A pivotal enzyme in the inflammatory cascade is prostaglandin-endoperoxide synthase, also known as cyclooxygenase (COX). COX produces prostaglandins (PGs) from arachidonic acid [9,10]. There are two functionally important isoforms of COX, each regulated and acting in distinct manners: COX-1 and COX-2. COX-1 is an enzyme whose activity is mostly associated with normal physiological functions such as protection of the stomach mucosa and preservation of kidney function. The activity of COX-2, an enzyme generally undetectable in most tissues, is linked predominantly with pathological conditions such as inflammation, pain and cancer [9–14]. However, it is important to emphasize that although COX-1 is generally regarded as a "house-keeping" enzyme responsible for *physiological* functions, it is also recognized as contributing to certain *pathological* processes including inflammation in select body tissues such as the brain [15–18]. Moreover, the generally accepted notion that inhibition of COX-2 results in a potent *anti*-inflammatory effect is not necessarily true under specific conditions; sometimes it can conversely lead to a *pro*-inflammatory response [19].

Most previous studies which tested the role of the COX-PGs pathway in the pathophysiology and treatment of ischemic stroke focused on COX-2 (inhibition), however, there is accumulating evidence suggesting that COX-1 also contributes to the post-ischemic inflammatory process [15,16,20,21]. The precise role of COX-1 in the post-ischemic stroke processes remains enigmatic [15,16]. A study on post-mortem brains of post-stroke patients revealed that COX-1 contributes to the post-ischemic inflammatory process [15]. This study showed a signifi- cant accumulation of COX-1-expressing microglial cells/macrophages and endothelial cells during brain injury in the necrotic core and in the peri-necrotic areas [15]. Candelario-Jalil et al. [16] also reported that both COX-1 and COX-2 are involved in the post-ischemic brain damage in gerbils.

As mentioned, inflammation contributes to the neuronal damage that occurs in post-stroke brain. Therefore, the primary objective of this study was to examine the effects of a selective COX-1 inhibitor – mofezolac – on clinical outcomes and brain inflammatory markers in post-ischemic stroke rats. Mofezolac was reported to show anti-neuro- inflammatory effects [22]. To the best of our knowledge, the present study is the first that examined the efficacy of mofezolac in post-ischemic stroke animals. We particularly focused on determining the levels of interleukin (IL)-6, prostaglandin (PG)E2 and tumor necrosis factor (TNF)-α, because several studies revealed that they are *increased* in post-ischemic stroke animals [23–26]. The levels of these inflamma- tory mediators were examined in three brain regions – the frontal cortex (FC), hippocampus (HC) and hypothalamus (HT). This is because the FC, HC and HT are repetitively linked to the pathophysiology of ischemic stroke [23–25,27] as well as other neurological disorders [28–30].

2. Materials and methods

2.1. Animals

Male and female Sprague-Dawley rats were used throughout the studies. The animals were housed 3 per cage and maintained under controlled environmental conditions (ambient temperature 22 ± 1 °C, relative humidity 55–58 %, and photoperiod cycle 12 h light: 12 h dark), fed Purina Lab Chow and water *ad libitum*. Only animals with no overt pathology were included in this study. The procedures of the study were in accordance with the guidelines of the Committee for the Use and Care of Laboratory Animals in Ben-Gurion University of the Negev, Israel (Authorization number – IL-58—08-2019(E)).

2.2. Mofezolac safety/toxicity study

Prior to conduction of efficacy experiments in post-stroke rats, the mofezolac safety/toxicity profile in control (non-stroke) male and female rats was examined. Mofezolac effects were determined based on basic biochemical and clinical parameters, including blood cell count (red blood cells [RBC], hemoglobin [HB], white blood cells [WBC], platelets [PL]), plasma creatinine and urea levels, body weight (BW), gastric mucosal integrity, and mortality. Rats (8 rats per group – 4 males and 4 females) were treated intraperitoneally (*ip*) with mofezolac 1, 5, 10, 50, 100 or 500 mg/kg, once daily, for 8 days (during which BW was daily measured). Control animals were treated with the vehicle (dimethyl sulfoxide [DMSO], 0.1 mL/rat). At the end of mofezolac treatment, rats were euthanized, blood was collected, and stomachs were extracted for assessment of gastric mucosal integrity. Blood cell count was determined in the *Biochemistry Laboratory* in Soroka Univer- sity Medical Center, Beer-Sheva, Israel. Evaluation of gastric mucosal integrity/damage was performed by an experienced investigator. Based on these results (see *Results* section), we decided to use a daily dose of 50 mg/kg mofezolac in the efficacy experiments.

2.3. Surgical procedure for inducing ischemic stroke

In this process of the experiment, we used only male rats because our preliminary testing revealed that post-surgical recovery time in female rats extended significantly longer as compared to males. This gender-inconsistency complicated the possibility of administering the treatment compound (after surgery) at the same time to all animals. Permanent mid cerebral artery occlusion (MCAO) was performed according to the method of Longa et al. [31] with slight modifications [23,32]. Briefly, rats were anesthetized with 75 mg/kg ketamine and 3 mg/kg midazolam (both given *ip*). Anesthetized rats were subjected to the surgical procedure which lasted 25—30 min during which they were able to spontaneously breathe. The right common carotid artery (CCA) was exposed through a middle neck incision and carefully dissected from surrounding tissues from its bifurcation to the base of the skull. The occipital artery branches of the external carotid artery (ECA) were then isolated, dissected, and coagulated. The ECA was further dissected distally and coagulated along with the terminal lingual and maxillary artery branches. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve and the pterygopalatine artery was ligated close to its origin with a 4—0 silk suture. The MCA was then blocked by inserting a 3.5 cm long 4—0 nylon silicon-coated filament 18.0–18.5 mm before the bifurcation of the CCA. The filament then extended into the circle of Willis, effectively occluding the MCA (a procedure hereafter referred to as MCAO). The ICA and CCA were temporarily blocked. Subsequently, a 4—0 silk suture was tied loosely around the CCA before its bifurcation. The silk suture around the CCA stump was fastened around the intraluminal filament to prevent bleeding. The filament was then fixed by tying up a silk suture over the CCA. The suture was left in place permanently. Sham-operated (control) rats were anesthetized and subjected only to a middle neck skin incision.

2.4. Mofezolac treatment in post-MCAO rats

Mofezolac was dissolved in DMSO at concentrations adjusted to animals' BW. The mofezolac solution was then filtered using 0.22 μm filters to produce a sterile solution. Rats were treated once daily with either mofezolac (50 mg/kg, *ip*) or DMSO (0.1 mL/rat, *ip*) for 14 days, beginning at the day of the surgical procedure (the first injection was given at 2 h after the surgery).

2.5. Measurement of body temperature

Rectal BT was determined using a plastic-coated thermocouple probe (HL 600 Thermometer, Anristu Meter Co., Japan).

2.6. Assessment of postsurgical neurological deficits and mortality

To confirm the accuracy of the MCAO procedure, animals were tested for the presence of neurological deficits (NDs) [32]. An experienced observer, who was blinded to the type of surgical procedure administered, examined each animal for visible NDs. Any of the following features were regarded as a ND: forelimb flexion; contralateral forelimb gripping weakly (the operator placed the rat on an absorbent pad and gently pulled the tail); circling to the paretic side only when pulled by the tail (the rat was allowed to move freely on the absorbent pad); and spontaneous circling. Rats in the MCAO group needed to present at least two of the ND features above in order to be included in this group. In contrast, rats in the sham group were excluded if they presented any visible ND. Mortality was monitored for 14 days following the surgical procedures.

2.7. Preparation of brain homogenates

At 14 days after the surgical procedures, surviving rats were anesthetized briefly (with a mixture of 4% isoflurane in 100 % oxygen) and immediately euthanized by decapitation. Brains were quickly extracted and washed in ice-cold 0.9 % saline. Their FCs, HTs and HCs were gently excised on ice, cleaned, and immediately transferred to – 80 °C. Each sample was then weighed and manually homogenized for 10 s in 500 μL of a cold phosphate-buffered saline solution containing protease/phosphatase inhibitors (homogenizing buffer). Subsequently, tissue homogenates were centrifuged at 10,000 rpm, 4 °C for 10 min. Supernatants were collected and immediately transferred to –80 °C for further analysis.

2.8. Determination of PGE2 and cytokines levels

Levels of PGE_2, IL-6 and TNF-α in brain samples (supernatants) were determined using specific ELISA kits according to the manufacturer's instructions (R&D Systems; Minneapolis, MN, USA). For samples in which the level of the detected mediator was below the lowest detection limit of the assay, results were marked as "below detection limit" and calculated as zero.

2.9. Statistical analysis and presentation of data

Initially, normality tests were performed to determine whether the examined variable distributed normally, using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Based on the results of the normality tests, appropriate statistical tests were chosen. For evaluation of changes in BT (Fig. 1), a paired-samples T test was used for in-group comparisons, whereas an independent-samples T test was used for between-group comparisons. For evaluation of differences in neurological score (Fig. 2), the Wilcoxon Signed Ranks test was used for in-group comparisons, and the Chi-square test was used for intergroup comparisons. The one-way ANOVA test was used for determining intergroup differences in brain cytokine levels (Fig. 3), followed by Student's T test. Differences in mortality rates were determined by using the Fisher exact test. Values of $P < 0.05$ were considered statistically significant. Results in figures of IL-6, PGE_2, and TNF-α were calculated as follows: ELISA result (pg/mL) divided by sample weight in milligrams. Results are presented as pg/mg wet weight.

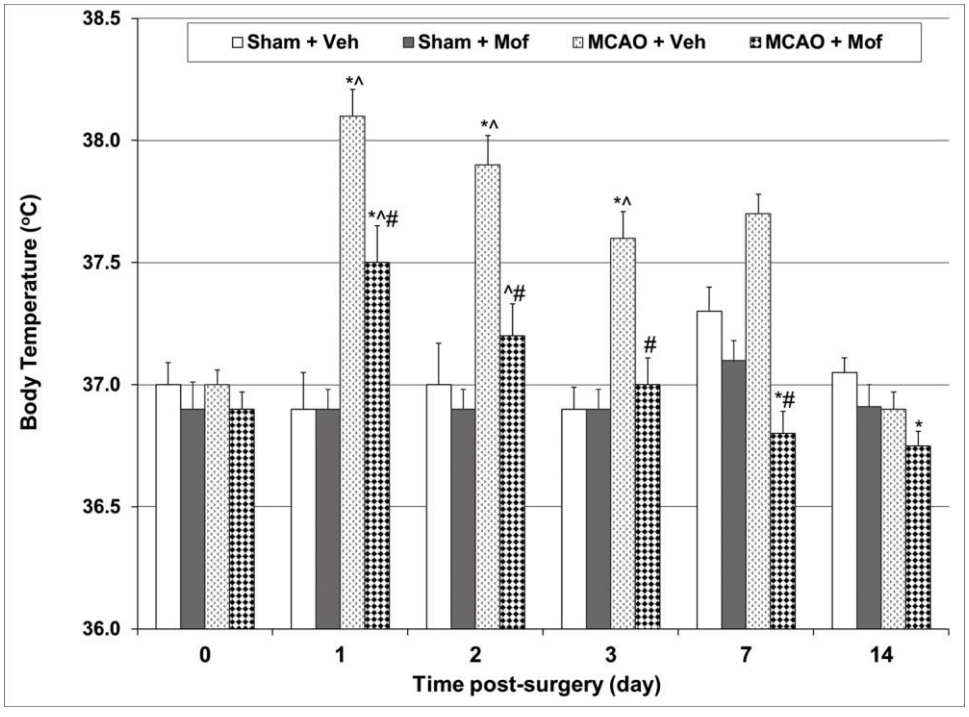

Fig. 1. Effects of Mofezolac on BT of Post-stroke Rats. BT was measured before (time zero) and at 1, 2, 3, 7 and 14 days post-surgery. Each column is the mean ± SEM of 12 to 24 rats per group, at the different measurement time- points. Paired-samples T-test: § $P < 0.05$ *vs.* same group at time zero. Independent-samples T-test: * $P < 0.05$ *vs.* sham + vehicle; ^ $P < 0.05$ *vs.* sham + mofezolac; # $P < 0.05$ *vs.* MCAO + vehicle – between-group comparisons for the same time-point. Abbreviations: MCAO, middle cerebral artery occlusion; Mof, mofezolac; Veh, vehicle.

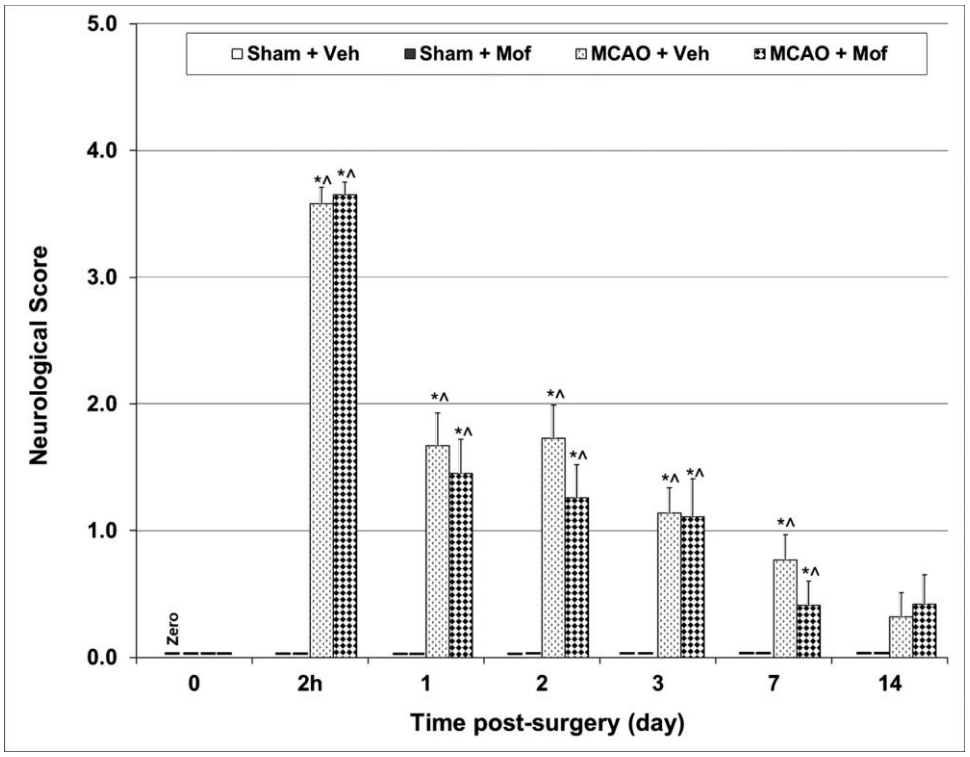

Fig. 2. Effects of Mofezolac on NS of Post-stroke Rats. NS was assessed before (time zero), at 2 h, and at 1, 2, 3, 7 and 14 days post-surgery. Each column is the mean ± SEM of 12 to 24 rats per group, at the different measurement time-points. Wilcoxon Signed Ranks test: § $P < 0.05$ *vs.* same group at time zero. Chi- square test: * $P < 0.05$ *vs.* sham + vehicle; ^ $P < 0.05$ *vs.* sham + mofezolac – between- group comparisons for the same time-point. Abbreviations: MCAO, middle cerebral artery occlusion; Mof, mofezolac; NS, neurological score; Veh, vehicle.

3. Results

3.1. Safety/toxicity experiment

First, we examined the effects of mofezolac treatment at 1, 5, 10, 50, 100 or 500 mg/kg (7–8 rats per group) on general appearance, BW and mortality of naïve rats. We found that treatment with either vehicle (control) or mofezolac 1, 5, 10, 50 or 100 mg/kg for 8 days did not cause any visible alteration in animals' external appearance, did not cause significant changes in rats' BW, and did not impact mortality (100 %

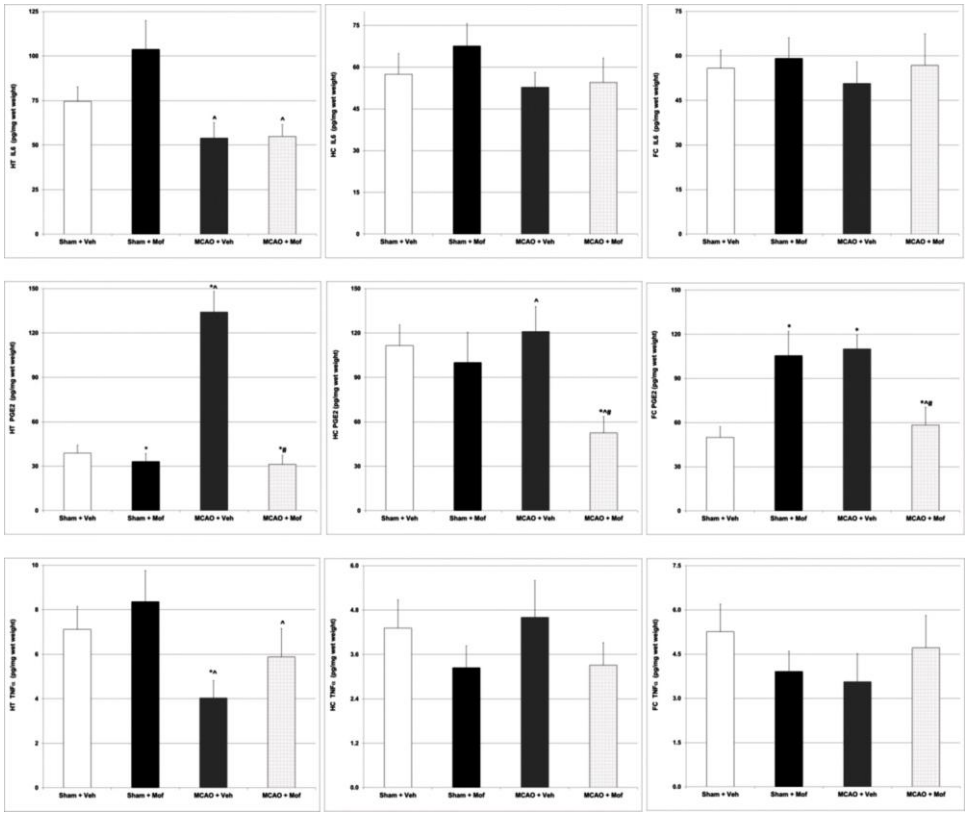

Fig. 3. Effects of Mofezolac on levels of IL-6, PGE2 and TNF-α in Brain of Post-stroke Rats. Brain regions were extracted as described in "*Materials and Methods*". IL-6 (A, B, C), PGE2 (D, E, F) and TNF- α (G, H, I) levels in HT (A, D, G), HC (B, E, H) and FC (C, F, I) were determined by ELISA at 14 days post-surgery. Each column is the mean ± SEM of 12 to 14 samples per group. One-way ANOVA test: (A) [$F(345) = 4.621$, $P = 0.007$], (B) [$F(348) = 0.789$, $P = 0.506$], (C) [$F(346) = 0.218$, $P = 0.884$], (D) [$F(348) = 32.763$, $P = 0.00001$], (E) [$F(349) = 3.396$, $P = 0.025$], (F) [$F(349) = 7.366$, $P = 0.00001$], (G) [$F(347) = 2.753$, $P = 0.0529$], (H) [$F(349) = 0.788$, $P = 0.506$], (I) [$F(349) = 0.710$, $P = 0.551$]. Student's T-test: * $P < 0.05$ *vs.* Sham + Veh; ^ $P < 0.05$ *vs.* Sham + Mof; # $P < 0.05$ *vs.* MCAO + Veh – between-group comparisons. Abbreviations: MCAO, middle cerebral artery occlusion; Mof, mofezolac; Veh, vehicle.

survival, data not shown). In contrast, mofezolac 500 mg/kg had a highly toxic affect and resulted in 100 % mortality. The mofezolac 500 mg/kg-treated animals began to appear sick on the first day of treatment and mostly died within 2–3 days after commencement of treatment.

Treatment with mofezolac 1, 5, 10, 50 and 100 mg/kg did not significantly alter blood cell count or plasma creatinine and urea levels (Table 1).

Moreover, treatment with mofezolac 1, 5, 10, 50 and 100 mg/kg did not cause clinically significant gastric mucosal damage (Table 2). Rats in all groups did not have visible gastric ulcers or bleeding.

3.2. *Efficacy experiments*

3.2.1. *Effects of mofezolac on BT of post-stroke rats*

Before surgery, BT did not significantly differ between any of the treatment groups. Post-stroke rats had a significantly higher BT during the 3 days after surgery, as compared to sham-operated rats (Fig. 1). Treatment with mofezolac (50 mg/kg) significantly decreased BT in post-stroke rats in comparison to those vehicle-treated at most measurement time-points after surgery.

3.2.2. *Effects of mofezolac on post-stroke neurological deficits*

Before surgery, rats did not have any visible NDs. At 2 h after surgery, none of the sham-operated rats exhibited any visible NDs and were assigned a score of zero (Fig. 2). Contrarily, all rats that underwent MCAO surgery had at least two visible NDs (Fig. 2). Furthermore, at 2 h post-surgery, the NS was very similar in the two MCAO-operated rats. Treatment with mofezolac (50 mg/kg) did not significantly alter the NS at any measurement time-points (Fig. 2).

3.2.3. *Effects of mofezolac on mortality of post-stroke rats*

Mortality was followed for 14 days after surgery. Animals that died *during* the surgical procedure were not counted as part of the MCAO groups. Out of the 47 rats that underwent MCAO surgery in the two experimental stages of the study, one died during the surgical procedure (2% perioperative mortality). Thus, mortality was assessed in the remaining 46 MCAO rats. Five out of 24 vehicle-treated MCAO rats died during the 14-days of follow-up (20.8 % mortality, Table 3). Eleven out of 23 MCAO-mofezolac-treated rats died during the 14 day follow-up period (47.8 % mortality, Table 3). These results indicate that treatment with mofezolac non-significantly (*P* value = 0.184) increased the rate of post-stroke mortality. As for the sham-operated groups, two out of 15 mofezolac-treated rats died during the 14-days of follow up, while in the vehicle-treated group all the rats survived (Table 3, *P* value = 0.485 for difference between the groups).

3.2.4. *Effects of mofezolac on levels of brain inflammatory mediators in post-stroke rats*

There were no significant differences in IL-6 and TNF-α levels between any of the experimental groups in the HC and FC (Fig. 3B, C, H, I).

Treatment with mofezolac (50 mg/kg) altered the levels of IL-6 and TNF- α in these brain regions. In the HT, levels of IL-6 and TNF- α were generally lower in post-stroke subjects as compared to those who were sham-operated (Figure 4A, G). PGE_2 levels were significantly higher in post-stroke rats than in sham-operated rats both in the HT and FC (Fig. 3D, F). Treatment with mofezolac significantly decreased PGE_2 levels in post-stroke rats in all tested brain regions (Fig. 3D, E, F). Of note, the prominent decrease in PGE_2 levels in the HT of post-stroke rats (Fig. 3D) may explain the significant attenuation of post-stroke fever in these animals (Fig. 3).

4. Discussion

Table 2
Dose-dependent Effects of Mofezolac on Gastric Mucosal Integrity/Damage. Naïve rats were treated with vehicle (DMSO 0.1 mL/rat) or mofezolac 1, 5, 10, 50 or 100 mg/kg for eight days through a single daily *ip* injection. Then, rats were euthanized, and stomachs were ousted for evaluating gastric mucosal integrity/damage.

Gastric Mucosa					
		Gastritis			Group (n = 8)
Active Bleeding	Ulcer	Severe	Mild-Moderate *	Normal	
–	–	–	1	7	**Control**
–	–	–	1	7	**1 mg/kg**
–	–	–	2	6	**5 mg/kg**
–	–	–	1	7	**10 mg/kg**
–	–	–	1	7	**50 mg/kg**
–	–	–	2	5	**100 mg/kg****

* No significant differences between the groups (*Fisher Exact* test).
** n = 7.

deficits in rats that were subjected to an ischemic stroke. On the other hand, mofezolac significantly attenuated the elevated BT (fever) in post-stroke rats.

Despite being a selective COX-1 inhibitor, mofezolac significantly attenuated the elevated BT in post-stroke rats (Fig. 1). This is consistent with the fact that a medication such as naproxen, which is clearly a COX-1 preferential inhibitor [33,34], also exerts antipyretic effects [34–36]. It is likely that the decrease in post-stroke fever was derived from the prominent reduction in HT PGE_2 levels in these animals (Fig. 3). The prominent reduction in PGE_2 levels in the HT (as well as in FC and HC) by mofezolac seems to result mainly from inhibition of COX-1 within the brain. Consistent with these findings, mofezolac has been shown to inhibit neuro-inflammation *in-vivo* [22]. Moreover, a previous *in vitro* study suggested that mofezolac presents a low BBB permeability [37].

Fever has been repetitively associated with the pathophysiology and prognosis of post-ischemic stroke patients [38–40]. The mechanism underlying post-stroke fever is not fully understood. Several studies reported different findings that may explain the pathogenesis of post-stroke fever; however, none of these hypotheses are beyond doubt [41–43]. It is not clear if post-stroke fever is derived from over-production of PGs in the brain. In contrast, lipopolysaccharide (LPS)-induced fever is associated with increased PGE2 production in the brain, especially through induction of COX-2 expression [44–46], whereas COX-1 seems to contribute only to LPS-induced hypothermia (a short-term decline in BT that occurs after systemic administration of LPS) [46]. As for stroke-induced fever, a previous study showed that

Table 3
Effects of Mofezolac on Cumulative Mortality of Post-stroke Rats. Mortality was monitored for 14 days post-surgery. Shown are the cumulative numbers of dead animals at each time-point and the rate (percentage) of death in parenthesis.

	Sham + Veh, number dead (%) N at baseline	Sham + Mof, number dead (%)	MCAO + Veh, number dead (%)	MCAO + Mof, number dead (%)
Time-point (day)	15	15	24	23
0	0 (0)	0 (0)	0 (0)	0 (0)
2	0 (0)	0 (0)	2 (8.3)	4 (17.4)
4	0 (0)	0 (0)	2 (8.3)	4 (17.4)
6	0 (0)	0 (0)	2 (8.3)	4 (17.4)
8	0 (0)	0 (0)	2 (8.3)	8 (34.8)
10	0 (0)	0 (0)	4 (16.7)	9 (39.1)
12	0 (0)	1 (6.7)	4 (16.7)	9 (39.1)
14	0 (0)	2 (13.3)	5 (20.8)	11 (47.8)

The present study demonstrated that treatment with the selective COX-1 inhibitor mofezolac did not reduce mortality and neurological

Abbreviations: MCAO, middle cerebral artery occlusion; Mof, mofezolac; Veh, vehicle.

Table 1
Dose-dependent Effects of Mofezolac on Blood Cell Count, Plasma Creatinine and Urea Levels. Naïve rats were treated with vehicle (DMSO 0.1 mL/rat) or mofezolac 1, 5, 10, 50 or 100 mg/kg for eight days through a single daily *ip* injection. Then, rats were euthanized, and blood was collected for determination of blood cell count and renal function parameters. Results are presented as mean ± SEM of 8 rats per group.

	Control	1 mg/kg Mofezolac	5 mg/kg Mofezolac	10 mg/kg Mofezolac	50 mg/kg Mofezolac	100 mg/kg Mofezolac
RBC [10^3 µL	7.89 ± 0.12	7.75 ± 0.34	7.93 ± 0.98	7.92 ± 0.16	7.83 ± 0.14	7.61 ± 0.17
HB [gr/dl]	15.24 ± 0.2	15.11 ± 0.59	15.64 ± 0.31	15.26 ± 0.25	15.14 ± 0.16	15.09 ± 0.41
WBC [10^6 µL	11.11 ± 0.89	11.24 ± 1.07	11.70 ± 0.98	11.03 ± 1.07	11.41 ± 0.81	12.70 ± 1.82
PL [10^3 µL	997.9 ± 86.2	833.9 ± 110.4	971.6 ± 85.1	818.7 ± 115.5	992.3 ± 69.5	936.0 ± 243.9
Creatinine [mg\dL]	0.24 ± 0.01	0.22 ± 0.01	0.23 ± 0.02	0.22 ± 0.01	0.21 ± 0.01	0.20 ± 0.01
Urea [mg\dL]	51.11 ± 1.82	48.6 ± 3.02	51.7 ± 2.17	49.69 ± 1.80	49.09 ± 3.15	50.87 ± 3.34

Abbreviations: RBC, red blood cells; HB, hemoglobin; WBC, white blood cells; PLC, platelets cells. Based on these results, we decided to use a daily dose of 50 mg/kg mofezolac in the efficacy experiments.

acute treatment with lithium – a mood stabilizer – did not alter post-stroke fever in rats despite causing a prominent reduction in HT PGE2 levels [23]. This suggests that an increase in HT PGE2 levels is not the sole factor which leads to post-stroke fever. Consistently, aspirin, a NSAID that inhibits PGE2 production, did not reduce post-MCAO fever in rats [47]. Therefore, it is possible that the reduction in post-stroke fever by mofezolac (Fig. 1) occurred either due to inhibition of PGE2 production in the FC and HC (Fig. 3), or through a COX-1-PGE2-independent mechanism.

The pathophysiology of stroke comprises a prominent inflammatory response, particularly in the post-ischemic zone. The ischemic cascade leads to dysregulation of brain function and homeostasis due to a profound oxidative stress and subsequent inflammation, both of which leading to cell death and aggravation of tissue damage [6–9]. An integral enzyme in the inflammatory cascade is COX. In the brain, COX-1 and COX-2 are constitutively expressed in particular brain regions such as the HT and blood vessels, respectively [48]. Most previous studies that tested the role of the COX-PGs pathway in ischemic stroke settings and focused on COX-2 inhibition revealed inconsistent results. COX-1 also seems to contribute to the post-ischemic inflammatory process [15,16]. Therefore, it was interesting to determine the efficacy of a highly selective COX-1 inhibitor such as mofezolac [49] in an established animal model of ischemic stroke. As a nonsteroidal anti-inflammatory drug that is used for the treatment of various inflammatory-associated conditions, including neuro-inflammation [22,50], we hypothesized that mofezolac may attenuate post-stroke morbidity and mortality due to its anti-inflammatory effects. However, under the experimental conditions used in the present study, mofezolac caused a modest anti-inflammatory effect in the brain of post-stroke animals reducing PGE_2 levels, but, generally, not affecting IL-6 and TNF-α levels (Fig. 3).

In ischemic stroke, the resultant brain tissue damage manifests in various pathological features and deficits. We examined the effects of mofezolac on post-surgical NDs (as evaluated by assessing the NS). All rats that underwent MCAO surgery and survived had prominent visible NDs after surgery (Fig. 2). The severity of NDs gradually decreased during follow-up time. Mofezolac treatment did not influence the severity of NDs at any measurement time-points (Fig. 2). Furthermore, mofezolac did not significantly ($P = 0.184$) reduce the cumulative mortality in post-stroke rats (Table 3). However, in contrast to our prediction, mofezolac treatment led to a mild, non-significant increase in cumulative mortality (~ 48 % *versus* ~ 21 % mortality in vehicle-treated MCAO-operated rats, Table 3). The exact reasoning behind this result is unclear. It is possible that it is a coincidental occurrence. However, it is also conceivable that mofezolac altered the function of the immune system in a way that it harmed the body's defense mechanisms that are important for protecting the brain from the ischemic damage [8]. Of note, mofezolac did not cause gastric damage, similarly to P6, another highly selective COX-1 inhibitor [14]. Collectively, these results suggest that mofezolac does not have prominent beneficial effects in post-ischemic stroke animals, and that inhibition of COX-1 is not an effective therapeutic strategy for this clinical condition.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Authors' contribution

I.S.R. conducted the *in-vivo* experiments in rats, contributed a major part to the biochemical analysis of the samples, and participated in the analysis of the data and writing of the manuscript. M.B. participated in the design of the study, performed the surgical procedures in sham and stroke-operated rats, participated in the analysis of the data and writing of the manuscript. S.F. participated in the design of the study and contributed a major part to the chemical synthesis of the experimental drug (mofezolac). A.S. participated in the design of the study, contributed to the chemical synthesis of mofezolac, and participated in the analysis of the data and writing of the manuscript. M.G.P. participated in the design of the study, contributed to the chemical synthesis of mofezolac, and participated in the analysis of the data and writing of the manuscript. J.K. participated in the design of the study, assisted in the *in-vivo* experiments, and participated in the analysis of the data and writing of the manuscript. A.Z. participated in the design of the study, served as an advisor regarding the conduction of the surgical procedures, and helped in the analysis of the data and writing of the manuscript. A.N.A. designed all study protocols and coordinated all study procedures including the *in-vivo* experiments and biochemical analysis of the samples, analysis of the data and writing the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgement

The authors would like to thank Dr. Muhammad Abu Tailakh for his assistance in conducting the statistical analysis of the data.

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