Physiological and Biochemical Changes in NRF2 Pathway in Aged Animals Subjected to Brain Injury

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Key Words
Nrf2 • Oxidative stress • Aging • Inflammation • Brain injuries

Abstract
Background/Aims: Oxidative stress plays a key role in aging, which in turn represents a substantial risk factor for brain injuries. The aim of the present study was to investigate the differences in physiological and biochemical changes in the brain during injury-related inflammation and oxidative stress, comparing young and old mice. Methods: Young and old mice were subjected to focal cerebral ischemia induced by transient middle cerebral artery occlusion or to traumatic brain injury performed by a controlled cortical impactor. At the end of both experiments, mice were sacrificed 24h after injuries and brains were collected to perform biochemical analysis. Results: In both ischemic stroke and traumatic brain injury, aging has not only led to damage-induced worsening of motor function and behavioural changes but also increased of infarct area compared to young animals. Moreover, aged mice show increased evidence of oxidative stress and reduced antioxidant capacity when compared to younger animals, as demonstrated by Nrf2-Keap1 signalling pathway and lower expression of antioxidant enzymes, such as HO-1, SOD-1 and GSH-Px. Additionally, brain tissues collected from elderly mice showed an increased IκB-α degradation into the cytoplasm and consequently NF-κB translocation into the nucleus, compared to young mice subjected to same injuries. The elderly mice showed significantly higher levels of iNOS and CoX-2 expression than the young mice, as well as higher levels of inflammatory cytokines such as TNF-α and IL-6.

M. Cordaro and R. D’Amico contributed equally to the research.
as TNFα, IL-1β, and IL-6 after MCAO and TBI. **Conclusion:** Preserving and keeping the NRF-2 pathway active counteracts the onset of oxidative stress and consequent inflammation after ischemic and traumatic brain insult, particularly in the elderly. Not only that, NRF-2 pathway could represent a possible therapeutic target in the management of brain injuries.

**Introduction**

Nrf2 (nuclear erythroid 2-related factor 2) is a basic region leucine-zipper transcription factor, that represent a key molecule regulating the cellular antioxidant response [1]. Under physiological conditions, Nrf2 is located into the cytoplasm bound to its negative regulator, Kelch-like ECH associating protein 1 (Keap1). However, upon exposure to reactive oxygen species (ROS), Nrf2 is released from the Keap1-Nrf2 complex and translocates to the nucleus, where it sequentially binds to the antioxidant response element (ARE), a regulatory enhancer region within gene promoters. This binding induces the production of several detoxifying and antioxidant enzyme genes such as heme oxygenase 1 (HO-1), SOD-1 and glutathione peroxidase 1 (GPx1), which protect cells from oxidative stress and a broad range of other toxins [2, 3]. Moderate or low amounts ROS are neutralized by the antioxidant system and, in this manner, tissues effectively regulate its oxygen consumption and redox generation capacity. The endogenous antioxidant system (i.e., glutathione peroxidase, superoxide dismutase, catalase, and uric acid) aims to convert/neutralize ROS to less toxic derivatives [4]. When production of ROS exceeds scavenging capacity of antioxidant response system, extensive lipid peroxidation and protein oxidation occurs, causing oxidative damage, cellular degeneration, and even functional decline [5]. ROS also interact with nuclear factor κB (NF-κB) that is known to be activated by the redox state of the cell in a number of disease pathologies. Such activation can be inhibited by the use of antioxidants [6]. Additional downstream gene targets of NF-κB also include inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and cytokines which are involved in a number of detrimental pathways such as apoptosis and inflammation [7-9].

As such, it is not surprising that Nrf2 dysfunction is a key feature of a wide variety of pathologies, including aging and age-related diseases. Traditionally, aging is associated with a gradual decrease in cell signaling, increased protein dysfunction/misfolding/aggregation, and an increased risk of cell death. In aging, oxidant production from various sources is increased while antioxidant enzymes, the primary lines of defense, are decreased. Repair systems, including the proteasomal degradation of damaged proteins also declines. With advancing age, particularly as organisms become frail, susceptibility to oxidants and other toxicants increases [10]. Importantly, the adaptive response to oxidative stress declines with aging. Accumulating data suggest that this age-dependent decline in the antioxidant enzyme response is caused by declining efficiency of Nrf2/ARE signaling. Understanding accurately how aging increases the risk of disease can help better address this growing problem. Aging is a complex process associated with structural and physiological changes, in particular in the brain that can account for age-related behavioral changes and increased incidence of neuropsychiatric disorders.

It is clear that ROS play an important pathophysiological role and that their accumulation increases the susceptibility of tissues to injury, in particular of brain tissue. The brain becomes an easy target of excessive oxidative insult due its rich lipid content, high energy demand and weak antioxidant capacity [11]. Indeed, phospholipids in the brain are especially vulnerable to peroxidation mediated by ROS, but proteins and DNA also are targeted by ROS.

Recently, many evidences have demonstrated that activation of the Nrf2-ARE pathway is able to protect the brain from oxidative stress in both *in vitro* and *in vivo* models of neurodegenerative diseases, stroke and traumatic brain injury (TBI) [12-16]. Nevertheless, until today, no one have compared the variation in Nrf-2 expression in young and aged mice subjected to brain injury. Over the past several decades, several experimental models have been implemented to study the mechanisms of stroke and TBI in rodents [17-20]. These
animal models have been well defined with predictable histological, neurological and physiological alterations similar to those observed in clinical brain injury and help us to determine the underlying mechanisms of acute injury and establish treatment strategies. For these reasons, we evaluated and compared the possible physiological and biochemical changes related to inflammation and oxidative stress injury in brain of young and aged mice.

**Materials and Methods**

**Animals**

Young CD1 mice (male, 8 week old, 18-24g) and old mice (male, 24 month old, 25-30g) were acquired from Envigo (Milan, Italy) and posted in a controlled environment. The University of Messina Review Board for animal care (OPBA) approved the study (protocol number n° 617/2017-PR dated 8/2/2017). All animal experiments were in compliance with the new Italian regulations (D.Lgs 2014/26), the EU regulations (EU Directive 2010/63) and the ARRIVE guidelines.

**Middle Cerebral Artery Occlusion (MCAo)**

Focal cerebral ischemia was performed by transient MCAo in the right hemisphere. The mice were anesthetized with inhaled 1.0–2.0% isoflurane and 5.0% isoflurane (Baxter International) in air by a mask. Body temperature was conserved at 37°C with a heating pad. MCAo was provided by introducing a 6–0 nylon monofilament (Ethilon; Johnson & Johnson, Somerville, NJ, USA), precoated with silicone (Xantopren; Heraeus Kulzer; Germany) via the external carotid artery into the internal carotid artery to occlude the MCA. The thread was prudently withdrawn 60 min after MCAo to induce I/R injury. At the end of the procedure, anaesthesia was discontinued, and the mice were returned to a prone position. Laser Doppler flowmetry (PeriFlux System 5000; Perimed AB, Stockholm, Sweden) with a flexible probe over the skull was used to monitor regional cerebral blood flow (rCBF), as previously described [21].

**Traumatic brain injury (TBI)**

Animals were anesthetized with an injection of ketamine and xylazine (2.6 and 0.16 mg/kg body weight, respectively, intraperitoneal). TBI was induced by a controlled cortical impactor (CCI) as described by Gugliandolo et al. [22]. Briefly, a craniotomy was made between the sagittal suture and the coronal ridge of the right hemisphere, using a Micro motor hand piece and drill. A cortical contusion was produced on the exposed cortex using the controlled impactor device Impact OneTM Stereotaxic impactor for CCI (Leica, Milan, Italy) [17]. Subsequently, the skin incision was sutured, and 2% lidocaine jelly was applied to the lesion to minimize any possible discomfort.

**Experimental groups**

For MCAo model, mice were randomized into several groups of 10 mice each:

- **MCAo young group**: mice were subjected to MCAo as described above;
- **MCAo old group**: mice were subjected to MCAo as described above;
- **Sham young group**: mice were subjected to the same procedure, but the filament was introduced into the internal carotid artery and suddenly withdrawn;
- **Sham old group**: mice were subjected to the same procedure, but the filament was introduced into the internal carotid artery and suddenly withdrawn.

For TBI model, all animals were randomized in the indicated groups of 10 mice each:

- **TBI young group**: mice were subjected to CCI as describe above;
- **TBI old group**: mice were subjected to CCI as describe above;
- **Sham young group**: mice were subjected to the surgical procedures as above (anaesthesia and craniotomy) except that the impact was not applied;
- **Sham old group**: mice were subjected to the surgical procedures as above (anaesthesia and craniotomy) except that the impact was not applied.

At the end of both experiments, mice were sacrificed 24h after MCAo and TBI and brains were collected to perform biochemical analysis.
Behavioural testing

In a separate set of experiments, 5 additional animals for each group of both models were used for behavioural testing. The mice were placed in behaviour rooms 5 min for 2 days for acclimation prior to the start of behavioural testing. The behavioural tests were conducted by expert observers blinded to the injury status of the mice. Tests are described below:

**Morris water maze (MWM) test**

MWM test was used to evaluate hippocampal-dependent spatial learning and memory function [23, 24]. The MWM test was conducted as previously described [17]. The device was a sink of stainless steel, 50 cm in height and 100 cm in diameter, containing four quadrants. In the centre of the platform quadrant was a circular platform with a height of 27 cm and a diameter of 9 cm, and the position did not change throughout the experiment. To make the water opaque, milk was added and temperature was kept at 23°C. On the first day, a visual platform experiment was performed. The platform was placed 1 cm above the water surface. Each animal was allowed to swim for 2 minutes for acclimation. During the following 2–5 days, the navigation experiment was performed. The platform was placed 1 cm under water surface. The mouse was located into the water in each of the three different quadrants and allowed to swim for 1 minute each time. When the mouse found the platform remained there for 5 seconds, or the 1-minute test time lapsed. When the animal did not find the platform, it was guided to the platform and allowed to stay there for 15 seconds. One day after the navigation experiment, the platform was removed for the test. The mouse was located in the water in the same quadrant. The latency to find the location of the platform and the time spent in the target quadrant were recorded.

**Novel Object Recognition (NOR) Test**

The NOR test evaluates cognition, particularly recognition memory [25]. The spontaneous inclination of mice to spend time investigating a novel object or a familiar one was examined. The experiment was conducted as previously described and performed in a black empty box in a quiet environment [26]. The mouse was replaced in the box, and its behaviour was observed for 10 min. The total time the mouse spent exploring each object was recorded. The exploration time comprised the distance between the object and the nose tip when the mouse sniffed the object from less than 2 cm, and the times the front paw or nose directly touched the object. Walking near the object was not considered exploratory behaviour. A solution of 90% ethanol was used to eliminate odours between different animals (to avoid olfactory cues from affecting the exploratory behaviour of other animals).

**Social Interaction Test**

The social interaction test was performed using a three-chambered apparatus (polycarbonate 80 cm x 31.5 cm) divided into three compartments. It consisted of three trials of ten minutes. Initially, a mouse was acclimated in an empty arena for 5 min. In the second phase, the experimental mouse was exposed to an object, one of the empty wired cages and a wired cage covering a stimulus mouse. The frequency of the investigatory behaviour with the novel mouse was recorded [27, 28].

**Elevated Plus Maze (EPM)**

The EPM test was used to measure anxiety-like behaviour, as described previously [29, 30]. The apparatus of the EPM consisted of a cross-shaped maze elevated above the floor with 2 enclosed arms (protecting), 2 open arms (stressful), and a centre area. Mice were positioned in the centre of the apparatus facing an open arm and behaviour was videotaped with a camera fixed above the EPM. The percentage of time spent on the open arms was scored by a researcher blind of the animal treatment. Anxiety was indicated by a decrease in the proportion of time spent in the open arms and a decrease in the proportion of entries into the open arms.

**Open Field**

The Open Field Test was used for evaluating locomotor activity [17, 31]. The apparatus consisted of a Plexiglas box 50 cm x 50 cm with its floor separated into 16 squares. The centre was defined by four squares and the squares along the wall defined the periphery. During the test, the animal was located in the centre of the box, and the movement of the mouse was observed for 5 min. The movement was scored as a line
crossing when a mouse removed all paws from one square and entered another. The number of crossings and the time spent in the centre were calculated and scored.

**TTC staining**

The 2,3,5-triphenyltetrazolium chloride (TTC) staining technique was performed as previously described [32]. Briefly, the brains were cut into coronal slices of 2-mm thickness that were incubated in a 2% solution of TTC for 30 min at 37°C, processed, and quantified as previously described [33–35]. TTC stains the viable brain tissue red while infarcted tissue remains unstained. For quantification of infarcted area and volumes, the brain slices were photographed and then image analysis was performed on a personal computer with an image analysis software program using ImageJ. All analyses were carried out by two observers blinded to the treatment.

**Western Blot Analysis of Cytosolic and Nuclear Extracts**

Cytosolic and nuclear extracts were prepared as previously described [36, 37]. Brain tissues from each mouse were suspended in an extraction’s buffer containing 0.15 µM pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and 20 µM leupeptin, which was homogenized at the highest setting for 2 min and centrifuged at 1000 × g for 10 min at 4°C. Supernatants contain the cytosolic fractions, while the pellets represent the nuclear ones. Pellets were re-suspended in a second buffer containing 150 mM sodium chloride (NaCl), 1% Triton X-100, 1 mM ethylene glycol tetraacetic acid (EGTA), 10 mM trischloridric acid (HCl) pH 7.4, 0.2 mM PMSF, 1 mM Ethylenediaminetetraacetic acid (EDTA), 0.2 mM sodium orthovanadate, and 20 µm leupeptin. After centrifugation at 4°C and 15,000 × g for 30 min, the nuclear protein contained the supernatants were stored at −80°C for further analysis. The following primary antibodies were used: anti-NRF-2 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc-365949), anti-Heme Oxigenase 1 (HO-1; 1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc-136960), anti-Keap 1 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc-365626), anti-κB-α (1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc1643), anti-NF-κB p65 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc8008), anti-iNOS (inducible-Nitric oxide synthases; 1:500, BD transduction, San Jose, CA, USA), anti-COX-2 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc-376861) in 1× PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 at 4°C overnight. To ensure that blots were loaded with equal amounts of proteins, they were also probed with antibody against β-actin protein for cytosolic fraction (1:500; Santa Cruz Biotechnology) or lamin A/C for nuclear fraction (1:500 Sigma-Aldrich Corp., Milan, Italy). Signals were examined with an enhanced chemiluminescence (ECL) detection system reagent according to the manufacturer’s instructions (Thermo, Monza, Italy). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDocTM XRS + software and standardized to the β-actin and lamin A/C levels.

**Enzyme-linked immunosorbent assay (ELISA)**

The supernatant of homogenate of brain tissue was centrifuged and operated [38, 39]. The expression of TNF-α, IL-1β, IL-6, SOD-1, GSH-PX and MDA were measured using ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions. The absorbance value of each well was measured at 450 nm by a microplate reader.

**Materials**

Unless otherwise stated, all compounds were purchased from Sigma-Aldrich.

**Statistical evaluation**

All values are expressed as mean ± standard error of the mean (S.E.M.) of N observations. For in vivo studies, N represents the number of animals used. The results were analysed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A P value less than 0.05 was considered significant.
Results

Difference between young and old mice on spatial learning and memory function

First, we evaluated how aging negatively affects post-injury behaviours. In particular, to determine the impact of aging on memory and learning following MCAo and TBI, the MWM test was performed. The results revealed that, for both experimental models, the injury resulted in overall spatial learning deficits as demonstrated by increased latency to find the platform during the final training days compared to sham groups (Fig. 1A and E). In addition, we observed that young mice continued to show reduced latency time compared to aged mice, in particular after MCAo and TBI (Fig. 1A and E, respectively). Furthermore, spatial memory was measured in a probe test. During this test, the platform was removed and time spent in target quadrant of the MWM was recorded, in which more time spent in the target quadrant indicates better memory. Consistent with the above findings, we observed that ischemic mice, namely old mice, spent less time on the platform location and target quadrant after removing the platform (Fig. 1B). These findings indicated that MCAo impaired cognitive function in a significant manner in aged mice compared to young animals. Similar cognitive alterations were also observed in mice following TBI (Fig. 1F).

In order to evaluate impairments in their social interaction and exploring behaviour, important clinical features of dementia, we performed the NOR test and the social interaction test. Rodents have the natural habit of exploring new objects and interacting with other mice. In line with this, we did not see any difference in sham animals in both experiments (Fig. 1C and G). In the NOR test, we observed that exploration time spent on the novel or familiar objects was not statistically different across experimental groups after MCAo or TBI (Fig. 1C and G, respectively), indicating compromise of cognitive function, while sham mice had more exploratory behaviour and spent more time with the novel object. At the same way, in the social interaction test, we observed that the number of contacts was significantly decreased in the MCAo old and TBI old groups compared to the MCAo young and TBI young groups (Fig. 1D and H). These findings suggest that older mice have a greater predisposition to develop dementia-like symptoms.

Difference between young and old mice on post-injury anxiety and locomotor activity

Mice were subjected to the EPM test to evaluate post-injury anxiety and risk-taking behaviours. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behaviour, as demonstrated in sham animals. After MCAo, aged mice showed a significant decrease in duration and entries in open arms compared to MCAo young group (Fig. 2A, B). Similar results were also observed in mice following CCI (Fig. 2E, F).

To further assess the locomotor activity in young and old mice, the Open Field test was performed. We found that the number of crossings is higher in young mice than in aged animals (Fig. 2C and G). The number of crossing decreased after MCAo and TBI injuries, but in a significant manner in old mice compared to young group (Fig. 2C and G). At the same way, MCAo old group and TBI old group exhibited a decreased time spent in the centre compared to younger mice (Fig. 3D and H).

Difference between young and old mice on injury volume

TTC staining offers a rapid method in quantification of infarction volume after MCAo [40]. We found that 24 h after ischemia/reperfusion, the animals showed a large area of infarct affecting the striatum and cortex (Fig. 3C, D), unlike sham groups that showed no infarct area (Fig. 3A, B). However, young mice (Fig. 3C) had significantly less tissue damage 24 h after transient MCAo compared to elderly animals (Fig. 3D).

In the CCI brain injury model, TTC staining was used to determine the relationship between changes of infarction volume and impact strength [41]. Sham-control animals had no cortical lesion (Fig. 3E, F) that was detectable with TTC staining. Instead, the largest injury volume was found in aged mice compared to young mice (Fig. 3G, H).
Fig. 1. Behavioural assessment of spatial learning and memory function. Behavioural analysis after MCAo: Water Maze Test (training A and probe trial B), Novel object recognition test (C), Social Interaction Test (D). Behavioural analysis after TBI: Water Maze Test (training E and probe trial F), Novel object recognition test (G), Social Interaction Test (H). *p <0.05 vs sham young; **p <0.01 vs sham young; ***p <0.001 vs sham young; ###p <0.01 vs sham old; ####p <0.001 vs sham old; ° p <0.05 vs TBI young.
Fig. 2. Behavioural assessment anxiety and locomotor activity. Behavioural analysis after MCAo: Elevated Plus Maze test (time in open arms A and number of entries B), Open Field (time in the centre C and number of crossing D). Behavioural analysis after TBI: Elevated Plus Maze test (time in open arms E and number of entries F), Open Field (time in the centre G and number of crossing H). *p < 0.05 vs sham young; **p < 0.01 vs sham young; ***p < 0.001 vs sham young; ##p < 0.01 vs sham old; ###p < 0.001 vs sham old; ° p < 0.05 vs TBI young.
Difference between young and old mice on NRF-2 pathway

To understand the molecular mechanisms underlying the natural antioxidant activity related to age, we decided to investigate the Nrf2 pathway, which plays a key role in orchestrating cellular antioxidant defences and in maintaining cellular redox homeostasis. Our results, performed by Western blot analysis, showed low Nrf2 expression levels in sham groups, while slightly higher in young mice (Fig. 4A, A' and D, D'). A significant increase in Nrf2 expression was induced by stroke injury in a significant manner in young animals compared to old mice (Fig. 4A, A'). The increase in Nrf2 expression was also detected in young animals 24h after TBI, in a significant manner compared to TBI old group (Fig. 4D, D').

Under homeostatic conditions, Nrf2 is bound to Keap-1 in the cytoplasm. Western blot analysis showed basal levels of Keap-1 in sham mice of both experimental models, while keap-1 expression was significantly reduced after MCAo and TBI in both young and old mice (Fig. 4B, B' and E, E').

Additionally, HO-1 is an Nrf2-regulated gene that plays a critical role in maintaining antioxidant/oxidant homeostasis. In line with this, a significant increase of HO-1 expression was observed in young MCAO and young TBI groups compared to aged animals (Fig. 4C, C' and F, F').

**Fig. 3.** Difference between young and old mice on injury volume. TTC staining of brain section after MCAo: sham young (A), sham old (B), MCAo young (C) and MCAo old (D), quantification of infarct area (I). TTC staining of brain section after TBI: sham young (E), sham old (F), TBI young (G) and TBI old (H), quantification of infarct area (J). The red arrows indicated infarct area. ***p <0.001 vs sham young; ###p <0.001 vs sham old; °° p <0.01 vs TBI young.
Fig. 4. Western blots of NRF-2 pathway. Western blots and respectively quantification after MCAO of NRF-2 (A and A'), Keap-1 (B and B') and HO-1 (C and C'). Western blots and respectively quantification after TBI of NRF-2 (D and D'), Keap-1 (E and E') and HO-1 (F and F'). *p < 0.05 vs sham young; **p < 0.01 vs sham young; ***p < 0.001 vs sham young; #p < 0.05 vs sham old; ##p < 0.01 vs sham old; ###p < 0.001 vs sham old; ° p < 0.05 vs TBI young.
**Difference between young and old mice on oxidative markers**

Moreover, to confirm the previous results, we have studied some markers of oxidative stress, such as MDA levels, SOD-1 activity and GSH-Px levels. As illustrated in Fig. 5A, the results showed that MDA levels were significantly increased after MCAo, in particular in brain from aged mice compared to MCAo young group. Furthermore, we observed that SOD-1 activity (Fig. 5B) was significantly decreased as well as GSH-Px levels (Fig. 5C) in brain collected from MCAo old group compared to MCAo young group.

These oxidative markers were also determined in animals subjected to TBI. Also in this case, aging negatively affects oxidative stress, as demonstrated by the increase of MDA levels (Fig. 5D) and the decrease of both SOD-1 activity (Fig. 5E) and GSH-Px levels (Fig. 5F) in brain tissue from elderly mice compared to young rodents.

![Fig. 5. Difference between young and old mice on oxidative markers. Oxidative markers evaluation after MCAo: MDA (A), SOD-1 (B) and GSH-Px (C). Oxidative markers evaluation after TBI: MDA (D), SOD-1 (E) and GSH-Px (F). *p <0.05 vs sham young; **p <0.01 vs sham young; #p <0.05 vs sham old; ##p <0.01 vs sham old; ###p <0.001 vs sham old; °p <0.05 vs TBI young.](image-url)
Difference between young and old mice on NF-κB pathway

Since ROS are known to interact with NF-κB, we assessed NF-κB pathway activation by Western blot analysis. The results showed a basal expression of IκB-α in the brain tissue taken from sham groups (Fig. 6A, A' and C, C'), while in samples from MCAo and TBI group the levels of IκB-α decreased significantly. However, younger mice showed a low reduction of degradation of IκB-α induced by stroke and traumatic brain injuries compared to older animals (Fig. 6A, A' and C, C').

Vice versa, levels of NF-κB p65 significantly increased after MCAo and TBI damage compared with sham group (Fig. 6B, B' and D, D'). Importantly, the young mice showed a markedly reduction of NF-κB translocation compared to aged animals group (Fig. 6B, B' and D, D'). These data suggest that young age offers greater prevention also in the inflammatory pathway.

Fig. 6. Western blots of NF-κB pathway. Western blots and respectively quantification after MCAO of IκB-α (A and A') and Nf-κB (B and B'). Western blots and respectively quantification after TBI of of IκB-α (C and C') and Nf-κB (D and D'). *p <0.05 vs sham young; **p <0.01 vs sham young; ###p <0.01 vs sham old ; ####p <0.001 vs sham old; ° p <0.05 vs TBI young.
Difference between young and old mice on iNOS and COX-2 expression

To further focus on the impact of aging in inflammation, we performed Western blot analysis on iNOS and COX-2 expression after stroke and traumatic injuries. The findings displayed a low expression of iNOS in brain taken from both sham groups, whereas in MCAo and TBI groups, it was increased (Fig. 7A, A' and C, C'). In addition, it was also observed that the elderly mice showed significantly higher levels of iNOS expression than the young mice (Fig. 7A, A' and C, C').

The same trend was observed for the COX-2 expression in both experimental models' group (Fig. 7B, B' and D, D').

Difference between young and old mice on pro-inflammatory cytokines

Since additional downstream gene targets of NF-κB also include cytokines, we investigated the levels of TNF-α, IL-6 and IL-1β by ELISA kits. A substantial increase of TNF-α was found in brain tissues collected from MCAo mice and TBI mice (Fig. 8A and D, respectively), while low levels of this cytokine were observed in sham group.

Similar results have been observed for other two cytokines, IL-6 (Fig. 8B and E) and IL-1β (Fig. 8C and F). It is important to point out that in both experimental models, young mice offer greater protection than older mice.

Fig. 7. Western blots of iNOS and COX-2. Western blots and respectively quantification after MCAO of iNOS (A and A') and Cox-2 (B and B'). Western blots and respectively quantification after TBI of of iNOS (C and C') and Cox-2 (D and D'). *p <0.05 vs sham young; ***p <0.001 vs sham young; ##p <0.01 vs sham old; ###p <0.001 vs sham old; ° p <0.05 vs TBI young.
Fig. 8. Difference between young and old mice on pro-inflammatory cytokines. Cytokines expression after MCAo: TNF-α (A), IL-6 (B) and IL-1 (C). Cytokines expression after TBI: TNF-α (D), IL-6 (E) and IL-1β (F). **p <0.01 vs sham young; ***p <0.001 vs sham young; ###p <0.001 vs sham old; °p <0.05 vs TBI young.
Discussion

Cells are permanently exposed to a variety of oxidative stressors so they must be able to trigger antioxidative signaling pathways in order to maintain redox homeostasis. To this end Nrf2 is activated, it is shuttled to the nucleus where it binding to the antioxidant response elements (AREs) [42, 43]. The activated complex in turn promotes the transcription of genes involved in the modulation of both antioxidant and anti-inflammatory signaling [44].

The brain is particularly vulnerable to the effects of ROS due to its high demand for oxygen, and its abundance of highly peroxidisable substrates. The last two decades has witnessed accumulating evidences in favour of oxidative stress as a causative link between normal brain aging and several neuropathological conditions. Oxidative stress becomes particularly problematic during aging, as aged brains have been reported to exhibit high levels of mitochondrial DNA mutations caused by oxidative stress [45]. In the aging brain, as well as in the case of several neurodegenerative diseases, there is a decline in the normal antioxidant defense mechanisms, which increases the vulnerability of the brain to the harmful effects of oxidative damage [46, 47]. The impact of age on brain and behaviour has been explored in a variety of animal studies, leading to understanding of the brain mechanisms behind developmental changes associated with normal and pathological aging [48, 49]. Despite these studies comparing young and old animals, there is still relatively little data on age-related changes in behaviour and their impact on oxidative stress, in particular after stroke and traumatic injuries.

Ischemic stroke is a major cause of disability in the elderly worldwide, over 80% of ischemic strokes occur in people aged 65 years and older. The risk of stroke increases with the increase of age, and older patients suffer more severe functional disability and slower recovery after stroke when compared to younger patients [50]. Therefore, it is becoming increasingly clear that the aged brain responds differently to experimental stroke than the young. Following an ischemic event, older people are more likely to have serious neurological conditions, greater number of infarctions, higher morbidity and mortality rates [51, 52]. The elderly not only have a higher incidence of stroke but also less than optimal post-stroke recovery compared with their younger counterparts [53]. Previous studies have proven that the brain of young people has the capacity to initiate repair processes itself after ischemic stroke [50, 54, 55]. On contrary, aging dampens the activity of brain remodelling and results in exacerbated long-term neurological deficits following stroke [56, 57]. Thus, it is necessary to investigate accessible strategies to protect against ischemic stroke in aging animals to understand the underlying mechanisms.

At the same way, elderly people are particularly vulnerable to TBI; starting at age 65 years, TBI incidence doubles each additional 10 years of age [58]. Elderly individuals have clinically worse performance after TBI, including increased morbidity and mortality [59], and reduced functional recovery [60, 61]. Following TBI, the aging brain is more vulnerable to chronic inflammatory / degenerative changes. TBI’s adverse outcomes include loss of motor control, cognitive impairment, diminished quality of life, and death in extreme cases, all of which worsen with age [62, 63]. Secondary damage mechanisms after TBI have been well investigated in experimental models. However, it is poorly known how aging impacts secondary injury cascades and how it leads to worsened neurological function following injury. In addition, cognitive dysfunction and oxidative stress are normal occurrences in old age and are frequently traceable to events such as increased lipid peroxidation and protein oxidations in specific cognitive regions of the brain, the hippocampus and cerebral cortex [64]. Indeed, ROS production is considered a main factor contributing to decline in brain function with aging.

The present study was conducted in order to examine the role of Nrf2 pathway after ischemic stroke and brain traumatic injury in young and aged mice. We demonstrate that aging not only increased oxidative stress response but also inflammation and cytokine release in both experimental models. Moreover, we investigated the impact of aging on behavioural alterations in young and aged mice following ischemic stroke and traumatic injury.
Several behavioural tests have been used to evaluate learning and memory, anxiety and locomotor activity in young and aged mice. Behavioural tests showed the impaired cognitive and social functions in old mice subjected to MCAo and TBI compared to young mice. In particular, aged animals displayed reduced memory and learning capacity, as demonstrated in the MWM test. Moreover, in the EPM, Open Field, social interaction and novel object recognition tests, old mice showed anxiety-like behaviour and a reduced exploratory activity compared to mice with the same lesions. In both injury paradigms, aging has not only led to damage-induced worsening of motor function and behavioural changes but also increased oxidative stress response.

It is well established that the oxidative stress is associated with the aging process [65]. In response to oxidative stress, cells upregulate antioxidant pathways, including activation of the transcription factor Nrf2, the principal regulator of antioxidant defences and the proposed "master regulator" of the aging process [10, 66]. As mentioned above, under homeostatic conditions, Nrf2 is inhibited in the cytoplasm by the binding with Keap1. However, Nrf2 dissociates from Keap1 and translocates to the nucleus to bind to ARE upon stimulus or oxidative stress. Activation of the Nrf2-ARE pathway has a protective effect against various diseases via antioxidative mechanisms [67, 68]. Nrf2 signalling is important for maintaining antioxidant / oxidant homeostasis and for defending against ROS by modulating a variety of protective enzymes, including HO-1, SOD-1 and GSH-Px, all of which have strong antioxidant properties [69]. In line with above, our findings clearly demonstrated that aged animals show increased evidence of oxidative stress and reduced antioxidant capacity when compared to younger animals. In particular, we found a significant increase in Nrf2 levels accompanied by a markedly decreased Keap-1 expression following MCAO and TBI damages. Consequently, the modulation of the Nrf2-Keap1 signalling pathway led to an increase in antioxidant enzymes, such as HO-1, SOD-1 and GSH-Px, as well as to higher levels of MDA. Importantly, young mice show a greater antioxidant action compared to old mice, confirming the impact of aging on oxidative stress.

It is well known the cross-talking between Nrf2 and NF-κB [70]. Both are regulated by redox sensitive factors and the absence of Nrf2 is associated with increased oxidative stress, leading to increase of cytokine production, as NF-κB is more readily activated in oxidative conditions [71]. Furthermore, NF-κB has been directly involved in the aging process. In fact, biologic pathways implicated in aging, including immune responses, metabolism, apoptosis and cell senescence are all regulated at least in part by NF-κB [72].

Given that inflammation is critical driver of both ischemic stroke and brain trauma, we evaluated the effects of aging on the NF-κB pathway in both experimental models. Brain tissues collected from elderly mice showed an increased κB-α degradation into the cytoplasm and consequently NF-κB translocation into the nucleus, compared to young mice subjected to same injuries. Through this modulation, the aging increases the transcription of the NF-κB target genes involved in the enhancement of the inflammatory process. Thus, age might be an important intrinsic factor determining production of inflammatory cytokines such as TNFα, IL-1β, and IL-6 after MCAO and TBI. We detected that old mice exhibited higher inflammatory cytokines levels after injuries compared to young mice.

**Conclusion**

In conclusion aging represents an important risk factor in functional recovery after ischemic and traumatic brain damage. Not only that, preserving and keeping the NRF-2 pathway active could counteract the onset of oxidative stress and consequent inflammation after damage.

Therefore, the NRF-2 pathway could represent a possible therapeutic target in the management of ischemic and traumatic insult in particular in the elderly.
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