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ORIGINAL ARTICLE

Repetitive hyperbaric oxygen therapy provides better effects on brain inflammation and oxidative damage in rats with focal cerebral ischemia

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KEYWORDS
cerebral ischemia;
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Background/Purpose: Repetitive hyperbaric oxygen (HBO_2) therapy may cause excessive generation of reactive oxygen species. This study assessed whether repetitive or 2–4-day trials of HBO_2 therapy (2 treatments daily for 2–4 consecutive days) provides better effects in reducing brain inflammation and oxidative stress caused by middle cerebral artery occlusion (MCAO) in rats than did a 1-day trial of HBO_2 therapy (2 treatments for 1 day).
Methods: Rats were randomly divided into four groups: sham; MCAO without HBO_2 treatment; MCAO treated with 1-day trial of HBO_2; and MCAO treated with 2–4-day trials of HBO_2. One treatment of HBO_2 (100% O_2 at 253 kPa) lasted for 1 hour in a hyperbaric chamber.
Results: Therapy with the 2–4-day trials of HBO_2 significantly and dose-dependently attenuated the MCAO-induced cerebral infarction and neurological deficits more than the 1-day trial of HBO_2 therapy. The beneficial effects of repetitive HBO_2 therapy were associated with: (1) reduced inflammatory status in ischemic brain tissues (evidenced by decreased levels of tumor necrosis factor-α, interleukin-1β, and myeloperoxidase activity); (2) decreased oxidative damage in ischemic brain tissues (evidenced by decreased levels of reactive oxygen and nitrogen species, lipid peroxidation, and enzymatic pro-oxidants, but increased levels of enzymatic antioxidant defenses); and (3) increased production of an anti-inflammatory cytokine, interleukin-10.

Conflicts of interest: The authors declare that they have no conflict of interest.

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Conclusion: The results provide the apparently contradictory finding that heightened oxygen tension reduced oxidative stress (and inflammation), which was reflected by increased antioxidant and decreased oxidant contents under focal cerebral ischemia.

Introduction

Hyperbaric oxygen (HBO_2) therapy at a single dose is associated with decreased brain neurtrophil infiltration[1] and myeloperoxidase activity[2] in middle cerebral artery occlusion (MCAO). In experimental MCAO, one dose of HBO_2 therapy is also associated with downregulation of cyclooxygenase-2 mRNA and protein levels (an inducible enzyme responsible for elaboration of inflammatory prostanoids, prostaglandins, prastacyclins, and thromboxane),[3] suggesting that single HBO_2 therapy may improve outcomes of MCAO by reducing brain inflammation. In addition, inhibition of reactive oxygen species (ROS) production by an antioxidant is found to be beneficial in treating MCAO rats.[4] However, prolonged or repetitive HBO_2 therapy may cause excessive generation of ROS in rat lung[5] and in patients.[6] Neuroprotection by HBO_2 after MCAO is not found to be associated with decreased lipid peroxidation.[7] It remains unclear whether repetitive HBO_2 therapy provides better effects on brain inflammation and oxidative damage in rats with focal cerebral ischemia.

This study was conducted in order to evaluate the efficacy of a 1-day trial (2 HBO_2 treatments in one day) or 2—4-day trial (2 HBO_2 treatments daily and consecutively for 2—4 days) of HBO_2 therapy on both brain inflammation and oxidative stress caused by transient focal cerebral ischemia in rats. Therefore, by using the transient MCAO rat model, brain levels of proinflammatory cytokines [interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α)], an anti-inflammatory cytokine (IL-10), and a leukocyte accumulation indicator [myeloperoxidase (MPO) activity] were measured as indicators for brain inflammation. By contrast, brain levels of malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase, nitric oxide (NO), and 2,3-dihydroxybenzoic acid (2,3-DHBA) were measured as indicators for oxidative stress. The expected results would elucidate whether heightened oxygen tension by repetitive HBO_2 reduced oxidative stress, which was reflected by increased antioxidant and decreased oxidant contents under focal cerebral ischemia.

Methods

Animals

Adult male Sprague—Dawley rats (Animal Research Center of the National Science of the Republic of China (Taipei, Taiwan)) (weight, 246 ± 8 g) were housed under environmental conditions with ambient temperature of 22 ± 1°C, relative humidity of 65% and 12-hour light/dark cycle, with free access to food and water. Brain focal ischemia was induced by MCAO in rats by intraluminal filament, using the relatively noninvasive technique previously described by Belayev et al.[8] To allow reperfusion, the nylon filament was withdrawn 90 minutes after MCAO. The anesthetized animals were allowed to awaken and were kept in their cages with free access to food and water. All protocols, designed to minimize discomfort in the animals during surgery and in the recovery period, were approved by the Institutional Animal Care and Use Committee of Chi Mei Medical Center (Tainan, Taiwan) with a reference number of IACUC Approval No: 100120717.

HBO_2 therapy and animal groups

There were 192 rats randomly assigned to one of six groups: MCAO0T (MCAO rats untreated and euthanized 7 days post-MCAO; $n = 32$); MCAO1T (MCAO rats treated 3 hours after surgery with HBO_2 twice for 1 day and euthanized 7 days post-MCAO; $n = 32$); MCAO2T (MCAO rats treated 3 hours after surgery with HBO_2 twice daily for consecutive 2 days and euthanized 7 days post-MCAO; $n = 32$); MCAO3T (MCAO rats treated 3 hours after surgery with HBO_2 twice a day for consecutive 3 days and killed 7 days post-MCAO; $n = 32$); MCAO4T (MCAO rats treated 3 hours after surgery with HBO_2 twice a day for consecutive 4 days and euthanized 7 days post-MCAO; $n = 32$); and sham (sham-MCAO rats untreated and euthanized 7 days post-MCAO; $n = 32$). All tests were done with researchers blinded to which groups the rats were in; group codes were revealed only after all behavioral and histologic analyses had been completed. In MCAO groups treated with HBO_2, rats were placed in a custom-made pressure chamber of transparent acrylic plastic (Space Research Institute, Beijing, China) and given 1 hour of HBO_2 at 2.0 ata in 100% O_2 twice a day for 1—4 days. The chamber was flushed with 100% oxygen at a rate of 5 L/minute to avoid carbon dioxide accumulation. Decompression was done at 0.2 kg/cm^2/minute. During HBO_2 exposure, oxygen and carbon dioxide content were continuously monitored and maintained at ≥98% O_2 and ≤0.03% CO_2. The pressure chamber temperature was maintained between 22°C and 25°C. To minimize the effects of diurnal variation, all HBO_2 exposures were started at approximately 2:00 PM. MCAO-0 T or sham-MCAO was treated with normobaric air at 1.0 ata in 21% oxygen at an ambient temperature of 22—25°C.

Neurological and motor function evaluation

All rats were evaluated using a neurological severity score (NSS),[9] which is a composite of the motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive) and reflex tests. The inclined plane was used to measure limb strength. Animals were placed, facing right and left, perpendicular to the slope of a 20 cm × 20 cm ruffer ribbed surface of an inclined plane starting at angle

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of 55°.[10] Measurements of both NSS and motor deficits were conducted the day prior to surgery and 7 days after surgery.

Cerebral infarction assessment

Rats with deep anesthesia were transcardially perfused with heparinized 0.05 M phosphate-buffered saline followed by ice-cold 15% sucrose in phosphate-buffered saline. The brains were rapidly removed and frozen in liquid and then sectioned for immunohistochemistry. Eight serial sections from each brain were cut at 2-mm intervals from the frontal pole using a rat brain matrix (Harvard Apparatus, Holliston, MA, USA). To measure ischemic change, brain slices were stained in a solution containing 2% 2,3,5-triphenyltetrazolium chloride in saline at 37°C as detailed previously.[11] We also made correction of the distortion of infarct volume caused by brain edema in reference to the report of Lin et al.[12]

Assessment of free radical compounds

All animals were sacrificed at 7 days after MCAO. Under deep anesthesia (sodium pentobarbital, 100 mg/kg, intraperitoneally), animals were perfused intracardially with saline. The injured brain tissue proper (core region) was then removed and dissected out the cortex for determination of MDA, GPx, GR, and SOD, contents. As demonstrated in Fig. 1, the marked infarction focus was dissected out of the brain for the above-mentioned laboratory tests for free radical compounds.

Lipid peroxidation was assessed by measuring the levels of MDA with 2-thiobarbituric acid to form a chromophore absorbing at 532 nm.[13] The values of lipid peroxidation are expressed as nanomoles of 2-thiobarbituric acid-reactive substance (MDA equivalent)/mg of protein.[14]

SOD was analyzed with a SOD activity commercial kit (Oxis Research, Portland, OR, USA). The SOD activity is determined from the ratio of the autoxidation rates in the presence (Vx) and the absence (Vc) of SOD. One unit is defined as the activity that doubles the autoxidation rate of the control bland (Vx/Vc = 2). Protein concentration was determined by the method of Lowry et al.[15] NO levels were measured using an high performance liquid chromatography-nitric oxide (HPLC-NO) detector system (ENO-10; Eicom, Kyoto, Japan) as reported previously.[16] These oxidative NO products were also evaluated as NOx. The concentrations of hydroxyl radicals were measured by a

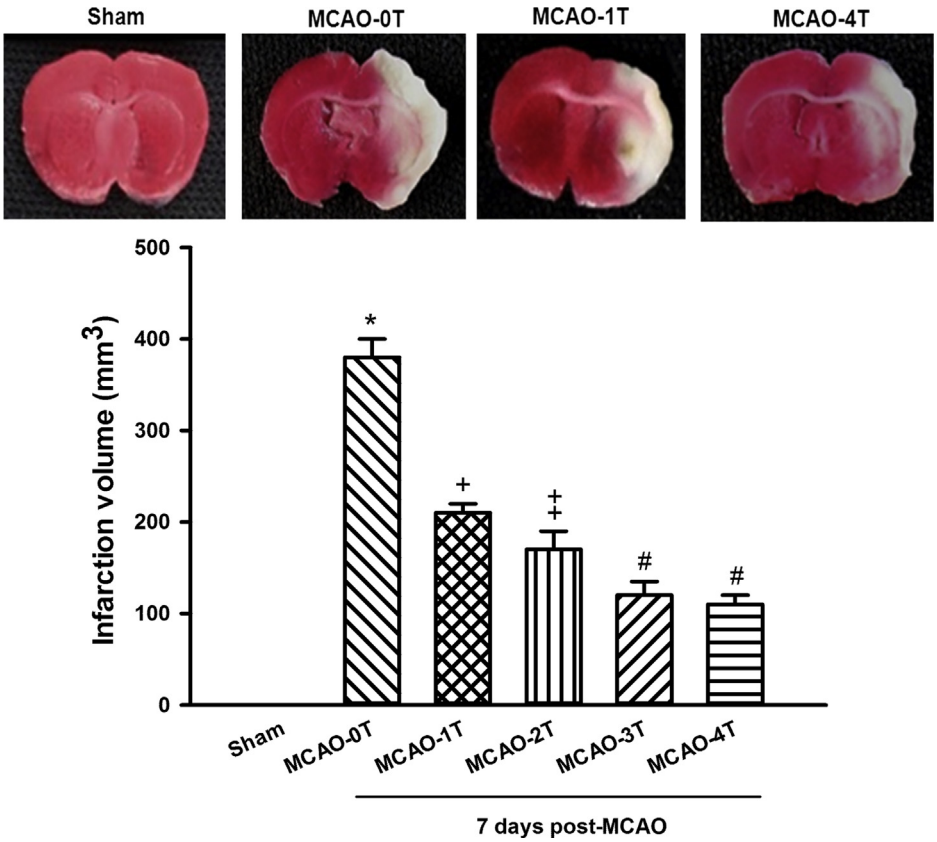

Figure 1 2,3,5-triphenyltetrazolium chloride-stained infarction volume by middle cerebral artery occlusion (MCAO) in rats ($n = 8$ in each group of different treatments). *The infarction volume of 7-day post-MCAO significantly ($p < 0.01$) increased for MCAO-injured rats treated with 0 trial of hyperbaric oxygen (HBO_2) therapy (MCAO-0T) compared with sham group. $^{+}$The infarction volume was significantly decreased for MCAO-injured rats treated with one trial of HBO_2 (MCAO-1T) of HBO_2 compared with the MCAO-0T group. ‡The infarction volume was significantly ($p < 0.05$) decreased for MCAO-injured rats treated with two trials of HBO therapy (MCAO-2T) compared with MCAO-1T. $^{\#}$The infarction volume was significantly decreased for MCAO-injured rats treated with three or four trials of HBO_2 therapy (MCAP-3T or MCAO-4T) compared with MCAO-2T group ($p < 0.05$). Top panels depict photographs showing 2,3,5-triphenyltetrazolium chloride staining for a sham rat, a MCAO-0T rat, a MCAO-1T rat, and a MCAO-4T rat.

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modified procedure based on hydroxylation of sodium salicylates by hydroxyl radicals, leading to production of 2,3-DHBA and 2,5-DHBA.[17,18]

Cytokine contents of ischemic cerebral homogenate

Cerebral hemispheres were quickly dissected free and kept on ice in physiological salt solution containing 5 mM glucose. Segments of ipsilateral cerebral cortex (75 mg; i.e., approximately the weight of each cerebral hemisphere) were weighed, cut into small pieces, dispersed by aspiration into a pipette and suspended in 1 mL of physiological salt solution in a test tube. Samples were kept on wet ice for 20 minutes prior to use. The homogenates were centrifuged at 5150 $\times$ *g* for 20 minutes. The supernatants were used for measuring TNF-α, IL-1β, and IL-10 concentrations. Cytokine concentrations were measured using commercial enzyme-linked immunosorbent assay kits (Biosource International Inc., Boshide Company, Wuhan, China) and following the manufacturer's instructions. All samples were assayed in duplicate.

Assay of MPO activity

Segments of ipsilateral cerebral cortex were used for biochemical analysis of MPO activity. Samples were homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM phosphate buffer (pH 6.0; 5.0 mL HTAB solution/g tissue) ice in a Polytron homogenizer (NS-50; Teraoka, Osaka, Japan) for 30 seconds. The homogenate was centrifuged at 10,000 $\times$ *g* for 30 minutes at 4°C. The pellet was resuspended in HTAB solution, and sonicated with an ultrasonic homogenizer (USP-600; Shimadzu, Kyoto, Japan) for 90 seconds at full power. The sonicate was incubated in a water bath at 60°C for 2 hours, and then centrifuged again. A 100-μL sample of supernatant was added to 2.9 mL of 50 mM phosphate buffer, pH 6.0, which contained 0.167 mg/mL O-dianisidine and 0.0005% hydrogen peroxide. Absorbance at 460 nm was monitored for 3 minutes in a spectrophometer (DU-640; Beckman Instruments, Fullerton, CA, USA). Then the MPO activity/g of wet tissue was calculated as described previously.[19]

Statistical analysis

Results are expressed as the mean $\pm$ standard error of the mean for eight animals/group. Data were compared using the analysis of variances (ANOVA) test followed by a multiple-comparison test (Scheffe's test). A *p* value of <0.05 was considered to be statistically significant.

Results

HBO_2 attenuates MCAO-induced cerebral infarction

Fig. 1 depicts the 2,3,5-triphenyltetrazolium chloride-stained cerebral infarction volume by MCAO in rats of different treatments. The infarction volume of rats 7 days after MCAO injury was significantly ($p < 0.01$) increased for

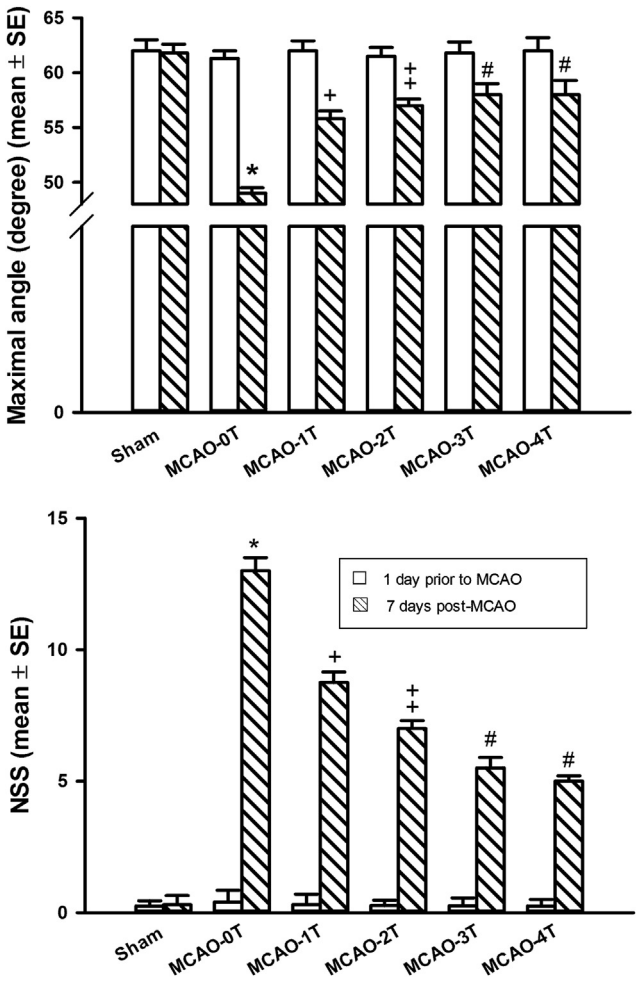

Figure 2 Changes in maximal angle or neurological severity score (NSS) by 1 day prior to MCAO or sham operation (□) or 7 days post-MCAO (▨) for different groups of rats ($n = 8$ each group). *The NSS and maximal angle were significantly increased and decreased respectively for MCAO-0T compared with sham-group ($p < 0.05$). $^{+}$The NSS and maximal angle were significantly decreased and increased respectively for MCAO-1T compared with MCAO-0T ($p < 0.05$). ‡The NSS and maximal angle were significantly decreased and increased respectively for MCAO-2T compared with MCAO-1T ($p < 0.05$). $^{\#}$The NSS and maximal angle was significantly decreased and increased respectively for MCAO-3T or MCOA-4T group compared with MCAO-2T group ($p < 0.05$). Please see the legend of Fig. 1 for abbreviations.

MCAO-0T compared with sham-MCAO. The infarction volume was significantly decreased for MCAO-1T compared with MCAO-0T. In addition, the infarction volume was significantly decreased for MCAO-2T, MCAO-3T, or MCAO-4T compared with MCAO-1T.

HBO_2 attenuates MCAO-induced increased NSS and decreased maximal angle

Fig. 2 summarizes the NSS and maximal angle changes by MCAO in rats of different treatments. The NSS and maximal angle were significantly increased and decreased respectively for MCAO-0T compared with the sham-MCAO. As

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compared with MCAO-0T, the NSS and maximal angle were significantly decreased and increased respectively for MCAO-1T. Additionally, the NSS and maximal angle were significantly decreased and increased respectively for MCAO-2T, MCAO-3T, or MCAO-4T compared with MCAO-1T.

HBO_2 attenuates MCAO-induced increased proinflammatory cytokines but stimulates IL-10

Fig. 3 depicts the changes in brain TNF-α, IL-1β, MPO, and IL-10 by MCAO in rats of different treatments. As compared with sham-MCAO, the brain levels of TNF-α, IL-1β, and MPO were significantly increased for MCAO-0T. However, the brain levels of TNF-α, IL-1β, and MPO were all significantly decreased for MCAO-1T compared with MCAO-0T. The brain levels of these cytokines were further decreased significantly for MCAO-2T, MCAO-3T, or MCAO-4T compared with MCAO-1T. As compared with MCAO-0T, MCAO-1T had significantly higher cerebral levels of IL-10. Furthermore, MCAO-2T, MCAO-3T, or MCAO-4T had significantly higher levels of IL-10 compared with MCAO-1T.

HBO_2 attenuates MCAO induced increased brain levels of NO and 2,3-DHBA

Fig. 4 summarizes the changes in brain levels of both NO and 2,3-DHBA by MCAO in rats of different treatments. The brain levels of both NO and 2,3-DHBA were significantly increased for MCAO-0T compared with sham-MCAO. As compared with MCAO-0T, the brain levels of both nitric oxide and 2,3-DHBA were significantly decreased for MCAO-1T. In addition, the brain levels of NO and 2,3-DHBA were significantly decreased for MCAO-2T, MCAO-3T, or MCAO-4T compared with MCAO-1T.

HBO_2 attenuates MCAO-induced increased brain levels of MDA and decreased brain levels of GPX, SOD, GR, and catalase

Fig. 5 summarizes the changes in brain levels of MDA, GPx, SOD, and GR. As compared with sham-MCAO, the brain levels of MDA were significantly increased for MCAO-0T,

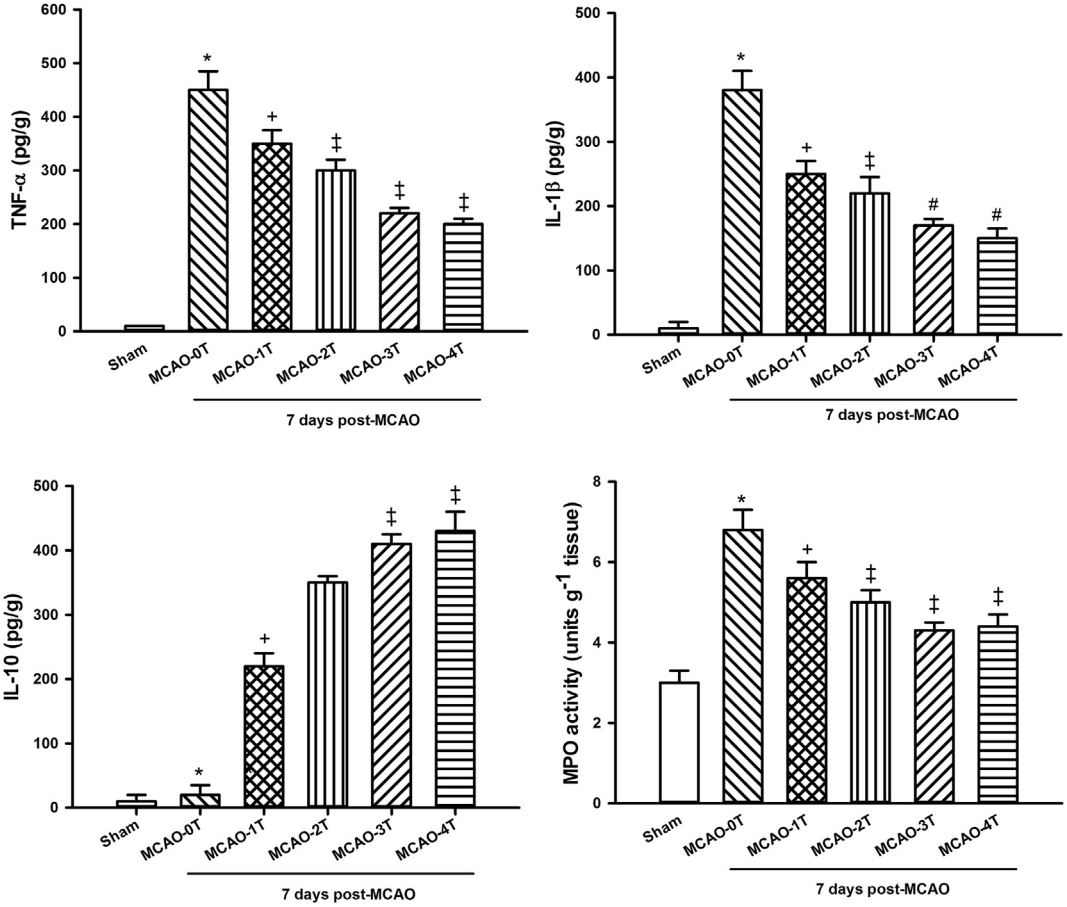

Figure 3 Changes in brain levels of cytokines and myeloperoxidase (MPO) activity by middle cerebral artery occlusion (MCAO) in different groups of rats ($n = 8$ each group). *The brain levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and MPO were significantly increased for MCAO-0T compared with sham-group ($p < 0.01$). $^{+}$The brain levels of TNF-α, IL-1β, and MPO were significantly decreased for MCAO-1T compared with MCAO-0T. By contrast, the brain levels of IL-10 were significantly increased for MCAO-1T compared with MCAO-0T (▨; $p < 0.05$). ‡The brain levels of TNF-α, IL-1β, and MPO for MCAO-2T were significantly decreased compared with MCAO-1T ($p < 0.05$). The brain levels of IL-10 were significantly increased for MCAO-2T compared with MCAO-1T ($p < 0.05$). $^{\#}$The brain levels of TNF-α, IL-1β, and MPO for MCAO-3T or MCAO-4T group were significantly decreased compared with MCAO-2T group ($p < 0.05$). Please see the legend of Fig. 1 for abbreviations.

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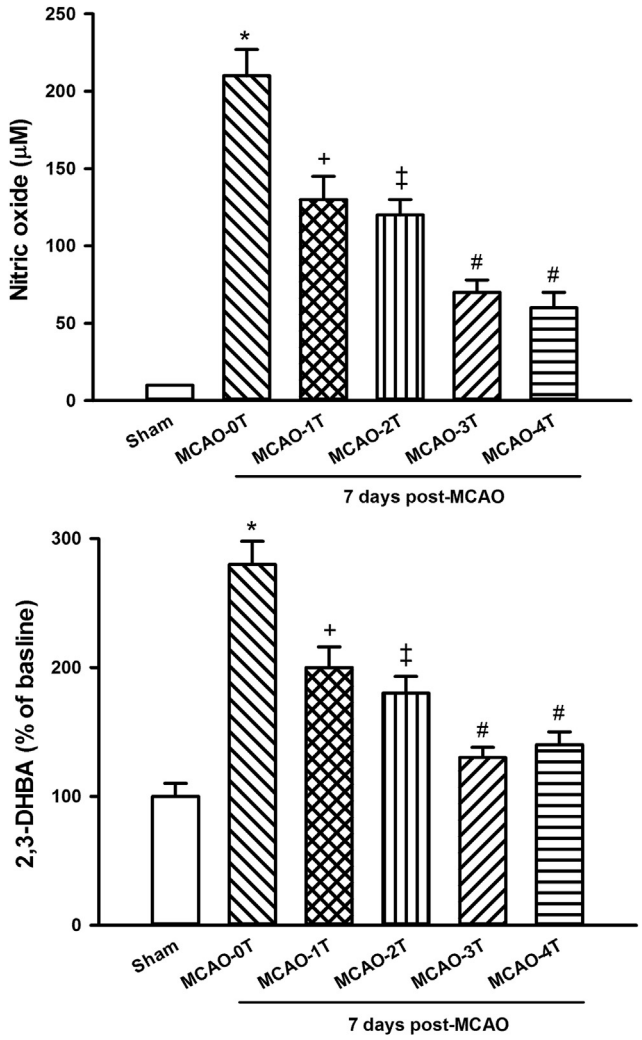

Figure 4 Changes in brain levels of nitric oxide (NO) and 2,3-dihydroxybenzoic acid (2,3-DHBA) by middle cerebral artery occlusion (MCAO) in rats ($n = 8$ each group). *The brain levels of NO and 2,3-DHBA were significantly increased for MCAO-0T compared with sham group ($p < 0.01$). $^{+}$The brain levels of NO and 2,3-DHBA were significantly decreased for MCAO-1T compared with MCAO-0T ($p < 0.05$). ‡The brain levels of NO and 2,3-DHBA were significantly decreased for MCAO-2T compared with MCAO-1T ($p < 0.05$). $^{\#}$The brain levels of NO and 2,3-DHBA were significantly decreased for MCAO-3T or MCAO-4T compared with MCAO-2T ($p < 0.05$).

whereas the brain levels of GPx, SOD, and GR were significantly decreased for MCAO-0T. The brain levels of MDA were significantly decreased whereas the brain levels of GPx, SOD, and GR, were significantly increased for MCAO-1T compared with MCAO-0T. In addition, the brain levels of MDA were significantly decreased and the brain levels of GPx, SOD, and GR, were significantly increased for MCAO-2T, MCAO-3T, or MCAO-4T compared with MCAO-1T.

Discussion

In the present study, MCAO rats were treated with HBO_2 3 hours after surgery. The neurological and motor deficits caused by MCAO in rats are significantly and dose-dependently attenuated by HBO_2 therapy. Our results are consistent with previous findings[20–22] showing that HBO_2 therapy after focal transient cerebral ischemia is frequently protective. However, focal permanent cerebral ischemia[4,23,24] is not affected by HBO_2 therapy. One report shows that HBO_2 is beneficial even if delayed as long as 24 hours after reperfusion,[25] whereas HBO_2 therapy, if adopted 6 hours after reperfusion, may have worse outcomes.[24,26] Although it has been demonstrated that HBO_2 therapy is beneficial in chronic symptomatic cerebrovascular diseases,[27] human studies in acute ischemic stroke have not shown the benefit of HBO_2 therapy.[28–30] There is an apparent discrepancy in therapeutic outcomes between clinical trials and animal studies, especially the current one with repetitive HBO_2 therapy. Probably, the most striking finding of the present study is that we demonstrate that repetitive (or multiple-day trials of) HBO_2 therapy display better beneficial effects on outcomes of focal transient brain ischemia in rats than did the 1-day trial of HBO_2 therapy.

It has been proposed that inflammation after ischemic stroke contributes to postischemic pathology.[31] In MCAO models, beneficial effect of HBO_2 therapy is associated with decreased neutrophil infiltration[1] and reduced brain myeloperoxidase activity.[2] Here we have demonstrated that, in addition to increasing MPO activity, MCAO-treated animals display higher levels of TNF-α and IL-1β in the ischemic areas, which can be attenuated by HBO_2 therapy. Additionally, our results demonstrate that HBO_2 therapy elevates brain levels of IL-10 in MCAO rats. IL-10 is an anti-inflammatory cytokine. For example, systemic administration of IL-10 protects mice from endotoxemia by reducing TNF-α release.[32] Neutralization of endogenously produced IL-10 causes an increased production of proinflammatory cytokines and enhanced mortality after sepsis.[33] IL-10 knockout mice have an increased likelihood of inflammatory illness[34] and higher mortality rates after experimental sepsis.[35] Our present results showed that HBO_2 therapy induces an increased production of IL-10 but a decreased production of TNF-α and IL-1β in the ischemic brain in MCAO rats. Thus, it appears that HBO_2 therapy improves outcomes of MCAO in rats by stimulating IL-10 production but inhibiting production of proinflammatory cytokines such as TNF-α and IL-1β. In fact, our hypothesis can be applied to other neurodegenerative disease models. For example, animals with heatstroke injury have increased brain levels of TNF-α, IL-1β, and MPO, which can be attenuated by HBO_2 therapy in diabetic rats.[36] HBO_2 therapy is shown to attenuate traumatic brain injury by reducing both TNF-α production and MPO activity but stimulating IL-10 production in rats.[37]

Extreme hyperbaric conditions (4—5 ata $\times$ 1 hour) may cause lipid peroxidation and altered enzymatic anti-oxidative process,[38,39] upregulation of NO synthase,[39] and central nervous system oxygen toxicity.[40,41] HBO_2 therapy at lower pressure (for example HBO_2 therapy pressure of no greater than 3 ata), is associated with few side effects and was therefore used in the present study.[42] Ischemic brain injury is associated with the overproduction of free radicals and ROS in rats.[43] Our current results further show that MCAO injured rats display significantly higher brain levels of ROS (NO and 2,3-DHBA), lipid peroxidation (MDA), and enzymatic pro-oxidants (oxidized glutathione (GSSG)/

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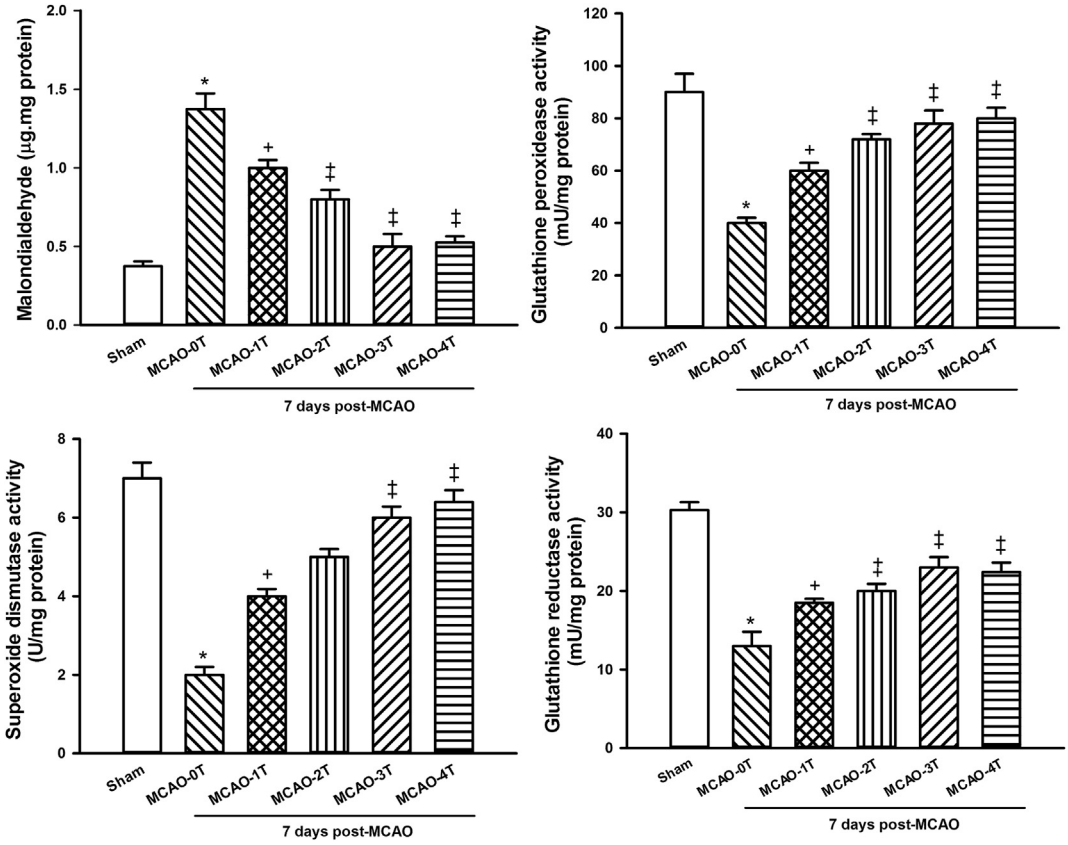

Figure 5 Changes in brain levels of malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione reductase (GR), by middle cerebral artery occlusion (MCAO) in rats ($n = 8$ each group). *The brain levels of malondialdehyde were significantly increased for MCAO-0T compared with MCAO-sham ($p < 0.05$). By contrast, the brain levels of GPx, SOD, and GR for MCAO-0T were significantly decreased compared with MCAO-sham ($p < 0.05$). [+]The brain levels of MDA for MCAO-1T were significantly decreased compared with MCAO-0T, whereas the brain levels of GPx, SOD, and GR for MCAO-1T were significantly increased compared with MCAO-0T ($p < 0.05$). [‡]The brain levels of MDA were significantly decreased for MCAO-2T compared with MCAO-1T, whereas the brain levels of GPx, SOD, and GRT for MCAO-4T were significantly increased compared with MCAO-1T ($p < 0.05$). [‡]The brain levels of MDA for MCAO-2T were significantly decreased compared with MCAO-1T, whereas the brain levels of GPx, SOD, and GR for MCAO-2T increased compared with MCOA-1T ($p < 0.05$). [#]The brain levels of MDA for MCAO-3T or MCAO-4T were significantly decreased whereas brain levels of GPx, SOD, and GR for MCAO-3T or MCAO-4T were significantly decreased compared with MCO-2T, whereas the brain levels of GPs, SOD, and GR for MCAO-3T or MCAO-4T were significantly increased compared with MCAO-2T ($p < 0.05$).

reduced glutathione (GSH)), but lower brain levels of enzymatic antioxidant defenses (GR and GPx) during MCAO. Increased production of ROS has been reported to be directly involved in oxidative damage with cellular macromolecules in ischemic brain tissues, which lead to cell death.[44] In MCAO, we first demonstrate that HBO_2 therapy attenuates ischemic stroke in rats by reducing the increased brain levels of ROS, lipid peroxidation, and enzymatic pro-oxidants but enhancing brain levels of enzymatic antioxidant defenses. In particular, we address apparent contradictory findings that heightened oxygen tension reduced oxidative stress, which was reflected by increased antioxidant and decreased oxidant contents under cerebral ischemia. An antioxidant is also found to be more beneficial than HBO_2 on permanent MCAO in rats.[4] Again, brain oxidative stress caused by other neurodegenerative disease models such as heatstroke can also be suppressed by HBO_2 therapy.[36]

In summary, we have demonstrated that treatment of MCAO-injured rats with HBO_2 protects against MCAO-induced cerebral infarction and neurological and motor dysfunction. The beneficial effects of HBO_2 may be attributed to: (1) inhibition of activated inflammation (increased brain levels of IL-1β, TNF-α, and MPO); (2) inhibition of oxidative stress (increased brain levels of ROS, lipid peroxidation, and enzymatic pro-oxidants, but decreased brain levels of enzymatic antioxidant defenses); and (3) overproduction of an anti-inflammatory cytokine (IL-10) in MCAO-injured brain. The beneficial effects exerted by multiple (2—4) day trials of HBO_2 therapy are superior to those of a 1-day trial of HBO_2 therapy in reducing MCAO-induced brain NO-related oxidative stress, inflammation, and functional deficits in rats. Thus, it can be deduced that HBO_2 therapy may improve outcomes of MCAO by reducing oxidative stress as well as inflammation in ischemic brain tissues.

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References

1. Atochin DN, Fisher D, Demchenko IT, Thom SR. Neutrophil sequestration and the effect of hyperbaric oxygen in a rat model of temporary middle cerebral artery occlusion. *Undersea Hyperb Med* 2000;**27**:185–90.
2. Miljkovic-Lolic M, Silbergleit R, Fiskum G, Rosenthal RE. Neuroprotective effects of hyperbaric oxygen treatment in experimental focal cerebral ischemia are associated with reduced brain leukocyte myeloperoxidase activity. *Brain Res* 2003;**971**:90–4.
3. Yin W, Badr AE, Mychaskiw G, Zhang JH. Down regulation of COX-2 is involved in hyperbaric oxygen treatment in a rat transient focal cerebral ischemia model. *Brain Res* 2002;**926**:165–71.
4. Acka G, Sen A, Canakci Z, Yildiz S, Akin A, Uzun G, et al. Effect of combined therapy with hyperbaric oxygen and antioxidant on infarct volume after permanent focal cerebral ischemia. *Physiol Res* 2007;**56**:369–73.
5. Topal T, Oter S, Korkmaz A, Sadir S, Metinyurt G, Korkmazhan ET, et al. Exogenously administered and endogenously produced melatonin reduce hyperbaric oxygen-induced oxidative stress in rat lung. *Life Sci* 2004;**75**:461–7.
6. Benedetti S, Lamorgese A, Piersantelli M, Pagliarani S, Benvenuti F, Canestrari F. Oxidative stress and antioxidant status in patients undergoing prolonged exposure to hyperbaric oxygen. *Clin Biochem* 2004;**37**:312–7.
7. Schäbitz WR, Schade H, Heiland S, Kollmar R, Bardutzky J, Henninger N, et al. Neuroprotection by hyperbaric oxygenation after experimental focal cerebral ischemia monitored by MRI. *Stroke* 2004;**35**:1175–9.
8. Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. *Stroke* 1996;**27**:1616–22.
9. Shohami E, Novikov M, Bass R. Long-term effect of HU-211, a novel non-competitive NMDA antagonist, on motor and memory functions after closed head injury in the rat. *Brain Res* 1995;**674**:55–62.
10. Chang MW, Young MS, Lin MT. An inclined plane system with microcontroller to determine limb motor function of laboratory animals. *J Neurosci Methods* 2008;**168**:186–94.
11. Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* 1986;**17**:1304–8.
12. Lin TN, He YY, Wu G, Khan M, Hsu CY. Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. *Stroke* 1993;**24**:117–21.
13. Wang JL, Ke DS, Lin MT. Heat shock pretreatment may protect against heatstroke-induced circulatory shock and cerebral ischemia by reducing oxidative stress and energy depletion. *Shock* 2005;**23**:161–7.
14. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;**95**:351–8.
15. Lowry H, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265–75.
16. Togashi H, Mori K, Ueno K, Matsumoto M, Suda N, Saito H, et al. Consecutive evaluation of nitric oxide production after transient cerebral ischemia in the rat hippocampus using *in vivo* brain microdialysis. *Neurosci Lett* 1998;**240**:53–7.
17. Obata T. Use of microdialysis for *in vivo* monitoring of hydroxyl free-radical generation in the rat. *J Pharm Pharmacol* 1997;**49**:724–30.
18. Yang CY, Lin MT. Oxidative stress in rats with heatstroke-induced cerebral ischemia. *Stroke* 2002;**33**:790–4.
19. Kira S, Daa T, Kashima K, Mori M, Noguchi T, Yokoyama S. Mild hypothermia reduces expression of intercellular adhesion molecule-1 (ICAM-1) and the accumulation of neutrophils after acid-induced lung injury in the rat. *Acta Anaesthesiol Scand* 2005;**49**:351–9.
20. Hou H, Grinberg O, Williams B, Grinberg S, Yu H, Alvarenga DL, et al. The effect of oxygen therapy on brain damage and cerebral pO(2) in transient focal cerebral ischemia in the rat. *Physiol Meas* 2007;**28**:963–76.
21. Huang ZX, Kang ZM, Gu GJ, Peng GN, Yun L, Tao HY, et al. Therapeutic effects of hyperbaric oxygen in a rat model of endothelin-1-induced focal cerebral ischemia. *Brain Res* 2007;**1153**:204–13.
22. Lou M, Zhang H, Wang J, Wen SQ, Tang ZQ, Chen YZ, et al. Hyperbaric oxygen treatment attenuated the decrease in regional glucose metabolism of rats subjected to focal cerebral ischemia: a high resolution positron emission tomography study. *Neuroscience* 2007;**146**:555–61.
23. Veltkamp R, Sun L, Herrmann O, Wolferts G, Hagmann S, Siebing DA, et al. Oxygen therapy in permanent brain ischemia: potential and limitations. *Brain Res* 2006;**1107**:185–91.
24. Lou M, Eschenfelder CC, Herdegen T, Brecht S, Deuschl G. Therapeutic window for use of hyperbaric oxygenation in focal transient ischemia in rats. *Stroke* 2004;**35**:578–83.
25. Yin D, Zhang JH. Delayed and multiple hyperbaric oxygen treatments expand therapeutic window in rat focal cerebral ischemic model. *Neurocrit Care* 2005;**2**:206–11.
26. Badr E, Yin W, Mychaskiw G, Zhang JH. Dual effect of HBO on cerebral infarction in MCAO rats. *Am J Physiol Regul Integr Comp Physiol* 2001;**280**:R766–70.
27. Vila JF, Balcarce PE, Abiusi GR, Dominguez RO, Pisarello JB. Improvement in motor and cognitive impairment after hyperbaric oxygen therapy in a selected group of patients with cerebrovascular disease: a prospective single-blind controlled trial. *Undersea Hyperb Med* 2005;**32**:341–9.
28. Rusyniak DE, Kirk MA, May JD, Kao LW, Brizendine EJ, Welch JL, et al. Hyperbaric oxygen therapy in acute ischemic stroke: results of the Hyperbaric Oxygen in Acute Ischemic Stroke Trial Pilot Study. *Stroke* 2003;**34**:571–4.
29. Nighoghossian N, Trouillas P, Adeleine P, Salord F. Hyperbaric oxygen in the treatment of acute ischemic stroke. A double-blind pilot study. *Stroke* 1995;**26**:1369–72.
30. Anderson DC, Bottini AG, Jagiella WM, Westphal B, Ford S, Rockswold GL, et al. A pilot study of hyperbaric oxygen in the treatment of human stroke. *Stroke* 1991;**22**:1137–42.
31. Feuerstein G. Inflammation and stroke: therapeutic effects of adenoviral expression of secretory leukocyte protease inhibitor. *Front Biosci* 2006;**11**:1750–7.
32. Gérard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, et al. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J Exp Med* 1993;**177**:547–50.

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33. Standiford TJ, Strieter RM, Lukacs NW, Kunkel SL. Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. *J Immunol* 1995;**155**:2222–9.
34. Rennick DM, Fort MM, Davidson NJ. Studies with IL-10-/- mice: an overview. *J Leukoc Biol* 1997;**61**:389–96.
35. Berg DJ, Kühn R, Rajewsky K, Müller W, Menon S, Davidson N, et al. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J Clin Invest* 1995;**96**:2339–47.
36. Lee KL, Niu KC, Lin MT, Niu CS. Attenuating brain inflammation, ischemia, and oxidative damage by hyperbaric oxygen in diabetic rats after heat stroke. *J Formos Med. Assoc* 2013;**112**:454–62.
37. Lin KC, Niu KC, Tsai KJ, Kuo JR, Wang LC, Chio CC, et al. Attenuating inflammation but stimulating both angiogenesis and neurogenesis using hyperbaric oxygen in rats with traumatic brain injury. *J Trauma* 2012;**72**:650–9.
38. Pablos MI, Reiter RJ, Chuang JI, Ortiz GG, Guerrero JM, Sewerynek E, et al. Acutely administered melatonin reduces oxidative damage in lung and brain induced by hyperbaric oxygen. *J Appl Physiol* 1997;**83**:354–8.
39. Zhang JH, Lo T, Mychaskiw G, Colohan A. Mechanisms of hyperbaric oxygen and neuroprotection in stroke. *Pathophysiology* 2005;**12**:63–77.
40. Chavko M, Xing G, Keyser DO. Increased sensitivity to seizures in repeated exposures to hyperbaric oxygen: role of NOS activation. *Brain Res* 2001;**900**:227–33.
41. Blenkarn GD, Schanberg SM, Saltzman HA. Cerebral amines and acute hyperbaric oxygen toxicity. *J Pharmacol Exp Ther* 1969;**166**:346–53.
42. Hampson NB, Bakker DJ, Camporesi EM. *Hyperbaric Oxygen Therapy Committee Statement*. Durham, NC: Undersed and Hyperbaric Medicine Society; 1999. Available at: http://www.uhm.org. [accessed 22.08.99].
43. Nita DA, Nita V, Spulber S, Moldovan M, Popa DP, Zagrean AM, et al. Oxidative damage following cerebral ischemia depends on reperfusion - a biochemical study in rat. *J Cell Mol Med* 2001;**5**:163–70.
44. Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci* 1997;**20**:132–9.

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