Methotrexate Induces an Antioxidant Hormetic Response in Primary Rat Astrocytes

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Abstract
Neurodegenerative diseases have increased worldwide in recent years. Their relationship with oxidative stress has motivated the research to find therapies and medications capable of suppressing oxidative damage and therefore slowing the progression of these diseases. Glutathione (GSH) is the most important cellular antioxidant in living beings and is responsible for regulating the cellular redox state. However, GSH cannot be administered by any route of administration, so molecules that increase its levels by activating Nrf2-ARE signaling pathway are explored; since Nrf2 regulates the main genes involved in GSH de novo synthesis and recycling. Astrocytes are the most important cell-type in the antioxidant cell response and are responsible for providing GSH and other substrates for neurons to have an efficient antioxidant response. Methotrexate (MTX) is an anti-inflammatory agent that has different cellular effects when administered at low or high concentrations. So in this study, we used MTX different concentrations and exposure times to induce a hormetic antioxidant response in rat primary astrocytes. Our results showed that 20 nM MTX pre-conditioning for 12 h augmented the GSH/GSSG ratio and protected cellular viability against a toxic MTX and H₂O₂ insult, which was abrogated when Nrf2 was inhibited by brusatol. Hence, MTX subsequent studies as a drug to counteract the progression of some stress-associated neurodegenerative diseases are suggested.

Keywords
methotrexate, astrocytes, hormesis, glutathione, redox state, neurodegeneration

Introduction
Astrocytes represent the most abundant population of glial cells in the brain. They perform important roles in brain’s physiology, such as ion and water homeostasis, neurotransmitters recycling, blood-brain barrier (BBB) formation and maintenance, immune signaling, and neuronal synaptogenesis regulation. Likewise, in situations of neuronal injury, disease and/or aging, these glia have the ability to generate a multifactorial inflammatory response providing defense against a variety of aggressions aimed at eliminating harmful agents. It has been reported that when neurons are cultured in the presence of astrocytes, they become more resistant to the oxidative damage induced by reactive oxygen species (ROS), than when cultured alone. Some astrocytes neuroprotective effects are mediated by the transfer of GSH or its precursors to neurons. Furthermore, astrocytes are capable of producing the antioxidant enzymes superoxide dismutase (SOD) and secreting them into the extracellular space.
In recent years, interventions to counteract oxidative stress in various types of pathologies have been developed; among the most prominent are those that promote or induce an adaptive response to changes in the physiological or cellular homeostasis, such as the hormesis. The hormetic response can be defined as “A process in which exposure to low doses of a chemical agent or a slight change in environmental factors induce a beneficial adaptive effect on the cell or organism, which protects them when exposed to higher doses of the same compound.”

The hormetic response is induced by the imbalance of homeostasis. To this end, a wide variety of stressors have been used, including chemical treatments, electromagnetic radiation, changes in temperature, and exercise. The hormetic response is characterized by increased damage repair mechanisms and augmented cellular defenses; in particular, the hormetic response to oxidative stress is characterized by increased antioxidant cellular response mechanisms and cell survival proteins. Hormetic antioxidant stimuli are generally characterized by increasing the levels of ROS/RNS, and some other metabolites that can directly or indirectly modify the cellular redox state. Among the most studied molecules are sulforaphane, resveratrol, curcumin, fumaric acid, tert-butyl hydroquinone, as well as others capable of inducing an adaptive response to stress at sublethal doses, which, however, at high doses can be extremely toxic or lethal.

Methotrexate (MTX), a folic acid antagonist, is commonly used as a chemotherapeutic agent in cancer treatment and chronic inflammatory diseases such as dermatomyositis, sarcoidosis, psoriasis, and rheumatoid arthritis. MTX is one of the most recommended drugs for patients with rheumatoid arthritis and is used to compensate for the low efficacy of glucocorticoids or other antirheumatic drugs. However, administration of high doses of MTX (for example, in leukemia) or long-term use can cause fibrosis and liver damage that progress to cirrhosis. Clinically, the hepatotoxicity that occurs in MTX long-term use remains an important factor that restricts its usage. Both the therapeutic and the hepatotoxic effects of MTX are attributed to the inhibition of folic acid conversion into tetrahydrofolate, but there are also studies that conclude that MTX toxicity occurs due to different mechanisms.

MTX administration has been found to suppress enzymatic and non-enzymatic antioxidant response, by decreasing reduced glutathione (GSH) and superoxide dismutase (SOD), while elevating enzymatic and non-enzymatic oxidants, such as myeloperoxidase (MPO) and malondialdehyde (MDA), in liver, kidney, and intestinal tissues of experimental animals. Furthermore, MTX was reported to cause DNA oxidative damage. On the other hand, it has been shown that low doses of MTX were beneficial when treating Wistar rat’s spinal cord injuries. MTX decreased inflammation, MPO activity, and MDA levels, thus preventing the appearance and development of a secondary lesion. Another interesting study showed that low doses of MTX reduced lipid peroxidation and apoptosis in rabbits in which ischemia/reperfusion damage was induced. Therefore, MTX’s mechanism of action and effects regarding oxidative stress are still controversial.

MTX diverse effects when used at different doses make it an excellent candidate to induce the hormetic antioxidant response, since high and chronic doses induce oxidative damage, while at low doses it can have beneficial antioxidant properties. In addition, an advantage of MTX as a hormetic inducer is that it decreases the systemic inflammatory state. Hence, the aim of this study was to evaluate the hormetic response induced by MTX against oxidative stress cytotoxicity in primary astrocytes isolated from Wistar rats.

Materials and Methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO). The reagents obtained from other sources are detailed throughout the text.

Astrocytes Isolation and Characterization

Astrocytes primary culture was obtained from the cerebral cortex of neonatal (3–7 days old) albino rats of the Wistar strain (Rattus norvegicus) according to the previously established protocol. Rats were provided by the Universidad Autónoma Metropolitana-Iztapalapa animal facility. A total of 24 neonatal rats were used for the study and three animals were used for each culture. Cells were grown in MEM medium supplemented with 10% fetal bovine serum (BSA), .11% glutamine, .15% glucose, and .1% penicillin-streptomycin and were subsequently incubated at 37°C and 5% CO2. The medium was replaced every third day. Cells were divided when they reached confluency and reseeded continuously. All procedures with animals were strictly carried out in accordance with the guide for handling and care of laboratory animals of the National Institutes of Health, and the official Mexican standard for handling animals NOM: 062-ZOO-1999.

The glial fibrillary acidic protein (GFAP) detection by immunocytochemistry was performed to confirm culture purity as described before. Single plane images were obtained in a LSM-META-Zeiss Axioplan-2 confocal microscope at 40X magnification; the 405 nm Laser Diode was used for DAPI and the Ar/ML 458/488/514 nm for the ALEXA 594. Images were analyzed using the ZEN 2010 version 6.0 program from Carl Zeiss.

Antioxidant Hormetic Response Determination

To establish the antioxidant hormetic response model (AHRM), astrocytes were seeded at $1 \times 10^5$ density into 24-well plates and treated with diverse concentrations of MTX (10, 20, 50, 100, and 200 nM), at different time points (1, 2, 3,
6, 9, 12, and 24 h). Cellular viability and redox state were evaluated to select the AHRM. As discussed later, two concentrations (10 and 20 nM) maintained a high redox state and cellular viability and were therefore chosen for the AHRM experiments for a short (1 h) and a long (12 h) hormetic induction. MTX (500 nM) or H$_2$O$_2$ (200 μM) were used to induce an oxidative stress challenge to the AHRM.

**Cellular Viability**

To determine cellular viability, astrocytes were trypsinized and a 20 μL aliquot was stained with an equal volume of a .4% trypan blue physiological solution (trypan blue exclusion). The number of living cells in 10 μL of this suspension was scored using five fields of a hemocytometer, under a phase-contrast optical microscope, as described elsewhere. The results were normalized with respect to total viable cells of the control group.

**Redox State Determination (GSH/GSSG Ratio)**

The GSH/GSSG ratio was determined by high-performance liquid chromatography (HPLC) following the protocol described by Hernández-Alvarez with some modifications. Treated cells were trypsinized and homogenized mechanically in 1 mL of perchloric acid/BPDS 1 mM. The homogenate was centrifuged at 14,000 rpm at 4°C for 5 min. Supernatants were separated and diluted with PBS (1:10 v/v). Each sample (100 μL) was injected on a binary pump (Waters 1525) coupled to a UV/visible detector (Waters 2489) at 210 nm. The stationary phase was performed in a 4.6 × 250 mm Eclipse XDB-C18 column, and a 5 μm particle size using KH$_2$PO$_4$ 20 mM and 1% pH 2.7 acetonitrile as mobile phase with a 1 mL/min flux. The area under the curve was determined through a standard curve using 10, 25, 50, 100, 200, and 400 μM GSH and GSSH concentrations.

**Nrf2 Immunolocalization**

Astrocytes were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Cells were actually astrocytes, so the presence of glial fibrillary acidic protein (GFAP) was evaluated. The confocal microscopy results in Figure 2A show that 500 nM MTX treatment at 10 and 20 nM MTX showed a survival greater than 70%, so those concentrations were considered as potential treatments to induce the hormetic response.

**Results**

**Antioxidant Hormetic Response Model (AHRM)**

To establish the AHRM, cellular viability was evaluated after MTX treatment at diverse concentrations and time intervals. Previously, we verified that the isolated cells in the primary cultures were actually astrocytes, so the presence of glial fibrillary acidic protein (GFAP) was evaluated. The confocal microscopy results in Figure 1A show that more than 90% of the cells were positive for GFAP, confirming the purity of the primary cultures.

Cellular viability gradually decreased with respect to exposure time at 50, 100, and 200 nM MTX (Figure 1B). At 24 hours, only 40% astrocytes survived. Conversely, astrocytes treated with 10 and 20 nM MTX showed a survival greater than 70%, so those concentrations were considered as potential treatments to induce the hormetic response.

The redox state (GSH/GSSG ratio) was also quantified to select the AHRM (Figure 1C). Of the five evaluated treatments, three were discarded (50, 100, and 200 nM) because besides the low cellular survival previously presented, the GSH/GSSG index decreased with respect to time and MTX concentration. On the other hand, the 10 and 20 nM treatments showed a typical “J” shape hormetic curve. This type of curve displays a gradual decrease in the response at short time points (6 h in these experiments), and then an important increase at longer times (9 and 12 h for 10 and 20 nM respectively). Intriguingly, at both concentrations (10 and 20 nm), there was a rapid and transient GSH/GSSG increase at 1 h, which was interesting and worthy to explore. So, four treatments were considered for the AHRM: 10 and 20 nM MTX for 1 h (short AHRM) and 10 and 20 nM MTX for 12 h (long AHRM).

**Short-Term Antioxidant Hormetic Response**

To assess the short-term antioxidant hormetic response, we used the AHRM model and challenged it with 500 nM MTX or 200 μM H$_2$O$_2$ for 1 h after the hormetic MTX treatment (inset in Figure 2A). Figure 2A shows that 500 nM MTX decreased 43% cellular viability ($P < .05$), while 200 μM H$_2$O$_2$ decreased it by 90% ($P < .001$). Pre-conditioning with 10 nM MTX for 1 h protected the cells, so that the survival only decreased 25% when challenged with 500 nM MTX ($P < .05$); however, the hormetic treatment did not protect the cells when challenged with 200 μM H$_2$O$_2$. Similar results

**Statistical Analysis**

Data are reported as the means ± SD for five independent experiments performed by triplicate. The differences in the viability and redox state analyses were determined by an ANOVA followed by a multiple comparison test of Tukey–Kramer. In all cases, the significance used was * $P < .05$, ** $P < .01$, and *** $P < .001$.
were observed during the pre-conditioning with 20 nM MTX, where astrocytes were protected from the 500 nM MTX challenge \((P < .05)\) and only decreased their survival by 28%. No protection was observed against the \(\text{H}_2\text{O}_2\) challenge. Figure 2B shows cellular morphology and survival after MTX and \(\text{H}_2\text{O}_2\) insults.

The antioxidant hormetic response was corroborated by evaluating the GSH/GSSG ratio. The results in Figure 2C...
indicate that the 500 nM MTX challenge reduced the redox index by 62% ($P < .01$), while it decreased by 84% with 200 μM H$_2$O$_2$ ($P < .001$). Pre-conditioning astrocytes with 10 nM MTX for 1 h reduced the GSH/GSSG ratio by only 46% after the 500 nM MTX challenge, thus generating a protection of more than 15% compared to the decrease observed without hormesis; while pre-conditioning with 20 nM MTX for 1 h decreased the GSH/GSSG ratio by 26% with the same MTX challenge ($P < .01$), giving a 36% protection. After both hormetic treatments, no protection was found in the redox state when challenged with H$_2$O$_2$.

**Long-Term Antioxidant Hormetic Response**

The results in Figure 3A show that pre-conditioning astrocytes with 10 nM MTX for 12 h induced a protection of 28.5% ($P < .05$) in cellular viability when challenged with 500 nM MTX when compared to the viability of cells without the hormetic treatment (figure 3A). However, no significant protection was found when challenged with 200 μM H$_2$O$_2$. Pre-conditioning with 20 nM MTX for 12 h protected viability by 37.22% vs the challenge with 500 nM MTX ($P < .05$). Interestingly, this hormetic treatment induced a protection of 2.7 times ($P < .05$) since it maintained 37% of cell viability after H$_2$O$_2$ challenge compared to the 10% viable cells determined without the hormetic treatment. Figure 3B shows representative micrographs of the viability assays to confirm the previous data.

As in the AHRM at 1 h, the redox state was evaluated form the long-term hormetic response. Figure 3C illustrated that pre-conditioning with 10 nM MTX for 12 h protects the redox index by 84% when challenged with 500 nM MTX ($P < .05$), but no significant differences were observed after the H$_2$O$_2$ insult (Figure 3C). Whereas, pre-conditioning with 20 nM MTX for 12 h protected the GSH/GSSG ratio by 110% when challenged
Figure 2. Continued.

Figure 3. Long-term antioxidant hormetic response. Primary astrocytes were pre-conditioned for 12 h with 10 on 20 nM MTX (long-term Antioxidant hormetic response model) and then challenged with 500 nM MTX or 200 μM H2O2. Cellular viability was determined as described in materials and methods. Representative micrographs of cellular morphology and survival after MTX and H2O2 insults. GSH/GSSG ratio. Values plotted are the mean ± SD of three independent experiments. Statistically significant differences are marked as *P < .05 and **P < .01 with respect to control; & P < .05 with respect to MTX or H2O2. Abbreviations: MTX, Methotrexate.
Figure 3. Continued.

Figure 4. Nrf2 immunolocalization. Nrf2 nuclear translocation was evaluated by confocal microscopy as described in materials and methods in cells pre-treated with 20 nM MTX for 12 h after MTX and H2O2 insults. Representative confocal micrographs of treated astrocytes. Nrf2 is shown in red and the nuclei are stained in blue with DAPI. Quantitative determination of Nrf2 nuclear translocation. Values plotted are the mean ± SD of three independent experiments. Statistically significant differences are marked as *P < .05 and **P < .01, ***P < .001.
Abbreviations: MTX, Methotrexate.
with 500 nM MTX ($P < .05$) and more than twice when challenged with H$_2$O$_2$ ($P < .05$). These results indicate that the long-term AHRM induces greater hormetic protection than the short-term AHRM and that pre-conditioning with 20 nM MTX for 12 h induced the best hormetic antioxidant response.

**Nrf2 Nuclear Translocation**

Once it was established that the best antioxidant hormetic response was pre-conditioning the cells with 20 nM MTX for 12 h, Nrf2 nuclear translocation was evaluated to confirm its participation in the antioxidant hormetic response. In Figures 4A and 4B it is shown that the Nrf2 nuclear translocation is 3.83 times more after the 500 nM MTX challenge compared to the control ($P < .05$) and 2.26 times more after the 200 μM H$_2$O$_2$ insult compared to the control ($P < .05$), thus confirming Nrf2 participation in this response.

**Nrf2 Regulates the Antioxidant Hormetic Response**

To confirm that Nrf2 participates in the antioxidant hormetic response regulation, we inhibited this transcription factor by

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**Figure 5.** Nrf2 regulate long-term antioxidant hormetic response (AHRM). Primary cultures of astrocytes were incubated with Brusatol, an inhibitor of Nrf2 synthesis to demonstrate the involvement of Nrf2 in long-term AHRM. The cells were treated according to the procedure described in the insert of figure A and C. Cellular viability was determined as described in materials and methods. Representative micrographs of cellular morphology and survival after MTX and H$_2$O$_2$ insults. GSH/GSSH ratio was determined by HPLC. Values plotted are the mean ± SD of three independent experiments. Statistically significant differences are marked as *$P < .05$ and **$P < .01$. 

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incubating the cells with Brusatol (Bru) 40 nM, 4 h before the hormetic treatment with MTX. Bru is a quassinoid compound that specifically inhibits Nrf2 signaling pathway. Figure 5 shows that incubation with Bru decreased astrocyte’s viability by 31% ($P < .05$) (Figure 5A and 5B) and reduced the GSH/GSSG ratio by 36.78% ($P < .05$) with respect to control group (Figure 5C). While treating the cells only with MTX for 12 h decreased cellular viability by 21% ($P < .05$) but increased the GSH/GSSG ratio by 22.65% ($P < .05$) compared to control. When astrocytes were treated with Bru before long AHRM (cells incubated with 20 nM MTX for 12 h and challenged with 500 nM MTX or 200 μM H$_2$O$_2$ for 1 h), reductions in viability of 84.75% ($P < .001$) and 91.05% ($P < .001$) were observed. The GSH/GSSG ratio also decreased by 65.31% ($P < .01$) and 71.89% ($P < .01$), respectively (Figure 5C).

Figures 6A and 6B show Nrf2 levels in Bru-treated astrocytes. Nrf2 decreased by 36% with respect to the control ($P < .05$). While in astrocytes incubated with Bru+MTX (20 nM)+MTX (500 nM) or with Bru+MTX (20 nM)+H$_2$O$_2$ (200 μM), the decrease determined was 76.59% ($P < .001$) and 73.36% ($P < .001$), respectively. Astrocytes that were only exposed to 20 nM MTX for 12 h increased Nrf2 levels by 58.80% ($P < .05$) compared to the control (Figure 6A and 6B).

**Cell Viability During Long MTX Treatments**

To demonstrate the protective effect of MTX at longer times, we performed a survival assay in which astrocytes were incubated with 20 nM MTX for 12 h, and subsequently re-exposed to 50 nM MTX for another 24 h. The results showed that pre-conditioning with 20 nM for 12 h increased cell viability by 68.15% ($P < .05$) compared to subsequent treatment with 50 nM MTX for 24 h. However, the viability was lower than the control at 34.8% ($P < .05$), as can be seen in Figure 7. These results and the previous ones suggest that the hormetic treatment with MTX is able to protect the cells.

**Discussion**

In recent years age-related neurodegenerative diseases such as Alzheimer’s and Parkinson’s have increased worldwide, so it has become necessary to develop therapies and drugs that are capable of suppressing the progression of these diseases. The brain is very susceptible to oxidative damage, since it has a deficient capacity of antioxidant response and a high energy demand, which induces elevated mitochondrial ROS production. Therefore, exploring alternative methods to reduce oxidative stress is one of the challenges in the field to reduce neurodegenerative disorders.

MTX is a drug used to treat various diseases related to chronic inflammation and cancer. Intriguingly, its effects vary depending on the concentration used. High doses of MTX have been reported to generate a state of oxidative stress capable of damaging biomolecules, inducing cancer cells death; this mechanism is independent of folic acid conversion to tetrahydrofolate by inhibiting the dehydrofolate reductase (DHFR) activity, an essential enzyme in DNA synthesis mechanisms. On the other hand, treatment with low concentrations of MTX is known to reduce inflammation, decrease apoptosis, and induce proliferation by inhibiting NF-kB and PI3K/Akt. Studies using HUVEC cells treated with TNF-α have shown that low concentrations of MTX induce the expression of survival genes such as SOD-Mn, OH-1, UCP-2, which are regulated by AMPK-CREB. That is, low doses of MTX can induce an antioxidant and survival response. This duality in the cellular response to different concentrations of the same compound is characteristic of the hormetic response. Our group and others have studied the antioxidant hormetic response through treatment with
different compounds like H$_2$O$_2$ or sulforaphane through a mechanism that involves the transcription factor Nrf2. Here, we corroborate the participation of this transcription factor and report for the first time an increase in Nrf2 nuclear translocation in response to low concentrations of MTX, which rise the GSH/GSSG ratio.

GSH is the cellular main antioxidant and is responsible for maintaining tissue homeostasis by regulating the redox state. GSH depletion has been reported in the brain of patients with neurodegenerative diseases. That is why much of the research has focused on therapeutic strategies to develop drugs that increase GSH levels in the brain, since it is known that GSH direct

**Figure 6.** Nrf2 immunolocalization with Brusatol in AHRM. Nrf2 expression levels were determined by confocal microscopy as described in materials and methods. Astrocytes were incubated with 40 nM brusatol for 4 h. Subsequently, the cells were incubated with the AHRM treatment (20 nM of MTX/12 h). Finally, the cells were challenged with lethal treatments of MTX (500 nM/1 h) or H$_2$O$_2$ (200 μM/1 h). A. Representative confocal micrographs of treated astrocytes. Nrf2 is shown in red and the nuclei are stained in blue with DAPI. B. Quantitative determination of Nrf2 levels expression. Values plotted are the mean ± SD of three independent experiments. Statistically significant differences are marked as *P<.05 and **P<.01. Abbreviations: MTX, Methotrexate; AHRM, Antioxidant hormetic response model.
administration has had little or null effects because this tripeptide hardly crosses the blood-brain barrier. Additionally, GSH cannot be directly absorbed orally since it is degraded by gastrointestinal peptidases and intravenously it is also metabolized by GGT in the blood, so it has a very short half-life, which is not sufficient for clinically effective administration. 45,46

Using MTX we developed a short and long-time antioxidant hormetic response model (AHRM) in astrocytes primary cultures, in which the GSH/GSSG ratio improved preserving the cellular capacity against a toxic challenge of the drug. Treatment with 20 nM MTX for 12 h turned out to be the most efficient AHRM, since a protective effect against lethal doses of oxidizing agents such as MTX (500 nM) and H2O2 (200 μM) was observed.

GSH is de novo synthesized in the cytosol by two enzymes, γ-glutamyl-cysteine ligase (γ-GCL) and GSH-synthetase (GS), the former being the rate-limiting enzyme. 47,48 Another mechanism for maintaining optimal GSH levels is the recycling pathway. This mechanism is regulated by the enzyme glutathione reductase (GR). GR is a homodimeric flavoprotein of 55 kDa per unit, and it is responsible for regulating the cellular GSH homeostasis by catalyzing the reduction of GSSG to GSH using NADPH as a reducing cofactor. 49,50

In Parkinson’s and Alzheimer’s diseases, the main target for to induce the molecular mechanisms related to GSH synthesis are glial cells, especially astrocytes. Mainly because in vitro studies have shown that GSH concentrations in neurons are lower in astrocytes and astrocytes supply the precursor molecules for GSH synthesis in neurons. 51,52 Nrf2 is a transcription factor that regulates the transcription of more than 250 genes that contain the ARE sequence, and that participate in the organisms response to stress. Among the main genes that are regulated by Nrf2 are the ones that participate in GSH metabolism, such as glutathione sulfhydryl transferase (GST), GR, Glutathione peroxidase (GPx), GCL, and GS. Therefore, Nrf2-ARE signaling pathway activation has been related to increase the redox potential and reduce oxidative damage 53,54.

Here we have shown that pre-conditioning astrocytes with 20 nM MTX for 12 h induces a hormetic antioxidant response, which correlates with Nrf2 nuclear translocation. This is consistent with the increase in GSH/GSSG ratio observed after the challenge with MTX and H2O2. These results agree with our previous findings where Nrf2 activation occurred 30 min after tBHQ treatment in primary rat astrocytes, and was sustained for 6 h. 15 The increase in GSH/GSSG ratio suggests that GSH augment might be due de novo synthesis because this pathway is limited by the enzyme GCL, which is regulated by Nrf2. 55-58

Additionally, incubating astrocytes with Brusatol, an inhibitor of the Nrf2 pathway, abrogates the hormetic antioxidant response, confirming Nrf2 participation in the hormetic antioxidant response. 59,60 Hence, the hormetic treatment with MTX, either in a short- or long-term AHRM, is capable of protecting the astrocytes against chronic MTX exposures. This is very interesting, since normally the antioxidant protection induced with hormetic treatments is evaluated with acute and lethal exposures to various chemical or physical agents. In the AHRM Nrf2 participation might be regulating a large number of vitagens, and Nrf2 might be increased as long as 24 h. 15,42,61,62 Nrf2-mediated antioxidant hormetic protection against lethal chronic challenges has been previously reported, Posadas et al. in
2020 showed that tBHQ pre-conditioning increases Nrf2 levels and GSH/GSSG ratio in L6 rat myocytes, and that this effect protected the cells against exposure to chronic lethal doses of palmitate for 24 h. Hormetric antioxidant treatments have also been shown to protect against chronic toxic treatments in animal models; CD-1 mice pre-conditioned with RBT3 for 18 h, increased levels of nuclear Nrf2 and antioxidant enzymes, which conferred a 72 h protective effect on the kidney against chronic treatment with cisplatin.

In conclusion, to our knowledge, this is a first approach of using MTX as an inducer of the antioxidant hormeric response. There is no doubt that there are other experiments to be carried out to demonstrate the effectiveness of these concentrations in various models of neurodegenerative diseases related to oxidative stress, where MTX could be used in in vivo and in vitro models, to evaluate not only antioxidant, but also anti-inflammatory effects.

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References
1. Giovannoni F, Quintana FJ. The role of astrocytes in CNS inflammation. Trends Immunol. 2020;41(9):805-819. doi:10.1016/j.it.2020.07.007.
2. Chen Y, Swanson RA. Astrocytes and brain injury. J Cereb Blood Flow Metab. 2003;23(2):137-149. doi:10.1097/01.WCB.0000044631.80210.3C.
3. Escartin C, Galea E, Lakatos A, et al. Reactive astrocyte nomenclature, definitions, and future directions. Nat Neurosci. 2021;24(3):312-325. doi:10.1038/s41593-020-00783-4.
4. Lee KH, Cha M, Lee BH. Crosstalk between Neuron and Glial Cells in Oxidative Injury and Neuroprotection. Int J Mol Sci. 2021;22(24):13315. doi:10.3390/ijms222413315.
5. Takano K, Tanaka N, Kawabe K, Moriyama M, Nakamura Y. Extracellular superoxide dismutase induced by dopamine in cultured astrocytes. Neurochem Res. 2013;38(1):32-41. doi:10.1007/s11064-012-0882-2.
6. Asanuma M, Miyazaki I. Glutathione and related molecules in parkinsonism. Int J Mol Sci. 2021;22(16):3869. doi:10.3390/ijms22168689.
7. Berry R 3rd, López-Martínez G. A dose of experimental hormesis: When mild stress protects and improves animal performance. Comp Biochem Physiol Mol Integr Physiol. 2020;242:110658. doi:10.1016/j.cbpa.2020.110658.
8. Calabrese V, Cornelius C, Dinkova-Kostova AT, Calabrese EJ, Mattson MP. Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. Antioxid Redox Signal. 2010;13(11):1763-1811.
9. Luna-López A, González-Puertos VY, López-Diazguerrero NE, Königsberg M. New considerations on hormeric response against oxidative stress. J Cell Commun Signal. 2014;8(4):323-331. doi:10.1007/s12079-014-0248-4.
10. López-Diazguerrero NE, González Puertos VY, Hernández-Bautista RJ, Alarcón-Aguilar A, Luna-López A, Hormesis K-FM. Lo que no te mata te hace más fuerte. Gac Med Mex. 2013;149(4):438-447.
11. Ristow M, Schmeisser S. Extending life span by increasing oxidative stress. Free Radic Biol Med. 2011;51:327-336.
12. Hoffmann GR, Moczula AV, Laterza AM, Maeneil LK, Tartaglione JP. Adaptive response to hydrogen peroxide in yeast: induction, time course, and relationship to dose-response models. Environ Mol Mutagen. 2013;54(6):384-396.
13. Speciale A, Chirafisi J, Saija A, Cimino F. Nutritional antioxidants and adaptive cell responses: an update. Curr Mol Med. 2011;11(9):770-789.
14. Calabrese V, Cornelius C, Cuzzocrea S, Iavicoli I, Rizzarelli E, Calabrese EJ. Hormesis, cellular stress response and vitagenes as critical determinants in aging and longevity. Mol Aspects Med. 2011;32(4-6):279-304.
15. Alarcón-Aguilar A, Luna-López A, Ventura-Gallegos JL, et al. Primary cultured astrocytes from old rats are capable to activate the Nrf2 response against MPP+ toxicity after tBHQ pretreatment. Neurobiol Aging. 2014;35(8):1901-1912.
16. Zhang Q, Wang DW, Shu HS. Outcome of primary central nervous system lymphoma treated with combined surgical resection and high- dose methotrexate chemotherapy: A single-institution retrospective study. Turk Neurosurg. 2022;32(1):1-5. doi:10.5137/1019-5149.TNN.24054-19.2.
17. Mailankody S, Kumar VS, Khan SA, Banavali SD, Bajpai J. Resource-appropriate selection of osteosarcoma treatment protocols in low- and middle-income countries. Pediatr Blood Cancer. 2021;69:e29540. doi:10.1002/pbc.29540.
18. Choonhakarn C, Chaowattanapanit S, Julanon N, Limpawattana P. Comparison of the clinical efficacy of subcutaneous versus oral administration of methotrexate in patients with psoriasis vulgaris: a randomized controlled trial. Clin Exp Dermatol. 2022;47:942-948. doi:10.1111/ced.15102.
19. Boussaid S, Makhlof Y, Rekek S, et al. The effects of autoimmune rheumatic-related diseases on male reproductive health: A systematic review. J Reprod Immunol. 2022;150:103472. doi:10.1016/j.jri.2021.103472.
20. Cetinkaya A, Bulbuloglu E, Kurutas EB, Kantarceken B. N-acetylcysteine ameliorates methotrexate-induced oxidative liver damage in rats. *Med Sci Monit*. 2006;12(8):BR274-BR278.

21. Kaeley GS, MacCarter DK, Goyal JR, et al. Similar improvements in patient-reported outcomes among rheumatoid arthritis patients treated with two different doses of methotrexate in combination with adalimumab: results from the MUSICA trial. *Rheumatol Ther*. 2018;5(1):123-134.

22. Hytiroglou P, Tobias H, Saxena R, Abramidou M, Papadimitriou CS, Theise ND. The canals of hering might represent a target of methotrexate hepatic toxicity. *Am J Clin Pathol*. 2004;121:324-329.

23. Sener G, Eksioglu-Demiralp E, Cetiner M, Ercan F, Yegen BC. Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. *Eur J Pharmacol*. 2006;542:170-178.

24. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. *New Eng J Med*. 1983;309:1094-1104.

25. Kremer JM, Galivan J, Streckfuss A, Kamen B. Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients. Association with hepatic folate deficiency and formation of polyglutamates. *Arthritis Rheum*. 1986;29:832-835.

26. Jahovic N, Cevik H, Sehirli AO, Yegen BC, Sener G. Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. *J Pineal Res*. 2003;34:282-287.

27. Huang HY, Helzlouer KJ, Appel LJ. The effects of vitamin C and vitamin E on oxidative DNA damage: results from a randomized controlled trial. *Cancer Epidemiol Biomarkers Prev*. 2000;9:647-652.

28. Marnett LJ. Oxyradicals and DNA damage. *Carcinogenesis*. 2000;21:361-370.

29. Sanli AM, Serbes G, Sargon MF, et al. Methotrexate attenuates early neutrophil infiltration and the associated lipid peroxidation in the injured spinal cord but does not induce neurotoxicity in the uninjured spinal cord in rats. *Acta Neurochir*. 2012;154:1045-1054.

30. Bakar B, Kose EA, KupanaAyva S, Sarkarati B, Kasimcan MO, Kilince K. Effects of low-dose methotrexate in spinal cord injury in rats. *Ulus Travma Acil Cerrahi Derg*. 2013;19:293.

31. Kertmen H, Gurer B, Yilmaz ER, et al. The protective effect of low-dose methotrexate on ischemia-reperfusion injury of the rabbit spinal cord. *Eur J Pharmacol*. 2013;714:148-156.

32. Guler A, Sahin MA, Yuelc O, et al. Proanthocyanidin prevents myocardial ischemic injury in adult rats. *Med Sci Monit*. 2011;17(11):BR326-31.

33. Uraz S, Tahan V, Aygun C, et al. Role of ursodeoxycholic acid in prevention of methotrexate-induced liver toxicity. *Dig Dis Sci*. 2008;53:1071-1077.

34. López-Diazguerrero NE, López-Araiza H, Conde-Perezprevina JC, et al. Bel-2 protects against oxidative stress while inducing premature senescence. *Free Radic Biol Med*. 2006;40(7):1161-1169. doi:10.1016/j.freeradbiomed.2005.11.002.

35. Hernández-Álvarez D, Mena-Montes B, Toledo-Pérez R, et al. Long-term moderate exercise combined with metformin treatment induces an hormetic response that prevents strength and muscle mass loss in old female wistar rats. *Oxid Med Cell Longev*. 2019;2019:3428543. doi:10.1155/2019/3428543.

36. Mattson MP. Hormesis and disease resistance: activation of cellular stress response pathways. *Hum Exp Tox*. 2008;27:155-162.

37. Cav Si, Liu Y, Yang C, et al. Brusatol, an NRF2 inhibitor for future cancer therapeutic. *Cell Biosci*. 2019;9:45. doi:10.1186/s13578-019-0309-8.

38. Hess JA, Khasawneh MK. Cancer metabolism and oxidative stress: Insights into carcinogenesis and chemotherapy via the non-dihydrofolate reductase effects of methotrexate. *BBA Clin*. 2015;3:152-161. doi:10.1016/j.bbacli.2015.01.006.

39. Lodhi MS, Khalid F, Khan MT, et al. A novel method of magnetic nanoparticles functionalized with anti-folate receptor antibody and methotrexate for antibody mediated targeted drug delivery. *Molecules*. 2022;27(1):261. doi:10.3390/molecules27010261.

40. Liu X, Zhang R, Fu G, et al. Methotrexate therapy promotes cell coverage and stability in in-stent neo-intima. *Cardiovasc Drugs Ther*. 2021;35(5):915-925. doi:10.1007/s10557-020-07121-7.

41. Thornton CC, Al-Rashed F, Calay D, et al. Methotrexate-mediated activation of an AMPK-CREB-dependent pathway: a novel mechanism for vascular protection in chronic systemic inflammation. *Ann Rheum Dis*. 2016;75(2):439-448. doi:10.1136/annrheumdis-2014-206305.

42. Luna-López A, Triana-Martínez F, López-Diazguerrero NE, et al. Bel-2 sustains hormetic response by inducing Nrf2 nuclear translocation in L929 mouse fibroblasts. *Free Radic Biol Med*. 2010;49(7):1192-1204. doi:10.1016/j.freeradbiomed.2010.07.004.

43. He C, Buongiorno LP, Wang W, et al. The inhibitory effect of sulforaphane on bladder cancer cell depends on GSH depletion-induced by Nrf2 translocation. *Molecules*. 2021;26(16):4919. doi:10.3390/molecules26164919.

44. Brosnan JT, Brosnan ME. The sulfur-containing amino acids: an overview. *J Nutr*. 2006;136(6 suppl l):1636S-1640S. doi:10.1093/jn/136.6.1636S.

45. Cornford EM, Braun LD, Crane PD, Oldendorf WH. Blood-brain barrier restriction of peptides and the low uptake of endoplasmic-reticulum-generated oxidative stress. *Endocrinology*. 1978;103(4):1297-1303. doi:10.1210/endo-103-4-1297.

46. Aebi S, Assereto R, Lauterburg BH. High-dose intravenous glutathione in man. Pharmacokinetics and effects on cyst(e)ine in plasma and urine. *Eur J Clin Invest*. 1991;21(1):103-110. doi:10.1111/j.1365-2362.1991.tb01366.x.

47. Chakravarthi S, Jessop CE, Bulleid NJ. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO Rep*. 2006;7(3):271-275. doi:10.1038/sjembr.7400645.

48. Bjørklund G, Peana M, Maes M, Dadar M, Severin B. The glutathione system in Parkinson’s disease and its progression. *Neurosci Biobehav Rev*. 2021;120:470-478. doi:10.1016/j.neubiorev.2020.10.004.

49. Fuji T, Endo T, Fuji J, Taniguchi N. Differential expression of glutathione reductase and cytosolic glutathione peroxidase,
GPX1, in developing rat lungs and kidneys. *Free Radic Res.* 2002;36(10):1041-1049. doi:10.1080/1071576021000006725.

50. Fujii J, Ito JI, Zhang X, Kurahashi T. Unveiling the roles of the glutathione redox system in vivo by analyzing genetically modified mice. *J Clin Biochem Nutr.* 2011;49(2):70-78. doi:10.3164/jcbn.10-138SR.

51. Dringen R, Kassmaul L, Gutterer JM, Hirrlinger J, Hamprecht B. The glutathione system of peroxide detoxification is less efficient in neurons than in astroglial cells. *J Neurochem.* 1999;72(6):2523-2530. doi:10.1046/j.1471-4159.1999.0722523.x.

52. Kim K. Glutathione in the nervous system as a potential therapeutic target to control the development and progression of amyotrophic lateral sclerosis. *Antioxidants (Basel).* 2021;10(7):1011. doi:10.3390/antiox10071011.

53. Hirotsu Y, Katsuoka F, Funayama R, et al. Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. *Nucleic Acids Res.* 2012;40(20):10228-10239. doi:10.1093/nar/gks827.

54. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun.* 1997;236(2):313-322. doi:10.1006/bbrc.1997.6943.

55. Griffith OW. Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med.* 1999;27(9-10):922-935. doi:10.1016/s0891-5849(99)00176-8.

56. Rahman I, MacNee W. Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. *Free Radic Biol Med.* 2000;28(9):1405-1420. doi:10.1016/s0891-5849(00)00215-x.

57. Kawakami K, Moritani C, Uraji M, et al. Sake lees hydrolysate protects against acetaminophen-induced hepatotoxicity via activation of the Nrf2 antioxidant pathway. *J Clin Biochem Nutr.* 2017;61(3):203-209. doi:10.3164/jcbn.17-21.

58. Seelig GF, Simonsen RP, Meister A. Reversible dissociation of gamma-glutamylcysteine synthetase into two subunits. *J Biol Chem.* 1984;259(15):9345-9347.

59. Nguyen BT, Shin EJ, Jeong JH, et al. Ginsenoside Re attenuates memory impairments in aged Klotho deficient mice via interactive modulations of angiotensin II AT1 receptor, Nrf2 and GPx-1 gene. *Free Radic Biol Med.* 2022;189:2-19. doi:10.1016/j.freeradbiomed.2022.07.003.

60. Kannan S, Irwin ME, Herbrich SM, et al. Targeting the Nrf2/HO-1 antioxidant pathway in FLT3-ITD-positive AML enhances therapy efficacy. *Antioxidants (Basel).* 2022;11(4):717. doi:10.3390/antiox11040717.

61. Narayanan SV, Dave KR, Perez-Pinzon MA. Ischemic preconditioning protects astrocytes against oxygen glucose deprivation via the nuclear erythroid 2-related factor 2 pathway. *Transl Stroke Res.* 2018;9(2):99-109. doi:10.1007/s12975-017-0574-y.

62. Alfieri A, Srivastava S, Siow RCM, et al. Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood-brain barrier disruption and neurological deficits in stroke. *Free Radic Biol Med.* 2013;65:1012-1022. doi:10.1016/j.freерadbiomed.2013.08.190.

63. Posadas-Rodríguez P, Posadas-Rodríguez NE, González-Puertos VY, et al. tBHQ induces a hormetic response that protects L6 myoblasts against the toxic effect of palmitate. *Oxid Med Cell Longev.* 2020;16:20203123268. doi:10.1155/2020/3123268.

64. Zager RA, Johnson ACM, Therapeutics R. Iron sucrose (‘RBT-3’) activates the hepatic and renal HAMP1 gene, evoking renal hepcidin loading and resistance to cisplatin nephrotoxicity. *Nephrol Dial Transplant.* 2021;36(3):465-474. doi:10.1093/ndt/gfaa348.