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Original Article

MSM ameliorates HIV-1 Tat induced neuronal oxidative stress via rebalance of the glutathione cycle

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Abstract: HIV-1 Tat protein is a key neuropathological element in HIV associated neurogcognitive disorders (HAND); a type of cognitive syndrome thought to be at least partially mediated by increased levels of brain reactive oxygen species (ROS) and nitric oxide (NO). Methylsulfonylmethane (MSM) is a sulfur-containing compound known to reduce oxidative stress. This study was conducted to determine whether administration of MSM attenuates HIV-1 Tat induced oxidative stress in mouse neuronal cells. MSM treatment significantly decreased neuronal cell NO and ROS secretion. Further, MSM significantly reversed HIV-1 Tat mediated reductions in reduced glutathione (GSH) as well as HIV-1 Tat mediated increases in oxidized glutathione (GSSG). In addition, Tat reduced nuclear translocation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), a key nuclear promoter of antioxidant activity, while MSM increased its translocation to the nucleus in the presence of Tat. These results suggest that HIV-1 Tat reduces the resiliency of neuron cells to oxidative stress which can be reversed by MSM. Given the clinical safety of MSM, future preclinical *in vivo* studies will be required to further confirm these results in effort to validate MSM as a neuroprotectant in patients at risk of, or who are already diagnosed with, HAND.

Keywords: HIV, cognitive, methylsulfonylmethane, oxidative, glutathione

Introduction

HIV-1 enters the central nervous system (CNS) soon after infection where it is largely resistant to combination antiretroviral therapy (cART). As survival time with chronic HIV-1 infection continues to increase, there are larger numbers of patients harboring the virus within their CNS. As such, the prevalence of HIV-associated neurocognitive disorder (HAND) is rising and, less severe forms of HAND including minor neurocognitive/motor disorder (MCMD) have became more common. HAND continues to be a significant independent risk factor for HIV infection related deaths [1-6].

A major contributing factor to HAND development is the neurotoxicity of proteins secreted by the virus [7-14]. Neurons are not productively infected by HIV-1, thus neuronal damage is largely through indirect mechanisms. The HIV-1 transactivator of transcription protein, Tat, is necessary for viral replication, but is also a significant promoter of neurotoxicity [15-18]. It has been reported that HIV-1 Tat freely penetrates neuron cell membranes and increases levels of lipid peroxidation [19] by generating the reactive oxygen species (ROS) superoxide ($O2^{-}$) and hydrogen peroxide (H_2O_2). In addition, HIV-1 Tat can activate inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO), which binds superoxide anion yielding the highly reactive peroxynitrite ($ONOO^{-}$) [20] which can have deleterious central nervous system (CNS) effects [21]. This condition of oxidative stress is a state in which the existing balance between free radical generation and their subsequent neutraliza-

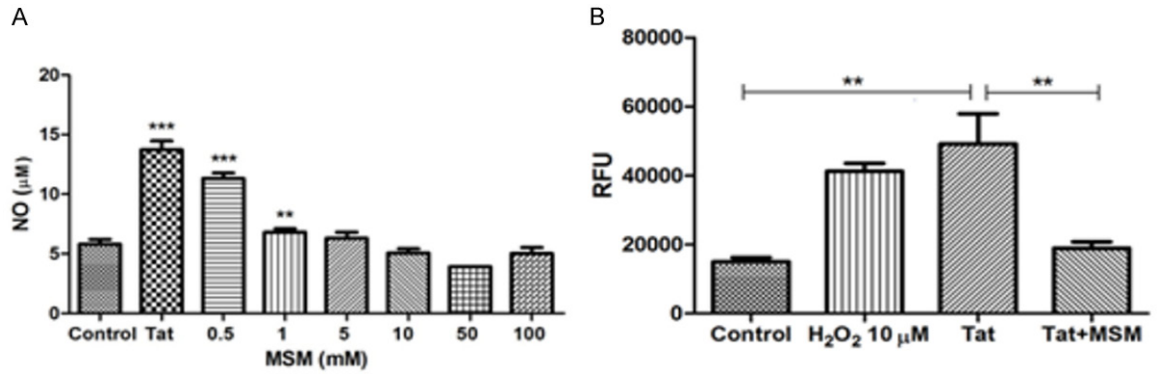

Figure 1. MSM decreases HIV-1 Tat promoted neuronal ROS and NO release. A. Mouse N2a cells were treated with HIV-1 Tat (250 ng/ml) or HIV-1 Tat (250 ng/ml) in combination with various concentrations of MSM (0.5, 1, 5, 10, 50 or 100 mM) for 24 h. Cell cultured media were subjected to NO assays. Results are representative of three separate experiments and indicated the control group produced basal levels of NO while the HIV-1 Tat treated group produced highest levels of NO. The MSM treatments decreased NO production dose dependently and at 1 mM, the levels of NO were similar to control. Values are means ± standard deviation. B. Using the most effective MSM dose to reduce NO secretion (1 mM), cell cultured media were subjected to ROS assay. Results are representative of three separate experiments and indicated that when 1 mM MSM was treated together with HIV-1 Tat, ROS production was decreased at the similar level of control. Values are means ± standard deviation. Statistical significance was analyzed by ANOVA with Tukey's *post hoc test* (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

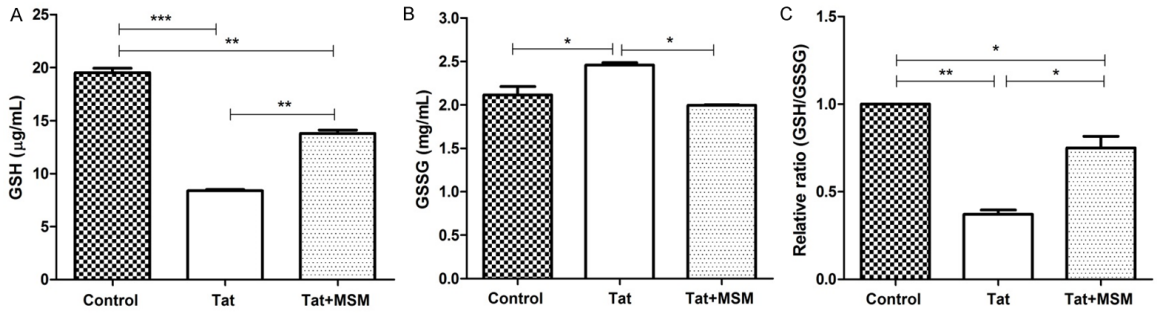

Figure 2. Lowered reduced glutathione (GSH) and increased oxidized glutathione (GSSG) are promoted by HIV-1 Tat and reversed by MSM. N2a cells were treated with PBS (negative control), HIV-1 Tat (250 ng/ml), or HIV-1 Tat (250 ng/ml) in combination with various concentrations of MSM (1 mM) for 3 h. (A) Cell lysates were subjected to reduced glutathione (GSH) and (B) oxidized glutathione (GSSG) detection assays. Results are representative of three separate experiments and indicated HIV-1 Tat treated cells had significantly increased GSSG. When 1 mM MSM was added, it attenuated GSSG levels similar to control group. (C) The ratio of GSH and GSSG were compared. Values are means ± standard deviation. Statistical significance was analyzed by ANOVA with Tukey's *post hoc* test (**$p < 0.01$, ***$p < 0.001$).

tion via the antioxidant defense system becomes shifted to favor free radical expression [21-23].

Indeed, during HIV infection, the antioxidant defense system is likely compromised by insufficiency of endogenous antioxidants, especially in the CNS. More importantly, several reports indicate that oxidative stress may play a key role in CNS damage and cognitive symptoms in HIV infected individuals [24-27]. HIV-1 Tat protein has been reported to be a key mediator of oxidative stress in the brain of HIV-1 infected patients with HAND [28, 29]. Therefore, given the potential involvement of ROS in HAND, research has focused on the possible beneficial effects of antioxidant therapy [8, 9, 30]. It has been reported that supplementation with antioxidants may oppose the damage of the body induced by oxidative stress in HIV infected patients [31, 32].

The antioxidant, methylsulfonylmethane (MSM), is a sulfur-containing compound. It has a good safety profile with low toxicity and is naturally occurring in an array of human foods

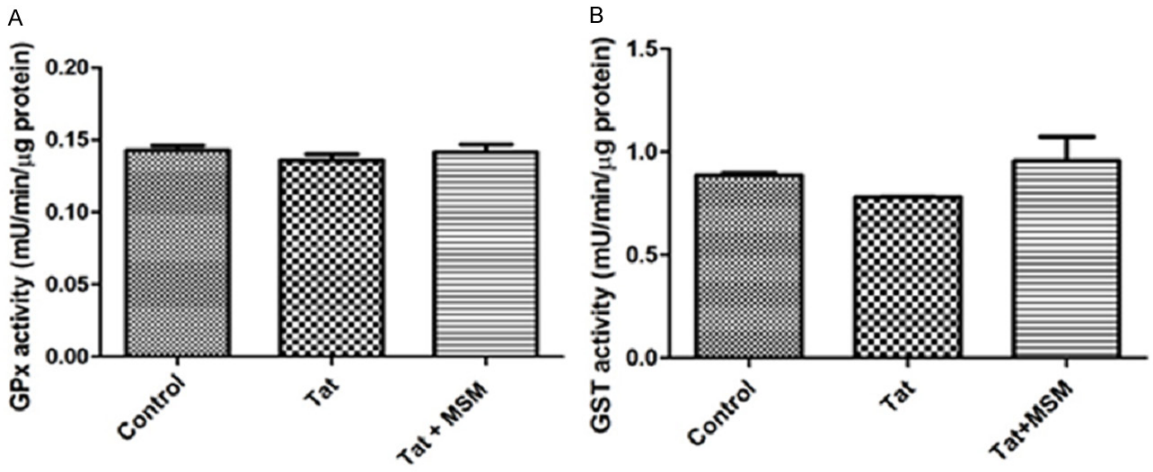

Figure 3. Effect of HIV-1 Tat and MSM on GPx and GST activity. N2a cells were treated with PBS (negative control), HIV-1 Tat (250 ng/ml), or HIV-Tat (250 ng/ml) in combination with MSM (1 mM) for 3 h. Cell lysates were subjected (A) GPx and (B) GST activity assays. Values are means ± standard deviation. Data suggest HIV-1 Tat treatment promoted lowered GPx and GST activity compared to control which was improved by MSM, but it was not statistically significant. ($p > 0.05$). Statistical significance was analyzed by ANOVA with Tukey's *post hoc* test.

including fruits, vegetables, grains, and beverages [33, 34]. Recently, the compound received wide attention for the clinical treatment of osteoarthritis [33, 35, 36]. It has also been found clinically, in humans, that MSM is effective in seasonal allergic rhinitis [37], interstitial cystitis [38], and oxidative stress following acute exhaustive exercise [21]. It also promotes anti-inflammatory in addition to anti-oxidant effects [21, 39]. However, to our knowledge little is known about the mechanism by which MSM may exert its protective effect against HIV-1 Tat-induced oxidative stress in neuronal cells.

Materials and methods

Cell culture

Murine neuroblastoma 2a (N2a) cells (ATCC) were cultured in Dulbecco's Minimum Essential Medium with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell growth was maintained as monolayer in 75 cm^2 tissue culture flask. The cell culture was maintained in humidified, 5% CO_2 incubator at 37°C.

Nitric oxide production

Nitric oxide (NO) production was measured by using Griess reagent (Progema) following the manufacturer's instructions. Nitrite (NO_2^-), a stable metabolite of NO, levels were measured by *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic conditions generated with sulfanilamide. Neuronal cells were seeded at 2.5×10^4 cells per well in 96-well plates overnight. Cells were treated with 250 ng/mL Tat and various concentration of MSM for 24 hours. Next, 50 µL of media samples were transferred onto a new 96-well plate. Fifty µL of sulfanilamide solution was added and incubated for 10 minutes in the dark. Finally, 50 µL of NED solution was added and the plate was incubated for 10 minutes in the dark. Absorbance was measured at 520 nm using a Biotek Synergy H1 micro plate reader.

Reactive oxygen species production

Intracellular ROS production was measured by using 2', 7'-Dichlorofluorescein diacetate (H_2DCFDA, Sigma). The H_2DCFDA is permeable to the cell membrane and oxidized to fluorescent 2', 7'-dichlorofluorescein by various ROS including hydrogen peroxide, hydroxyl radicals and peroxynitrite. Neuronal cells were seeded at 2.5×10^4 cells per well in 96-well plates, and 25 µM of H_2DCFDA was added prior to Tat and/or MSM administration and incubated for 45 min. H_2DCFDA containing media was discarded and washed once with PBS. 100 µL of HIV-1 Tat (final concentration 250 ng/mL) and/or MSM (1 mM) containing phenol red free DMEM was added. ROS production was recorded after 3 hours at Ex/Em = 485/535 nm using a Biotek Synergy H1 micro plate reader.

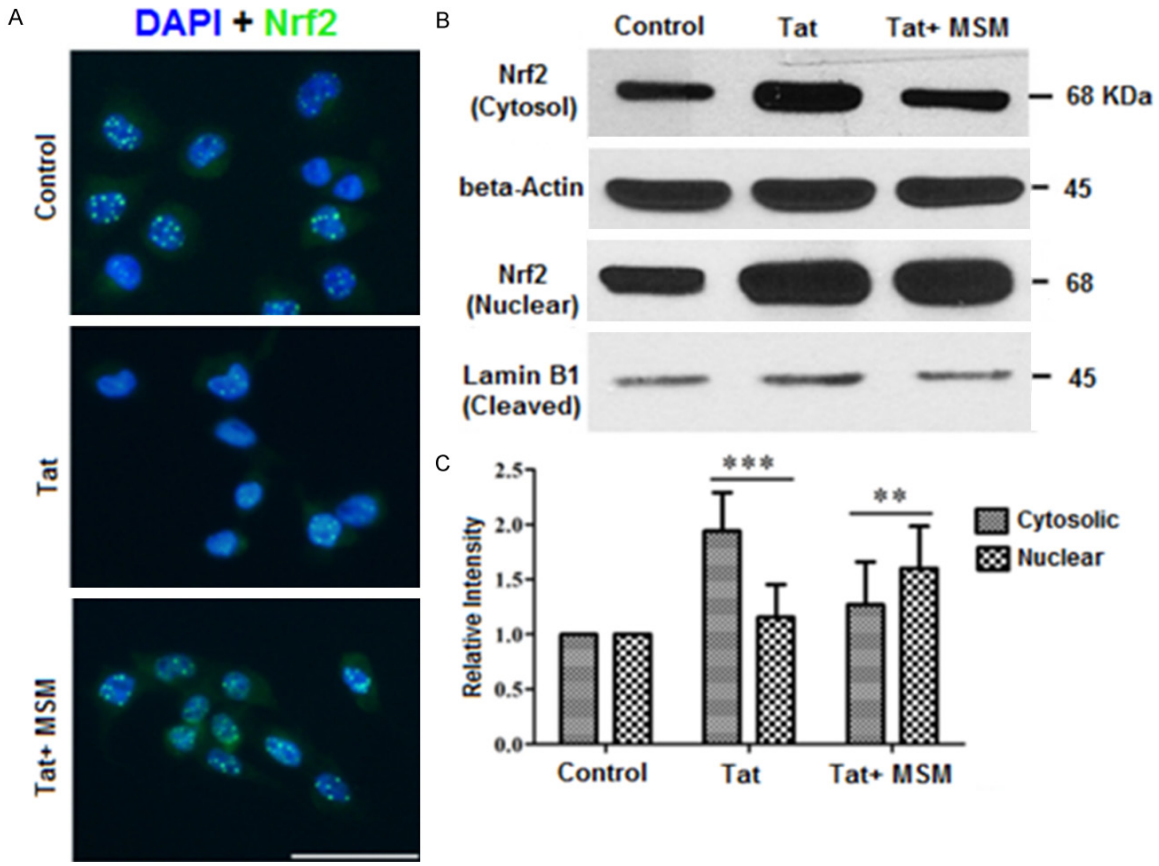

Figure 4. Effect of HIV-1 Tat and MSM nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). N2a cells were treated with PBS (negative control), HIV-1 Tat (250 ng/ml), or HIV-1 Tat (250 ng/ml) in combination with MSM (1 mM) for 3 h. A. Cells were subjected to immunocytochemistry for detection of Nrf2. As shown, there appears to be a reduction in Nrf2 in the nucleus compared to the cytoplasm that appears to be reversed by MSM. B. Western blot analysis of nuclear vs. cytoplasmic Nrf2. C. Densitometric analysis of nuclear vs. cytoplasmic Nrf2 indicates HIV-1 Tat significantly reduced nuclear translocation of Nrf2 ($p < 0.001$) while MSM opposed this effect ($^{**}p < 0.01$). Values are means ± standard deviation. Data were statistically analyzed with a two-way ANOVA with Bonferroni's *post hoc* test.

Reduced (GSH) - and oxidized (GSSG) - glutathione assay

GSH levels was measured by o- phthalaldehyde (OPA) derived fluorescence and GSSG levels was measured by reducing GSSG to GSH after quenching existing GSH using a fluorescent GSH/GSSG assay kit (Biovision). Cells were seeded at 2 × 10^5 cells per well in 6-well plates and allowed to attach the plate bottoms overnight. GSH/GSSG assay was carried out according to manufacturer's instructions.

Glutathione peroxidase (GPx) activity assay

GPx plays an important role in reducing oxidative stress by converting GSH to GSSG. GPx activity assay was instead of has carried out by colorimetric GPx activity assay (Biovision) according to manufacturer's instruction. In this assay, GPx reduces Cumene hydroperoxide while oxidizing GSH to GSSG. The GSSG is reduced back to GSH by glutathione reductase (GR) using NADPH as a proton donor. The decreased NADPH level was instead of is measured at 340 nm and it is proportional to GPx activity. Neuronal cells were seeded at 2 × 10^5 cells per well in 6-well plates overnight. Cells were treated with 250 ng/mL Tat and/or 1 mM MSM for 24 hours. Next, 200 µL of ice-cold assay buffer was added after removing the media and incubated for 10 minutes with vigorous shaking. Cell were collected by pipetting and centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatant was collected and used for assay. Ten µL of samples were transferred into 96-well plate and 40 µL of assay buffer was added. Forty µL of reaction

mix (containing 33 μL assay buffer, 3 μL 40 mM NADPH solution, 2 μL GR solution and 2 μL GSH solution) was added to deplete GSSG in the samples and incubated for 15 minutes. Ten μL cumene hydroperoxide solution was added to start the GPx reaction. GPx activity was measured at 340 nm at 5 minutes and 140 minutes. NADPH levels were calculated based on standard curve generated and GPx activity was calculated by following equation: (the decreased NADPH/time × sample volume) × sample diluon = mU/mL. Each value was normalized with its protein amount.

Glutathione-S-transferase (GST) activity assay

GST is the enzyme that catalyzes detoxification by conjugating xenobiotics with GSH. To determine if there is any change in GST activity in neuronal cells by Tat and MSM, GST activity was measured after Tat and/or MSM administration using a GST fluorometric activity assay (Biovision). In this assay, non-fluorescent monochlorobimane (MCB) is conjugated with GSH by GST reaction and produces fluorescent MCB-GSH. Neuronal cells were seeded at 2 × 10^5 cells per well in 6-well plates overnight. Cells were treated with 250 ng/mL Tat and/or 1 mM MSM for 24 hours. Cells were washed with ice-cold PBS once and collected in microcentrifuge tubes after trypsinization. One hundred μL of ice-cold assay buffer was added after removing the media and incubated for 10 minutes with vigorous shaking. Samples were centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatant was collected and used for assay. According to manufacturer's instruction, 10 μL of samples were plated onto 96-well plates and 90 μL of GST sample buffer was added. In order to start the GST reaction, 100 μL of reaction mix (containing 2 μL MCB solution and 98 μL GST assay buffer) was added and fluorescent MCB-GSH was measured at Ex/Em = 380/461 nm. GST activity was calculated based on standard curve and all values were calculated by following equation: sample GST activity/(reaction time × sample volume) × dilution factor = mU/min/mL. Each value was normalized with its protein amount.

Western blotting

N2a cells were treated with Tat and/or MSM for 24 hours. Cell lysates were collected using Cell lysis buffer (Cell signaling) with protease inhibitors cocktail (Thermo scientific). Cytosolic and nuclear fractionation was carried out by subcellular protein fractionation kit for cultured cells (Thermo scientific) according to manufacturer's instruction. Cytosolic and nuclear fractions were obtained and used for western blotting. Samples were run in 10% SDS gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk dissolved in 0.01% TBST for 1 hour at room temperature. Membranes were probed with mouse anti-Nrf2 antibody (Abcam, ab89443, 1:2000) and mouse anti-beta actin (Cell signaling, 8H10D10, 1:5000) was diluted with blocking buffer and incubated overnight at 4°C. After washing with TBST 3 times for 5 minutes, membranes were incubated with anti-mouse, peroxidase-liked species-specific secondary antibody (Amersham, 1:5000) for 1 hour at room temperature. After washing with TBST for 5 minutes, membranes were visulized by using chemiluminescence detection with ECL Plus Western Blotting Detection Reagents (Amersham). Densitometry analysis was represented by the ratio of Nrf2 to β-actin. Data from three separate experiments were used to assess statistical significance.

Immunocytochemistry

Neuronal cells were seeded at 5 × 10^4 cells per well in 24-well plates and allowed to attach overnight. Cells were then treated with 250 ng/mL Tat and/or 1 mM MSM for 24 hours. Cells were fixed with 4% paraformaldehyde for 5 minutes at room temperature and washed with PBS for 3 times. Cells were permeabilized with 1% Triton X-100 in PBS for 15 minutes and washed with PBS for 5 minutes 3 times. After washing, cells were blocked with 1% bovine serum albumin in 0.02% Tween 20 containing PBS (PBST) for 45 minutes at room temperature with gentle shaking. Cells were then incubated with anti-Nrf2 antibody for 3 hours at room temperature with gentle shaking. After washing with 0.02% PBST for 5 min 3 times, cells were incubated with goat anti-mouse Alexa Fluor 488 (1:250, green) for 1 hour at room temperature with gentle shaking. Nuclei were visualized by mounting with VECTASHEILD fluorescent mounting media with DAPI (Vector laboratory, Burlingame, CA). Fluorescence images were obtained by using an Olympus IX73P1F-30W microscope.

Statistics

NO, ROS, GSH/GSSG, GST and GPx data were statistically analyzed by one-way ANOVA with Tukey's *post hoc* test. Nrf2 data were statistically analyzed with a two-way ANOVA with Bonferroni's *post hoc* test. Alpha levels were set at 0.05 and GraphPad Prism 5 was used for all data analyses.

Results

MSM dependently decreases HIV-1 Tat promoted neuronal ROS and NO release

HIV-1 Tat induced cellular damage may be in part due to the accumulation of oxidative damage induced by ROS and reactive nitrogen species (RNS) to cells and macromolecules. One molecule involved in oxidative damage and inflammatory response is NO. It can act as both inflammatory mediator and RNS, either directly or through peroxynitrites generated by its interaction with O_2^- [40, 41]. Because oxidative injury is seen in the brains of patients with HAND, we tested for increased secretion of NO and ROS induced by Tat application to mouse neuronal cells. HIV-1 Tat application elicited more significantly more NO (*$^{**}p < 0.01$, $^{***}p < 0.001$*) and ROS (*$^{**}p < 0.01$*) than did the control which was reversible to control levels with MSM treatment (*$^{**}p < 0.01$, $^{***}p < 0.001$*). For ROS analysis (**Figure 1B**), we used a dose of 1 mM MSM based on the NO response (**Figure 1A**).

Lowered reduced glutathione (GSH) and increased oxidized glutathione (GSSG) are promoted by HIV-1 Tat and reversed by MSM

GSH is the major antioxidant in aerobic cells. It is important for neuroprotection from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation. The ratio of reduced GSH to oxidized GSH (GSSG) is a measure of cellular health, with reduced GSH constituting up to 98% of cellular GSH under basal conditions. On the other hand, the GSH/GSSG ratio is reduced in neurodegenerative diseases. Measuring the GSH/GSSG ratio in in our model system in comparison to the results in controls is an efficient way to assess potential MSM efficacy in maintaining cellular redox potential [42]. The endogenous antioxidant, GSH, in neuronal cells was decreased significantly by HIV-1 Tat treatment as compared to the levels measured in the control group (*$^{***}p < 0.001$;* **Figure 2A**). However, MSM co-administration significantly reversed the HIV-1 Tat-induced GSH reduction (*$^{**}p < 0.01$*) to levels comparable to that the control group. GSSG was increased by Tat and reduced by MSM to control levels (*$^{*}p < 0.05$;* **Figure 2B**). The ratio of GSH to GSSG was significantly improved by MSM (*$^{*}p < 0.05$*; **Figure 2C**).

Effect of HIV-1 Tat and MSM on glutathione (GPx) and glutathione-s-transferase (GST) activities

The role of this enzymatic antioxidant system was further analyzed. GSH serves as a substrate for GPx to catalyze ROS conversion to H_2O and O_2 using NADPH as a proton donor. Results showed that MSM treatment at the concentration to reduce intracellular ROS and NO did not significantly decrease the activities of, GPx or GST, however a trend towards this was evident (**Figure 3**).

HIV-1 Tat reduces nuclear translocation of Nrf2 that is opposed by MSM

Nrf2 is a master transcription factor that controls the antioxidant response element (ARE)-mediated transcription of genes. In turn, this can mediate the regulation of the synthesis and conjugation of GSH [43, 44]. During non-stimulated activities, Nrf2 is sequestered in the cytoplasm, while Nrf2 is translocated into the nucleus and activates the electrophilic response element/antioxidant response element (EpRE/ARE) upon exposure to oxidative insults [43-45]. Immunocytochemistry using DAPI (blue) to stain neuronal nuclei and green (Alexa Flour 488 conjugated goat anti mouse antibody for green fluorescence) to stain Nrf2 indicated that HIV-1 Tat disrupted the nuclear translocation of Nrf2 which is appears to be brought back to control levels by MSM (**Figure 4A**). Densitometric analysis of western blot (**Figure 4B**) of cytosolic to nuclear Nrf2 indicated that Tat significantly inhibited nuclear translocation of Nrf2 (*$^{***}p < 0.001$*) while MSM significantly increased nuclear translocation of this antioxidant transcription factor (*$^{**}p < 0.001$*) in the presence of Tat. (**Figure 4C**).

Discussion

Oxidative stress plays a role in the development of HAND, and other neurodegenerative

disorders [46-48]. In the case of HIV-1 infection, Tat can elicit such oxidative stress as has been shown by this report as well as others [10, 49]. Such oxidative stress can damage neurons and cause cognitive dysfunction [50]. From our data it is unclear as to whether HIV-1 Tat directly promotes neuronal oxidative stress or whether it is an indirect promotion through activation of inflammatory responses *via* expression of NO, creating a feed forward cycle. Another possibility is that direct interactions of HIV-1 Tat with neurons triggers oxidative stress and NO production [51], which was observed in our study.

Several lines of evidence support the hypothesis that oxidative stress induced modifications of neuronal lipids, proteins, and nucleic acids may be primarily an early step in HIV-1 Tat induced neurotoxicity [51, 52]. The control of ROS is important in relation to HAND because they interfere with the many CNS processes involved in cellular repair. Therefore, many studies have focused their attention on the search of substances that could reduce this increase in not only HIV-1 Tat, but other HIV-1 protein induced oxidative stressors including gp120.

MSM is a known antioxidant that can scavenge ROS, thus preventing tissue damage [53]. It is an endogenous cellular metabolite that acts as a sulfur donor. MSM is also able to act as an antioxidant and free radical scavenger. It has also been shown to promote salutary effects in other biological states in which free radicals and ROS are involved, such as hyperacidity, parasitosis, musculoskeletal pain, arthritis, allergies, and Ehlers-Dantos syndrome [54-56]. Thus, we examined its potential to be protective against the effects of HIV-1 Tat protein, an important mediator of HAND [15].

In the current study, we found that MSM can significantly reduce NO and ROS in cultured mouse neuronal cells at clinically relevant doses (**Figure 1**). Being that both are contributors to HAND, this would indicate MSM as a possible novel neuroprotectant. Underlying this phenomenon was a significant increase in GSH and significant decrease in GSSG, which suggests that ROS and NO induced by Tat are efficiently cleared by GSH conversion to GSSG, promoted by MSM (**Figure 2**).

Two enzymes responsible for detoxification of ROS *via* GSH conversion to GSSG are GST and GPx. A trend toward decreases in these enzyme activities after HIV-1 Tat stimulation has been found in this study (**Figure 3**). Although it was not significant, we surmise the decrease was enough to be responsible for the increase in the ratio of GSH to GSSG that was observed (**Figure 2**) since very small changes in enzyme activity can have much larger effects on their substrate levels. This data is in agreement with previous works, in which a decrease of these enzyme activities was observed under conditions that increased oxidative stress [57-58]. However, to our knowledge this is the first data showing a decrease in the GSH to GSSG ratio after HIV-1 Tat stimulation that can be reversed by MSM in neuronal cells. The observed HIV-1 Tat induced reduction in GPx and GST activities would lead to a decrease in GSH synthesis, which would affect an array of important metabolic pathways in which GSH is involved and is in agreement with previous data that HIV- Tat decreases levels of GSH available to relieve oxidant stress in endothelial cells [59].

The GSH antioxidant system is extremely important in terms of cellular protection. It is common for this molecule to become depleted as a result of increased formation of ROS during increased cellular activities [60]. Since HIV-1 Tat can be chronically expressed in the CNS from integrated HIV-1, this situation would lead to a self-perpetuating cycle, in which the free radicals generated by Tat would induce GSH depletion, thus increasing oxidative stress that would reduce antioxidant enzyme levels, which would further reduce GSH synthesis.

It has been previously shown that HIV-1 deregulates neuronal glutathione redox status [10] and these reports are in accordance with our present findings. In our study, treatment with MSM induced an increase in GSH levels, as could be expected, since MSM metabolism delivers one of the precursors needed for GSH synthesis, therefore counteracting HIV-1 Tat mediated GSH depletion. Our finding of increased ROS and NO (**Figure 1**) supports the hypothesis of augmented oxidative stress due to HIV-1 Tat. The Tat-induced increase in NO and ROS content was reduced by treatment with MSM, suggesting an improvement in the oxidative status of the neuron cells, especially

in light of the MSM-induced increase of GSH previously mentioned. In addition, MSM has been suggested to act as a direct free radical scavenger, another mechanism that could be underlying its antioxidant properties [61].

As mentioned earlier, HIV-1 Tat can also trigger the expression of iNOS, leading to the over production of NO, which can react with superoxide anion to form peroxynitrite, a highly reactive endogenous oxidant. NO can increase glutamate release from astrocytes, enhancing NMDA excitotoxicity [62]. Tat activates several signaling pathways, in one of which superoxide acts as an intermediate, while in others peroxide is utilized [10].

HAND is thought to be driven by chronic inflammatory processes in the CNS. In fact, we and others previously found that pro-inflammatory molecules increase with Tat expression in model mice previously [18, 63]. NO has been reported to be one molecular mediator involved in both the inflammatory response and oxidative damage [64]. Overexpression of NO is believed to contribute to neuronal abnormalities in HAND [65, 66]. In the present study, neuronal NO secretion was found to be increased after HIV-1 Tat treatment, in accordance with other reports which show that iNOS activity and NO release are increased by HIV-1 Tat in several cell types [67, 68]. Our study shows that treatment with MSM normalized increases in NO levels to those observed under control conditions suggesting that MSM may also be able to modulate the neuronal inflammatory response.

Nrf2 is a master transcription factor that controls the antioxidant response element (ARE)-mediated transcription of genes that mediate the regulation of the synthesis and conjugation of GSH [43-45]. Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein (Keap1) under unstimulated conditions, while Nrf2 is translocated into the nucleus and activates the electrophilic response element/antioxidant response element (EpRE/ARE) upon exposure to oxidative insults [43-45]. There should be further translocation to the nucleus by after MSM treatment, which would augment the antioxidant response as well as indicated by our immunocytochemistry data (**Figure 4A**). Upon densitometric analysis (**Figure 4C**) of western blot of Nrf2 (**Figure 4B**), we found that HIV-1 Tat inhibits it's translocation to the nucleus significantly, and that MSM significantly increased Nrf2 nuclear translocation in the presence of Tat. These observations would be in agreement with the observed ability of MSM to reduce oxidative stress (**Figure 1**) and improve the glutathione antioxidant system (**Figure 2**).

These findings suggest the necessity of further investigating the mechanisms of the protective effect of MSM in an *in vivo* model of HAND. As HIV-1 infection of the brain lasts the lifetime of affected individuals, and as eradication of CNS HIV-1 is currently not possible clinically, control of the neuronal dysfunction caused by the virus may represent a feasible approach to a treatment that would limit neuronal oxidative stress induced by HIV-1 Tat.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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References

[1] Major EO, Rausch D, Marra C, Clifford D. HIV-associated dementia. Science 2000; 288: 440-442.

[2] Mattson MP, Haughey NJ, Nath A. Cell death in HIV dementia. Cell Death Differ 2005; 12 Suppl 1: 893-904.

[3] McArthur JC, Brew BJ, Nath A. Neurological complications of HIV infection. Lancet Neurol 2005; 4: 543-555.

[4] Nath A, Sacktor N. Influence of highly active antiretroviral therapy on persistence of HIV in the central nervous system. Curr Opin Neurol 2006; 19: 358-361.

[5] Ances BM, Ellis RJ. Dementia and neurocognitive disorders due to HIV-1 infection. Semin Neurol 2007; 27: 86-92.

[6] Antinori A, Larussa D, Cingolani A, Lorenzini P, Bossolasco S, Finazzi MG, Bongiovanni M, Guaraldi G, Grisetti S, Vigo B, Gigli B, Mariano

A, Dalle Nogare ER, De Marco M, Moretti F, Corsi P, Abrescia N, Rellecati P, Castagna A, Mussini C, Ammassari A, Cinque P, d'Arminio Monforte A; Italian Registry Investigative Neuro A. Prevalence, associated factors, and prognostic determinants of AIDS-related toxoplasmic encephalitis in the era of advanced highly active antiretroviral therapy. Clin Infect Dis 2004; 39: 1681-1691.

[7] Giunta B, Ehrhart J, Townsend K, Sun N, Vendrame M, Shytle D, Tan J, Fernandez F. Galantamine and nicotine have a synergistic effect on inhibition of microglial activation induced by HIV-1 gp120. Brain Res Bull 2004; 64: 165-170.

[8] Giunta B, Obregon D, Hou HY, Zeng J, Sun N, Nikolic V, Ehrhart J, Shytle D, Fernandez F, Tan J. EGCG mitigates neurotoxicity mediated by HIV-1 proteins gp120 and Tat in the presence of IFN-gamma: Role of JAK/STAT1 signaling and implications for HIV-associated dementia. Brain Res 2006; 1123: 216-225.

[9] Rrapo E, Zhu YY, Tian J, Hou HY, Smith A, Fernandez F, Tan J, Giunta B. Green Tea-EGCG reduces GFAP associated neuronal loss in HIV-1 Tat transgenic mice. Am J Transl Res 2009; 1: 72-79.

[10] Agrawal L, Louboutin JP, Reyes BAS, Van Bockstaele EJ, Strayer DS. HIV-1 Tat neurotoxicity: A model of acute and chronic exposure, and neuroprotection by gene delivery of antioxidant enzymes. Neurobiol Dis 2012; 45: 657-670.

[11] Louboutin JP, Strayer DS. Blood-Brain Barrier Abnormalities Caused by HIV-1 gp120: Mechanistic and Therapeutic Implications. ScientificWorldJournal 2012; 2012: 482575.

[12] Choi JY, Hightower GK, Wong JK, Heaton R, Woods S, Grant I, Marcotte TD, Ellis RJ, Letendre SL, Collier AC, Marra CM, Clifford DB, Gelman BB, McArthur JC, Morgello S, Simpson DM, McCutchan JA, Richman DD, Smith DM, Charter Group. Genetic features of cerebrospinal fluid-derived subtype B HIV-1 tat. J Neurovirol 2012; 18: 81-90.

[13] Hui L, Chen XS, Haughey NJ, Geiger JD. Role of endolysosomes in HIV-1 Tat-induced neurotoxicity. Asn Neuro 2012; 4: 243-252.

[14] Hui L, Chen XS, Bhatt D, Geiger NH, Rosenberger TA, Haughey NJ, Masino SA, Geiger JD. Ketone bodies protection against HIV-1 Tat-induced neurotoxicity. J Neurochem 2012; 122: 382-391.

[15] Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 2001; 410: 988-994.

[16] van de Bovenkamp M, Nottet HSLM, Pereira CF. Interactions of human immunodeficiency virus-1 proteins with neurons: possible role in the development of human immunodeficiency virus-1-associated dementia. Eur J Clin Invest 2002; 32: 619-627.

[17] Giunta B, Hou HY, Zhu YY, Rrapo E, Tian J, Takashi M, Commins D, Singer E, He J, Fernandez F, Tan J. HIV-1 Tat Contributes to Alzheimer's Disease-like Pathology in PSAPP Mice. Int J Clin Exp Pathol 2009; 2: 433-443.

[18] Jin JJ, Lam L, Sadic E, Fernandez F, Tan J, Giunta B. HIV-1 Tat-induced microglial activation and neuronal damage is inhibited via CD45 modulation: A potential new treatment target for HAND. Am J Transl Res 2012; 4: 302-315.

[19] Haughey NJ, Cutler RG, Tamara A, McArthur JC, Vargas DL, Pardo CA, Turchan J, Nath A, Mattson MP. Perturbation of sphingolipid metabolism and ceramide production in HIV-dementia. Ann Neurol 2004; 55: 257-267.

[20] Nicotera P, Bonfoco E, Brune B. Mechanisms for nitric oxide-induced cell death: Involvement of apoptosis. Adv Neuroimmunol 1995; 5: 411-420.

[21] Nakhostin-Roohi B, Niknam Z, Vaezi N, Mohammadi S, Bohlooli S. Effect of Single Dose Administration of Methylsulfonylmethane on Oxidative Stress Following Acute Exhaustive Exercise. Iran J Pharm Res 2013; 12: 845-853.

[22] Fisher-Wellman K, Bloomer RJ. Acute exercise and oxidative stress: a 30 year history. Dyn Med 2009; 8: 1.

[23] Ahmadvand H, Tavafi M, Khalatbary AR. Hepatoprotective and Hypolipidemic Effects of Satureja Khuzestanica Essential Oil in Alloxan-induced Type 1 Diabetic Rats. Iran J Pharm Res 2012; 11: 1219-1226.

[24] Velazquez I, Plaud M, Wojna V, Skolasky R, Laspiur JP, Melendez LM. Antioxidant enzyme dysfunction in monocytes and CSF of Hispanic women with HIV-associated cognitive impairment. J Neuroimmunol 2009; 206: 106-111.

[25] Viviani B, Corsini E, Binaglia M, Galli CL, Marinovich M. Reactive oxygen species generated by glia are responsible for neuron death induced by human immunodeficiency virus-glycoprotein 120 in vitro. Neuroscience 2001; 107: 51-58.

[26] Mastroianni CM, Lichtner M, Mascia C, Zuccala P, Vullo V. Molecular mechanisms of liver fibrosis in HIV/HCV coinfection. Int J Mol Sci 2014; 15: 9184-9208.

[27] Ferrell D, Giunta B. The impact of HIV-1 on neurogenesis: implications for HAND. Cell Mol Life Sci 2014; 71: 4387-4392.

[28] Cassol E, Misra V, Dutta A, Morgello S, Gabuzda D. Cerebrospinal fluid metabolomics reveals altered waste clearance and accelerated aging in HIV patients with neurocognitive impairment. AIDS 2014; 28: 1579-1591.

[29] Zhang Y, Wang M, Li H, Zhang H, Shi Y, Wei F, Liu D, Liu K, Chen D. Accumulation of nuclear

and mitochondrial DNA damage in the frontal cortex cells of patients with HIV-associated neurocognitive disorders. Brain Res 2012; 1458: 1-11.
[30] Tang H, Lu D, Pan R, Qin X, Xiong H, Dong J. Curcumin improves spatial memory impairment induced by human immunodeficiency virus type 1 glycoprotein 120 V3 loop peptide in rats. Life Sci 2009; 85: 1-10.
[31] Winter FS, Emakam F, Kfutwah A, Hermann J, Azabji-Kenfack M, Krawinkel MB. The Effect of Arthrospira platensis Capsules on CD4 T-Cells and Antioxidative Capacity in a Randomized Pilot Study of Adult Women Infected with Human Immunodeficiency Virus Not under HAART in Yaound, Cameroon. Nutrients 2014; 6: 2973-2986.
[32] Nguyen D, Hsu JW, Jahoor F, Sekhar RV. Effect of increasing glutathione with cysteine and glycine supplementation on mitochondrial fuel oxidation, insulin sensitivity, and body composition in older HIV-infected patients. J Clin Endocrinol Metab 2014; 99: 3468-3468.
[33] Kim LS, Axelrod LJ, Howard P, Buratovich N and Waters RF. Efficacy of methylsulfonylmethane (MSM) in osteoarthritis pain of the knee: a pilot clinical trial. Osteoarthritis Cartilage 2006; 14: 286-294.
[34] Magnuson BA, Appleton J, Ames GB. Pharmacokinetics and distribution of [35S]methylsulfonylmethane following oral administration to rats. J Agric Food Chem 2007; 55: 1033-1038.
[35] Usha PR, Naidu MUR. Randomised, double-blind, parallel, placebo-controlled study of oral glucosamine, methylsulfonylmethane and their combination in osteoarthritis. Clin Drug Investig 2004; 24: 353-363.
[36] Gregory PJ, Sperry M, Wilson AF. Dietary supplements for osteoarthritis. Am Fam Physician 2008; 77: 177-184.
[37] Barrager E, Veltmann JR, Schauss AG, Schiller RN. A multicentered, open-label trial on the safety and efficacy of methylsulfonylmethane in the treatment of seasonal allergic rhinitis. J Altern Complement Med 2002; 8: 167-173.
[38] Childs SJ. Dimethyl sulfone (DMSO2) in the treatment of interstitial cystitis. Urol Clin North Am 1994; 21: 85-88.
[39] Amirshahrokhi K, Bohlooli S, Chinifroush MM. The effect of methylsulfonylmethane on the experimental colitis in the rat. Toxicol Appl Pharmacol 2011; 253: 197-202.
[40] Sastre J, Pallardo FV, Vina J. Mitochondrial oxidative stress plays a key role in aging and apoptosis. IUBMB Life 2000; 49: 427-435.
[41] Reiter RJ, Tan DX, Cabrera J, D'Arpa D, Sainz RM, Mayo JC, Ramos S. The oxidant/antioxidant network: role of melatonin. Biol Signals Recept 1999; 8: 56-63.
[42] Owen JB, Butterfield DA. Measurement of oxidized/reduced glutathione ratio. Methods Mol Biol 2010; 648: 269-277.
[43] Hayes JD, McMahon M, Chowdhry S, Dinkova-Kostova AT. Cancer Chemoprevention Mechanisms Mediated Through the Keap1-Nrf2 Pathway. Antioxid Redox Signal 2010; 13: 1713-1748.
[44] Copple IM. The Keap1-Nrf2 cell defense pathway--a promising therapeutic target? Adv Pharmacol 2012; 63: 43-79.
[45] Sharma B. Oxidative stress in HIV patients receiving antiretroviral therapy. Curr HIV Res 2014; 12: 13-21.
[46] Beal MF. Aging, energy, and oxidative stress in neurodegenerative diseases. Ann Neurol 1995; 38: 357-366.
[47] Mollace V, Nottet HS, Clayette P, Turco MC, Muscoli C, Salvemini D, Perno CF. Oxidative stress and neuroAIDS: triggers, modulators and novel antioxidants. Trends Neurosci 2001; 24: 411-416.
[48] Giunta B, Fernandez F, Nikolic WV, Obregon D, Rrapo E, Town T, Tan J. Inflammaging as a prodrome to Alzheimer's disease. J Neuroinflammation 2008; 11: 51.
[49] Buccigrossi V, Laudiero G, Nicastro E, Miele E, Esposito F, Guarino A. The HIV-1 Transactivator Factor (Tat) Induces Enterocyte Apoptosis through a Redox-Mediated Mechanism. PLoS One 2011; 6: e29436
[50] Bruce-Keller AJ, Li YJ, Lovell MA, Kraemer PJ, Gary DS, Brown RR, Markesbery WR, Mattson MP. 4-hydroxynonenal, a product of lipid peroxidation, damages cholinergic neurons and impairs visuospatial memory in rats. J Neuropathol Exp Neurol 1998; 57: 257-267.
[51] Aksenov MY, Hasselrot U, Wu GH, Nath A, Anderson C, Mactutus CF, Booze RM. Temporal relationships between HIV-1 Tat-induced neuronal degeneration, OX-42 immunoreactivity, reactive astrocytosis, and protein oxidation in the rat striatum. Brain Res 2003; 987: 1-9.
[52] Aksenov MY, Hasselrot U, Bansal AK, Wu GH, Nath A, Anderson C, Mactutus CF, Booze RM. Oxidative damage induced by the injection of HIV-1 Tat protein in the rat striatum. Neurosci Lett 2001; 305: 5-8.
[53] Maranon G, Munoz-Escassi B, Manley W, Garcia C, Cayado P, de la Muela MS, Olabarri B, Leon R, Vara E. The effect of methyl sulphonyl methane supplementation on biomarkers of oxidative stress in sport horses following jumping exercise. Acta Vet Scand 2008; 50: 45.
[54] Jacob SW, Herschler R. Dimethyl sulfoxide after twenty years. Ann N Y Acad Sci 1983; 411: 13-17.
[55] Mantle D, Wilkins RM, Preedy V. A novel therapeutic strategy for Ehlers-Dantos syndrome

based on nutritional supplements. Med Hypotheses 2005; 64: 279-283.
[56] Avellini L, Silvestrelli M, Gaiti A. Training-Induced Modifications in Some Biochemical Defenses against Free-Radicals in Equine Erythrocytes. Vet Res Commun 1995; 19: 179-184.
[57] Balogh N, Gaal T, Ribiczeyne PS, Petri A. Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses before and after exercise. Vet Clin Pathol 2001; 30: 214-218.
[58] Vijay KK, Naidu M. Effect of oral melatonin on exercise-induced oxident stress in healthy subjects. Indian Journal of Pharmacology 2002; 34: 256-259.
[59] Price TO, Ercal N, Nakaoke R, Banks WA. HIV-1 viral proteins gp120 and Tat induce oxidative stress in brain endothelial cells. Brain Res 2005; 1045: 57-63.
[60] Droge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. Exp Gerontol 2002; 37: 1333-1345.
[61] Parcell S. Sulfur in human nutrition and applications in medicine. Altern Med Rev 2002; 7: 22-44.
[62] Steiner J, Haughey N, Li W, Venkatesan A, Anderson C, Reid R, Malpica T, Pocernich C, Butterfield DA, Nath A. Oxidative stress and therapeutic approaches in HIV dementia. Antioxid Redox Signal 2006; 8: 2089-2100.
[63] Zhao L, Pu SS, Gao WH, Chi YY, Wen HL, Wang ZY, Song YY, Yu XJ. Effect of HIV-1 Tat on Secretion of TNF-alpha and IL-1 beta by U87 Cells in AIDS Patients with or without AIDS Dementia Complex. Biomedical and Environmental Sciences 2014; 27: 111-117.
[64] Szabo C. Multiple pathways of peroxynitrite cytotoxicity. Toxicol Lett 2003; 140: 105-112.
[65] Krogh KA, Wydeven N, Wickman K, Thayer SA. HIV-1 protein Tat produces biphasic changes in NMDA-evoked increases in intracellular Ca2+ concentration via activation of Src kinase and nitric oxide signaling pathways. J Neurochem 2014; 130: 642-656.
[66] Bokhari SM, Yao H, Bethel-Brown C, Fuwang P, Williams R, Dhillon NK, Hegde R, Kumar A, Buch SJ. Morphine enhances Tat-induced activation in murine microglia. J Neurovirol 2009; 15: 219-228.
[67] Liu X, Jana M, Dasgupta S, Koka S, He J, Wood C, Pahan K. Human immunodeficiency virus type 1 (HIV-1) tat induces nitric-oxide synthase in human astroglia. J Biol Chem 2002; 277: 39312-39319.
[68] Pingle SC, Jajoo S, Mukherjea D, Sniderhan LF, Jhaveri KA, Marcuzzi A, Rybak LP, Maggirwar SB, Ramkumar V. Activation of the adenosine A1 receptor inhibits HIV-1 tat-induced apoptosis by reducing nuclear factor-kappaB activation and inducible nitric-oxide synthase. Mol Pharmacol 2007; 72: 856-867.