STUDY THE EFFECT OF HEMOXYGENASE-1 INDUCTION AND SUPPRESSION ON LIPOPOLYSACCHARIDE- BRAIN INJURY IN MALE RATS

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Abstract

**Background:** The neurodegenerative changes occurring during aging is associated with oxidative stress and inflammatory responses. The role of induction or suppression of heme-oxygenase-1 (HO-1) in these changes still a mystery.

**Aim:** This study was designed to investigate the effect of hemoxygenase-1 (HO-1) induction and suppression on lipopolysaccharide- brain injury in male rats.

**Materials and Methods:** Forty male albino rats were divided into four equal groups: a) control group, received saline at a dose of 10 ml/kg, intraperitoneally (IP) for 2 weeks; b) LPS group, received LPS (5mg/kg/day) IP for 2 weeks; c) Hemin group, received Heminat a dose of 15 mg/kg/day IP along withLPS for 2 weeks; d) Nimodipine group, received Nimodipineat a dose of 30 mg/kg/day IP along with LPS for 2 weeks. At the end of the experimental period, all animals were tested for cognitive tests (two-way active avoidance test). Then, the animals were sacrificed by decapitation. Brains of experimental groups were harvested for measurement of HO-1, NO, BDNF, Glu, MDA levels and CAT activity.

**Results:** LPS significantly decreased avoidance number in a 10 block trials and significantly increased the highest avoidance latencies, associated with a significant decrease in BDNF, GLU levels & CAT activity, and a significant increase in brain HO-1, NO, MDA levels compared to control group. Treatment with Hemin deteriorated all these parameters and treatment with Nimodipine significantly improved all these parameters.

**Conclusion:** Suppression of HO-1 by Nimodipine can have a beneficial effect on neurodegenerative changes associated with aging.

Introduction:

Human neurodegenerative conditions, including Alzheimer disease (AD), Parkinson disease (PD), and lateral sclerosis, is associated with altered Fe mobilization and mitochondrial insufficiency, which participates within a pathological triad depending possibly on upregulation of HO-1 by astroglial cells. 

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The HO-1 promoter has a highly responsive system to be induced by several inflammatory and pro-oxidant stimuli as, heme, Aβ, LPS, IL-1β, TNF-α and H2O2. Lipopolysaccharide (LPS), a component of gram-negative bacterial wall, inducing host inflammatory responses and tissue injury associated with significant alteration of brain OS as a primary contributing factor to neurodegenerative changes during aging. So, we can use it as a model of neurodegeneration.

Oxidative and nitrosative stress are involved in pathogenesis of various degenerative disorders, when generation of reactive oxygen species exceeds the ability of defensive enzymes. Several signal transduction pathways may mediate HO-1 gene expression in response to multiple cellular disturbances including oxidative stress (OS) conditions.

Excess formation of nitric oxide (NO), acts as an important mediator of neurotoxicity in neurodegeneration and neuroinflammation. Also, NO has been observed to induce HO-1 expression in different in vitro models of stress-induced cellular injury. The loss of several synaptic protein caused by OS, leads to synaptic degeneration, especially at postsynaptic regions liable to high levels of Ca2+ influx via local activation of glutamate receptors, leading to alterations in cognitive functions.

Furthermore, Hemin, the ferric form of heme with a chloride ligand, is released from Hb at high concentrations in intracranial hematomas. Hemin is a highly reactive compound having direct cytotoxic effects in its high concentrations, via oxidative and non-oxidative mechanisms, leading to cell injury in adjacent tissue due to its potential HO-1 overexpression. Also, hemin causes release of redox active Fe and its breakdown.

Nimodipine (NM) a 1,4-dihydropyridine drug that blocks Ca2+ influx through L-type Ca2+ channels can crossblood brain barrier due to its lipophilic properties. NM has been reported to improve learning in animal models and AD. NM protects neuronal cells against OS and toxic effects of ethanol and heat in a dose dependent attitude.

Moreover, NM may suppress the hippocampal HO-1 expression in aluminum neurotoxicity in mice. Also, NM has shown inhibition of NO production, cytokines and prostaglandin E2 secretion from LPS-stimulated microglia and reduced degeneration of dopaminergic (DA) neurons. The possible protective mechanism for NM, is maintaining Fe homeostasis through suppression of HO-1 expression.

The aim of the present study was to investigate the effect of both chronic over-expression and suppression of HO-1 in LPS brain injury, studying the possible role of NO-dependent signal pathway and impaired glutamate neuroplasticity.

**Materials and Methods:**

**Chemicals and Reagents:**

LPS, Hemin and Nimodipine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) as a powder. All drugs were freshly prepared.

**Experimental design:**

This current work was performed at Tanta Faculty of Medicine, from December 2017 to May 2018, and all experiments were approved by the Ethical Committee of Medical Research, Tanta Faculty of Medicine, Egypt (approval number: 31844/10/17).

The study was carried out on forty male albino rats weighting 200 to 250 grams were purchased from the Experimental Animal House of Faculty of Science, Tanta University. The rats were housed in animal cages (5/cage), under controlled environmental conditions, at room temperature, with free access to water and food.

Animals were acclimatized for two weeks, and then randomly classified into four equal groups;

**Group I; (control group):** received saline at a dose of 10 ml/kg, intraperitoneally (IP) for 2 weeks.

**Group II; (LPS group):** received LPS IP at a dose of 5 mg/kg/day for 2 weeks.

**Group III; (Hemin group):** received LPS as group II, in addition to IP injection of Hemin at a dose of 15 mg/kg/day for 2 weeks.

**Group IV; (Nimodipine group):** received LPS as group II, in addition to IP injection of Nimodipine at a dose of 30 mg/kg/day for 2 weeks.
Cognitive tests:
At the end of the experimental protocol, all animals were tested for cognitive tests (two way active avoidance test) in a shuttle box apparatus, using conditioned stimuli (in the form of light) on the background of unconditioned stimuli (electrical shock), through pre-training, acquisition phase ad retention test described by Bures et al., 1976[18].

Tissue sampling:
Then, the animals were sacrificed by decapitation. Brains of experimental groups were harvested, snap-frozen in liquid nitrogen and subsequently used for measurement of oxidative stress, inflammatory markers and neurotransmitters.

Brain tissue HO-1, malondialdehyde (MDA) and brain derived neurotrophic factor (BDNF) were determined using Biodiagnostic ELISA Kits according to the methods described by Ozawa et al., [19], Zhao et al., [20] and Binder and Scharfman., [21] respectively and following the manufacturers’ instructions.

Nitric Oxide (NO), glutamate (Glu) and catalase (CAT) activity were determined in brain homogenate using colorimetric assay kits (Biodiagnostic Chemical Company, Giza, Egypt), according to the methods described by Montgomery and Dymock.,[22] Pérez-De la Mora et al.,[23] and Johansson and Borg.,[24] respectively and following the manufacturers’ instructions.

Tissues of the dissected brains (after perfusion with a PBS solution, PH 7.4) are homogenized in 5-10 ml of ice cold buffer per gram tissue, and then centrifuged (at 10000 x g for 15 minutes at 4°C). The supernatant was removed for assay and store on ice.

The sacrificed rats were packed in a special package according to safety precautions and infection control measures and sent with hospital biohazards.

Statistical analysis:
Collected data were statistically analyzed by Graphpad Instat software, version 3.10, using one-way ANOVA, followed by Tukey-Kramer's test. Statistical significance was considered at p-value ≤ 0.05, for all statistical tests.

Results:
Two way active avoidance test:
A significant decrease in avoidance number in a 10 block trials and a significant increase the highest avoidance latencies in LPS-treated group. These results were deteriorated with addition of Hemin. Addition of NM revealed a significant increase in avoidance number in a 10 block trials and a significant decrease in the highest avoidance latencies compared to the LPS-treated group (table 1).

| Group Parameters                | Control group | LPS group | Hemin group | Nimodipine group |
|--------------------------------|---------------|-----------|-------------|------------------|
| The highest avoidance latencies (seconds) | 12.69±1.60 | 19.45±1.52* | 27.35±1.68# | 14.08±1.50## |
| Number of avoidances (in 10-block trials) | 8.2±1.03 | 4.7±1.06* | 0.8±0.79# | 7.2±0.63## |

Brain HO-1, NO, BDNF and Glu:
Brain HO-1 and NO were significantly increased while brain BDNF and Glu were significantly decreased in LPS-treated group. These results were deteriorated with addition of Hemin. Addition of NM revealed a significant decrease in brain HO-1 and NO levels, together with a significant increase in brain BDNF and Glu levels compared to the LPS-treated group (table 2).

| Group Parameters                  | Control group | LPS group | Hemin group | Nimodipine group |
|-----------------------------------|---------------|-----------|-------------|------------------|
| Brain HO-1 (ng/ml)                | 0.888±0.299   | 1.964±0.263* | #2.997±0.306# | 1.054±0.351## |
| Brain NO (μmol / gm protein) | 0.942±0.200 | 1.828±0.157* | 2.329±0.761# | 1.000±0.191## |
|----------------------------|-------------|-------------|-------------|-------------|
| Brain BDNF (ng/gm protein) | 3.963 ± 0.329 | 1.851±0.315* | 0.503±0.312# | 3.921±0.338## |
| Brain Glu (μmol / gm protein) | 35.11±0.578 | 31.27±0.747* | 27.75±0.574# | 34.59±0.751## |

Brain oxidative stress markers:
Fig. 1(A,B) showed a significant increase of MDA levels and decrease of CAT activity in the brain tissues following LPS treatment. These results were deteriorated with addition of Hemin. With NM addition a significant decrease in brain MDA and a significant increase in brain CAT activity were detected in LPS + NM group versus LPS-treated group.

Discussion:
The results of the current study revealed that, LPS-treated male rats showed high brain concentrations of HO-1 and NO. These findings could be attributed to the LPS-induced OS. These results were significantly deteriorated after treatment with Hemin in addition to LPS. However, treatment with NM significantly decreased HO-1 and NO brain levels in the LPS + NM treated group.

The mechanism of brain damage induced by LPS may be due to generation of free radicals, which may produce lipid peroxidation and release of ROS.

The increase in HO-1 can be explained by increased OS due to LPS injection. LPS increased HO activity significantly, especially in substantia nigra and hippocampus, which was associated with increased both NOS activity and expression, showing brain OS indicated by regional distribution of lipid peroxides, that may lead to permanent dysfunction, this is according to Núñez and Hidalgo, 2019 [25].

Increased HO-1 activity in microglia induced by LPS, shows severely increased inflammatory mediators. In contrast, decreased cytokines has been shown with inhibition of HO-1 activity (Dodd and Filipov, 2011) [26]. Also, inflammation may induce HO-1 mRNA via Janus kinase /signal transducers & activators of transcription (JAK/STAT) signaling pathway in the brain (Ahmed et al., 2017) [27].

The significant increase in NO in LPS induced brain injury and in hemin group, may be secondary to direct activation of release of NO from glial cells, and may be indirectly due to increased HO-1 activity which increases both NOS expression and activity. Wegiel et al., 2014 [28], recorded that the stress inducible HO-1 generates CO, which is included within the regulation of the cellular NO signal pathways.

The significant decrease of NO by NM may be due to decreased NO production from glial cells, NM may be suggested to suppress the HO-1 expression in apoptosis caused by aluminum neurotoxicity in mice, possibly via...
maintaining Fe homeostasis (Saberzadeh et al., 2016)\(^{[26]}\). Also, NM has shown inhibition of NO production and prostaglandin E2 secretion from LPS-stimulated microglia and reduced degeneration of DA neurons (Espinosa-Parrilla et al., 2015)\(^{[30]}\).

The significant decrease in BDNF levels in LPS induced brain injury and in hemin group may be secondary to increased inflammatory cytokines especially IL-1β. This effect was significantly decreased after NM administration that inhibits the release of inflammatory cytokines and subsequently stimulating the production of BDNF. This is supported by Kranjac et al., 2012\(^{[31]}\), who has reported that administration of LPS suppresses of BDNF secretion from glial cells, and diminished neuroplasticity resulting in impaired learning and memory.

Also, BDNF levels were significantly decreased in the rat hippocampus and in several cortical regions after injection of LPS. Also, neurotrophins as nerve growth factor and neurotrophin-3 levels were decreased even with different peaks (Boschen and Klintsova1, 2017)\(^{[32]}\).

Tong et al., 2018\(^{[33]}\), reported that NM may inhibit apoptosis of hippocampal neurons after brain radiotherapy and upregulate BDNF expression which is associated with the survival of neurons enhancing cognitive function.

The present study shows significant decrease in Glu in LPS induced brain injury and in hemin group, may result from exaggerated release of inflammatory mediators and neuronal loss, which can be modulated by NO. This effect which was significantly abolished after NM administration which can decrease the release of both inflammatory cytokines and NO.

Astroglial NMDA-mediated Ca\(^{2+}\) influx occurs after releasing a Mg\(^{2+}\) block by sufficient depolarization, providing a variety of synaptic plasticity (Spalloni et al., 2013)\(^{[34]}\). According to Chen et al., 2012\(^{[35]}\), Glu binds to NMDA receptors that activates cAMP response element-binding protein, increasing BDNF gene expression, facilitating neuronal survival. Also, according to Greene, 2011\(^{[36]}\), NO can also react with thiols producing nitrosothiols which can inactivate NMDA receptor. Thus, NO can modulate glutamate neurotransmission.

Toll-like receptor-4 (TLR-4) triggered by LPS administration, activates sarcoma (Src) family kinases, which phosphorylate GluN2B subunit (NMDA receptor) enhancing GluN2B-dependent Ca\(^{2+}\) influx, promoting excitotoxicity. Also, neuronal loss can be induced by LPS progressively (Frühauf et al., 2015)\(^{[37]}\).

Choi et al., 2011\(^{[38]}\), stated that NM can decrease neurotoxic Glu release, as it is a L-type Ca\(^{2+}\) channel blocker at both presynaptic and postsynaptic neuronal membranes, and may be also an intracellular Ca\(^{2+}\) antagonist, with a probable protective effect on surgical ischemic conditions of brain from Glu-induced neuronal damage.

The present study shows significant increase in pro-inflammatory MDA brain tissue levels and the significant decrease in redox CAT activity, in LPS induced brain injury and in hemin group, which were reversed by NM. can be explained by increased ROS leading to exhaustion of antioxidant enzymes. Also, NM can effectively increase antioxidant capacity and inhibit OS expression.

Kaminska et al., 2016\(^{[39]}\), reported that LPS administration resulted in the increased mRNA expression of pro-inflammatory cytokines as IL-1β, IL-6 and TNF-α due to increased binding of nuclear factor-xB to these cytokines gene promoters in microglia. Also, Sharma and Nehru, 2015\(^{[40]}\), reported significant increase in MDA levels in the brain (compared to control animals) after single systemic injection of LPS.

Wang et al., 2017\(^{[41]}\), showed MDA elevation and reduction of CAT levels in early brain injury after subarachnoid hemorrhage in rats, in addition to increased tissue levels of TNF-α and IL-6. These increased cytokines and decreased antioxidant enzymes are due to activation of several signal cascades, such as NF-xB and nuclear factor erythroid 2-related factor/heme oxygenase-1 (2Nrf2/HO-1). This is supported by Kajimura et al., 2010\(^{[42]}\). There is a strong relationship between the activity of NOS, SOD, and other redox-active enzymes, and HOs reactions. Also, NO has other heme protein targets as CAT, cytochrome-c, hemoglobin and peroxidase.

NM can inhibit Ca\(^{2+}\) influx and reduce the apoptosis of cells. NM combined with edaravone (a potent free radical scavenger and antioxidant) can effectively increase antioxidant capacity and inhibit OS expression within brain cells. Thus, MDA levels were significantly lower in treated venous blood samples (Xie et al., 2016)\(^{[43]}\).
In the present study, there were observed memory and behavioral impairments as evidenced via active avoidance test in LPS and hemin groups. This may be explained by the long term inflammatory conditions and accumulation of cytokines. This neurotoxicity leads to learning deficits.

Sharma et al., 2017[44], observed memory and behavioral impairments via active avoidance test in LPS treated rats, which escaped maximum number of trails in active avoidance test after prenatal LPS exposure as compared to the control rats. Also, many individuals have been observed for cognitive impairment in long term inflammatory conditions or treatment with cytokine based therapies.

According to Koh and Liang, 2017[45], neurotoxicity leading to learning deficits (decreased step through latency time and increased escape latency time in behavioral tests), was shown to be under several mechanisms including increased ROS formation, through enzymes involving as Nrf2, HO-1, and NADPH oxidase-4 enzyme (NOX4). Inhibited ROS formation is related to down-regulated NOX4, Nrf2, and HO-1.

In the present study, NM improves the two ways avoidance test. This is evidenced by usage of NM as antidepressant and can be explained by its action as Ca\(^{2+}\) channel blocker.

Koskimäki, 2015[46], reported that the NM used as antidepressant, as in its subacute treatment in helpless rodents which failed to avoid the resistant stress (e.g. mild electrical shock), which is a freezing behavior, leads to improvement of the learning behavior of these rodents. Also, Dominguez, 2011[47], reported that the Ca\(^{2+}\) hypothesis of brain aging and AD can be proved by the role of Ca\(^{2+}\) antagonists in treatment of age related cognitive disorders and also symptoms of depressive mood.

Conclusion:-
Chronic HO-1 has a strong effect in a LPS brain injury rat model, through chronic inflammation, oxidative stress and modulation in NO signals, which can be exaggerated by Hemin and attenuated by Nimodipine, providing a new therapeutic intervention for neurodegenerative diseases, as assessed by biochemical findings.

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

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