Protective effect of hesperidin against ammonium chloride-induced hyperammonemia in rats

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Abstract: The current study aimed to investigate the protective effect of hesperidin against hyperammonemia-induced inflammation and oxidative stress. Hyperammonemia was induced by daily intraperitoneal injection of 100 mg/kg body weight ammonium chloride for 8 weeks. Administration of ammonium chloride induced hyperammonemia as evident by the significant increase in blood ammonia and urea levels. In addition, hyperammonemic rats exhibitedsignificantly increased brain lipid peroxidation as well as declined glutathione content and glutathione peroxidase and superoxide dismutase activities. Moreover, hyperammonemia induced a significant increase in serum tumor necrosis factor alpha (TNF-α) accompanied with significant upregulation of brain TNF-α mRNA expression. Concomitant administration of the flavonoid hesperidinsignificantlyameliorated the altered parameters. In conclusion, hesperidinprotects against hyperammonemia-induced oxidative stress and inflammation, demonstrating its antioxidant and anti-inflammatory efficacies.

1.Introduction:

The neurotoxicity of ammonia is well recognized and was first thought to play a significant role in the development of hepatic encephalopathy (HE) in the 1890s (Cichoż-Lach and Michalak, 2013). HE is a medical phenomenon that is described as a neuropsychiatric manifestation of chronic or acute liver disease that is characterized by psychomotor, intellectual and cognitive abnormalities with emotional/affective and behavioral disturbances (Cichoż-Lach and Michalak, 2013).In HE, complications from the hepatic detoxification of ammonia expose the brain to high ammonia concentrations. Consequently, these complications lead to neurotoxicity, which results in neuropsychiatric syndromes and abnormal cerebral blood flow (Detry*et al.,* 2006; Norenberg*et al.,* 2009).

The peripheral immune system communicates with the brain in response to infection and inflammation. This response results in neutrophil adhesion, migration across the BBB and release of chemokines, pro-inflammatory cytokines, proteases and reactive oxygen species (ROS) as well as inflammatory gene transcription (Shawcross*et al.,* 2010). Researchers have proven that the increased blood level of tumor necrosis factor alpha (TNF-α), which occurs during inflammation, stimulates the glial cells to excrete the cytokines [interleukin (IL)-1, IL-6] and influences the permeability of the blood brain barrier (BBB) (Montoliu*et al.,*2009; Prakash*et al.,*2010; Seyan*et al.,*2010). Therefore, natural remedies with antioxidant and anti-inflammatory efficiencies might protect brain against the deleterious effects of ammonia.

Flavonoids are non-nutritive dietary components that are widely distributed in plants (Mahmoud, 2012). It has been reported that compounds extracted from natural plants or traditional medicinal plants have drawn increasing attention for their clinical efficacy with minimal side effects (Moon *et al.,*2006). Multiple studies showed that flavonoids are very reactive towards reactive oxygen species, and they inhibited the activities of many enzymes, such asmetalloproteinases (MMPs), nuclear transcription factor kappa B (NF-κB) and also the expression of genes associated in chronic inflammatory disease (García-Lafuente*et al.,*2009). Hesperidin is a naturally occurring flavanone that exists in citrus and other plants and can be isolated in large amounts from the peels of *Citrus aurantium* (bitter orange), *Citrus sinensis* (sweet orange), and *Citrus unshiu* (Satsuma mandarin) (Crozier *et al.,* 2009). It has also been demonstrated that hesperidin can protect neurons against various types of insults associated with many neurodegenerative diseases (Cho, 2006). Therefore, the current study was designed to investigate the protective effect of hesperidin against hyperammonemia-induced inflammation and oxidative stress.

2.Materials and methods:
Chemicals:

Hesperidin (Figure 1) and ammonium chloride were purchased from Sigma Chemicals Co., St. Louis, MO, USA and all other chemicals were obtained from standard commercial supplies.
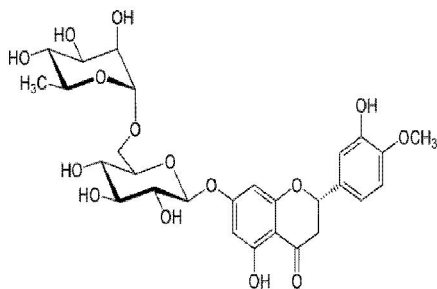
Figure 1: Chemical structure of hesperidin.

Experimental animals:

White male albino rats weighting about 130-150 g were used as experimental animals in the present investigation. The animals were housed in standard polypropylene cages (4 rats/cage) and maintained under controlled room temperature (22±2 °C) and humidity (55%±5%) with 12 h light and 12 h dark cycle and were fed a standard diet of known composition, and water *ad libitum*.

Experimental design:

The experimental animals were divided into three groups, each group comprising six rats designated as follows:

Group 1: Normal rats

Group 2: Hyperammonemic rats, received 100 mg/kg ammonium chloride intraperitoneally for 8 weeks (Mahmoud, 2012).

Group 3: concurrently received 100 mg/kg ammonium chloride intraperitoneally and 50 mg/kg hesperidin orally for 8 weeks (Mahmoud *et al.,*2012).

Biochemical assays:

At the end of 8 weeks, the animals were killed by decapitation under ether anesthesia. Blood samples were taken for the determination of ammonia (Wolheim, 1984) and urea (Varley*et al.*, 1998) using reagent kits purchased from Spinreact (Spain).

Serum levels of the pro-inflammatory cytokine, TNF-α, was determined by specific ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. The concentration of TNF-α was determined spectrophotometrically at 450 nm. Standard plot was constructed by using standard cytokine and the concentrations for unknown samples were calculated from the standard plot.

Brain lipid peroxidation, reduced glutathione (GSH), and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured according to the methods of Preuss*et al*. (1998), Beutler*et al*. (1963), Marklund and Marklund (1974) and Kar and Mishra (1976), respectively.

RNA isolation and quantitative real-time polymerase chain reaction (qPCR):

Total RNA was extracted from brain samples using the Triazol reagent method (Chomkczynski and Sacchi, 1987). RNA concentration and purity were measured using a UV spectrophotometer, with A260/A280 ratios of ≥ 1.6 considered acceptable. First strand of cDNA was synthesized from 2 µg of total RNA by using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, USA).

Quantitative PCR using SYBR Green RT-PCR Kit (Applied Biosystems, USA) was performed to analyze the mRNA levels of TNF-α and β-actin. The following primer sets were used;

TNF-α	Up 5′- AAATGGGCTCCCTCTCATCAGTTC-3′
	Down 5′-TCTGCTTGGTGGTTTGCTACGAC-3′
β-actin	Up 5′-AAGTCCCTCACCCTCCCAAAAG-3′
	Down 5′-AAGCAATGCTGTCACCTTCCC-3′

The samples were subjected to PCR cycles as follows: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60 s. Fluorescent values were converted into threshold cycle (CT) values and the expression of target genes was analyzed using the $2^{-\Delta\Delta Ct}$ method following the normalization through β-actin.

Statistical analysis:

Statistical analysis was performed using SPSS v.20. Results were articulated as mean ± standard error (SE) and all statistical comparisons were made by means of one-way ANOVA test followed by Duncan's multiple range test post hoc analysis. A *P* value <0.05 was considered significant.

3.Results:

The effect of ammonium chloride administration on blood ammonia and urea levels was summarized in Table 1. The recorded data showed a significantly elevated blood ammonia (*P*<0.001) and urea (*P*>0.01) in hyperammonemic rats when compared to the normal control ones. Supplementation of hesperidin significantly alleviated the altered blood ammonia and urea.

Data concerning the effect of hesperidin supplementation on brain lipid peroxidation level, estimated as malondialdehyde, were represented in Figure 2. Hyperammonemic rats exhibited a significant elevation in brain malondialdehydeconcentration when compared to normal control rats. On the other hand, concurrent administration of hesperidinproduced a potential amelioration of the altered malondialdehyde (*P*>0.001) level.

Table 1: Effect of hesperidinon blood ammonia and urea levels of hyperammonemic rats.

Parameter / Group	Ammonia (μmol/L)	Urea (mg/dl)
Normal	57.41 ± 4.26^{b}	13.66 ± 2.01^{b}
Hyperammonemic	302.23 ± 10.74^{a}	61.87 ± 4.62^{a}
Hyperammonemic + HES	88.06 ± 8.11^{b}	29.35 ± 2.89^{c}
F-Prob.	$P<0.001$	$P<0.01$

Data are expressed as Mean ± SE. Means which share different superscript symbol(s) are significantly different.

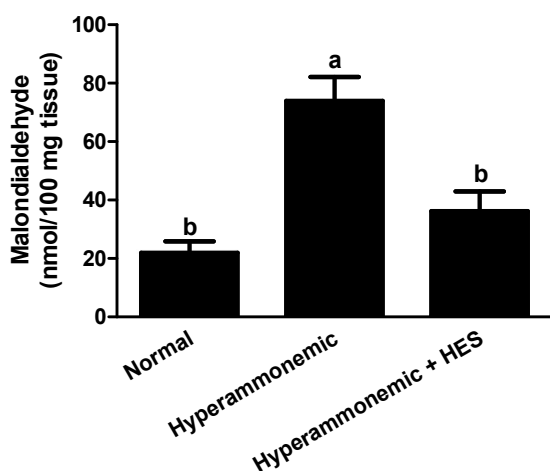

Figure 2: Effect of hesperidinon brain lipid peroxidation of hyperammonemic rats.

In contrary, brain glutathione exhibited a different behavioral manner. Ammonium chloride administration significantly ($P<0.001$) decreased reduced glutathione content when compared to normal control rats. Hesperidin supplementation significantly ($P<0.001$) protected rats from hyperammonemia-induced glutathione depletion, as depicted in Figure 3.

Activities of the antioxidant enzymes, superoxide dismutase and glutathione peroxidase, showed a significant ($P<0.001$) decrease in brain tissues of hyperammonemic rats compared to normal control ones (Figures 4 & 5). Concurrent administration of hesperidin potentially alleviated superoxide dismutase and glutathione peroxidase activities.
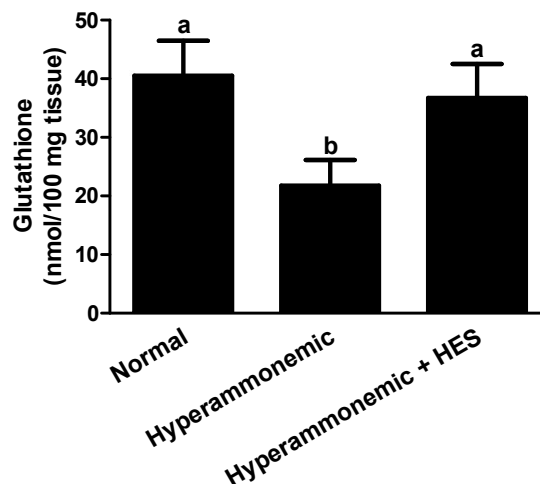

Figure 3: Effect of hesperidinon brain reduced glutathione content of hyperammonemic rats.

The effect of hyperammonemia on serum TNF-α and mRNA expression of brain TNF-α are represented in Figures 6 and 7, respectively. The recorded data showed significantly increased serum TNF-α concentration accompanied with a significant ($P<0.001$) upregulation of TNF-α mRNA expression in hyperammonemicrats when compared to the normal control ones. Hesperidin supplementation significantly decreased the elevated serum TNF-α and the hyperammonemia-induced expression of TNF-α.
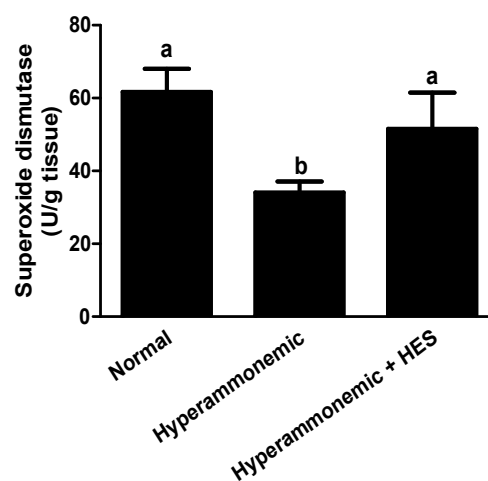

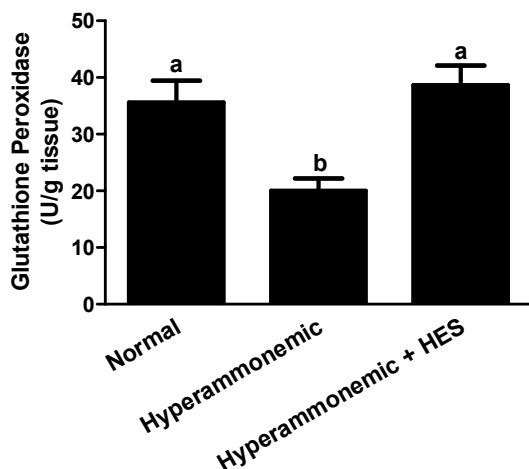Figure 4: Effect of hesperidinon brain superoxide dismutase activity of hyperammonemic rats.

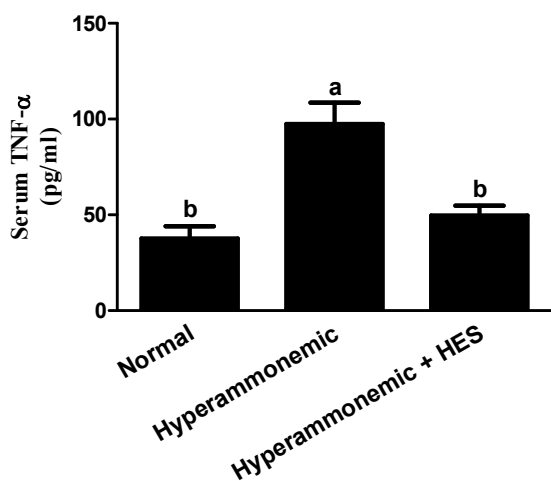Figure 5: Effect of hesperidinon brain glutathione peroxidase activity of hyperammonemic rats.

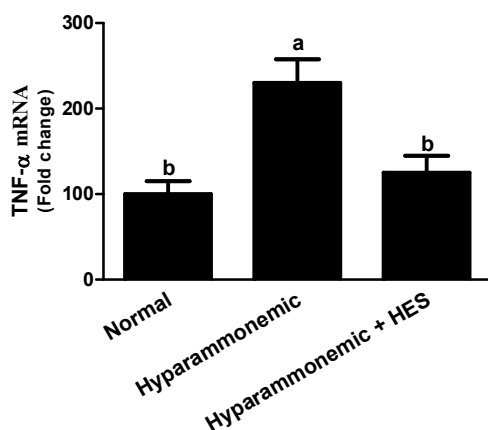Figure 6: Effect of hesperidinon serum TNF-α levels of hyperammonemic rats.

Figure 7: Effect of hesperidinon brain TNF-α mRNA expression of hyperammonemic rats.

4.Discussion:

Hyperammonemia is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy (Mahmoud *et al.,* 2014). The serious adverse effects, toxicity and reappearance of symptoms after discontinuation are the common disadvantages of the currently available conventional or synthetic antihyperammonemic agents (Srinivasan *et al.*, 2001). Therefore, there is a growing need to find protective agents against hyperammonemia from traditional medicinal plants (Mahmoud *et al.,*2014). In the current study, administration ammonium chloride induced a state of hyperammonemia as evident by the increased blood ammonia and urea levels. Our findings are in agreement with the studies of Essa and Subramanian (2007), Mahmoud (2012) and Mahmoud *et al.* (2014) who demonstrated that increased levels of circulatory ammonia and urea indicate hyperammonemic condition in ammonium chloride supplemented rats, which may be due to liver damage caused by ammonia intoxication. On the other hand, concurrent supplementation of hesperidin significantly protected rats against ammonium chloride induced blood ammonia. In this regard, Shirwaikar *et al.* (2003) reported that flavonoids offer ammonia detoxification by removing excess ammonia, urea, uric acid and creatinine during various disease conditions, such as hyperammonemia.

Oxidative stress is believed to play a role in the pathogenesis of HE because acute doses of ammonia lead to the induction of oxidative stress (Norenberg *et al.,*2004). In addition, ammonia neurotoxicity has been reported to induce oxidative stress and subsequently to brain edema (astrocyte swelling) (Norenberg *et al.,*2004). In the present study, a significant elevation in brain lipid peroxidation has been reported in the hyperammonemic rats. Accordingly, numerous previous studies reported the elevation of lipid peroxides in hyperammonemic animals (Vidhya and Subramanian, 2003; Essa and Subramanian, 2006; Mahmoud, 2012). Moreover, the studies of Murthy *et al.* (2001) and Jayakumar*et al.* (2006) revealed that cultured astrocytes acutely exposed to 5 mM ammonia showed an increase in reactive oxygen species and cell swelling, which are both prevented by antioxidant treatments.Swelling of cultured astrocytes treated with ammonia is invariably associated with intracellular accumulation of reactive oxygen and nitrogen species (Häussinger and Schliess, 2008), including the highly toxic peroxynitrite (Master *et al.,*1999). Hesperidin supplementation protected the brain against hyperammonemia-induced lipid peroxidation. The protective effect of hesperidin can be attributed to its antioxidant effect that has been highlighted in

previous studies (Chen *et al.,*2010; Mahmoud *et al.,* 2012).

On the contrary, ammonium chloride administration significantly reduced GSH content as well as activities of the antioxidant enzymes, superoxide dismutase and glutathione peroxidase. These results are in agreement with those of previous studies, which demonstrated a significant decrease in levels of reduced glutathione in brain of hyperammonemic rats (Essa and Subramanian, 2006; Mahmoud, 2012; Mahmoud *et al.,* 2014). Reduced glutathione is regarded as being the first line of defense, and neutralizes the hydroxyl radical and plays an important role against inflammation and oxidative stress (Circu and Aw, 2011). Therefore, we propose that the protective effect of hesperidin against hyperammonemia-induced oxidative stress is mediated through preventing gluathione depletion and potentiating the antioxidant enzymes.

Neuroinflammation is a new element in the pathogenesis of HE described in animal models, which seems to play an important role in the development of cognitive impairment, that can persist after liver transplantation (García-Martínez and Córdoba, 2011). In addition, a study conducted by Shawcross *et al.*(2004) in patients with liver cirrhosis have shown that inflammation and inflammatory mediators may significantly modulate ammonia influence on central nervous system (Shawcross*et al.,*2004). The inflammation is an important factor determining the presence and severity of neuropsychological dysfunction in minimal hepatic encephalopathy caused by ammonia, that is, more significant in more severe inflammation (Shawcross*et al.,*2007).

The current study demonstrated a significant increase in serum TNF-α as well as increased expression of TNF-α mRNA in brain of hyperammonemic rats. Increased circulatory cytokines in hyperammonemic rats has been recently recorded (Mahmoud *et al.,*2014). In addition, a significant increase of TNF-α and IL-6 pro-inflammatory cytokines in serum of patients with minimal hepatic encephalopathy was noticed (Srivastava*et al.,*2011). Moreover, Alvarez *et al.*(2011) study on the astrocyte cultures indicated that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IF-γ and ammonia induce increase of the mitochondria permeability and may be an important factor in the pathogenesis of hepatic encephalopathy. Concurrent administration of hesperidin markedly protected against hyperammonemia associated TNF-α over expression. In accordance, Mahmoud *et al.*(2012) demonstrated that hesperidin significantly decreased serum levels of TNF-α and IL-6 in type 2 diabetic rats.

In conclusion, the current study revealed that concurrent administration of hesperidin protected against the deleterious effects of hyperammonemia through its antioxidant and anti-inflammatory efficacies.

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