Protective and healing promotion effect of heat shock protein on stress induced gastric ulceration in rats

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ABSTRACT

Background: *Heat shock proteins play an important role in the maintenance of normal cell integrity and also serve to protect cells from the cytotoxic effects of aggregated proteins produced by various stresses. Water immersion and restraint stress (WRS) induces gastric ulceration. Treatment with non steroidal anti-inflammatory drugs or blocking nitric oxide "NO" synthesis by L-NAME, a NO synthase inhibitor, aggravates gastric ulceration. On the other hand heat preconditioning or treatment with NO donor, L-arginine, act as cyto-protective factors protecting the gastric mucosa and enhance gastric mucosal healing. However, whether the pathogenic effects of aspirin and L-NAME, and the cyto-protective effect of NO and heat preconditioning are related to their effect on expression of heat shock protein-70 (HSP70) in gastric mucosa or not is still undetermined.* ***Aim of work:*** *The present study was established to investigate the potential participation of HSP70 in the protection against acute gastric mucosal damage and its role in recovery, also the effect of acetyl salicylic acid, NO and heat preconditioning on its release and effect.* ***Methods:*** *128 male albino rats were divided into 6 groups, 24 rats each (except group-I comprised of 8 rats); Group-I: control non stressed group, rats received oral vehicle (saline) 1ml/rat, the rats in the stressed groups II-VI were pretreated 30 min. before the start of WRS by different factors. Group-II: rats received oral vehicle (saline 1ml/rat). Group-III: rats are heat preconditioned at 42°C for 30 min. Group-IV: rats received acetyl salicylic acid (ASA, aspirin) orally (40mg/kg). Group-V: rats received oral L-arginine (250 mg/kg). Group-VI: rats received oral L-NAME (10 mg/kg). All rats were exposed to WRS (except group-I) for 3.5 hours, then all rats were sacrificed, including group-I, then number of gastric ulcers, gastric malondialdehyde (MDA) content "an indicator for lipid peroxidation in gastric mucosa", and expression of inducible NO synthase (iNOS) and HSP70 in the stomach were determined at 0, 6, 24 hours after the end of WRS.* ***Results:*** *WRS caused typical bleeding erosions and that were aggravated by ASA and L-NAME pretreatment and this was accompanied by a significant rise in MDA, and a significant decrease in the expression of HSP70. Pretreatment by heat preconditioning or L-arginine resulted in a significant reduction in the number gastric ulceration and promotion of healing with a significant decrease in gastric level of MDA and significant increase in the expression of iNOS and HSP70 in the gastric mucosa.* ***Conclusion:*** *HSP70 has a healing promotion effect on induced gastric ulcer in WRS rats and the increase in its*

expression resulted in a protective effect against the development of the ulcer. It appears that HSP 70 expression alteration is one of the mechanisms by which heat preconditioning, aspirin, L-arginine, and L-NAME induced their effects on gastric mucosa.

Keywords: *Stress ulcers, L-NAME, L-arginine, Acetyl salicylic acid, Heat shock proteins (HSP).*

INTRODUCTION

Recent advances in molecular biology have demonstrated that most cells are able to alter their metabolism and function by modulating protein synthesis at both transcriptional[(1)] and translational levels[(2)]. In response to environmental or physiological stress, such as heat shock, ethanol, heavy metals, or amino acid analogues, cells increase synthesis of intracellular protein called heat shock proteins (HSPs) or stress protein.[(3)]

Certain HSPs are expressed under non stressful condition and play an important role in the maintenance of normal cell integrity. One such HSP function is represented by its role as a "molecular chaperone", folding polypeptides into mature tertiary structures. [(4)]

HSPs are classified into several families according to their apparent molecular weights and respective inducers. In addition HSP70 is the most inducible HSP[(5)] and closely linked to cytoprotection from hyperthermia, water immersion stress, and drugs induced gastric ulcers.[(6)]

One of the most famous drugs induced gastric ulcers are non steroidal anti-inflammatory drugs (NSAIDs), such as *aspirin*, which also interferes with the healing of acute or chronic gastric ulceration[(7)] and induces pathogenic events resulting from suppression of prostanoid synthesis, such as reduction in gastric microcirculation and the leukocyte-endothelium adherence.[(8)]

In contrast, nitric oxide (NO), which is synthesized from amino acid *L-arginine* by enzyme NO synthase (NOS), is considered to act as cytoprotective agent by many mechanisms enhancing the gastric mucosal defense ability as regulation of mucus/alkaline secretion, gastric motility and microcirculation.[(9)]

However, whether the pathogenic effect of *ASA* and the cytoprotective effect of NO are related to their effect on expression of HSP in gastric mucosal cells or not is still undetermined.

Among various stress models used in animals, the most reproducible results can be obtained by water immersion and restraint stress (WRS) which appear to act synergistically in the production of gastric ulceration.[(10)] Another form of stress can be induced is the thermal injury[(4)] which is considered as a powerful stressful condition. HSP70 is closely linked to heat shock.[(6)]

Given this back ground, the present study was established to investigate the potential participation of HSP70 in the protection against acute gastric mucosal damage and its role in recovery. Thus, the effects of heat preconditioning, *ASA*, NO donor

(*L-arginine*) and NO scavenger *(L-NAME)* on the stress induced by WRS in rats as regards the gastric ulcers number, lipid peroxidation in gastric mucosa as indicated by measuring malondialdehyde (MDA) and gastric mucosal content of inducible nitric oxide synthase (iNOS) were studied. In addition, the expression of HSP70 in gastric mucosa and its relation to the protection against WRS gastric ulcer and mucosal recovery were studied.

MATERIAL & METHODS

A) TEST DRUGS:

1. ***L-arginine*** **hydrochloride** (C8H14-N4O2.HCl) (*Sigma Chemical Company)* supplied as a white powder soluble in distilled water.
2. **N^G-nitro-L-arginine methyl ester** *(L-NAME) (Sigma Aldrich, St. Louis)* (MW=269.7) supplied as a white powder soluble in distilled water.
3. **Acetyl salicylic acid** (ASPÉGIC® Injectable vial, AMRIYA FOR PHARMACEUTICAL INDUSTRIES under license of LABORATORIES SYNTHELABO FRANCE) supplied as a white powder soluble in distilled water.

B) ANIMALS:

The study was conducted on 128 normal adult male albino rats, which had approximately the same body weight (150-200g), were kept in individual cages and kept on a 12:12 light dark cycle in a room temperature at 28 °C ± 1°C and given *ad libitum* access to water and rodent chow *(El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt)*. All rats were fasted for 18 hrs before and during the experiment, they allowed only water ad libitum. Rats were randomly assigned to one of 6 groups, each group comprised of 24 rats (except group-I contains 8 rats), as follows:

- **Group-I:** Control group. They are normal non stressed rats, and they received saline vehicle 1 ml/rat by gastric gavage 30 min. before the experiment.
- **Group-II:** Vehicle (saline) pretreated stressed group. Rats received saline vehicle 1 ml/rat by gastric gavage.
- **Group-III:** Heat preconditioned and stressed rats. Rats were exposed to mild whole body heating at 42°C for 30 minutes [11].
- **Group-IV:** Acetyl salicylic acid (*ASA*, *aspirin*) pretreated stressed group. The rats received *ASA* in a dose of 40 mg/kg [10] by gastric gavage.
- **Group-V:** *L-arginine* (NO donor) pretreated stressed rats. The rats received *L-arginine* in a dose of 250 mg/kg [12] by gastric gavage.
- **Group-VI:** *L-NAME* (NOS inhibitor) pretreated stressed rats. Rats received *L-NAME* in a dose of 10 mg/kg [13] by gastric gavage.

After 30 min. of the pretreatment, all rats, except the control group-I, were exposed to WRS by placing the rats in immobilization cages and immersing them in water at 23°C to the xiphoid process for 3.5 hours [10]. Eight animals of each group (II, III, IV, V and VI) were sacrificed at 0, 6, 24 hrs after the end of WRS.

All animals in control group-I were sacrificed 4 hours after receiving oral vehicle.

The rats were killed by a blow on the head and bled out by cutting their throat. The abdomen was opened, the stomachs were removed and the gastric mucosal ulcers were counted and the average number of ulcers in the stomach per animal was calculated. The stress lesions were defined as a round or linear mucosal defects of at least 0.1 mm in diameter.[10] Then, in all groups, each rat's gastric mucosal HSP70, iNOS and MDA content was estimated.

C) LABORATORY MEASUREMENTS:

Measurement of gene _expression of HSP70, iNOS and MDA levels in gastric mucosa:

Gastric mucosa was scraped then homogenized in 175µl RNA lysis solution which contains mercaptoethanol and guiandium thiocynate and was centrifuged at 10.000 rpm for 10min, and then the supernatants were kept frozen at –80 °C till examined for expression of HSP70 and iNOS by RT-PCR and measurement of MDA by colorimetery.

1. Measurement of MDA:

Malondialdehyde (MDA) was measured in tissue homogenate after precipitation of protein by addition of trichloroacetic acid (TCA), then thiobarbituric acid (TBA) reacted with (MDA) to form thiobarbituric acid reactive product, which was measured at 532 nm according to ***Draper and Hadley***[14].

2. Measurement of HSP70 and iNOS:

Quantitative competitive reverse transcription-polymerase chain reaction RT-PCR:

RNA was extracted from tissue homogenate by using SV-total RNA isolation system *(Promega- Madison, USA)* according to ***Chomezynnski and Sacchi,*** [15] and the extracted RNA was measured by spectrophotometer at 280nm.

About 5µg of RNA was reverse transcribed by using 12.5µl of oligonucleotides primer denaturated at 70°C for 2 min. The denaturated RNA was placed on ice for 5 min., 1 mmol KCl, 50 mmol HCl, 0.5 mmol deoxy nucleoside triphosphate (dNTPs) and 200 unit of moleny murine leukemia virus (MMLV) reverse transcriptase was used. The reaction conditions were: 42°C for 1 hour followed by heating at 95°C for 5min to stop the reaction. PCR reaction was performed by adding the PCR mix to about 5µl of cDNA, the mixture contained 10 mmol HCl, 50 mmol KCl, 100 mmol dNTPs and 2.5 unit of taq polymerase and about 10 µmol of each of sense and antisense primer of HSP70 and iNOS; with the following sequence for *HSP*; "sense: 5`- GTGAAG ATC TGCGTCTGCTTG-3` antisense 5`-TTTGACAACAGGCTGGTGAACC-3`. *iNOS* has the following primer sequence; sense: 5`-TTAGAGTTG CT-TACGTGTG-3`; antisense:5`-AC CTGAGAGCGGGTGACCAGGGT-3`.

The PCR cycling conditions were 94°C for 1 min. for denaturation followed by 57°C for 1 min. and 72°C for 45 seconds; for 40 cycles with final extension at 72°C for 12 min.

Gel electrophoresis:

10µl of PCR product was analyzed on 2% agarose gel with ethidium bromide staining and the product was visualized on ultraviolet transilluminator, then gel documentation was performed. PCR products were semi-quantitated by using gel documentation system *(Bio Doc Analyze)* supplied by *Biometra*.

D) STATISTICAL ANALYSIS:

Values are measured as mean ± SD. Comparison of data was performed by using ANOVA test (analysis of variance test). $p<0.05$ is considered statistically significant[16].

RESULTS

●***Effect of vehicle pretreatment, heat preconditioning, Acetylsalicylic acid (ASA), L-arginine and L-NAME pretreatment in male rats -30 minutes before WSR- on the number of gastric ulcers at 0 time after the end of WRS:***

All animals in group-I (control non-treated non-stressed) showed no gastric ulcers at the end of the experiment. Vehicle (saline, 1 ml orally) pretreated group-II developed a mean gastric ulcers of 16 ± 2 at 0 time after WRS. Giving oral *ASA* (40mg/kg) in group-IV and oral *L-NAME* (10mg/kg) in group-VI significantly increased the mean gastric ulcers to 29 ± 3 and 22 ± 3 respectively compared to group-II. On the other hand, heat preconditioning at 42°C for 30 min. before the start of WRS (group-III) and oral *L-arginine* (250 mg/kg) in group-V significantly reduced the mean rats' gastric ulcers to 7 ± 0.7 and 5 ± 0.6 respectively compared to group-II. [*Table (1&2), Fig. (1&5)*]**.**

●***Effect of vehicle pretreatment, heat preconditioning, ASA, L-arginine and L-NAME pretreatment in male rats -30 minutes before WSR- on the level of MDA in the gastric mucosa at 0 time after the end of WRS:***

MDA content, measured as an index lipid peroxidation at the end of WRS. In control group-I, MDA level was 0.0. In WRS vehicle pretreated group-II the MDA concentration reached the value of 12±1.25 nmol/g gastric mucosal tissue and it appears that exposure to WRS is a strong stimulus for lipid peroxidation. Oral *ASA* (40mg/kg) in group-IV and oral *L-NAME* (10mg/kg) in group-VI, significantly increased the mean MDA to 16±3 and 14±2 nmol/g gastric mucosal tissue respectively, compared to group-II. However, heat preconditioning at 42°C for 30 min. before the start of WRS (group-III) and oral *L-arginine* (250 mg/kg) in group-V significantly reduced the mean rats' MDA to 0.29±0.05 and 3±0.7 respectively compared to group-II. [*Table (1&2), Fig. (2&6)*].

●***Effect of vehicle pretreatment, heat preconditioning, ASA, L-arginine and L-NAME pretreatment in male rats -30 minutes before WSR- on the level of inducible nitric oxide synthase "iNOS" in the gastric mucosa at 0 time after the end of WRS:***

In control non stressed group-I the level of iNOS was 198±7 µg/mg tissue. In vehicle pretreated stressed group-II there was no significant alteration in level of iNOS (195±9

µg/mg tissue) compared to intact gastric mucosa in control group-I.
In heat preconditioned stressed group-III and pretreatment with *L-arginine* (group-V), 30 min. before exposure to WRS, there was a highly significant increase in the level of iNOS as compared to vehicle pretreated stressed group-II reaching 722±28 and 1051±29 µg/mg tissue respectively. There was no significant changes in the level of iNOS concentration in *ASA* pretreated stressed group-IV as compared to vehicle pretreated stressed rats (group-II) and the value was 190±14 µg/mg tissue. In contrast There was a significant attenuation in the level of iNOS in *L-NAME* pretreated stressed group reaching 75±5 µg/mg tissue compared to gastric mucosa in intact non stressed group-I and vehicle stressed group-II. [*Table (1&2), Fig. (3&7)*].

- ***Effect of vehicle, heat preconditioning, ASA, L-arginine and L-NAME pretreatment -30 minutes before WSR- on the expression of tissue HSP70 in male rats in the gastric mucosa, at zero time after the end of the WRS:***

Table (1&2), Fig. (4&8) show that; in the control rats, the mean level of HSP70 was 195±19 µg/mg tissue. The exposure to stress in vehicle pretreated group-II appeared as a strong stimulus for induction of expression HSP70 compared to the intact group-I reaching a mean value of 385±25 µg/mg tissue. Compared to vehicle pretreated group-II, there was a highly significant increase in the level of HSP70 in the heat preconditioned stressed rats (in group-III) as well as rats pretreated with *L-arginine* (in group-V) with a mean value 647±33 and 523±41 µg/mg tissue respectively. On the other hand, in rats pretreated with *ASA* (in group-IV) or *L-NAME* (in group-VI) before WRS, the mean HSP70 was significantly attenuated compared to those exposed to vehicle pretreated stressed group-II with a value of 68±12 and 62±9 µg/mg tissues respectively.

Table (1): Mean number of rats' gastric ulcers and the mean level of Malondialdehyde (MDA), inducible nitric oxide synthase (iNOS) and heat shock protein (HSP70) in different studied groups at 0 time after the end of water immersion restraint stress "WRS" compared to non-stressed group-I and stressed group-II. (n= 8)

Groups		**Mean gastric ulcers number** lesions/rat	**Mean MDA level** nmol/g gastric mucosal tissue	**Mean iNOS level** µg/mg gastric mucosal tissue	**Mean HSP70 level** µg/mg gastric mucosal tissue
Non-stressed intact Control Group-I (Vehicle-treated "1ml/rat")		0	0	198 ± 7^{a}	195 ± 19^{a}
Stressed rats (received medications 30 min. before WRS)	Group-II Vehicle treated (1ml/rat)	12 ± 1.25^{a}	16 ± 2^{a}	195 ± 9^{a}	385 ± 25^{b}
	Group-III Heat precondition (42°C)	0.29 ± 0.05^{b}	7 ± 0.7^{b}	722 ± 28^{b}	647 ± 33^{c}
	Group-IV *ASA* treated (40 mg/kg)	16 ± 3^{d}	29 ± 3^{c}	190 ± 14^{a}	68 ± 12^{d}
	Group-V *L-arginine* treated (250 mg/kg)	3 ± 0.7^{c}	5 ± 0.6^{b}	1051 ± 29^{c}	523 ± 41^{c}
	Group-VI *L-NAME* treated (10 mg/kg)	14 ± 2^{d}	22 ± 3^{d}	75 ± 5^{d}	62 ± 9^{d}

-Values are mean±SE.

-Significance differences when $p<0.05$

-Similar letters have the same significance and are significantly different from other letters within the same test column.

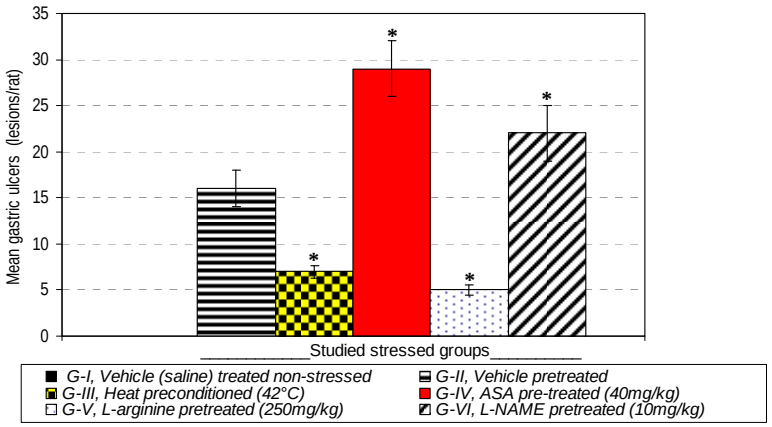


Fig (1): Mean number of rats' gastric ulcers in different studied groups at 0 time after water immersion and restraint stress "WRS".

- Data are mean±SD, n=8.
- = Significant changes compared to grouop-II "G-II" (**p**< 0.05).*
-G-I is hidden due to its 0 value.
-Group-II to Group-VI pretreated 30 min. before WRS.

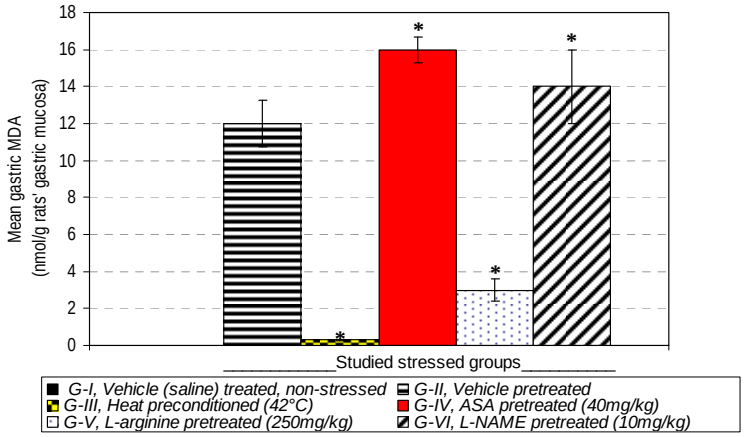


Fig (2): Mean malondialdehyde "MDA" in rats' gastric mucsa in different studied groups at 0 time after water immersion and restraint stress "WRS".

- Data are mean±SD, n=8.
- = Significant changes compared to grouop-II "G-II" (p< 0.05).*
-G-I is hidden due to its 0 value.
-Group-II to Group-VI pretreated 30 min. before WRS.

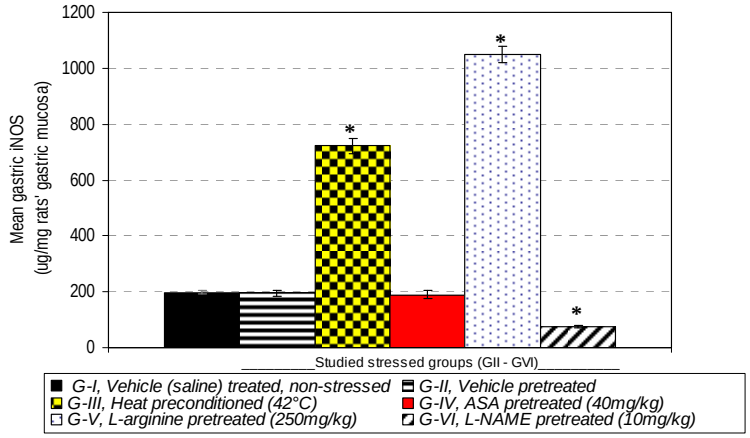
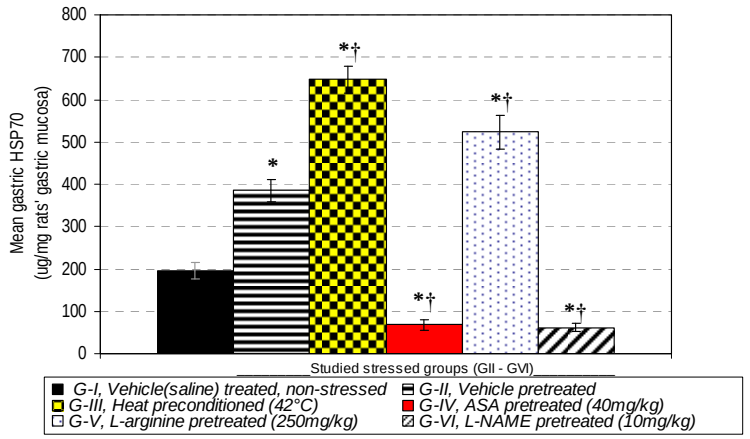


Fig (3): Mean inducible nitric oxide synthase (iNOS) in rats' gastric mucsa in different studied groups at 0 time after water immersion and restraint stress "WRS".

- Data are mean±SD, n=8.
- = Significant changes compared to grouop-I & II "G-I & G-II" (**p**<0.05).*
- Group-II to Group-VI pretreated 30 min. before WRS.



Fig(4): Mean heat shock protein-70 (HSP70) in rats' gastric mucsa in different studied groups at 0 time after water immersion and restraint stress "WRS".

- Data are mean±SD, n=8.
- = Significant changes compared to grouop-I (**p**<0.05).*
*-†= Significant changes compared to grouop-II (**p**<0.05).*
-Group-II to Group-VI pretreated 30 min. before WRS

The mean gastric ulcers number, gastric mucosal content of MDA and iNOS in rats exposed to WRS during recovery at 6h, 24h after the end of WRS:

6 and 24 hours after the end of WRS, at each time interval there was gradual significant reduction in the mean gastric ulcers, in rats that received vehicle (group-II), heat preconditioned (group-III) and *L-arginine* (group-V) compared to 0 time after WRS, within the same group. On the other hand, *ASA* and *L-NAME* pretreated rats in group IV and VI respectively, significantly increased the mean gastric ulcers at 6h and 24h after WRS compared to 0 time within the same group and to the corresponding values within the same times in group-II.

The mean gastric MDA level was significantly reduced in *L-arginine* treated group-V 24h after WRS compared to 0 time within the same group, however, there was no change in the mean MDA in heat preconditioned (group-II) during the whole experiment time. In contrast, 6 and 24 hours after WRS, the mean gastric MDA level significantly increased in vehicle (group-II), *ASA* (group-IV) and *L-NAME* (group-IV) treated rats compared to 0 time after WRS within the same group.

The mean iNOS insignificantly decreased in vehicle treated group-II, at the whole experiment time, compared to control group-I. The mean iNOS significantly increased at 6 and 24 hours after WRS in rats received *L-arginine*, inversely, it was significantly reduced in *L-NAME* pretreated rats at the same intervals after WRS in comparison to 0 time after WRS within the same group. However there was an insignificant difference in the mean iNOS at 6 and 24 hours after WRS compared to 0 time after WRS within the same group in rats of group II, III and IV. [*Table (2), Figures (5, 6 &7)*].

Expression of HSP70 during the mucosal recovery from WRS-induced gastric ulcers in different studied groups at 6h, 24h after the end of WRS:

Table (2), Fig. (8) showed that HSP70 was 195±19 µg/mg tissue in intact gastric mucosa. In vehicle pretreated stressed group-II of rats, a significant increase in HSP70 expression was observed in the mucosa at time 0 after WRS as compared to gastric mucosal content in intact group of rats, and progressively increased at 6h following the termination of WRS reached the top value. Furthermore, at 24h interval after WRS exposure still HSP70 was significantly above the level of intact animal but significantly less than that detected at 6h after the end of exposure to WRS.

In heat preconditioning pretreated stressed rats (group-II) there was a significant increase in level of HSP70 at both time interval 6h & 24h after the end of WRS exposure as compared to values recorded at 0 time in the same group and as compared to the corresponding values recorded in all studied groups at these time interval with maximum rise at 6 h.

In *ASA* pretreated stressed group-IV HSP70 progressively decreased with significant reduction compared to vehicle pretreated stressed group-II at time 6h, 24h. In *L-arginine* pretreated stressed group-V there was a progressive increase in the level of HSP70 at time 6h and reached the top but with gradual decrease at time of 24 h after WRS exposure but still significantly higher than the level detected at 0 level and than values recorded in vehicle pretreated stressed group. Lastly, in *L-NAME* pretreated stressed group there was no significant change in the level of HSP70 at time interval 6h, 24 h after WRS exposure compared to 0 time after the end of exposure to WRS.

Table (2): Effect of vehicle (saline) pretreatment, heat preconditioning, Acetyl salicylic acid *(ASA)*, *L-arginine* and *L-NAME* pretreatment; on the number of gastric lesions, malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), and heat shock protein 70 (HSP70) levels in the gastric tissue at 0, 6, and 24h after the end of water immersion restraint stress (WRS).

Groups			Mean gastric ulcers (lesions/rat) (n=8)	Mean MDA levels (nmol/g rats' gastric mucosa) (n=8)	Mean iNOS levels (µg/mg rats' gastric mucosa) (n=8)	Mean HSP70 levels (µg/mg rats' gastric mucosa) (n=8)
Intact Group-I: (Control Vehicle-treated & non-stressed) 1ml /rat			0	0	198±7	195±19
Stressed rats in different studied groups (received medications 30 min. before WRS) at different times after WRS	***Group-II:*** Vehicle treated (1ml/rat)	0.0 Time	16±2	12±1.25	195±9 §	385±25 ¶
		6 h.	9.4±1.2 ‡	14±2 ‡	196±5 §†	554±15 ‡¶
		24 h.	7±2 ‡	14±3 ‡	195±3 §†	400±13 ‡¶
	Group-III: Heat precondition (42°C)	0.0 Time	7±0.7 *	0.29±0.05 *	722±28 *	620±28 *¶
		6 h.	3±0.5 *‡	0.29±0.012 $^{\dagger *}$	732±30 $^{\dagger *}$	985±20 *‡¶
		24 h.	0	0.29±0.05 $^{\dagger *}$	720±15 $^{\dagger *}$	647±33 *‡¶
	Group-IV: *ASA* treated (40 mg/kg)	0.0 Time	29±3 *	16±3 *	190±14	68±12 $^{*\P\|}$
		6 h.	32±5 *‡	18±3 *‡	193±7 †	124±3 $^{*\ddagger\P\|}$
		24 h.	32±4 *‡	19±4 *‡	195±8 †	72±7 $^{\dagger *\P\|}$
	Group-V: *L-arginine* treated (250 mg/kg)	0.0 Time	5±0.6 *	3±0.7 *	1051±29 *	523±41 $^{*\P\|}$
		6 h.	1±0.05 *‡	3±0.3 $^{\dagger *}$	1073±25 *‡	694.5±14 $^{*\ddagger\P\|}$
		24 h.	0	2±0.2 *‡	1077±18 *‡	590±18 $^{*\ddagger\P\|}$
	Group-VI: *L-NAME* treated (10 mg/kg)	0.0 Time	22±3 *	14±2	75±5 *	62±9 $^{*\P\|}$
		6 h.	24±5 *‡	19±3 *‡	63±3 *‡	84±8 $^{\dagger *\P\|}$
		24 h.	25±4 *‡	19±2 *‡	58±5 *‡	66±6 $^{\dagger *\P\|}$

-Values are mean±SE.

-Significant differences when $p<0.05$; ‡= Significant change compared to the values at 0 time after WRS within the same group and the same test column; †= Insignificant change compared to the values at 0 time after WRS within the same group and the same test column.

*= Significant change compared to the corresponding values in group-II within the same test column; ¶ = Significant change compared to the value of control group-I within the same test column; § = Insignificant change compared to the value of control group-I within the same test column.

$^{\|}$ = Significant change compared to the corresponding values in group-III within the same test column.

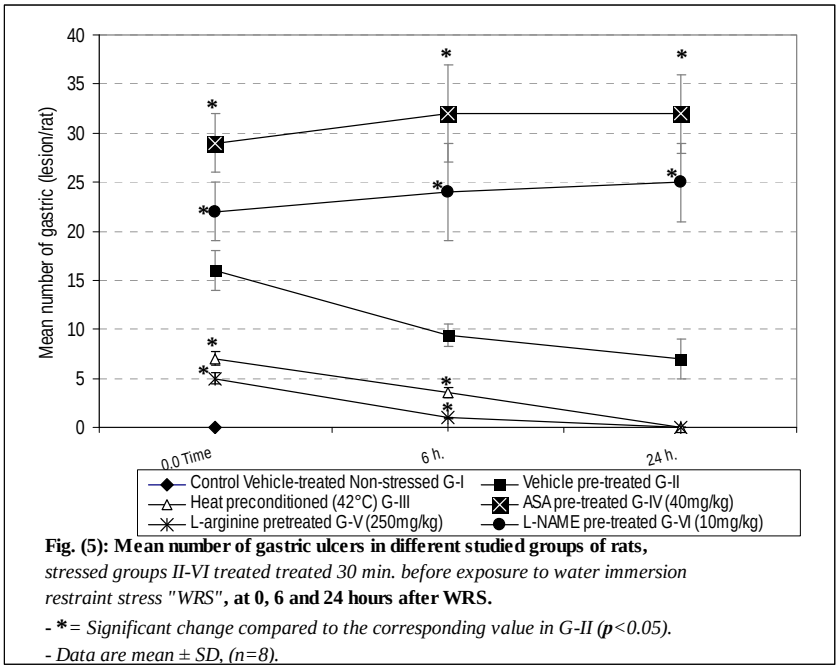


Fig. (5): Mean number of gastric ulcers in different studied groups of rats, *stressed groups II-VI treated treated 30 min. before exposure to water immersion restraint stress "WRS"*, **at 0, 6 and 24 hours after WRS.**

*- * = Significant change compared to the corresponding value in G-II (**p**<0.05).*

- Data are mean ± SD, (n=8).

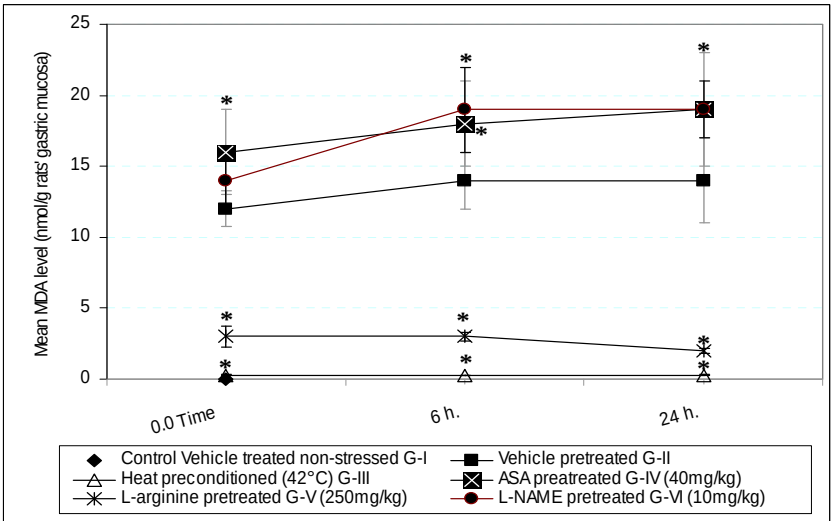


Fig. (6): Mean level of gastric malondialdehyde "MDA" in different studied groups of rats, *stressed groups II-VI treated 30 min. before exposure to water immersion restraint stress "WRS"*, **at 0, 6 and 24 hours after WRS.**

*- * = Significant change compared to the corresponding value in G-II (**p**<0.05).*

- Data are mean ± SD, (n=8)

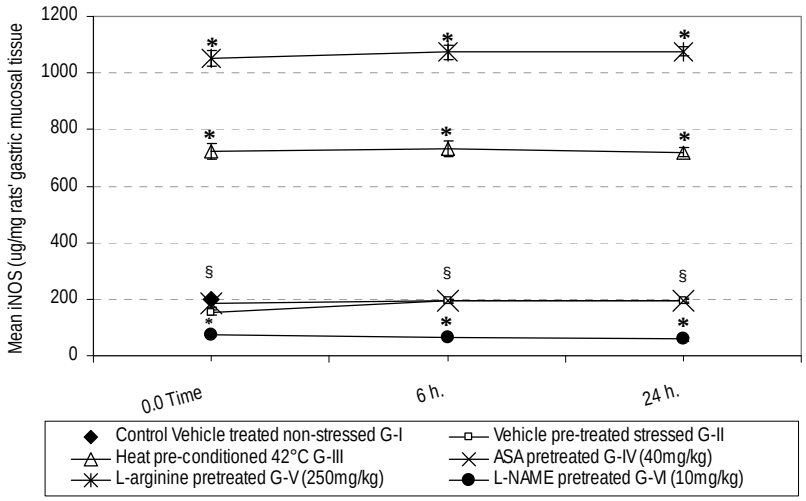


Fig.(7): The mean inducible nitric oxide synthase (iNOS) in different studied groups, *stressed groups (II-VI) received treatment 30min. before water immerssion restraint stress "WRS"***, at 0, 6, and 24 h. after WRS.**

- *§=Insignificant change compared to G-I (**p**<0.05).*
- ***=Significant change compared to the corresponding value in G-II (**p**<0.05).*
- *Data are mean ± SD, (n=8).*

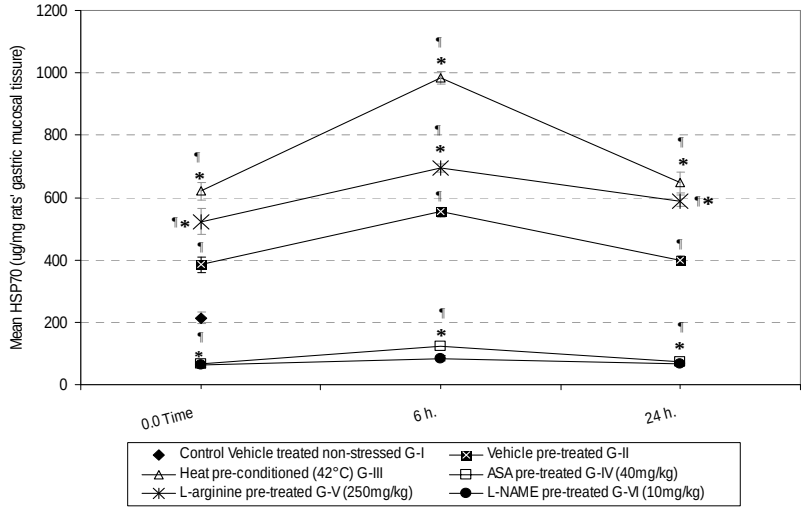


Fig.(8): The mean heat shock protein-70 (HSP70) in different studied groups, *stressed groups (II-VI) received treatment 30min. before water immerssion restraint stress "WRS"***, at 0, 6, and 24 h. after WRS.**

- *¶=Significant change compared to G-I (**p**<0.05).*
- ***=Significant change compared to the corresponding value in G-II (**p**<0.05).*
- *Data are mean ± SD, (n=8).*

DISCUSSION

Heat shock proteins (HSPs) are necessary for essential cellular events, such as folding, assembly and transport of proteins, as molecular chaperones and also serve to protect cells from the cytotoxic effect of aggregated proteins produced by various stress.[17] Expression of HSPs is generally induced following exposure to pathophysiological stresses in wide range of living organism.[18] They have been implicated in the defense mechanisms of gastro-intestinal mucosa. [19]

Stress ulcerations are defined as acute gastric mucosal lesions occurring as complications in severely ill patients.[10] Non steroidal anti-inflammatory drugs are known to induce gastric mucosal damage and interfere with the healing of acute or chronic ulcerations.[7] Nitric oxide donors help protection of gastric ulceration by producing gastric hyperemia and attenuation of lipid peroxidation in the gastric mucosa. [10]

In the present study, it was found that there was a significant increase in HSP70 mRNA expression in gastric mucosa of rats pretreated with vehicle and exposed to WRS at 0, 6 and 24 hrs time compared to control non stressed group with maximum rise at 6 hrs.

In accordance with these results, it was reported that in cultured gastric mucosal cells, ethanol and H_2O_2, as stress factors, induce major HSPs expression.[20&6]

These results were supported by results of ***Konturek et al.***, [21] and ***Tsukimi et al.***,[17] who reported that HSP70 in the gastric ulcer base was dramatically increased during the time of ulcer development and its expression in the ulcer margin gradually increased with ulcer healing in rats exposed to stress.

The results of the present study showed that the maximum rise in HSP70 was obtained at 6 hours after WRS exposure, and the expression was still significantly high 24 hours after the end of WRS.

Ulcer healing can be divided into three consecutive phases; first, inflammation, then, re-epithelization, granulation tissue formation and angiogenesis and at last, remodeling of extra-cellular matrix.[4] We can suggest that HSP70 is involved in the mucosal regeneration in different phases of ulcer healing. Since several growth factors and cytokines are synthesized in the ulcer base at the time of healing, HSP70 might help to their synthesis in the ulcer base via molecular chaperon activity. [22&23] Also, there is a strong relationship between the expression of HSP70 and markers for cell proliferation such as proliferating cell nuclear antigen (PCNA) or silver staining nucleolar organizer regions (AgNoRs). [24] Consequently, the increased expression of HSP70 in the ulcer margin is suggested to be involved in mucosal regeneration in the late stage of ulcer healing.

To study the role of HSP70 in the protection of gastrointestinal tract mucosa against ulcer in stressful conditions, the rats were exposed to mild whole body heating before exposure to WRS and it was found that mild whole body heating at 42°C

significantly increased HSP70 expression and significantly reduced the number of stress-induced gastric lesions as compared to stressed group of rats without preheating. Thus it can be suggested that the mucosa with increased HSPs acquires tolerance to necrotizing stimuli and the increased mucosal HSP70 is related to increased mucosal resistance (the decreased number of gastric lesions) and ulcer healing promotion that was observed after preconditioning. This tolerance was through playing role as intracellular factors integral in the defense mechanism of gastrointestinal mucosa.

Expression of HSPs is induced following exposure to heat shock. ***Nakamura et al.***[20] were the first who indicated that cells inducing HSPs by heat shock treatment resist 7.5% ethanol which is a lethal concentration in cultured guinea pig gastric mucosal cells.

In accordance with these results, ***Itoh and Noguchi***, [25] reported that pretreatment with whole body heating was found to prevent gastric lesions induced by WRS stress in rats. They reported a positive relationship between HSP induction and mucosal protection as they detected a high level of HSP70 expression after WRS stress in animals treated with hyperthermia as compared to stress alone.

Similar results were reported by ***Hirakawa et al.*** [26] and ***Konturek et al.*** [27] who demonstrated that oral administration of geranyl acetone (GGA), or repeated administration of *aspirin* increase gastric mucosal resistance to damage by stressful conditions and this increased protection is correlated with increased expression of HSP70.

HSP chaperoning is a permanent cellular event during both non stressed and stressed condition.[28] Upon stress, up-regulation of the synthesis of HSP70 suggests that during evolution, tissues developed intrinsic defense mechanisms for rescuing unfolding proteins in various cellular compartments and protecting the cells from the cytotoxic effect of aggregated proteins. [29] In addition, HSPs are also considered to improve cellular recovery both by refolding partially damaged functional proteins and by increasing delivery of precursor proteins to important organelles, such as mitochondria. [30] Moreover, HSP70 is known to repair partially damaged proteins, and key enzymes involved in cytoprotection through its molecular chaperone activity.[4] Accordingly, it is suggested that HSP70 contributes to the protection of gastrointestinal mucosa and that over expression of HSP70 by heat preconditioning might be related to protection of the gastric mucosa against different stressful stimuli.

More evidence for the role of HSPs in gastric mucosal protection was shown by studying the relation between *ASA* and HSP70. In this study it was found that *ASA* pretreatment aggravated the gastric mucosal lesions caused by WRS and it significantly suppressed HSP70 expression as compared to vehicle (saline) pretreated-stressed group. These results can partially explain augmentation of WRS-induced gastric damage caused by *ASA*. *Aspirin* causes disruption of phospholipids layer that covers and protects the

gastric mucosal surface,[31] and suppression of prostanoid synthesis by inhibition of COX-1. [32] The suppression of HSP70 expression by *ASA* is one of the mechanisms that may contribute to the development of NSAIDs gastro-pathology. These results are in agreement with work of ***Konturek et al.,*** [33] who reported that *ASA* suppressed significantly the increased HSP70 mRNA expression in rats pretreated with vehicle before WRS.

On the other hand, ***Konturek et al.,*** [27] reported that long term administration of low dose of *ASA* that induced gastric adaptation to these NSAIDs, resulted in a decrease in ulcerogenic activity of active *ASA* and they attributed this result to enhancement in expression of HSP72. Also, ***Soncin and Calderwood***,[34] showed that 3 mmol of *aspirin* clearly activates heat shock factor-1 (HSF-1) , a transcription factor for hsp-genes, by direct action of *aspirin*. These results may be explained by the adaptive cytoprotection phenomenon. This means that; necrotizing materials, such as *aspirin*, in a low concentration which is not necrotizing for the gastric mucosa, could protect it against strong irritant.[4] In addition, ***Tanaka et al.,*** [35] reported that, more apoptotic cells were observed in the gastric mucosa of HSF1-null mice than in wild-type mice.

In the present study, we have demonstrated that *L-arginine* as NO donor-administered before exposure to WRS significantly protects the gastric mucosa against acute lesions caused by WRS and enhanced the speed of mucosal recovery, as detected by decreased number of gastric ulcers, compared to rats pretreated with vehicle and exposed to WRS.

Similar results were obtained by ***Konturek et al.***, [10] and ***Takeuchi et al.***, [36] who found that NO-releasing *aspirin* (NO-*ASA*) exerted much less gastric toxicity and attributed the improved gastrointestinal safety profile of these drugs to NO moiety released, from these drugs.

Also ***Wink et al***, [37] demonstrated that NO-*ASA* administrated before exposure to WRS does not aggravate but actually protects gastric mucosa against acute gastric lesions. In addition they reported that NO-*ASA* enhances healing of gastric lesions caused by WRS.

In this study NO-donor pretreatment was found to be associated with an increase in the gastric mucosal expression of HSP70 in the stressed rats compared to vehicle pretreated stressed rats. This indicates that NO released from *L-arginine* has a stimulatory effect on the expression of HSP70. Similar results have been reported by ***Konturek et al.*** [10] who reported a significant increase in HSP70 mRNA expression in rats pretreated with NO-*ASA* in contrast to *ASA* treated rats. These results are in keeping with the finding of ***Byrne and Hanson***, [38] who demonstrated that NO-donor, S-nitroso-N-acetyl-penicillamine, induced expression of HSP70 in gastric mucosal cell cultures in a dose-dependent manner. In accordance with these results ***Xu et al.***, [39] have demonstrated that NO generated from two different NO donors, sodium nitroprusside or S-nitorse-N-acetyl penicillamine, led to

induction of HSP70 in cultured vascular smooth muscle cells.

The crucial role of NO in this protection is supported in this study by the fact that *L-NAME* administration before WRS aggravates the gastric mucosal damage caused by WRS as compared to rats received vehicle and exposed to WRS. In accordance of our results ***Konturek et al.***, [10] reported that co-administration of carboyx-PTIO, a NO scavenger, prevented the protective and hyperemic effects of NO-*ASA* against WRS-induced gastric damage.

This important finding indicates that HSP70 induced by NO donor could represent an important mechanism underlying the gastro protective effect of this drug. NO induces heat shock protein 70 via activation of heat shock factor-1 and it is suggested that the response is regulated at the transcriptional level.[10] In addition, NO has numerous beneficial effects for the protection of gastric mucosa against stress ulcer. It causes gastric hyperemia, increased bicarbonate and mucous secretion and antioxidant properties,[40] resulting in limitation of neutrophil-endothelium interaction and it promotes healing, possibly due to suppression of the inflammatory response in the area of gastric ulcer. [4]

The results of our study showed that WRS caused a significant rise in oxidative stress as indicated by significant increase in MDA as compared to non stressed group of rats. Heat preconditioning which stimulated HSP70 expression resulted in a significant reduction in MDA as compared to stressed group. It can be suggested that one of the mechanisms underlying gastric protection by heat preconditioning is scavenging activity of HSP70. Many studies point to increased scavenging enzyme after activation of hsp-gene expression, involving such intracellular enzymes as catalase, SOD and glutathione peroxidase.[41] Enhanced HSP synthesis modifies the activity of scavenging enzymes more likely via posttranslational modifications rather than by stimulating the synthesis of these scavenging enzymes.[42]

In the present study, inducible NOS in gastric tissues, was measured. The results showed a significant rise in gastric iNOS in the group of rats pretreated with *L-arginine* and in the group with heat preconditioning as compared to the other groups of rats. NO is synthesized from amino acid *L-arginine* by the enzyme NO synthase. There are two types of NOS; the constitutive NOS (cNOS) which is expressed under normal conditions, and inducible NOS (iNOS) which is not constitutively expressed and is only induced by certain cytokines. The released NO by iNOS has biological actions. [4]

The reported rise in iNOS in the stressed groups pretreated with heat preconditioning and *L-arginine* indicates a significant increase in NO synthesis in these groups compared to the other groups. This increase in the group pretreated with heat preconditioning can suggests a link between HSPs and NO that needs more studies to prove and to know its details. ***Malyshev et al.***, [43] suggested that nitric oxide is probably involved in heat shock – mediated HSP70 synthesis, because they

reported an inhibition of hsp70-gene transcription in blood vessels by NOS inhibitor. They stated that the signal transduction pathway for the activation of hsp70-gene expression through NO is still unclear. On the other hand, ***Hauser et al.***, [44] found that sodium arsenite, known to induce HSP70 synthesis, significantly attenuated iNOS mRNA, leading to reduced NO levels in the blood.

CONCLUSION

From the results of the study we concluded that HSP70 has a physiological role in healing of gastric ulcer in WRS rats and increasing expression of HSP70 before exposure to the stress by heat preconditioning or by nitric oxide donors has a protective effect against development of the ulcer. Inhibition of expression of HSP70 by either *ASA* or *L-NAME* aggravated the development of gastric ulceration in stress condition, and we suggested that intake of *L-arginine* as a NO donor in suspected stressful conditions may be of value in protection against gastric mucosal damage through increasing HSP70 expression.

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الملخص العربى

دور بروتينات الصدمات الحرارية الوقائى وتعجيل شفاء قرح المعدة الناتجةعن حالات التوتر فى الجرذان

المقدمة: تلعب بروتينات الصدمات الحرارية فى الحفاظ على سلامة الخلية و تساعد على حمايتها من التأثيرات السمية من تجمعات البروتينات الناتجة عن حالات التوتر المختلفة. يؤدى التوتر الناتج عن تقييد الجرذان و غمسها فى الماء الى حدوث قرح المعدة. يزيد العلاج بمضادات الالتهاب الغير استرودية كالأسبرين من الاصابة بقرح المعدة. كما يؤدى وقف تصنيع أوكسيد النيتريك الى زيادة تكوين قرح المعدة. أما التكيف الحرارى والعلاج بالمواد المنتجة لأكسيد النيتريك فتعمل على حماية الغشاء المخاطى المبطن للمعدة كما تحفز التئام القرح. لكن لم يحدد بعد هل هذه التأثيرات النافعة لهذه المواد أو التأثيرات الضارة للأسبرين وموقفات تصنيع أكسيد النيتريك من خلال تأثيرهم على بروتينات الصدمات الحرارية أم لا.

هدف العمل: صممت هذه الدراسة لتبحث عن الدور المحتمل لبروتينات الصدمات الحرارية رقم ٧٠ فى الحماية من الاصابة بقرح المعدة والمساعدة فى التئامها و كذلك تأثير التكييف الحراري، الأسبرين و الأكسيد النيتريكي على هذه البروتينات و وظيفتها.

المادة و الطرق: أستخدم فى هذه الدراسة مائة و ثمان و عشرون من الجرذان وقسمت الى ستة مجموعات: *المجموعة الأولى* (٨ جرذان) : المجموعة الضابطة ولم تعرض لأى توتر و أعطيت محلول ملحي بالفم ١ملل/جرذ. المجموعات الأخرى (٢٤ جرذ/مجموعة) كلها تعرضت للتوتر الناتج عن تقييد الجرذان و غمسها فى الماء لمدة ثلاث ساعات ونصف وذلك بعد نصف ساعة من علاجها بالمواد المختلفة الآتية بالفم: *المجموعة الثانية* : أعطيت١ملل/جرذ محلول ملحي خالى من أى دواء. *المجموعة الثالثة*:تعرضت لتكيف حرارى ٤٢°م لمدة ثلاث ساعات. *المجموعة الرابعة*: أعطيت الأسبرين ٤٠ مجم/كجم. *المجموعة الخامسة*: عولجت بال ل-أرجينين (مطلق الأكسيد النيتريكي) ٢٥٠ مجم/كجم. *المجموعة السادسة*:تناولت الـ ل.ن.ا.م (مثبط تكوين الأكسيدِ النتريكي) ١٠مجم/كجم. ثم تم ذبح الجرذان وعد قرح المعدة،وقياس نسب المالوندهايد "مؤشر لأكسدة الدهون"، والانزيم المصنع لأكسيد النيتريك، وبروتينات الصدمات الحرارية فى المعدة فى الحال بعد انتهاء التوتر، بعد ستة ساعات، و بعد أربع وعشرين ساعة (وفي كل توقيت إستخدم ٨ جرذان من كل مجموعة)، أيضاً تم قياس هذه المؤشرات في المجموعة الضابطة بعد ذبحهم مباشرةً.

النتائج: نتج تعرض الجرذان للقييد والانغماس فى الماء الى حدوث قرح بجدار المعدة، وهذه القرح زاد عددها فى الجرذان التى عولجت بالأسبرين أو بال ل.ن.ا.م مع زيادة نسبة المالوندهايد، و نقص مؤثر فى بروتينات الصدمات الحرارية رقم ٧٠ فى جدار المعدة.

الاستنتاج: يمكن استنتاج أن بروتينات الصدمات الحرارية رقم ٧٠ لها تأثير شافى لقرح المعدة الناتج عن التوتر الناتج عن تقييد الجرذان و غمسها فى الماء وزيادة تصنيعها يؤدى الى الحماية من قرح المعدة. وأن تغيير مستوى بروتينات الصدمات الحرارية رقم ٧٠ فى جدار المعدة يلعب دورا فى تأثير الأسبرين، الـ ل – أرجينين و الـ ل.ن.ا.م على جدار المعدة.