Journal of Health, Medicine and Nursing
ISSN 2422-8419 An International Peer-reviewed Journal
Vol.47, 2018

Protective effect of L-arginine in experimentally induced Necrotizing Enterocolitis in rats

[1]Nada Sadaqah Asali, [2]Magda M. Hagras*, [3]Omar Ibrahim Saadah, [4]Raed Fahad Althagafi, [5]Soad Shaker Ali

[1,2,3,4]Pharmacology Department. Faculty of Medicine, King AbdulAziz University Jeddah Saudi Arabia
[5]Anatomy department, Faculty of Medicine, King AbdulAziz University Jeddah Saudi Arabia previously
Histology Department, Faculty of Medicine, Assuit University. Egypt
Suez Canal University, Ismailia, Egypt*

Abstract

Necrotizing enterocolitis (NEC) is a leading cause of mortality and morbidity in the neonatal intensive care unit, until now the etiology of NEC remains unclear, prevention and treatment strategies are often inadequate. The protective effect of L-arginine was examined in an experimental model of necrotizing entercolitis. The study was conducted on 46 male albino rats which were divided into three main groups: Control group, L-arginine group, and the experimental group which has been divided into two sub-groups; Group A: received Lipopolysaccharides to induce NEC, Group B: injected by L-arginine prior to the disease was induced by endotoxin, asphyxia and cold stress. After the animals had been scarified, histological changes in the ilium were evaluated, gene expression of both iNOS and IL-12 were measured, and apoptosis also was detected by flowcytometry technique. The findings observed a significant increase in the expression of iNOS gene and IL-12 gene and a noticeable decrease of the apoptosis index. In addition, administration of L-arginine attenuated body weight, body temperature, and the histological changes that was altered by LPS/asphyxia. As such, the study was able to demonstrate that L-arginine administration had significant protective effect against NEC, but further clinical studies are still required on preterm infant to confirm these results.

Key words: Necrotizing enterocolitis, L-arginine, Interleukin-12, Inducible nitric oxide synthase, Lipopolysaccharides, Messenger Ribonucleic acid (mRNA)

1. Introduction

Necrotizing enterocolitis (NEC) is the most common and acute life-threatening medical and surgical emergency of the intestine encountered in preterm infants. It had many symptoms that could be severe and injurious to one or more organs resulting in pulmonary, renal and hepatic failure (Mitidiero LF *et al.*, 2014). For developing NEC, multifactorial and complex factors can cause its development. In addition, the diagnosis of NEC is based on clinical and radiologic findings which included several medications. Assessing gene-expression patterns could be by transcriptomics that has a future promise in determining biomarkers in necrotizing enterocolitis researches field. Therefore, the main objective of this study is to evaluate the effectiveness of L-arginine administration in necrotizing enterocolitis model in rats using quantification analysis of inducible nitric oxide synthase (iNOS) and interleukin-12 (IL-12) genes.

2. Theoretical Background

NEC is an acute inflammatory disease that affects the neonatal intestine leading to intestinal necrosis, multi-system organ impairment and septicemia (Lin and Stoll, 2006). It is affecting 5% to 10% of premature infants less than 36 weeks of gestation and weighing under 1,500 g, with an associated mortality rate of 10% to 50% (Chu et al., 2013). Kastenberg et al. (2013) added that about 50% of the affected baby would develop progressive intestinal necrosis requiring an urgent operation. It is not considered only as a most serious clinical problem which affects preterm infants, but also one of the most challenging to treat.

The incidence of NEC has increased to7% in very-low-birth-weight infants. The overall mortality rate for infants with NEC ranging from 15-30% (Lee, 2011) but close to 100% in preterm infants who are suffering from the rapidly progressive disease. Accordingly, effective medications were evaluated including L-arginine which is a semi-essential cationic amino acid that is utilized as a sole substrate in the synthesis of Nitric Oxide (NO), through L-arginine oxidation, that catalyzed by the nitric oxide synthase enzyme (NOS). Nitric oxide acts as a neurotransmitter for enteric non-adrenergic non-cholinergic neurons and inadequacy of NO can alter intestinal motility; NO inhibits leucocytes actions and changes the inflammatory responses in the intestine.

Ford H et al. (1997) elaborated that iNOS gene is detected in specimens of infants surgically resected for NEC.

On the other hand, Dvorak B et al. (2003) discussed that despite the presence of endothelial NO synthase (eNOS) in specimens from infants undergoing surgical resection for NEC and control patients with intestinal atresia, eNOS function was impaired, resulting in decreased NO production in arterioles from NEC specimens compared with controls. Accordingly, NO with the normal concentration in the gastrointestinal (GI) tract is very important since patients with mild NEC require GI rest to facilitate resolution of the intestinal inflammatory process (Akisu, 2002).

Moreover, Transcriptomics is a global way of assessing gene-expression patterns since it involves all mRNA molecules studies, or transcripts, and evaluation of the changes in the initiation of transcription, processing, and degradation of proteins. Polymerase chain reaction (PCR) technique, based on transcriptomics, holds promise in discovering biomarkers in the area of NEC research in the future. The question of enteral versus parenteral administration should be carefully considered where one potential advantage of enteral administration is that the arginine could have significant beneficial effects on the intestinal mucosa itself, increasing proliferation and differentiation due to the production of other amino acids and polyamines. Moreover, preventing a catabolism-induced production of asymmetric dimethyl arginine (ADMA) by overall nutrition that may decrease NOS activity and NO production by need careful attention since its ratio can affect the intestinal microvasculature and potentially predisposing to NEC.

3. Methodology

3.1. Material

- **Animals:** The present study was conducted in King AbdulAziz University (KAU) where the investigation was divided into two phases which are: Pilot study phase and Experimental study phase. In the pilot study, fourteen male albino rats weighing (30-50 gram) were used, and forty-six male albino rats weighing (30-50 gram) were used throughout the experimental study. They were placed in plastic cages in animal house and subjected to standard settings and were given freely water at all times and standard laboratory chow and were exposed to a temperature from (20–25° C) and humidity of (50 ± 5%) on a 12 h light/dark cycle for one week acclimation before use.
- **Drugs:** Firstly, L-arginine (Amargine® 100 mg/mL) bought from King AbdulAziz University Hospital which was given orally (261mg/kg/day) (1,5mmol/kg/day) (Douglas, 2004). Secondly, Sodium pentobarbital purchased from Sigma Aldrich Co. as a white powder dissolved in sterile water to a concentration of 50 mg/mL solution for the intraperitoneal injection in a dose of 40 mg/kg/day (Chan *et al.*, 2009).
- **Chemicals:** The study used several chemicals which are: Lipopolysaccharides 10 mg (LPS) a powder 10 mL of phosphate buffer saline to make the final concentration is (1 mg/mL); Ethyl ether; Formaldehyde**;** Liquid nitrogen**;** Phosphate buffered saline powder (PPS)**;** TACS Annexin V-FITC Kit, for measurement of antiapoptic effect of L-arginine**;** Rat Interleukin 12 (IL-12/P70) ELISA Kit**;** RNeasy® Fibrous Tissue Mini Kit; RNase free DNase**;** High capacity cDNA Reverse Transcription Kit**;** Tagman® Gene expression Master Mix for both IL-12 and iNOS B genes.
- **Equipment:** Rat rectal temperature probe (MLT1403, ADI Instruments); 45-50 °C incubators; Microcentrifuge ≥ 12,000 x g; ELISA Reader of measuring absorbance at 530-532 nm; 7500 Fast Real-Time PCR; Beckman Coulter flow cytometry.

3.2. Methods

3.2.1. Pilot Study:

This study conducted a pilot to determine the appropriate dose of LPS to design an adequate experimental model for the study of necrotizing enterocolitis on full-term rats. Firstly, the study prepared the LPS (Lipopolysaccharides) that was supplied as lyophilized, γ-irradiated powders for reconstitution; 10 mL sterile balanced salt solution (BSS) which was added to the vial (10 mg) and swirled to make the final concentration is (1 mg/mL). Secondly, it prepared the L-arginine (Arginine solution) with (100 mg/mL) bottle that was kept out of light and at room temperature.

- **Pilot study design**

Fourteen male albino rats weighing (30-50 gm) were used throughout the study; they were divided into three groups. Group I contains two rats were injected with BSS (I.P.) as a control group. Group II contain 4 rats were injected with LPS **5** mg/kg/day (I.P.) for 2 days (Zhou W et al., 2011 and Lu H et al., 2006) and exposed to a 45 seconds of asphyxia (in carbon dioxide hypoxic chamber) followed by cold stress (4°C for 10 min). Group III: contain 4 rats were injected with LPS **4** mg/kg/day (I.P.) for 2 days then they were exposed to a brief episode of asphyxia (45 seconds in carbon dioxide hypoxic chamber) followed by cold stress (4°C for 10 min) (Dvorak B et

al., 2003). Group IV: contain 4 rats were injected with LPS **2** mg/kg/day (I.P.) for 2 days (Feng J et al., 2006) then they were exposed to a brief episode of asphyxia (45 seconds in carbon dioxide hypoxic chamber) followed by cold stress (4°C for 10 min) (Dvorak B et al., 2003). Also, the previous procedures were performed twice daily (at 07:00 a.m. and 19:00 p.m.) for two consecutive days which were according to the study of Dvorak B *et al*. (2003). NEC evaluation: small intestine was visually evaluated after the animals had been sacrificed for typical signs of NEC (discoloration of the intestine, intestinal hemorrhage, ileal distention, and ileal stenosis). Ileal and jejunal tissues were collected and stained with hematoxylin and eosin for histologic evaluation according to Zhou W *et al.* (2011)

3.2.2. Experimental induction of NEC:

NEC was induced by intraperitoneal injection of LPS (**2** mg/kg) (Feng J *et al*., 2006) to a full term neonatal rats then they were exposed to a brief episode of asphyxia (10 minutes in 10% carbon dioxide hypoxic chamber) followed by cold stress (4°C for 10 min) (Wei et al., 2007 & Zhou et al., 2011).

- **Experimental design:**

Forty-six rats were divided into 3 groups: Control (10 rats) which received BSS orally only for 10 days; second group: L-arginine only (12 rats) that received orally (261mg/kg/day) for 10 days; the third group which was subdivided into 2 subgroups (12 rats each). The first subgroup III a (LPS/asphyxia-induced rats) which received BSS orally for ten days before they were injected with intraperitoneal LPS (**2** mg/kg/day once daily for two days). The second subgroup III b (LPS/asphyxia + L-arginine): (12 rats) were received L-arginine orally (261mg/kg/day) for ten days before they were injected with intraperitoneal LPS (**2** mg/kg/day once daily for two days). Both sub-groups were exposed to a brief episode of asphyxia (45 seconds in carbon dioxide hypoxic chamber) followed by cold stress (4°C for 10 min), on the 11^{th} and 12^{th} day. This procedure was performed twice daily (at 7:00 a.m. and 19:00 p.m.) for two consecutive days.

After the animals had been sacrificed (on the 48^{th} hours after the last dose of LPS), the small intestine (ileal tissue) was immediately dissected then cut into many parts. One part (3 cm) immersed immediately in 10% neutral buffered formalin for light microscopy. It was stained with hematoxylin and eosin (H & A), Alcian blue and periodic acid chief (PAS) stains to study histopathological changes induced by necrotizing enterocolitis and the role of L- arginine as a protective agent. Another Fresh parts of intestine were sectioned into segments of 30 mg of each and were placed in liquid nitrogen until use for detecting iNOS and IL-12 gene levels. Parts of intestine were snap-frozen in phosphate buffer saline for enterocyte apoptosis and interleukin 12 measurements. The rest of intestinal tissue frozen at - 80°C for further biochemical analysis. Moreover, the study measured the animal body weight, temperature, and histological features.

- **Experimental procedure:**

The Sample was frozen immediately in liquid nitrogen and then ground to a fine powder under liquid nitrogen using mortar and pestle. The powder was transferred into polypropylene tube (RNase-free, liquid-nitrogen–cooled) Liquid nitrogen allowed evaporate, Lysis buffered was added, and homogenization was continued as quickly as possible using a QIAshredder spin column. The study prepared TaqMan Gene Expression Assay, TaqMan Gene Expression Master Mix, cDNA Template, and RNase-free water. Furthermore, the protocol of gene expression was started with allowing the component to thaw on ice, completely resuspend by gently vortexing, and then briefly centrifuge. For each sample (to be run in Triplicate), pipet the following into a nuclease-free 1.5-mL microcentrifuge tube:

PCR Reaction Mix Component		Volume per 20-µL Reaction	
		Single reaction	Three replicates #
1	20 x TaqMan Gene Expression Assay	1.0	4.0
2	2 x TaqMan Gene Expression Master Mix	10.0	40.0
3	cDNA Template	(1 to 100 ng)	-
4	RNase-free water	Up to 20 µL	Up to 80 µL

The study capped the tube and invert it several times to mix the reaction components, the centrifuge the tube briefly to transfer 20 µL of PCR reaction mix into each well 96-well reaction plate. Also, it sealed the plate with the appropriate cover and centrifuged the plate briefly. After that, the study created a plate document/experiment for the run. The plate was loaded into the instrument.

3.3. Data analysis

The data were analyzed, and amplification plots were generated using the software program, 7500 fast real-time PCR. The comparative CT method used ($2^{-\Delta\Delta CT}$) for calculating the relative quantification of the target gene was used as follows ΔΔCT = (CT of target gene – CT of ACTB) for χ – (CT of target gene – CT, ACTB) for y, where χ = treated sample and y = control sample. After validation of the method, results for each sample were expressed in N-fold changes in χ target gene copies, normalized to ACTB relative to the copy number of the target gene in control, according to the following equation: the amount of target = $2^{-\Delta\Delta Ct}$. All data are presented as means ±SDV. Statistical analysis was performed using SPSS. $P \leq 0.05$ was considered significant. Furthermore, all data were statistically processed with the SPSS program, version 21; both descriptive statistics and analytical statistics were done. Data were presented as means ± standard deviations. Comparison among different groups was carried out by non-parametric tests. The difference was considered statistically significant when $P < 0.05$

4. Results

4.1. Pilot study result

Three rats were injected with (LPS **5** mg/kg) and drop out within 12 hours were the last rat which died after 18 hours of the first LPS dose. Two rats were injected with (LPS **4** mg/kg) which were died after 24 hours. While all rats injected with (LPS **2** mg/kg) were survived for 48 hours from the last dose of endotoxin, and only one died after 48 hours. All rats developed diarrhea and exhibited abdominal distention after administration of LPS injection, and their appetite was lost. Locomotors activity of rats injected with the highest dose decreased after 2 hours from the first dose of LPS, unlike other rats who have decreased their activity obviously after 24 hours. Visually, it was observed that there were intestinal hemorrhage and ileal distention in the small intestine of remaining rats that were injected with LPS **2** mg/kg/day.

Histologically, a Photomicrograph showing the changes in the small intestine in response to LPS administration (2mg/kg/day) in LPS/asphyxia induced necrotizing enterocolitis (figure 4.1b) in comparative with the control group (figure 4.1a). Intestinal tissue in which severe coagulative necrosis was given a score of 3.

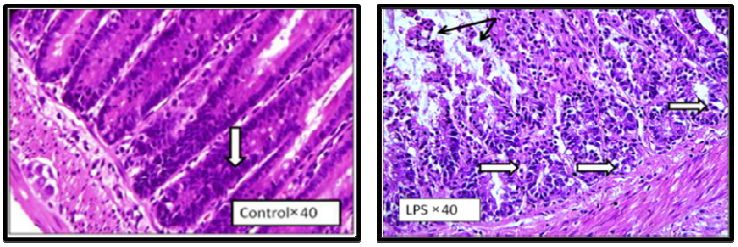

Figure 4.1: Photomicrograph of small intestine changes

4.2. Effect of L-arginine administration on body weight of LPS/asphyxia induced enterocolitis in rats

The result from table (4.1) and table (4.2) showed that the administration of L-arginine for 10 days before LPS/asphyxia improved the body weight by 23.7% compared to control before the LPS/asphyxia, however, LPS/asphyxia reduced the body weight from (59.01±10.05) before LPS/ asphyxia to (53.19±9.85) after LPS/asphyxia. After 48 hours of LPS-asphyxia induction, the body weight in LPS/asphyxia+ L-arginine group was decreased by 2.3% (average) compared to before LPS/asphyxia. However, in LPS group, the body weight was decreased by 10.9 % compared to before LPS/asphyxia.

Table (4.1): Effect of L-arginine on body weight in LPS/asphyxia induced enterocolitis in rats.

Rats weight (gm)	Control (n=10) Mean ± STD	L-arginine (n=12) Mean ± STD	LPS/asphyxia (n=12) Mean ± STD	LPS/asphyxia + L-arginine (n=12) Mean ± STD
Day (1)	40.63 ± 4.74	36.80 ± 2.49	38.85 ± 5.73	40.07 ± 4.38
Day (2)	42.18 ± 4.60	39.92±2.52	40.74 ± 6.21	42.14 ± 4.37
Day (3)	43.90 ± 4.81	43.93 ± 2.89	42.86 ± 6.96	44.92 ± 4.72
Day (4)	45.85 ± 4.89	48.80 ± 3.14	44.97 ± 7.42	48.62 ± 5.13
Day (5)	48.22 ± 5.24	51.55 ± 3.45	47.52 ± 8.14	52.59 ± 5.17
Day (6)	50.41 ± 5.84	55.29 ± 2.89	50.16 ± 8.95	56.62 ± 4.89
Day (7)	52.78 ± 6.35	58.77 ± 2.82	52.49 ± 9.70	60.28 ± 5.16
Day (8)	55.13 ± 6.51	63.08 ± 3.02	54.61 ±10.20	63.94 ± 5.08
Day (9)	56.95 ± 6.83	67.50 ± 3.08	56.43 ± 10.43	68.11 ± 4.88
Day (10)	59.14 ± 6.97	71.67 ± 3.05	58.32 ± 10.41	72.43 ± 5.50
Day (11) Before LPS	61.84 ± 6.69	75.86 ± 3.93	59.01 ± 10.05	76.50 ± 5.49
Day (13) Before sacrifice	65.81 ± 7.57 ↑	84.37 ± 4.41↑	53.19 ± 9.85 ↓	74.73 ± 5.57 ↓

Table (4.2): Multiple Comparisons of body temperature among all experimental groups

Groups Contrast		Averaged Variable
		The change of rats weights over time
L-arginine vs. Control	Mean Difference	16.288
	Std. Error	2.927
	P	**0.0001****
LPS/asphyxia vs. Control	Mean Difference	-7.725
	Std. Error	3.057
	Sig.	**0.016***
LPS/asphyxia + L-arginine vs. Control	Mean Difference	11.788
	Std. Error	2.927
	P	**0.0001****
L-arginine vs. LPS/asphyxia + L-arginine	Mean Difference	4.499
	Std. Error	2.790
	P	.115
LPS/asphyxia vs. LPS/asphyxia + L-arginine	Mean Difference	-19.513
	Std. Error	2.927
	P	**0.0001****

* The Mean difference is Significant at the 0.05 level
** The Mean difference is Significant at the 0.01 level

In the present study, An ANOVA with repeated measures statistical analysis was applied to compare the mean differences between the four groups that have been split on two within subjects' factors (The weight of rats on day 11 before LPS, The weight of rats on day 13 before sacrifice). In the table (4.2), the average change of rats' weights was statistically high which is significantly greater with *(P<0.0001)* in the L-arginine group and the LPS/asphyxia + L-arginine group comparing to the Control group. On the other hand, it was lesser with *(P=0.016),* in LPS/asphyxia group comparing to the Control group, and with *(P<0.0001)* in LPS/asphyxia + L-arginine group comparing to the L-arginine group.

4.3. Effect of L-arginine administration on body temperature of LPS/asphyxia induced enterocolitis in rats

Administration of LPS together with asphyxia increased the body temperature significantly from (38.80± 0.63 to 41.25±0.1°C); however, administration of L-arginine significantly lowered the body temperature nearly to the normal levels (41.25±0.1 °C in the LPS group to 39.37±0.5 °C in the L-arginine treated group) (Table 4.3).

Table (4.3): Effect of L-arginine administration on body temperature in LPS/asphyxia induced enterocolitis

Groups	Before LPS - day 11 Mean± STD (degrees centigrade)	After LPS - day 13 Mean± STD (degrees centigrade)
Control (n=10)	38.40 ± 0.40	38.50 ± 0.65**
LPS/asphyxia (n=12)	38.80 ± 0.63	41.25 ± 0.1* ↑
LPS/asphyxia+ L-arginine (n=12)	38.75 ± 0.49	39.37 ± 0.5**

*Significant change compared with the corresponding control group values
** Significant change compared with the corresponding LPS group values
Significance was considered at P<0.05

4.4. Effect of L-arginine administration on iNOS mRNA gene expression of LPS/ asphyxia induced enterocolitis in rats

LSD pair comparison table (4.4) and showed a significant increase in the expression of iNOS gene produced by oral administration of L-arginine (261 mg/kg/day) compared with control (P < 0.001) with mean± STD of and mean % change of 121.95 %. Also, the expression of iNOS gene in LPS/asphyxia group was statistically high significantly (5.812 ± 2.56) compared with Control group by 481.1%, *p<0.0001* also it was statistically high significantly greater than L-arginine group (1.910 ± 0.460), *p=0.001* and protected group (2.220 ± 0.7633), *p=0.002*. RT-PCR of ACTB mRNA was used as a housekeeping control. As such, highly significant differences statistical results were found between the groups of iNOS gene as determined by one-way ANOVA (F (3, 13) = 10.583, *p =0 .001*).

Table (4.4): Effect of daily oral administration of arginine for ten days on the expression of (iNOS) gene in experimental albino rats

(I) Group	Mean± STD	(J) Group	Mean Difference (I-J)	Sig	95% Confidence Interval	
					Lower Bound	Lower Bound
Control	1.00000 ± E-9	LPS/asphyxia	-4.81211*	0.000	-6.8112-	-2.81298-
		L-arginine	-.90607	.321	-2.8026-	0.990466
		LPS/asphyxia + L-arginine	-1.21954	0.210	-3.2186-	0.779587
LPS/ asphyxia	5.8121 ± 2.560	**Control**	**4.81211***	**0.000**	2.81298	6.811251
		L-arginine	**3.90604***	**0.001**	2.00949	5.802584
		LPS/asphyxia + L-arginine	**3.59257***	**0.002**	1.59343	5.591705
L-arginine	1.906 ± .4600	Control	.90607	0.321	-.99046-	2.802622
		LPS/asphyxia	-3.90604*	0.001	-5.8025-	-2.00949-
		LPS/asphyxia + L-arginine	-.313468	0.727	-2.2100-	1.583076
LPS/ asphyxia+ L-arginine	2.219 ± .7633	Control	1.21954	0.210	-.77958-	3.218679
		LPS/asphyxia	-3.5925*	0.002	-5.5917-	-1.59343-
		L-arginine	.31346	0.727	-1.5830-	2.210012

* The mean difference is significant at the 0.05 level

4.5. Effect of administration of L-arginine for ten days as a protective agent on the expression of IL-12 in experimental albino rats

LSD pair comparison table (4.5) represented a significant increase by 113 % in the expression of IL-12 gene produced by oral administration of L-arginine compared with control (P < 0.001). In addition, the expression of IL-12 gene in LPS/asphyxia group was statistically high significantly (3.246±0.948) compared with the control group by 224.6%, *p<0.0001*. As such, LPS/asphyxia has high significant statistically results which is greater than L-arginine group (1.442±0.322), P <*0.001* and protected group (2.130±0.690), *p<0.015*.

Table (4.5): Effect of daily oral administration of L-arginine for ten days on the expression of (IL-12) gene in experimental albino rats.

(I) Group	Mean± STD	(J) Group	Mean Difference (I-J)	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	1.00000 ± E-9	LPS/asphyxia	-2.246074-*	.000	-3.05773-	-1.434418-
		L-arginine	-.4417673-	.264	-1.25342-	.3698887
		LPS/asphyxia+ L-arginine	-1.130386-*	.013	-1.99127-	-.2694949-
LPS/ asphyxia	3.246 ± .94789	**Control**	**2.2460743***	**.000**	1.434418	3.0577305
		L-arginine	**1.8043069***	**.000**	.9926508	2.6159631
		LPS/asphyxia + L-arginine	**1.1156880***	**.015**	.2547967	1.9765794
L-arginine	1.4417 ±. 3220	Control	.441767380	.264	-.369888-	1.2534235
		LPS/asphyxia	-1.804306-*	.000	-2.61596-	-.9926508-
		LPS/asphyxia+ L-arginine	-.68861889-	.109	-1.54951-	.17227248
LPS/ asphyxia+ L-arginine	2.130 ± .69015	Control	**1.1303862***	**.013**	.2694949	1.9912776
		LPS/asphyxia	-1.115688-*	.015	-1.97657-	-.254796-
		L-arginine	.68861889	.109	-.172272-	1.5495102

* The mean difference is significant at the 0.05 level

4.6. Effect of L-arginine administration on apoptosis index of LPS/asphyxia induced enterocolitis in rats

Data in the table (4.6) elaborated that the apoptosis mean high significantly less in L-arginine group (0.15 ± 0.17) compared to LPS/asphyxia + L-arginine group (4.16 ± 1.8) *(P<0.001)*. Furthermore, there was no statistically significant difference between the means of late apoptosis based on the different groups. Necrotic cells means were statistically highly significant difference between the LPS/asphyxia group (9.17 ± 14.95) and LPS/asphyxia + L-arginine group (0.15 ± 0.24) (*P=0.009*). As such, the highest mean of early apoptosis was for the LPS/asphyxia + L-arginine = 4.16, and the lowest mean was for L-arginine = 0.15. by LSD test.

Table (4.6): Effect of L-arginine on apoptosis index in LPS/asphyxia induced enterocolitis in rats.

Degree of apoptosis	Parameter	Control	L-arginine	LPS/asphyxia	LPS/asphyxia + L-arginine
		(n=10)	(n=12)	(n=12)	(n=12)
Early apoptosis	Mean ± STD	3.76 ± 4.71	0.15 ± 0.17	2.22 ± 2.41	4.16 ± 1.80
	min-max	0.03 -14.53	0.01 - 0.58	0.04 - 5.94	1.30 - 6.93
	Significance		***p=0.003**	*p=0.280; **p=0.078	*p=0.725; ****p=0.001;** ***p=0.099
Late apoptosis	Mean ± STD	0.52 ± 0.99	0.15 ± 0.34	0.37 ± 0.83	0.03 ± 0.03
	min-max	0.00 -3.15	0.00 -1.2	0.00 -2.59	0.00 -0.12
	Significance		*p=0.183	*p=0.606; **p=0.422	*p=0.085; **p=0.667; ***p=0.228
Necrosis	Mean ± STD	2.73 ± 4.58	2.16 ± 3.80	9.17 ± 14.95	0.15 ± 0.24
	min-max	0.00 - 13.98	0.00 - 9.35	0.00 - 38.19	0.00 - 0.78
	Significance		*p=0.864	*p=0.068; ****p=0.039**	*p=0.437; **p=0.524; ****p=0.009**

*p: significance level for Control; **p: significance level for L-arginine; ***p: significance level for LPS/asphyxia.

4.7. Effect of L-arginine administration on IL-12 level using ELISA of LPS/asphyxia induced enterocolitis in rats

Multiple comparison table (4.7) showed a significant increase of IL-12 level in LPS/asphyxia group (1048 ± 202.09) greater than Control group (353.70 ± 167.28), *p<0.0001* also it was statistically high significantly greater than L-arginine group (327.37 ± 185.88), *p<0.0001* and LPS/asphyxia + L-arginine group (613.30 ± 253.78), *p<0.0001*.

Moreover, the IL-12 level of the protected group with L-arginine was relatively decreased by 41.5% than LPS/asphyxia group. Otherwise, there is a significant increase in LPS/asphyxia + L-arginine group mean (613.30±253.78) greater than Control group (353.70±167.28), *p=0.007*. Also, it is statistically high significantly greater than L-arginine group (327.37±185.88), *p=0.00*3.

Table (4.7): Effect of L-arginine on IL-12 level in experimentally rats using ELISA method

(I) Group	Mean± SD	(J) Group	Mean Difference (I-J)	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	353.701 ± 167.283	LPS/asphyxia	-694.898788-*	.000	-878.93-	-510.866-
		L-arginine	26.328727	.761	-148.25-	200.916
		LPS/asphyxia +L-arginine	-259.596455-*	.007	-443.62-	-75.564-
LPS/ asphyxia	1048.60 ± 202.09	**Control**	**694.898788***	**.000**	510.866	878.930
		L-arginine	**721.227515***	**.000**	537.195	905.2595
		LPS/asphyxia +L-arginine	**435.302333***	**.000**	242.287	628.3167
L-arginine	327.372 ± 185.87	Control	-26.328727-	.761	-200.91-	148.259
		LPS/asphyxia	-721.227515-*	.000	-905.25-	-537.195-
		LPS/asphyxia +L-arginine	-285.925182-*	.003	-469.95-	-101.893-
LPS/ Asphyxia + L-arginine	613.298 ± 253.77	**Control**	**259.596455***	**.007**	75.5644	443.628
		LPS/asphyxia	-435.302333-*	.000	-628.31-	-242.287-
		L-arginine	**285.925182***	**.003**	101.893	469.957

* The mean difference is significant at the 0.05 level

There is a highly significant difference between the groups of study F=26.477 and *p<0.0001*

4.8. Histological results

- ***Histological feature of rat ileum part in control group***

Sections of control rat ileum at the region of Payer's patch (figure 4.2) showed:

- Intact elongated villi (black arrows) which were absent over lymphoid aggregation (star).
- Villi have a normal connective central core and simple (black star) columnar covering epithelium with goblet cell (dotted arrows).
- The intestinal showed similar lining with few Panthers ill-defined cells at their bases.
- The covering epithelium cover the patch is of cubical type (arrows). Germinal center was ill-defined (star) (H&E stain)

Moreover, Sections from control rat ileum stained by (PAS) for neutral muco-polysaccharides (figure 4.3) showed positive staining of goblet cells (arrows) (PAS reaction) , while sections from control rat ileum stained by alcian blue stain for acidic muco-polysaccharides (figure 4.4) showed positive staining of goblet cells (arrows).

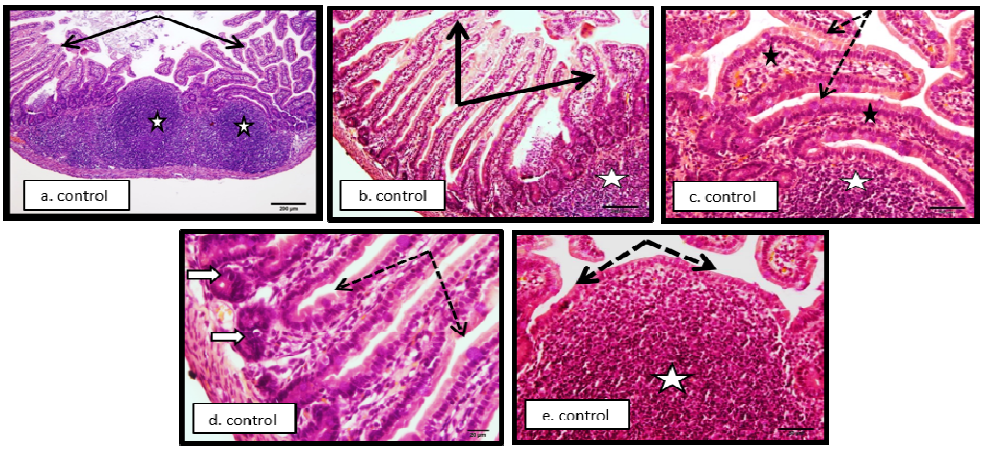

Figure (4.2): Photomicrograph of ileal sections from control group in experimentally rats using hematoxylin and eosin stain

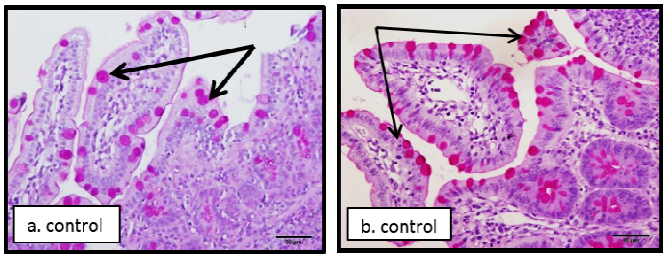

Figure (4.3): Photomicrograph of ileal sections from control group in experimentally rats using periodic acid chief stain

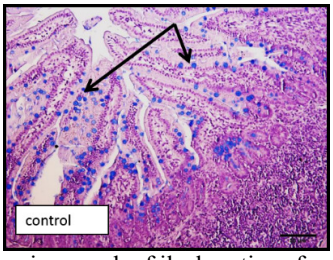

Figure (4.4) Photomicrograph of ileal sections from control group in experimentally rats using alcian blue stain

- ***Histological feature of rat ileum part in L-arginine group***

Sections of rat ilium after administration of L-arginine showed normal villi and payer's patches (star) (figure 4.5). Villi showed intact columnar and goblet cells (arrows). Payer's patches showed normal center (star). H & E stains for neutral and acid muco-polysaccharides showed normal goblet cells (arrows)

- ***Histological feature of rat ileum part in LPS/asphyxia group***

Sections from rat ileum of LPS/asphyxia group showed: a. Marked necrosis of villi (arrows) and hypertrophy of muscle layer (star). b. Increase the size of the lymphoid follicle and its germinal center (figure 4.6). On the other hand, sections from the ileum of LPS group showed a & b: marked degeneration (necrosis) of mucosal lining and glands (white arrows) also muscles (black stars); (figure 4.7), showed hydropic degeneration. C & d: increase size of germinal centers of lymphoid follicles (dotted circle).

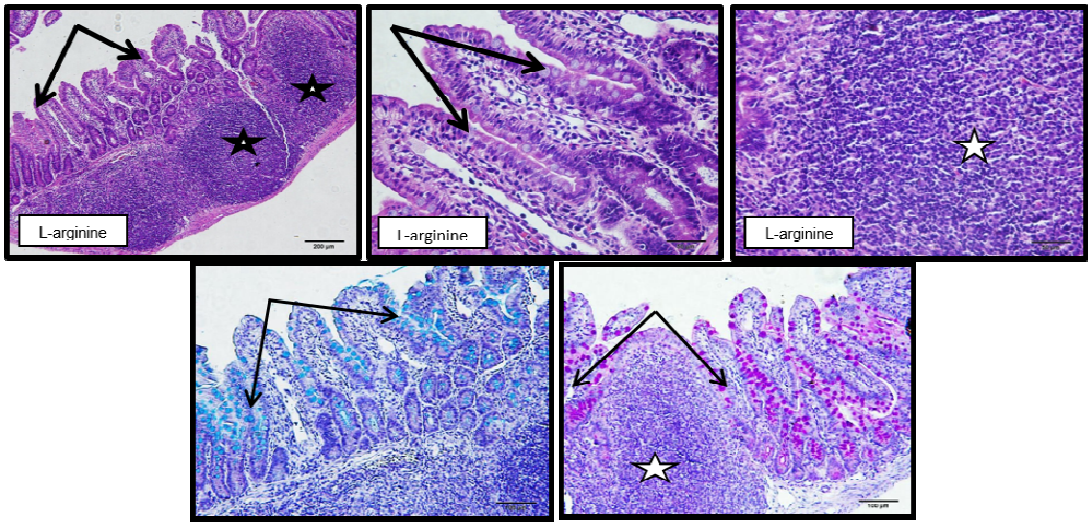

Figure (4.5) Photomicrograph of ileal sections from L-arginine group in experimentally rats

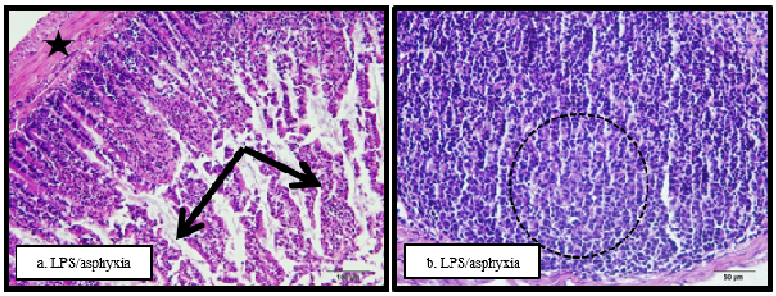

Figure (4.6) Photomicrograph of ileal sections from LPS/asphyxia group in experimentally rats using hematoxylin and eosin stain

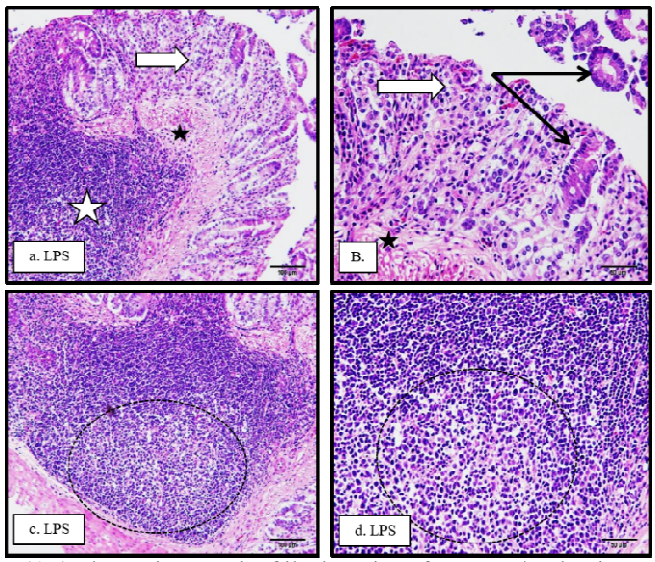

Figure (4.7) Photomicrograph of ileal sections from LPS/asphyxia group in experimentally rats with marked necrosis using H & E stain

- *Histological feature of rat ileum part LPS/asphyxia + L-arginine group*

A Photomicrograph was showed the changes in the small intestine in response to L-arginine administration in LPS/asphyxia induced enterocolitis in rats. Sections from rat ileum mucosa of LPS/asphyxia + L –arginine

showed: a. Intact villi with active goblet cells (arrows) slight inflammatory cells in villus core (star). b and c: Follicle of payer patch with decrease germinal center (star) and normal intact overlying villi (black arrows) and crypts thick (white arrows). Follicle showed vacuolated cells (white arrows) (figure 4.8).

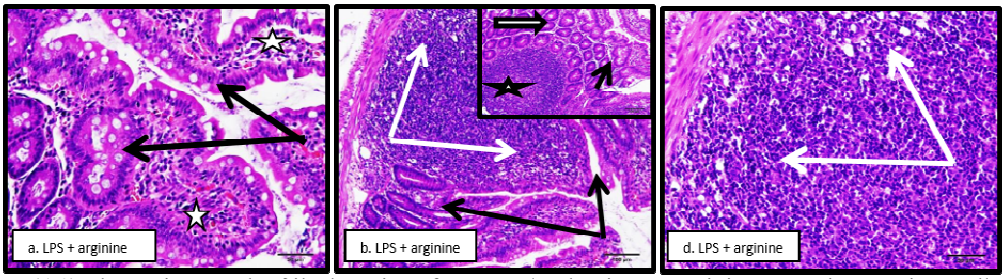

Figure (4.8) Photomicrograph of ileal sections from LPS/asphyxia + L-arginine group in experimentally rats using H & E stain

5. Discussion

The overall goal of the present study was to validate a claim that the administration of orally L-arginine for preterm infants has a protective effect against NEC in rat model. In the beginning of this study it was compared different doses of LPS injection to induce NEC and then disease induction was validated by histopathology. Results showed that both doses (**5** mg/kg/day and **4** mg/kg/day) of LPS injection after 24 hours are lethal and impracticable, in contrast with the collected data of the present study is the data of Zhou Wet al, (2011), who used a lethal dose of (LPS: **5** mg/kg) to induce intestinal injury. The mortality rate of NEC in groups 2 & 3 was 75% and 50%, respectively. There was no morbidity and mortality in control group. Compared with control group injury to the intestine was severe in the LPS group with **(2** mg/kg/day) dose. Based on the histological result, it showed that the optimal dose of LPS to induce NEC in term rats is **(2** mg/kg/day) in addition to asphyxia and cold stress. In consistent with this study, the data of Feng et al., (2006).

Moreover, the findings found that rats with enterocolitis produced by LPS/asphyxia showed reduced body weight by 10.9 % compared to before LPS/asphyxia, together had elevated body temperature significantly comparing to the control group. Administration of L-arginine as a protective agent before induction of necrotizing enterocolitis attenuated the body weight and body temperature to nearly normal value. In line with the current study was the data of Yao et al., (2008) that emphasized on the L-arginine supplementation ability to increase growth of neonatal pigs and promote weight gain which oppose the study of Osowska S et al. (2004) and Sukhotnik et al. (2005) who emphasized the non-significant alteration in final body weight of L-arginine rats. In addition, contrary to the related literature that indicated the NO possess a harmful role as an inflammatory mediator, the present study highlighted that NO production such as L-arginine may be beneficial in the prevention of NEC.

In the present study, histopathological examination of LPS/asphyxia group revealed focal destruction of villi. The intestinal crypts looked disorganized with numerous unstained degenerated cells. As regards to the effect of L-arginine administration, the results obtained demonstrated that L-arginine produced significantly decreased of the histopathological damage in LPS/asphyxia induced NEC. The present results could be explained by L-arginine provides NO that is known to have a protective role in the intestinal mucosa, as it prevents vasoconstriction by regulating of vascular perfusion and subsequently prevents the ischemia-induced damaging effects of mucosa (Fotiadis *et al.*, 2004).

6. Summary and Conclusion

In conclusion, this small experimentally study has shown that L-arginine afforded significant protective effect against NEC. L-arginine may not prevent NEC completely but its use from the first days of life for a preterm infant may delay the NEC progression and alleviate the severity of this deadly disease. Moreover, according to the results obtained in the present study, the rationale for using L-arginine as a protective agent for NEC was supported by; antiapoptotic effect of L-arginine (a NO synthase substrate) which was significantly decreased of apoptosis index and necrosis in protective group, L-arginine caused interruption of the inflammatory cascade and significant upregulation of both iNOS and IL-12 mRNA gene expression compared to control and LPS/asphyxia group. Also, significantly attenuated body weight & body temperature, histological changes, and intestinal injury which were altered by LPS/asphyxia. Moreover, it is recommended that L-arginine could be provided as a protective agent for the high-risk neonatal group. Further studies are highly required to

shed the lighter on the possible protective mechanism of L-arginine in NEC. Further evidence is required to confirm these results in large, prospective, randomized, placebo-controlled studies. Also, to focus on the possible adverse effects that may arise due to L-arginine administration in preterm infants.

References

Zhou W, Zheng XH, Rong X, Huang LG (2011). Establishment and evaluation of three necrotizing enterocolitis models in premature rats. *Mol Med Rep*.; 4(6):1333-8.

Feng J, El-Assal ON, Besner GE. (2006). Heparin-binding epidermal growth factor-like growth factor decreases the incidence of necrotizing enterocolitis in neonatal rats. J. Pediatr. Surg. 41, 144–149

Yao K, Yin YL, Chu W, et al. (2008): Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J Nutr. *138(5):867-72*

Osowska S, Moinard C, Neveux N, Loï C, Cynober L (2004).Citrulline increases arginine pools and restores nitrogen balance after massive intestinal resection. *Gut.53*(12):1781-6.

Sukhotnik I, Helou H, Mogilner J, Lurie M, Bernsteyn A, Coran AG, Shiloni E (2005). Oral arginine improves intestinal recovery following ischemia-reperfusion injury in rat. *Pediatr Surg Int.* 21(3):191-6.

Fotiadis C, Adamis S, Misiakos EP, Genetzakis M, Antonakis PT, et al. (2007). The prophylactic effect of L-arginine in acute ischaemic colitis in a rat model of ischaemia/reperfusion injury. Acta Chir Belg.;107(2):192-200.

Lu H, Zhu B, Xue X (2006). Role of neuronal nitric oxide synthase and inducible nitric oxide synthase in intestinal injury in neonatal rats. *World J Gastroenterol.*12(27): 4364-4368

Dvorak B, Halpern M, Holubec H, Dvorakova K, Dominguez JA, Williams CS, Meza YG, Kozakova H, McCuskey RS. (2003). Maternal milk reduces severity of necrotizing enterocolitis and increases intestinal IL-10 in a neonatal rat model. Pediatr. Res. 53, 426–433

Lin PW, Stoll BJ (2006). Necrotising enterocolitis. Lancet. 368:1271.

Chu A, Hageman J, Caplan M (2013). Necrotizing Enterocolitis: Predictive Markers and Preventive Strategies. NeoReviews (14):3

Kastenberg ZJ, Sylvester KG (2013).The surgical management of necrotizing enterocolitis. Clin Perinatol; 40:135–48

Lee H J (2011).An update on necrotizing enterocolitis: pathogenesis and preventive strategies. Korean J Pediatr; 54(9): 368–372

Akisu M, Ozmen D, Baka M, Habif S, Yalaz M, Arslanoglu S et al. (2002). Protective effect of dietary supplementation with Larginine and L-carnitine on hypoxia/reoxygenation-induced necrotizing enterocolitis in young mice. Biol Neonate 81:260–265

Ford H, Watkins S, Reblock K, Rowe M (1997). The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. Journal of Pediatric Surgery.32(2):275–282.

Mitidiero LF, Simões AL, Gonçalves FL, Figueira RR, Silva OC, Sbragia L ('2014). L-FABP and I-FABP expression in newborn rats changes inversely in the model of necrotizing enterocolitis. Acta Cir Bras.; 29 Suppl 2:43-9.