Asian Journal of Chemistry Vol. 22, No. 2 (2010), 1569-1576

Parameters of Some of Biochemistry on Ischemia-Reperfusion Injury in Newborn Rat Intestine

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The purpose of the present study was to determine the effects of treatment with thyroxin and dexamethasone separately and together on I/R injury in newborn rat intestine. Newborn rats were divided into five groups: (1) control (C) group, (2) sham group, (3) thyroxin (T_4) group, (4) dexamethasone (DEX) group and (5) thyroxin plus dexamethasone (T_4+DEX) group. Each group consisted of seven pups. Group T_4 received thyroxin 1 μg/g BW/day, group DEX received dexamethasone 5 μg/gBW/day and group T_4+DEX received 1 μg/gBW/day thyroxin and 5 μg/gBW/day dexamethasone i.p. for 7 days. Group C received only physiological saline (NaCl 0.9 %). Animals were sacrificed at the end of the reperfusion period and ileum samples were obtained. Malondialdehyde (MDA) levels, a product of lipid peroxidation, glutathione (GSH) levels, a key antioxidant were determined in ileum homogenates. In the jejunum, only T_4 caused a significant statistical elevation in GSH compared with control, sham and DEX groups ($p < 0.05$). There was significant statistical interaction between T_4 and DEX treatment, *i.e.* the effect of T_4 treatment was greater in both regions. Mucosal damage scores showed statistical significant effect of T_4 in the ileum compared with control and T_4+DEX group. The same significant statistical effect was seen with T_4+DEX group compared to the control group in the ileum. Overall, the comparison between the two regions shows a powerful effect of T_4 in both regions. There was statistical significance between the scores of T_4 in the ileum and of DEX and T_4+DEX in the jejunum. A beneficial effect of thyroxin in all samples was observed in this study supporting its protective effects against I/R injury which was attenuated by glucocorticoid administration.

Key Words: Ischemia-reperfusion, Thyroxin, Dexamethasone, Injury.

INTRODUCTION

Studies and experimental models regarding ischemia-reperfusion (I/R) injury and its prevention have suggested that any alteration in series of ongoing pathological and physiological mechanisms in the intestine tissue due to I/R could help to hinder the injury.

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The use of thyroxin with this aim in renal I/R models has shown beneficial effects on cell regeneration by preserving levels of tissue adenosine triphosphate (ATP) and membrane lipid organization[1]. Dexamethasone alone was shown to protect heart muscle cells and brain tissue together with mannitol infusion from I/R in animal experiments[2,3]. The purpose of the present study was to determine the effects of treatment with thyroxin and dexamethasone separately and together on I/R injury in newborn rat intestine.

EXPERIMENTAL

Five female and 5 male Swiss albino rats (*Rattua rattus*) from Hacettepe University Experimental Laboratories (Ankara) were received and housed as pairs in stainless-steel cages and provided with food and water *ad libitum*. Thirty-five Swiss albino pups were obtained and the birth date was regarded as day 0.

Experimental design: On the first day 35 pups were divided into five groups: (1) control (C) group, (2) sham group, (3) thyroxin (T_4) group, (4) dexamethasone (DEX) group and (5) thyroxin plus dexamethasone (T_4+DEX) group. Each group consisted of 7 pups. Group T_4 received T_4 1 μg/gBW/day i.p. for 7 days. Group DEX received DEX 5 μg/gBW/day i.p. for 7 days and group T_4+DEX received 1 μg/gBW/day T_4 and 5 μg/gBW/day DEX i.p. for 7 days. Group C received only physiological saline (NaCl 0.9 %). All pups were sacrified on day 7.

Mesenteric Ischemia and reperfusion method: At the end of the experimental period, rats were anesthetized with an intraperitoneal injection of pentobarbital (10 mg/kg). A midline laporotomy was performed and the superior mesenteric artery was exposed. After the collateral arcades were ligated as described by Megison *et al.*[4], an atraumatic micro vascular clamp was placed across the superior mesenteric artery. After 45 min of ischemia, the clamp was removed for 1 h of reperfusion.

Preparation of the tissue extracts: After completing the procedure, the animals were sacrified and tissue samples were obtained from the intestine. Pieces of jejunum and ileum were dissected for histology and fixed in 10 % formaldehyde and pieces for biochemical analysis were stored at -70 ºC.

Samples were sectioned and stained with H&E and PAS and mucosal lesions were graded on a scale from 0 to 5 as described by Chiu *et al.*[5]. Grade 0, normal mucosal villi; **Grade 1**, development of sub epithelial Gruenhagen's space, usually at the apex of the villus, often with capillary congestion; **Grade 2**, extension of the sub epithelial space with moderate lifting of epithelial layer from the lamina propria; **Grade 3**, massive epithelial lifting down the sides of villi (a few villus tips may be denuded); **Grade 4**, denuded villi with lamina propria and dilated capillaries exposed (increaed cellularity of lamina propria may be noted) and **Grade 5**, digestion and disintegration of lamina propria, haemorrhage and ulceration.

Enzyme assays: The levels of malonedialdehyde (MDA) were determined by the technique of Uchiama and Mihara with the aid of thiobarbituric acid reactive substance (TBARS) method[6] and the results were obtained in μmol/mg protein.

Levels of reduced glutathione (GSH) were determined by the method of Sedlak and Lindsay[7] and the results obtained in μmol/mg protein. The protein content of the homogenates was determined according to the method of Lowry.

The data are presented as the mean ± SEM. Mann-Whitney U statistical method was used for the histological and Dunnet t-test for the biochemical results; $p < 0.05$ was considered the limit for statistical significance.

RESULTS AND DISCUSSION

Tables 1 and 2 show the results of hormone treatment on TBARS values in the jejunum and ileum. There was no significant interaction between control and sham groups. Although, no significant statistical interaction between treatment and control groups was seen in the ileum, statistical significance between group DEX and Sham group ($p < 0.05$) existed in the jejunum.

TABLE-1
TREATMENT RESULTS ON TBARS (nmol/mg PROTEIN) IN THE JEJUNUM (A)

	Control (0.2 mL saline i.p.)	Sham (only laporotomy)	T_4 (1 μg/gBW/ day i.p.)	DEX (5 μg/gBW/ day i.p)	T_4+DEX
MDA	97.5±4.9	86.85±5.5	103.39±6.0	113.78±3.8[a]	98.06±3.6

Seven animals were used in every group; [a]Statistical significance.

TABLE-2
TREATMENT RESULTS ON TBARS (nmol/mg PROTEIN) IN THE ILEUM (B)

	Control (0.2 mL saline i.p.)	Sham (only laporotomy)	T_4 (1 μg/gBW/ day i.p.)	DEX (5 μg/gBW/ day i.p)	T_4+DEX
MDA	125.92±3.3	123.86±4.4	129.27±6.8	113.78±7.9	113.94±8.5

Tables 3 and 4 show the results of reduced glutathione (GSH) in the jejunum and the ileum. In the jejunum, only T_4 caused a significant statistical elevation in GSH compared with control, sham and DEX groups ($p < 0.05$). There was significant statistical interaction between T_4 and DEX treatment, *i.e.* the effect of T_4 treatment was greater in both regions.

TABLE-3
TREATMENT RESULTS ON GSH (μmol/mg PROTEIN) IN THE JEJUNUM (A)

	Control (0.2 mL saline i.p.)	Sham (only laporotomy)	T_4 (1 μg/gBW/ day i.p.)	DEX (5 μg/gBW/ day i.p)	T_4+DEX
GSH	86.86±1.3	89.55±2.4	96.85±1.8[a]	87.67±3.8	90.41±2.3

[a]Statistical significance.

TABLE-4
TREATMENT RESULTS ON GSH (μmol/mg PROTEIN) IN THE ILEUM (B)

	Control (0.2 mL saline i.p.)	Sham (only laporotomy)	T_4 (1 μg/gBW/ day i.p.)	DEX (5 μg/gBW/ day i.p)	T_4+DEX
GSH	69.35±2.6	68.86±0.9	78.56±3.6[a]	64.13±2.4	73.57±5.6

[a]Statistical significance.

Pathology: Tables 5 and 6 show the effects of hormone treatment on mucosal injury in the jejunum and the ileum. Statistical evaluation of mucosal damage scores showed statistical significant effect of T_4 in the ileum compared with control and T_4+DEX group. The same significant statistical effect was seen with T_4+DEX group compared to the control group in the ileum. Overall, the comparison between the two regions shows a powerful effect of T_4 in both regions. There was statistical significance between the scores of T_4 in the ileum and of DEX and T_4+DEX in the jejunum.

TABLE-5
EVALUATION OF SAMPLES FROM CONTROL AND TREATMENT GROUPS ACCORDING TO CHIU CRITERIA

	Control (0.2 mL saline i.p.)		Sham (only laporotomy)		T_4 (1 µg/gBW/ day i.p.)		DEX (5 µg/gBW/ day i.p)		T_4+DEX	
Case	A	B	A	B	A	B	A	B	A	B
1	3	4	0	0	2	1	2	2	4	3
2	3	3	0	0	1	2	4	4	3	2
3	2	3	0	0	3	2	2	2	3	2
4	3	3	0	0	1	1	4	3	2	3
5	3	4	0	0	2	1	3	4	1	2
6	3	4	0	0	2	1	3	3	2	3
7	3	4	0	0	2	1	3	3	5	3

TABLE-6
DISTRIBUTION OF PATHOLOGICAL CHANGES IN PERCENTAGES

	Control (0.2 mL saline i.p.)		Sham (only laporotomy)		T_4 (1 µg/gBW/ day i.p.)		DEX (5 µg/gBW/ day i.p)		T_4+DEX	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Grade 0	-	-	100	100	-	-	-	-	-	-
Grade 1	-	-	-	-	28.57	71.42	-	-	14.28	-
Grade 2	14.28	-	-	-	57.14	28.57	28.57	28.57	28.57	42.85
Grade 3	85.71	42.85	-	-	14.28	-	42.85	42.85	28.57	57.14
Grade 4	-	57.14	-	-	-	-	28.57	28.57	14.28	-
Grade 5	-	-	-	-	-	-	-	-	14.28	-

Ischemic injury following pathological changes has been described in details by many researchers[8-11]. Lipid peroxidation started by hydroxyl radicals enables the formation of lipid-based free radicals like conjugated dienes and lipid hydroxy-peroxide radicals, which play a key role in reperfusion injury[12,13]. Free oxygen radicals causing peroxidation of unsaturated fatty acids on the cell membranes result in oxidative mucosal injury[14]. End-products of lipid peroxidation include malonedi-aldehyde (MDA), hydrocarbon gases and conjugated dienes[15]. During this process, concentration levels of MDA and conjugated dienes are directly correlated with the duration of ischemia[16]. Antioxidants like superoxide dismutase (SOD), glutathion peroxidase (GSHPx), bilirubin, vitamin A, compound **IA** and catalase have shown to minimize mucosal injury following ischemia/reperfusion[17,18].

Protective effects of immunosuppressants like EGF NAC and steroids against ischemia-reperfusion injury in cremaster muscle flap at microcirculatory level has been reported[19].

Malonedialdehyde originated from peroxidation of membraneous lipids after ischemia/reperfusion is detected through measuring the substances acting with thiobarbituric acid and this method was used mainly in present research. Experimental studies have shown that accumulating MDA is a sensitive index related to ischemia/reperfusion[16]. Malonedialdehyde and thiobarbituric acid are forming a complex with a characteristic colour in acid environment which absorbs the light at 532 nm wavelength. Biological researchers and the food industry as a sensitive and specific measure of polyunsaturated auto oxidation of lipids[20] used this complex. After standardization, this test serves as a highly sensitive and specific method for measuring substances reacting with thiobarbituric acid (TBARS)[6].

Glutathione has antioxidant features and displays its effect by enhancing GSHPx enzyme activity[21]. Oxidative stress results in a decrease of reduced glutathione in the tissue and this measurement can be used as a parameter in ischemia/reperfusion[21-25].

We also measured tissue GSH besides TBARS because normal tissue concentrations of reduced glutathione indicate tissue preservation from ischemia/reperfusion[26].

Thyroxin (T_4) and dexamethasone (Dex) are shown to improve intestine mucosa maturation in several ways either individually or together with a synergistic outcome. Thyroidal hormones are vital for growth and development and they enhance metabolic activity in the tissue. Thyroxin increases oxygen utilization in the tissue. Dexamethasone is a synthetic derivate of cortisol but its immunosuppressive effects are stronger than its glucocorticoid properties[27]. McDonald *et al.*[28] have shown synergistic effects of T_4 and Dex on the postnatal development of the small intestine in rat studies[29]. Malo and Menard demonstrated that insulin, cortisone and thyroxin resulted in beneficial synergistic outcome on the proliferation and differentiation of epithelial cells in the small intestine of rats fed with breast milk and proved the effect of postnatal maturation[30]. It was shown that thyroxin stimulates the organization of phospholipids essential for the cell membrane integrity. Treatment with thyroxin in the post ischemic stage has shown to have positive effects on plasma membranes of renal proximal tubular cells[1].

Amer *et al.*[31] showed that following replacement of thyroxin in an infant with necrotizing enterocolitis (NEC) due to hypothyroidism regressed dramatically. Hence, it is known that I/R mechanisms also affect NEC. The present study supports this data. According to these findings, it may be postulated that thyroxin hormone has protective effects on tissue I/R injury.

In this study, no statistical significance was exposed between control and intervention groups related to TBARS measures in accordance with other publications (Tables 1 and 2). In addition, simultaneous use of thyroxin and dexamethasone did not result in statistical significance on TBARS values. Lack of difference of TBARS

measures between control and dexamethasone groups can be explained by that dexamethasone is ineffective on preventing I/R injury by its own as shown by another study[32]. Meanwhile, TBARS measures between groups where thyroxin was used showed no difference in this study, not in accordance with another study illustrating positive effects of thyroxin on renal ischemia/reperfusion processes[1].

Glutathione measures showed no statistical significance between control, dexamethasone and T_4+Dex groups whereby results of samples of ileum and jejunum from thyroxin and dexamethasone intervention groups revealed statistical significance (Tables 3 and 4) exposing a decrease in GSH measures in both groups treated with dexamethasone. According to these findings, one can state that dexamethasone is unsuccessful in preventing injury from I/R. On the other hand, high GSH values noticed in the thyroxin group imply protection from oxidative stress injury arising during reperfusion through the preservation of the reduced glutathione pool.

Samples from the jejunum of the thyroxin treated group demonstrate a statistical significant elevation for all GSH values. In addition, a considerable rise of GSH in the samples of the thyroxin treated ileum compared with the control group was observed. Although this elevation in the ileum is not statistical significant, this findings might be accepted as a supporting result that thyroxin protects the tissue from I/R injury.

Histological and pathological evaluation of the samples regarding tissue injury revealed no statistically significant difference between the control group and the dexamethasone and T_4+Dex groups. Evaluation of thyroxin and dexhametasone administered groups demonstrated a statistically significant difference between jejunal and ileal samples. There was also a statistically significant difference between samples belonging to control and thyroxin administered groups. This difference was in the form of less tissue injury in thyroxin-administered subjects. Grade 0 could not be seen in any groups other than Sham group according to the results of pathological investigations stating that thyroxin is only capable to reduce the amount of injury, not avoiding it completely (Table-5).

Evaluation of the ileal samples showed a statistically significant difference between thyroxin and T_4+Dex administered groups. Detection of a reduced amount of tissue injury in the thyroxin-administered group in comparison to T_4+Dex group demonstrates that the protective effect of thyroxin may decline with dexamethasone administration. Furthermore, pathological injury in the ileal samples of the T_4+Dex group was less than the control group, but this cannot be interpreted as inhibition of injury because the injury was condensed between grade 2 and 3 and it was higher than the thyroxin administered group (Table-5).

The synergistic actions of thyroxin and corticosteroid administration at the postnatal period on small bowel maturation, intestinal epithelial cellular proliferation and intestinal enzyme maturation[29,30,33] that are designated in the literature could not be demonstrated in present study. Although we are not able to show the exact mechanisms of the preventive effect(s) of thyroxin against I/R injury; higher levels

of reduced glutathione measurements in the thyroxin administered group compared to the additional groups and concurrent pathological demonstration of prominent attenuation of injury suggests possible inhibition of formation of free oxygen radicals. In this study, the effects of thyroxin and dexamethasone over small bowel I/R injury in newborn rats were investigated and following results were found. There was a faster weight gain and hair development in thyroxin group when compared to control and other groups during the experiment. A statistically significant difference for prevention of I/R injury according to TBARS values between control and drug administered groups could not demonstrated. It was reported chemical causes severe oxidative stress in gastric tissue manifested as stimulated lipid peroxidation by increasing MDA content and decreasing of gastric GSH concent[34]. The MDA determined was similar to that in the other studies[34].

There was a statistically significant difference between the thyroxin-administered group and the other groups according to the GSH values. This difference was related with the increase of GSH values in the thyroxin-administered group. In literature acute alcohol induced gastric mucosal injury in rats have been investigated and GSH value has been reduced[34]. These finding are in good agreement with a recent study by Kanter *et al.*[34]. Effects of applied some of biochemical parameters and on erythrocyte of rats have been investigated[35]. In the thyroxin administered group, tissue injury related to I/R injury was significantly reduced confirmed by pathological assessment.

Further investigations are essential to delineate the clinical use of thyroxin hormone to prevent I/R injury.

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(*Received*: 6 July 2009; *Accepted*: 3 November 2009) AJC-8016