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Effects of Epidermal Growth Factor Formulations on Liver Malondialdehyde and Reduced Glutathione Levels in Stress Ulcer Model

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Summary : *Gastric mucosal injury associated with hepatic dysfunction can be correlated with metabolic perturbation of antioxidant compounds such as reduced glutathione (GSH) and related compounds in the liver and stomach. The aim of this study was to determine the effects of Epidermal Growth Factor (EGF) administration routes on lipid peroxidation (MDA) and glutathione (GSH) metabolism in liver of cold-restraint stress rats. The study was performed on 7 groups: Group I: untreated rats (Control), Group II: Acute cold restraint stress effect on liver tissue (CRS), Group III: Untreated rats after cold restraint stress (CRS-UP), Group IV: Cold restraint stress with intragastric microemulsion treatment (ME), Group V: Cold restraint stress with intragastric EGF treatment in microemulsion (ME-EGF) (for 7 days, 6μg/kg), Group VI: Cold restraint stress with intraperitoneal physiologic saline injection (IP-PS), Group VII: Cold restraint stress with intraperitoneal EGF treatment in physiologic saline (IP-EGF) (for 7 days, 6μg/kg). The results indicated that the liver MDA levels were significantly increased in all experimental groups. The liver GSH levels of ME-EGF and IP-EGF treated groups were decreased significantly when compared to their control groups. Our results suggest that cold restraint stress caused an increase in hepatic lipid peroxidation levels and EGF treatment decreased hepatic GSH levels. These results might indicate that EGF plays a regulatory role independently to its dosage form or administration route on GSH metabolism in liver.*

Keywords : *Cold restraint stress, EGF, Liver, MDA, GSH*

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Stres Ülseri Modelinde Epidermal Büyüme Faktörünün Formulasyonlarının Karaciğer Malondialdehid ve Redükte Glutatyon Düzeylerine Etkisi

Özet : *Karaciğer yetmezliği ile birlikte oluşan mide mukozasının hasarı, mide ve karaciğerde redükte glutatyon (GSH) gibi antioksidan bileşiklerin metabolizmasındaki bozukluklar ile ilişkili olabilir. Bu çalışmanın amacı soğuk immobilizasyon stresinde tedavi amacıyla Epidermal Büyüme Faktörü'nün (EBF) değişik uygulama şekillerinin karaciğer lipid peroksidasyonu (MDA) ve GSH metabolizması üzerine olan etkilerini incelemektir. Çalışma 7 grup üzerinde gerçekleştirilmiştir: I. Grup: Herhangi bir uygulama yapılmayan kontrol grubu (Kontrol), II.Grup: Soğuk-immobilizasyon stresi uygulanıp hemen karaciğer dokusu çalışılan sıçan grubu (CRS), III.Grup: soğuk immobilizasyon stresi uygulanmasını takiben 7 gün boyunca herhangi bir tedavi uygulanmayan grup (CRS-UP), IV.Grup: Soğuk immobilizasyon stresi ve intragastrik mikroemulsiyon uygulanan grup (ME), V.Grup: Soğuk immobilizasyon stresi ve intragastrik yolla mikroemülsiyon formulasyonu içinde EGF uygulanan grup (ME-EGF) (6μg/kg, 7 gün boyunca), VI. Grup: Soğuk immobilizasyon stresi ve intraperitoneal olarak serum fizyojik uygulanan grup (IP-SF), VII. Grup: Soğuk immobilizasyon stresi ve intraperitoneal olarak serum fizyolojik içinde EGF uygulanan grup (IP-EGF) (6μg/kg, 7 gün boyunca). Sonuçlar incelendiğinde karaciğer MDA düzeyinin tüm deney gruplarında anlamlı olarak arttığı gözlenmiştir. Karaciğer GSH içeriğinin ME-EGF ve IP-EGF uygulanan gruplarda kendi kontrol grupları ile karşılaştırıldığında belirgin olarak azaldığı saptanmıştır. Sonuçlar soğuk immobilizasyon stresinin karaciğerde lipid peroksidasyonunu arttırdığını ve EGF uygulamasının karaciğer GSH içeriğini azalttığını göstermektedir. Ayrıca bu bulgular EGF'ün karaciğer GSH metabolizmasında uygulama formu veya yoluna bağlı olmaksızın regülatör rol oynayabileceğine de kanıt olabilir.*

Anahtar kelimeler : *Soğuk immobilizasyon stresi, EBF, Karaciğer, MDA, GSH*

INTRODUCTION

Cold restraint stress treatment causes gastric ulcers in rats. The major factors implicated in the development of stress ulcer include an increase in gastric acid secretion and a decrease in mucosal protection due to the reduction in mucus secretion, mucosal blood flow, and prostaglandin biosynthesis[1,2]. In addition,

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it has been reported that increase in gastric lipid peroxidation and decrease in gastric glutathione levels may be included among the aetiopathogenetic factors leading to stress-induced gastric ulcer[2-4].

Recent studies suggest that sulfhydryl compounds in the stomach may be important for the maintenance of gastric mucosal integrity[5-8]. It is known that glutathione is the predominant thiol of tissue in normal animals. The reduced form of glutathione (GSH) is the major endogenous antioxidant in living organism and is metabolised via organ cooperation in which hepatic synthesis and secretion of GSH into bile and plasma play an important role. The liver is known to export a large quantity of GSH into the plasma in response to stress conditions. Hepatic GSH is the major source of plasma GSH. Gastric mucosal injury associated with hepatic dysfunction would be correlated with metabolic perturbation of antioxidant compounds such as GSH and related compounds in the liver and stomach[5]. Water immersion restraint stress decreased both total thiol and GSH levels in the liver. Interestingly, gastric thiol levels markedly decreased during water immersion restraint treatment without appreciable decrease in its GSH levels[5].

The most important damaging effect of free radicals on tissues is lipid peroxidation. Oxygen free radicals cause cellular injury by inducing lipid peroxidation which results in functional and structural cell interactions. Lipid peroxidation can be evaluated by the formation of malondialdehyde (MDA)[9].

Epidermal Growth Factor (EGF) is a potent mitogenic peptide for many cell types. The liver is a target tissue for EGF action and liver contains a large number of EGF receptors in both fetal and adult life. The liver can also sequester high doses of intraperitoneally administered EGF[10-13]. EGF is a single-chain polypeptide that is secreted by submandibular glands and is a powerful inhibitor of gastric secretion[1]. Previous reports showed that EGF has a potent trophic action on the gastrointestinal mucosa and removal of the major source of EGF by sialoadenectomy resulted in the decrease of mucosal growth, integrity and protection against various ulcerogens[14-17].

Peptides are generally poorly absorbed from the gastrointestinal tract. Therefore, in recent years, much attention has been given to the design of new oral dosage forms of peptides. Much of the activity in this area has been focused on the development of microemulsions. Microemulsions can be defined in general as thermodynamically stable, isotropically clear dispersions. The formation of microemulsions is usually by mixing four components including a surfactant, a co-surfactant, oil and water[18]. Microemulsions may allow controlled drug release and they may increase systemic and topical absorption of peptides[19].

The objective of the present study was to investigate the effects of microemulsion formulation and intraperitoneal administration of EGF on the MDA, a marker of lipid peroxidation, and GSH levels in the liver of cold restraint stressed rats.

MATERIAL AND METHODS

Materials

Labrafil M 1944 CS (Unsaturated polyglycolyzed glycerides) was supplied by Gattefosse^{R} (France). Arlacel 186 (Glycerol-monoleate-propylene glycol) and Brij 35 (polyoxyethylene lauryl ether) were provided by ICI Pharmaceutics^{R} (England). Mouse Epidermal Growth Factor (m-EGF) was purchased from Sigma (USA). All other chemicals were of analytical grade.

Methods

Preparation of the microemulsion: The stable microemulsion was prepared as described in our previous study[20]. Briefly, Arlacel 186 and Brij 35 were used as surfactant (S) and Labrafil M 1944 CS was the oil phase. Absolute alcohol and distilled water were used as the cosurfactant (Co-S) and aqueous phase respectively. Pseudoternary phase diagrams were carried out by titration. The area enclosed by the microemulsion's existence field calculated and plotted as a function of S/Co-S, was 2,5. The aqueous and non-aqueous phases were prepared separately and then mixed with a stirring bar until a clear formulation was obtained. EGF was dissolved in water phase and then added to the other phase containing S,Co-S and oil.

In vivo studies:

Animals: In this study female Wistar albino rats weighing 210±10g were used. The animals were fasted for 24 h before the experiment but were allowed free access to water. They were assigned into seven groups and each experimental group contained six rats. Experimental processes followed were in accordance with Turkish Veterinary Research Council's guide for the care and use of laboratory animals.

Animal Model for Gastric Mucosal injury: Acute gastric lesions were induced by cold-restraint stress for 3 h in the refrigerator (4.0±0.5°C) . EGF was administered intragastrically in a dose of 6µg/kg/day in 0,2 ml microemulsion (ME) or intraperitoneally (IP) in physiologic saline (PS) for seven days. ME and PS without EGF administered intragastrically and intraperitoneally, served as control groups. Untreated 6 rats were used as normal controls. After 7 days the liver of the rats, were immediately excised (under nembutal anaesthesia) and frozen in liquid nitrogen. The MDA and GSH contents in the livers were determined by spectrophotometric methods given below using a Shimadzu UV-1208 spectrophotometer .

Lipid Peroxide Determination: Liver lipid peroxide levels were estimated by the method described by Casini et al[21]. Briefly, tissue samples were homogenised in ice-cold trichloroacetic acid (1 g tissue in 10 ml 10% trichloroacetic acid) in a tissue homogeniser (Heideloph Diax 900). Following centrifugation of the homogenate at 3000 rpm for 10 min (Hettich Universal 32 R), 750µl of supernatant was added to an equal volume of 0.67% (m/v) thiobarbituric acid and heated at 100 °C for 15 minutes. The absorbance of the samples were measured at 535 nm. Lipid peroxidation levels were expressed in terms of MDA equivalent using an extinction coefficient of $1.56x10^5\ M^{-1}\ cm^{-1}$.

Reduced Glutathione Determination: The GSH levels were determined by a modified Ellman method[22]. To the 0.5 ml of supernatant obtained above 2 ml 0.3 M $Na_2HPO_42H_2O$ solution was added. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml in 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. The GSH levels were calculated using an extinction coefficient of 13600 $M^{-1}\ cm^{-1}$.

Statistical analysis: One way analysis of variance (ANOVA) was used for statistical comparison of groups, with multiple post hoc comparison performed with the Tukey-Kramer test. P values of less than 0,05 were considered significant.

RESULTS

The results of MDA and GSH content in liver are shown in Table I. The hepatic MDA levels were sig-

Table 1: The liver MDA and GSH levels in ME-EGF, IP-EGF treated and non-treated control groups.

	PARAMETERS	
GROUPS	MDA (nmol/g) (Mean±SD)	GSH (µmol/g) (Mean±SD)
UT (n:6)	65,93±15,35	7,88±1.42
CRS (n:6)	73,58±17,95	7,73±1.03
CRS-UT (n:6)	96,13±15,86*,**	8,18±1.17
ME(n:6)	114,16±16,73 *,**	7.31±1.35
ME-EGF (n:6)	92,48±11,58 *	4,21±1.23 *,**,#
IP- PS(n:6)	109.08±24.24 *,**	9.80±1.63
IP-EGF (n:6)	124,05±14.13*,**	5.91±0.75+
	*p<0,05 with control, ** p<0,05 with CRS	* p<0,05 with control, ** p<0,05 with CRS, # p<0,05 with ME, + p<0,05 with IP-PS.

UT: Untreated rats (Control), CRS: Cold restraint stress (first day), CRS-UT: Untrated rats with cold restraint stress (7th day), ME: Microemulsion treatment after CRS, ME-EGF: EGF treatment in microemulsion after CRS, IP-PS: Physiological saline treatment after CRS, IP-EGF: EGF in physiological saline treatment after CRS.

nificantly increased in stress groups compared to control rats. The EGF treatment has no effect on decreasing lipid peroxidation levels in livers of stressed rats. The liver MDA level was found significantly higher in ME-EGF (92,48±11,58 nmol/g), ME (114,16±16,73 nmol/g), IP-EGF (124,05±14.13 nmol/g) and IP-PS treated group (109.08±24.24 nmol/g) and CRS-UT(96,13±15,86) than the control group (65,93±15,35 nmol/g).

Following 7 days , the exposure to 3 hours of stress was accompanied by a significant reduction in the content of GSH in the liver of EGF treated groups. The hepatic GSH levels were found to be significantly decreased in ME-EGF (4,21±1,23µmol/g) compared to control (7,88±1,42), CRS (7,73±1,03) and ME (7,31±1,35) groups. In the IP-EGF (5,91±0,75) treated group, the GSH levels decreased compared to the IP-PS groups (9,80±1,63)

MDA and GSH levels were not significantly different in the CRS group compared with the control group ($p>0,05$).

DISCUSSION

Gastric mucosal lesion sometimes occur in patient with hepatic dysfunction[23,24]. Recent studies suggest that sulfhydryl compounds in the stomach might be important for the maintenance of gastric mucosal integrity[5-8]. It has been reported that cold restraint stress resulted in a significant decrease in thiol levels both in the gastric mucosa and in the liver, but GSH content was reduced mainly in the hepatic tissue suggesting that extracellular and interorgan GSH metabolism, rather than gastric mucosal GSH level, plays a crucial role in protection against stress ulceration[5]. Extracellular glutathione and its interorgan metabolism might play a critical role in the protection of gastric mucosa particularly when animals were challenged with various stresses. The hepatic efflux of GSH occurs in water immersion restraint treated animals. Water immersion restraint treatment also inhibits hepatic synthesis of GSH[5].

In the present study, the hepatic lipid peroxidation levels were found significantly increased in cold restraint stress treated group compared with the control group. EGF treatment did not affect lipid peroxidation levels in liver. It was reported that EGF inhibits lipid peroxidation in the gastric mucosa[25]. Our previous study revealed that the gastric MDA levels of stressed animals increased. Contrary to stressed animals, gastric MDA levels decreased in EGF treated groups[26,27]. This observation suggested that cold restraint stress increased the gastric lipid peroxidation levels and EGF may prevent stress ulcer by means decreasing lipid peroxidation in gastric mucosa.

Increased levels of hepatic lipid peroxidation accompanied by decreased levels of GSH has been reported. It was demonstrated that EGF treatment increased GSH levels but did not change lipid peroxidation levels in lobectomized liver[28]. In this experimental protocol, gastric ulcer was induced by cold restraint stress. EGF was administered intragastrically and intraperitoneally. Ulcerated gastric tissue will be the target of EGF in both administration routes. Thus antioxidant effect of EGF will be more effective in ulcerative gastric mucosa than hepatic tissue. In the present study, we showed that cold restraint stress would be consistent with an increase in hepatic lipid peroxidation and EGF treatment did not affect lipid peroxidation in liver. In addition, there was no statistical relation between GSH and MDA levels in EGF treated groups.

ME-EGF and IP-EGF treatment decreased GSH levels in liver compared to their controls. Hirota et al.[5] demonstrated that hepatic GSH levels rapidly decreased during the initial 3 hours of the water immersion restraint treatments. They showed that water immersion restraint stress caused a decrease in the stomach GSH level. Nishida et al.[29] found that the water immersion restraint treatment causes an increase in gastric lipid peroxidation and a decrease in the non-protein GSH levels after 1,3,6 h of stress. In stressed rats treated with EGF, the liver content of GSH was markedly reduced. The decreased GSH levels in EGF treated groups may be caused by the effect of EGF on the interorgan cycle of GSH, especially to stomach during the 7 days period. However, in our previous study, we found that there was an increase in the gastric GSH levels of the same treated groups but it was not significant[26,27].

GSH metabolism occurs via interorgan cycles in which induced hepatic synthesis of GSH and its transfer to extrahepatic tissues in 3 hr play an important role[5]. On the 7th days of EGF treatment, the decreased GSH content in ME-EGF and IP-EGF treated groups may be due to the effect of EGF on interorgan cycle of GSH, especially to stomach in stress conditions.

In conclusion, cold restraint stress causes increased hepatic lipid peroxidation, and EGF treatment may play a regulatory role on GSH metabolism of liver independently of its dosage form or administration route. Thus, it can be speculated that EGF acts as an antioxidant in wounded tissues by regulating GSH mobilization from the liver.

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