Gastroprotective Potential Effects of Statins on Indomethacin-Induced Gastric Ulcers in Rats

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ABSTRACT

Statins (3-hydroxy-3-methyl glutaryl-CO A reductase inhibitors) exert favorable effects on lipoprotein metabolism but may, also, possess antioxidant and anti-secretory effects which have led to the interest in the use of that class of drugs outside treatment of cardiovascular diseases. Here, the effects of atorvastatin in experimently induced gastric acid secretion and ulcer formation and the mechanisms underlying that protection in rats were explored. Animals were randomly assigned to three experimental groups (control, indomethacin, and indomethacin+atorvastatin groups). Pyloric ligation was performed for collection of gastric juice, and gastric ulceration was induced by a single intraperitoneal injection of indomethacin (40mg/kg).The following parameters were assayed (volume of gastric secretion and acidity, the level of mucus, and proteolytic activity in gastric juice; lipid peroxides (MDA), nitric oxide (NO), and prostaglandin E_2 (PGE_2) in gastric mucosa). Pretreatment with atorvastatin (10 mg/kg orally for 7 days) caused significant reduction in gastric mucosal lesions, MDA and gastric acid secretion associated with significant increase in gastric juice mucin secretion. Also, atorvastatin significantly increased gastric NO and PGE_2 levels. These data illustrate the gastroprotective effects of atorvastatin which may be mediated by its anti-oxidant and anti-secretory properties.

INTRODUCTION

Gastric ulcer is a common health problem that affect a lot of population allover the world. Gastric ulcers are known as erosions of the gastric mucosa that may penetrate the muscle layer and perforate the stomach wall[(1)]. Ulcers are induced by predominance of aggressive factors to defensive factors, as the balance between both factors is necessary for maintaining intact and healthy gastric mucosa[(2)].

Regulation of gastric mucosal blood flow is very important to maintain normal circulation and prevent occurrence of peptic ulcer. Prostaglandins and nitric oxide have been recognized as important factors for the regulation of gastric mucosal microcirculation, repair, and integrity[(3,4)]. Inhibition of NO synthesis has been shown to produce acute gastric mucosal damge, whereas enhancement of NO synthesis exerts gastroprotective effects[(5)]. Ischemia of

gastric mucosa is accompanied by enhanced generation of oxygen-derived free radicals (ODFR) that may initiate a chain of reactions in membrane-bound lipids causing lipid peroxidation and cellular injury[6]. The most common lipid peroxide product is malondialdehyde (MDA), which is well known to cause cross-linking and polymerization of membrane components. Being diffusible, it also reacts with nitrogenous bases of DNA leading to more complicated sequelae[7]. The mucosal content of MDA (known alternatively as thiobarbituric acid reactive species, TBARS) was observed to increase with the development of gastric ulcers[8, 9].

Gastric ulcers associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) remain a major clinical problem[10]. The continuous generation of prostaglandins by cyclooxygenase isoenzymes in the gastric mucosa helps to maintain an adequate mucosal blood flow and, also, stimulates the generation of mucus[11]. NSAIDs inhibit cyclooxygenase and thereby reduce the intrinsic ability of the mucosa to resist injury induced by endogenous and exogenous aggressors. Indomethacin, one of NSAIDs family, causes gastric ulcers through various processes, including generation of reactive oxygen species, initiation of lipid peroxidation, infiltration of leucocytes, induction of apoptosis, and inhibition of prostaglandin synthesis[12]. Decreased prostaglandin level impairs almost all aspects of gastroprotection and increases acid secretions which in turn, aggravate the ulcer[13].

Statins are a group of drugs that are originally designed to lower serum cholesterol level and have been recognized as the most efficient drugs for the treatment of hyperlipidemia. Previous clinical trials have demonstrated that the therapeutic benefits of statins could not solely be explained by their inhibitory actions on cholesterol synthesis[14,15]. Interestingly, statins appear to have additional benefits beyond their lipid lowering effects. Besides the therapeutic use in hyperlipidemia, the antioxidant, anti-inflammatory, and immunomodulatory benefits of statins have been reported in many aspects[16]. Other investigators have suggested that protective effects of statins are mediated by enhancement of NO production, which is implicated in regulation of gastric acid secretion and modulation of gastric mucosal integrity together with endogenous prostaglandins[17]. **Birnbaum and his co-workers[18]** reported that oral pretreatment with atorvastatin caused an increase in NO and PGE_2 concentrations in the heart.

Therefore, the present study aimed to elucidate the possible gastroprotective effects of atorvastatin on gastric ulcer induced by indomethacin and the possible putative mechanisms underlying these effects.

MATERIALS & METHODS

1- Animals:

Adult male albino rats weighing 150-200 g were used (from animal house of National Research Center, Cairo). All experiments were performed during the same time of the

day to avoid variations due to diurnal rhythm of putative regulators of gastric function. Rats were fasted for 24 hours with free access to water in mesh bottomed cages to minimize coprophagia[19].

2. Experimental design:

Animals were randomly assigned to three experimental groups of eight animals included in each group:

a-Control group; in which animals received distilled water for 7 days before being subjected to pyloric ligation.

b-Indomethacin group (INDO); in which animals received distilled water for 7 days and then gastric ulceration was induced by a single intraperitoneal injection of indomethacin (Nile Co, Egypt) dissolved in sterile water in a dose 40mg/kg[20].

c-Atorvastatin(Ator)+INDO group; in which rats received 10 mg/kg of atorvastatin orally for 7 succesive days and there was another final dose one hour before indomethacin administeration[21].

3- Pyloric ligation:

In order to collect gastric juice, pyloric ligation was, carried out in each animal 2 hours after indomethacin administration[22]. Under light ether anesthesia, a mid-line abdominal incision was performed; the pyloric portion of the stomach was gently mobilized and carefully ligated with a silk ligature around the pyloric sphincter taking care not to interfere with gastric blood supply. The abdominal incision was sutured and the animals were allowed to recover from anesthesia.

4. Assessment of gastric mucosal lesions:

Three hours after pyloric ligation, administration rats were killed with ether overdose. Their stomachs were removed, opened along the greater curvature and the gastric content of each stomach was collected. The stomachs were washed with ice cold saline and scored for gross mucosal lesion. Gastric mucosal lesions were examined using a magnifier and were expressed in the form of ulcer index (U.I.) and the severity factor, were determined according to the method of **Peskar et al. 2002**[23]. The severity factor was measured by the gross lesion size. It was considered 0 if no lesions, 1 when lesion size is smaller than 1 mm, 2 when lesion size is 2-4 mm or 3 if the lesion exceeds 4 mm. The lesion score for each rat was calculated as the number of lesions multiplied by their respective severity factor .The mean lesion score for each group is the U.I of the group.

5-Collection and analysis of gastric juice:

The collected gastric juice after opening the stomach was centrifuged at 3000 rpm for 30 minutes to remove any solid debris and the volume of the supernatant was measured. Then, the supernatant was used for the analysis of pH, free and total acid-outputs, pepsin and mucin concentrations.

5.a. Determination of Acidity of the Gastric Juice:

The free acidity was determined by titration of a given volume of the gastric juice against 0.1N sodium hydroxide up to 5.5 as guided by a pH meter. The total acidity which is composed of both mineral and organic combined acids in the gastric juice

was determined by completing the titration in the above procedure for determining free acidity to pH 7 as guided by the pH meter[24].

5.b.The Colorimetric Assay for Mucins and Glycoproteins in the Gastric Juice:

It is a sensitive and specific method for saccharides, which are linked via N-acetylgalactosamine through O-glycosidic linkage to serine/threonine in mucins, the method is not affected by the carbohydrates present in other types of glycoproteins[25].

5. c.Determination of the Proteolytic Activity:

This was determined by a modified spectrophotometric method[26]. The pepsin activity is the major factor involved in the proteolytic activity of gastric secretion. This activity can be determined in terms of the amount of proteases produced after incubation of the substrate for ½ hour with standard pepsin of the juice.

6. Biochemical analysis of gastric mucosa:

6.a.Determination of Lipid Peroxides:

The total amount of lipid peroxides in the gastric mucosa, was assayed using kits for the colorimetric determination of thiobarbituric acid (Biodiagnostic, Egypt) method. This measures the malondialdehyde equivalent substances, which are the breakdown products of lipid peroxides[27].

6.b.Determination of Nitric Oxide in the Gastric Mucosa:

Gastric mucosal nitric oxide was determined using kits for the colorimetric determination of total nitrite (Biodiagnostic, Egypt). That assay determines the total nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction[28].

6.c.Determination of prostaglandin E_2 level in gastric mucosa:

Gastric mucosal prostaglandin E_2 (PGE_2) was determined by enzyme-linked immunosorbent assay (ELISA) using PGE_2 assay kit (R&D Systems,USA). The assay is based on the competitive binding technique in which PGE_2 present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled PGE_2 for sites on a mouse monoclonal antibody[29].

Statistical Analysis:

Results were expressed as mean ± S.E.M. The mean was that of 8 observations. Data sets were examined by one-or two-way analysis of variance, and individual group means were then compared with students unpaired t-test. The statistical significance between the two means was considered significant at p value ≤ 0.05 using the student´s "t" test.

RESULTS

1. Effect of indomethacin on the development of gastric lesions and its alterations by pretreatment with atorvastatin:

Indomethacin induced marked ulcerative lesions achieving an ulcer index of 32.25±1.13. Pretreatment with atorvastatin significantly attenuated the development of gastric lesions induced by indomethacin and decreased the ulcer index to 20.25±1.54. (Figure 1).

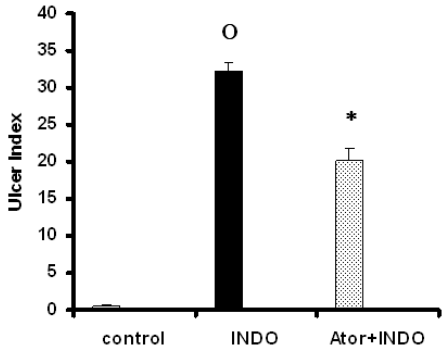

Figure (1): Effect of indomethacin on gastric lesions development and its alterations by pretreatment with atorvastatin.
O Significantly different from control at $p < 0.05$.
* Significantly different from indomethacin at $p < 0.05$. Values represent the mean ± S.E.M. of 8 observations.

2. Effect of indomethacin on gastric mucosal lipid peroxides and its alteration by pretreatment with atorvastatin:

Indomethacin significantly elevated the gastric mucosal malondialdehyde (MDA) concentrations compared to the control group (34±0.65 versus
12.4±0.86 nmol/g wet tissue in the control group). Pretreatment with atorvastatin significantly lowered the gastric MDA concentration in Ator+INDO groups (20.5±0.62 nmol/g/ wet/tissue). Figure (2).

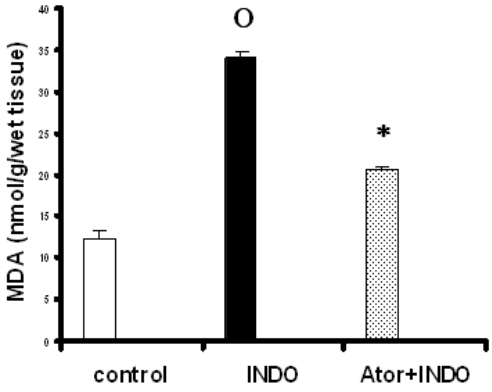

Figure (2): Effect of indomethacin on gastric mucosal lipid peroxides and its alterations by pretreatment with atorvastatin.
OSignificantly different from control at $p < 0.05$.
*Significantly different from indomethacin at $p < 0.05$.
Values represent the mean ± S.E.M. of 8 observations.

3. Effect of indomethacin on gastric mucosal nitrite concentration and its alteration by pretreatment with atorvastatin:

Figure (3) shows that and indomethacin significantly lowered the gastric mucosal nitrite concentrations compared to the control group (7.4±0.4 versus 27±0.54 nmol/g wet tissue in the control group). Atorvastatin significantly increased the gastric mucosal nitrite concentration in Ator+INDO (19.6±0.8 nmol/g wet tissue).

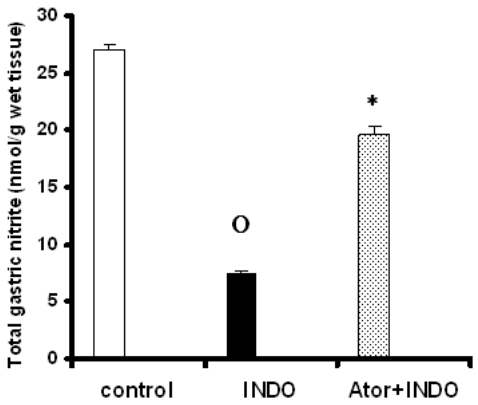

Figure (3): Effect of indomethacin on gastric mucosal nitrite concentration and its alterations by pretreatment with atorvastatin.
OSignificantly different from control at $p < 0.05$.
*Significantly different from indomethacin at $p < 0.05$.
Values represent the mean ± S.E.M. of 8 observations.

4. Effect of indomethacin on gastric mucosal PGE_2 concentration and its alteration by pretreatment with atorvastatin:

Indomethacin significantly lowered the gastric mucosal PGE_2 concentrations compared to the control group (16.3±0.57 versus 35±0.7 pg/mg wet tissue in the control group). Administration of atorvastatin significantly increased the gastric mucosal PGE_2 concentration in Ator+INDO group (28±0.73 pg/mg wet tissue) (Figure 4).

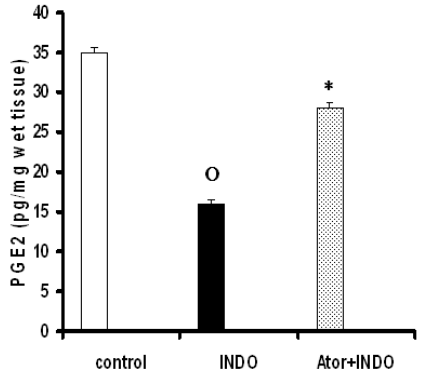

Figure (4): Effect of indomethacin on gastric mucosal PGE2 concentration and its alterations pretreatment with atorvastatin.
OSignificantly different from control at $p < 0.05$. *Significantly different from indomethacin at $p < 0.05$. Values represent the mean ±S.E.M. of 8 observations.

5-Effect of indomethacin on the volume and total acid output of gastric secretion and its alteration by pretreatment with atorvastatin:
In control rats, volume of gastric secretion was 2.25±0.16 ml/3hrs. There was no significant change in the volume of gastric secretion in Ator+INDO (0.83±0.05 ml/3hrs) as compared to 1.06±0.1 ml/3hrs in INDO group (figure 5). A significant decrease in total acid output was observed in Ator+INDO (93±1.4 mEq/3hrs) as compared to INDO (139±1.8 mEq/3hrs) figure (6).

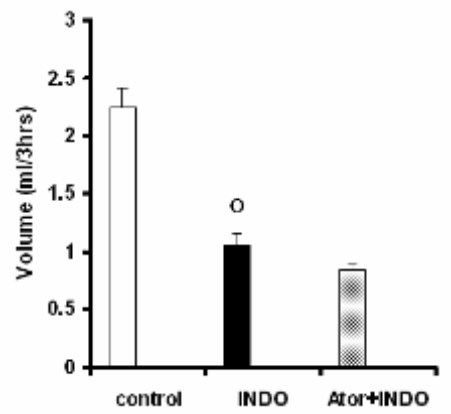

Figure (5): Effect of indomethacin on volume of gastric secretion and its alterations by pretreatment with atorvastatin.
O Significantly different from control at $p < 0.05$.
* Significantly different from indomethacin at $p < 0.05$.
Values represent the mean ± S.E.M. of 8 observations.

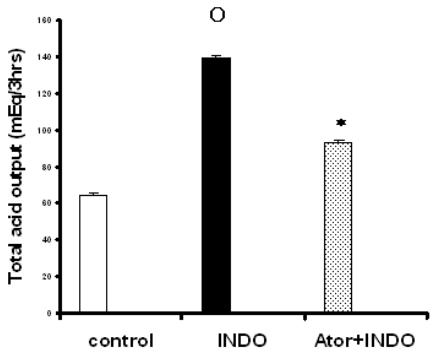

Figure (6): Effect of indomethacin on total acid output and its alterations by pretreatment with atorvastatin.
O Significantly different from control at $p < 0.05$.
* Significantly different from indomethacin at $p < 0.05$. Values represent the mean ± S.E.M. of 8 observations.

6. Effect of indomethacin on gastric juice proteolytic activity and its alteration by pretreatment with atorvastatin:
Indomethacin produced a significant increase in the pepsin level compared to the control rats 6.9±0.42 mg/ml for the control rats versus 13.6±0.5 mg/ml INDO. Administration of atorvastatin did not produce any significant change in the gastric juice pepsin activity when compared to indomethacin group (12.5±0.49 mg/ml). Figure (7)

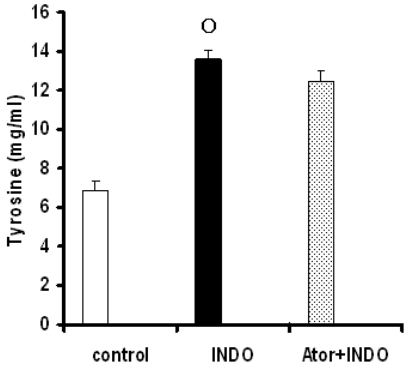

Figure (7): Effect of indomethacin on gastric juice proteolytic activity and its alterations by pretreatment with atorvastatin.
O Significantly different from control at $p < 0.05$.
* Significantly different from indomethacin at $p < 0.05$. Values represent the mean ± S.E.M. of 8 observations.

7. Effect of indomethacin on gastric juice mucin and its alterations by pretreatment with atorvastatin:

Figure (8): shows that indomethacin significantly lowered gastric juice mucin concentration from 13.6±0.4 mg/ml in the control group to 7±0.19 mg/ml. Administration of atorvastatin significantly elevated mucin concentration in the gastric juice in Ator+INDO group (10.48±0.22 mg/ml).

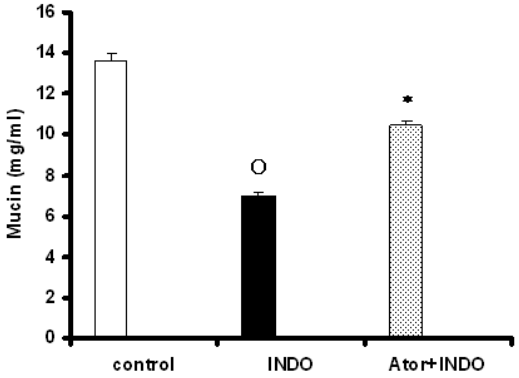

Figure (8): Effect of indomethacin on gastric juice mucin and its alterations by pretreatment with atorvastatin. ^{O} Significantly different from control at $p < 0.05$.
* Significantly different from indomethacin at $p < 0.05$.
Values represent the mean ± S.E.M. of 8 observations.

DISCUSSION

Since many years, NSAIDs are widely prescribed and used for their anti-inflammatory, antipyretic, and analgesic effects. The major limitation of their clinical application are their serious side effects such as induction of acute hemorrhagic erosions, potentiation of gastric ulcerogenic response to various stimuli and impairment of healing of pre-existing ulcer[13]. NSAIDs indirectly inhibit cyclooxygenase activity, thereby, suppressing the formation of prostaglandins. It has been shown that prostaglandins influence virtually every component of the mucosal defense: Stimulating mucus and bicarbonate secretion, maintenance of mucosal blood flow, enhancing the resistance of epithelial cells to injury induced by cytotoxins and inhibiting leukocyte recruitment[12].

One of these NSAIDs, is indomethacin, it was suggested to break the mucosal barrier, leading to back diffusion of hydrogen ions and extensive mucosal damage. Indomethacin was, also, reported to up-regulate the synthesis of pro-inflammatory molecules like TNF-α, contributing to mucosal injury[30]. It, also, decreases gastric mucosal blood flow, epithelial cell turnover, bicarbonate and mucous secretion[31].

The present finding revealed that indomethacin administration induced a significant and severe mucosal ulceration, associated with significant lipid peroxidation. This effect on mucosal oxidative stress in accordance with the report of **Valecheva-Kuzmanova et al.**[32]. Also, indomethacin administration induced significant increase in gastric acidity and significant decrease in gastric juice mucin and gastric mucosal NO and PGE_2 concentrations. The gastrotoxicity of the indomethacin in animals could be attributed to their ability to induce the reactive oxygen metabolites which may inturn promote lipid peroxidation. Ulceration due to NSAIDs is believed to occur because of non-selective inhibition of cyclooxygenases that hampers the release of mucus due to reduction in prostaglandin synthesis[33].

Drugs with the ability to reduce acid secretion[34] and/or improve microcirculation[35] have been shown to attenuate gastric lesions. Statins represent a well-established class of drugs that effectively lower serum cholesterol levels and widely prescribed for treatment of hypercholesterolemia. Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, reducing the availability of L-mevalonate and cholesterol biosynthesis **(36)**. Recent studies showed that atorvastatin (a commonly prescribed statin) stimulates NO release and has the potency to enhance NO/O^-_2 concentration ratio in the endothelial cells[37]. Also **Riad et al.**[38] found that low-dose treatment with atorvastatin (this means a dose too low to influence the lipid profile) leads to anti-oxidant and anti-inflammatory effects in diabetic rats.

Lipid peroxidation is one of several factors that act to produce observable gastric lesions. It initiates a series of complementary events disturbing the balance between the aggressive and defensive factors leading ultimately to gastric ulceration[39]**.** Pretreatment with atorvastatin in case of indomethacin induced ulcers showed a significant reduction in mucosal lipid peroxides. This suggested that free radical scavenging property of atorvastatin may be part of its mechanism against indomethacin-induced gastric ulcers, especially as free radical production has been proposed by more than one author to be a key detrimental factor in indomethacin-induced ulcers[12].

NO is a putative signaling molecule recognized for its ability to enhance gastric mucus/alkaline secretion, inhibiting gastric acid secretion and prevent neutrophil activation and adherence to vascular endothelium thus affording gastro protection. Earlier studies revealed that endogenous NO released from vascular endothelium, sensory nerves or gastric epithelium cooperates with endogenous prostaglandins in the maintenance of gastric mucosa integrity and microcirculation[5]. A previous report has shown that NO generated from transdermal nitroglycerin protected against indomethacin-induced gastric ulceration through maintenance of mucosal blood flow and reduction of leukocyte endothelial cell rolling and adherence[40]. In the present study,

indomethacin significantly reduced gastric mucosal NO level compared to control group. These findings are in accordance with that of **Trip and Tepperman** who reported a decrease in NO biosynthesis, as a result of decreased NOS activity that was associated with an increase in the extent of the damage[41]**.** Treatment with atorvastatin significantly increased mucosal NO level when compared to indomethacin-treated rats. The present findings is in agreement with earlier reports showing that statins can efficiently increase NO level, scavenge superoxide radicals and inhibit NAD (P)H oxidase-dependent superoxide generation[42]**.**

The role of PGE_2 in mediating the gastroprotective effect of atorvastatin was investigated. The results of the present study suggest that the gastroprotective effect of atorvastatin is mediated partially by PGE_2 as a direct measurement of its mucosal level confirmed that its biosynthesis was significantly enhanced by atorvastatin.

The increase in mucosal generation of PGE_2 observed after treatment with atorvastatin is probably mediated, at least in part, by NO. Thus, the gastroprotection afforded by atorvastatin could be attributed to the interaction of NOS and COX and their products, NO and PGE_2 in gastric mucosa. A mutual interaction was shown to exist between NOS and COX enzymes and has been confirmed by many investigators[43,44]. NO was reported to increase PGE_2 biosynthesis in vivo through a cGMP-independent mechanism and it is possible to assume that NO might regulate the release and/or the biosynthesis of PGE_2 in the stomach after damage[45].

Gastric mucus plays a critical role in the production of gastric barriers[46]. In the present study, the gastric juice mucin concentration was reduced in indomethacin-treated rats. Pretreatment with atorvastatin significantly increased gastric juice mucin concentration compared to non-treated indomethacin group. It has been shown that NO stimulates gastric mucus synthesis and secretion[47] and impairment of gastric mucus secretion occurs through a decrease in gastric mucosal NOS activity in indomethacin-treated rats[48]. Additionally, earlier studies showed that prostaglandins exert a gastroprotective action against gastric mucosal lesions through maintenance of gastric mucus synthesis and secretion[49]. Therefore, it could be stated that the increase in gastric juice mucin concentration afforded by atorvastatin depends on the increase of NO and PGE_2 levels, a finding which is supported by the data of the present study.

The increase in gastric acidity is considered an important contributing factor in the pathogenesis of gastric ulcers. In the present study gastric acidity was significantly increased in indomethacin-treated rats. Pretreatment with atorvastatin significantly reduced the acidity of gastric secretion. NO and prostaglandins play an important role in regulating acid secretion and maintaining the integrity of gastric mucosa against hyperacidity or exposure to ulcerogens **(50).** Therefore, reduction of gastric acidity

after treatment with atorvastatin could be attributed to enhancement formation of NO and PGE_2

In conclusion, the findings of the present study clearly demonstrate the gastroprotective effects of atorvastatin in experimental ulcers. Increased NO, PGE_2, mucin levels and decreased gastric acidity and lipid peroxides might explain these effects

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دراسة تقييمية للاليات المحتملة بالنسبة الاستاتينات فى حماية المعدة فى حالات قرحة المعدة الناتجة عن إعطاء الإندوميثاسين فى الجرذان

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ينتمى عقار الاتورفاستاتين الى مجموعة الاستاتينات التى تلعب دورا هاما فى تنظيم مستوى الدهون فى الجسم. ولكن ربما يكون لهذه العقارات تاثيرات مضادة للاكسدة ومضادة للالتهاب، ولهذا تم تجربتها فى علاج امراض أخرى.

وفى هذا البحث تم دراسة تأثير عقار الأتورفاستاتين على كمية العصارة المعدية وحدوث قرحة المعدة فى ذكور الجرذان البالغة. وقد وجد أن هناك زيادة فى نسبة تكوين جذور الأكسجين الحر النشط وحجم الحموضة الكلية فى حالة قرحة المعدة وأنهما من أسباب زيادة معدل حدوث هذه القرح. وقد تم تقسيم الجرذان إلى ثلاثة مجموعات، كل مجموعة تتكون من ثما نية جرذان١-مجموعة ضابطة:حيث أعطت الجرذان ماء مقطر لمدة سبعة أيام وذلك قبل ربط الفتحة البوابية للمعدة 2-مجموعة تم إعطائها ماء مقطر لمدة سبعة أيام ثم تم حقنها بجرعة واحدة من الاندوميثاسين ٣٠ ملليجرام م /كجم عن طريق الحقن فى الغشاء البريتونى للبطن ثم ربط الفتحة البوابية للمعدة بعد ساعتين من إعطاء الإندوميثاسين٣- مجموعة تم إعطائها عقار الأتورفاستاتين سبعة ايام بجرعة ١٠ ملليجرام /كجم عن طريق الفم، ثم إحداث قرحة المعدة فيها عن طريق إعطائها إندوميثاسين ٣٠ ملليجرام /كجم عن طريق الحقن فى الغشاء البريتونى للبطن ثم ربط الفتحة البوابية للمعدة بعد ساعتين من إعطاء الإندوميثاسين. وقد تم التضحية بالحيوانات بعد٣ ساعات من ربط الفتحة البوابية.

وقد تم قياس حجم عصارة المعدة وحجم الحموضة الكلية، الميوسين، والببسين فى العصارة المعدية، وكذلك قياس ثنائى الدهيد المالون وأكسيد النيتريك والبروستاجلاندين ه ٢ الغشاء المخاطى المبطن للمعدة.

وقد أدى عقار الأتورفاستاتين إلى إنخفاض ذى دلالة إحصائية فى حجم الحموضة الكلية وقرح وإصابات الغشاء المخاطى. وكذلك أدى إعطاء عقار الأتورفاستاتين إلى زيادة ذات دلالة إحصائية فى إفراز الميوسين والبروستاجلاندين ه ٢ وأكسيد النيتريك .ونقص ذو دلالة إحصائية فى إفراز ثنائى الدهيد المالون.

وتشير كل هذه النتائج إلى التأثير الوقائى لعقار الأتورفاستاتين فى حالات قرحة المعدة وذلك عن طريق زيادة تكوين اكسيد النيتريك والبروستاجلاندين ى٢ و قد أدت هذه الزيادة الى تأثير مضاد للأكسدة ونقص فى حجم الحموضة وزيادة فى إفراز الميوسين.