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Original research

Low dose aspirin prevents duodenoesophageal reflux induced mucosal changes in wistar rat esophagus by MAP kinase mediated pathways

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ABSTRACT

Background: Investigations of molecular mechanisms behind the progression of neoplastic changes in the esophagus have uncovered the role of the COX & 5-Lox pathways. Human squamous esophageal mucosa produces relatively large amounts of eicosanoids in the presence of inflammation. Laboratory and epidemiological data suggest that aspirin and non-steroidal anti-inflammatory drugs may be chemo preventive through their inhibitory effect on COX25, 10. Cell culture studies have shown that the members of the mitogen activated protein (MAP) kinase family plays an important role in the development of bile acid-induced carcinogenesis. Differences in MAPK pathways activated by bile exposure were also noted in esophageal squamous cell lines and biopsies from patients with GERD. The protective role of aspirin and its molecular mechanism is not well understood.
Aims:

1. The effect of duodenal reflux on esophageal mucosa.
2. The role of aspirin in preventing duodenal reflux induced esophageal mucosa changes.
3. If it is proven to be preventive, the mechanism of its action. A duodenal reflux rat animal model was used by an end- to-side esophago duodenostomy.

Methods: Total of 56 rats was included. 3 were "Naive control" animals which did not undergo the surgical procedure. The remaining animals were divided into two groups: Surgery alone (experimental) and Surgery + aspirin [therapy group], esophagoduodenostomy. At 40 weeks, the rats were euthanized and appropriate esophageal samples were analysed for histopathology and p38 & ERK MAP kinases, VEGF, protease activity and caspase 3 activities.
Results: The presence of gross mucosal nodularity was seen in 21 and 10 rats of the experimental and therapy group respectively ($p = 0.03$; Table 1). Reflux-associated changes such as basal cell hyperplasia were more common in the experimental group, however this association did not reach statistical significance ($p = 0.15$; Table 1). Epithelial hyperplasia was seen more in the experimental group, which was prevented by aspirin [$p < 0.01$]. Papillomatosis, as shown in Fig. 4 was more common in the experimental group ($p = 0.02$). Activation of p38 & ERK MAP kinases was prevented in aspirin group ($p < 0.05$, CI −1.796−−0.014). Examination of protease activity by zymographic analysis of the esophageal samples revealed a number of gelatinolytic bands in 50% rats of the experimental group, not observed in the therapy group. No significant changes were seen in Caspase 3 [Normal areas −99.74 & nodular areas − 100.34 percent of controls] or VEGF [mean 0.64, sd ± 0.76 Vs 0.69 ± 0.96] activity.
Conclusions: Our data demonstrated that low dose aspirin reduced the incidence of duodenoesophageal reflux induced histological changes in the esophagus by preventing activation of proliferative & anti-apoptotic MAP kinases such as p38 & ER as well as protease activity. Though Barretts' changes and

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adenocarcinoma have not developed, it could explain the role of duodenoesophageal reflux in the development of different histological but potential premalignant lesions and molecular level changes which are prevented by low dose aspirin.

1. Introduction

Esophageal squamous cell carcinoma is one of the most frequent malignancies worldwide.[1] It progresses through stages such as dysplasia, carcinoma in situ and then it becomes invasive carcinoma. In the development of disease, the common link between the dietary and environmental factors is a chronic inflammatory state which is associated with increased turnover of cells.[2] A significantly higher incidence of squamous cell carcinoma & preneoplastic disease of the larynx & pharynx was seen in patients undergoing Billroth II and total gastrectomy than Billroth I and Roux-en-Y resection[3]; the prevalence of histological esophageal papilloma or squamous cancer has shown to be increased in rats with combination of pancreatic and bile reflux.[4] A number of substances in duodenal refluxate such as lysolecithin, trypsin and secondary bile acids are possible co-carcinogens.[5]

Investigations of molecular mechanisms behind the progression of neoplastic changes in the esophagus have uncovered the role of the COX & 5-Lox pathways. Human squamous esophageal mucosa produces relatively large amounts of eicosanoids in the presence of inflammation.[6,7] Treatment with Omeprazole for GERD reduced the production of PGE_2 and LTB_4.[8,9] Laboratory and epidemiological data suggest that aspirin and non-steroidal anti-inflammatory drugs may be chemo preventive through their inhibitory effect on COX2.[5,10] Pooled results from human studies also support a protective association between aspirin and NSAID and esophageal cancer (of both histological types), and therefore provide evidence for a dose effect.[11]

Cell culture studies have shown that the members of the mitogen activated protein (MAP) kinase family plays an important role in the development of bile acid-induced carcinogenesis. Differences in MAPK pathways activated by bile exposure were also noted in esophageal squamous cell lines and biopsies from patients with GERD.[12] Considerable evidence exists to indicate that matrix metalloproteinases (MMPs) have an important role in tumor invasion and tumor spread.[13–16] Over expression of both MMP-2 and MMP-9 have been shown to be associated with esophageal squamous cell carcinoma.[17] VEGF has been associated with aggressive characteristics of esophageal squamous cell carcinoma, therefore possibly correlating with tumor stage, positive lymph nodes and patient outcome.[37]

2. Aim

1. The effect of duodenal reflux on esophageal mucosa
2. The role of aspirin in preventing duodenal reflux induced esophageal mucosa changes.
3. Mechanism by which aspirin exerts its preventive action. Duodenal reflux animal model was created by an end- to-side esophago duodenostomy.[18]

We hypothesized that the protective effect of aspirin on the metaplasia–dysplasia–carcinoma sequence in the esophagus could be mediated through modulation of the MAP kinase pathway.

3. Materials and methods

Animals and surgical procedure: The rats used for experiments were inbred wistar group of both sexes. They were housed in clean cages before and after the planned experiment. A total of 56 animals were included in the study. Of these, 3 were "Naive control" animals which did not undergo the surgical procedure. The remaining animals were divided into two groups: Surgery alone (experimental) and Surgery + aspirin (therapy group). The rats assigned for surgery were fasted overnight and surgeries were done under sterile techniques using intraperitoneal thiopentone sodium for anesthesia (75 mg/kg).

3.1. Procedure

A midline upper abdominal incision was made; the gastro esophageal junction was identified and suture ligated flush with the stomach. The distal esophagus was then transected proximal to the ligature. An enterotomy was made 3 cm distal to the pylorus on the anti-mesenteric border. The distal esophagus was then anastomosed to the duodenal enterotomy with accurate mucosa to mucosa opposition. The esophago-duodenal anastomosis was completed with six to eight interrupted full-thickness stitches of 7-0 polypropylene (Fig. 1).

At the end of the operation, 1 ml of 0.9% sodium chloride was left in the peritoneal cavity. Water was permitted after the rats were recovered from anesthesia; they were allowed to take standard solid chow ad libitum on post-operative day [POD] 1. Rats were on a 12 h light–12 h dark cycle at 21.6 °C. If they had poor intake, they were force fed in the immediate post-operative period. Animals were administered aspirin in the therapy group from POD 1; the brand used was disprin, the dispersible brand of aspirin from Rickitt & Collman of [I] Ltd which has 350 mg of acetyl salicylic acid as active ingredient. It was dissolved in their drinking water at a dose of 6 mg per kg per day and given daily till the end of the study. They were euthanized between 44 and 48 weeks using toxic doses of thiopentone. Then the abdomen was opened and esophagus resected from the cervical esophagus to the area just beyond the anastomosis in the duodenum. The specimens were examined grossly for the presence of ulcers, nodularity or tumor. Representative sections were taken from the lower esophagus and the tissue was routinely processed and paraffin-embedded. Five micron sections were cut and stained with hematoxylin and eosin. The slides were examined for the presence of reactive epithelial changes, dysplasia and malignancy.

3.2. Western blot analysis

Esophageal tissues were homogenized in buffer containing 250 mM sucrose, 5 mM Hepes and 1 mM EDTA pH-7.4 with protease inhibitor cocktail consisting of 2 mM AEBSF, 1 mM EDTA, 130 μM bestatin, 14 μM E–64, 1 μM leupeptin and 0.3 μM aprotinin using a Porter –Elvejhem homogenizer. Aliquots (20 μg) of esophageal homogenates were separated on an SDS-12% polyacrylamide gel followed by transfer onto polyvinyldine difluoride (PVDF) membranes (Millipore). Membranes were then blocked with 5% BSA in phosphate buffered saline tween-20 (PBST) following which they were probed using the following antibodies: - anti- P44/42 MAPK, anti-phosphorylated P44/42 MAPK, anti- P38 MAPK, anti-phosphorylated P38 MAPK, anti-VEGF Receptor 2 or anti-phospho-VEGF Receptor 2 (Cell Signaling Technology). Following incubation with horse radish peroxidase labeled secondary

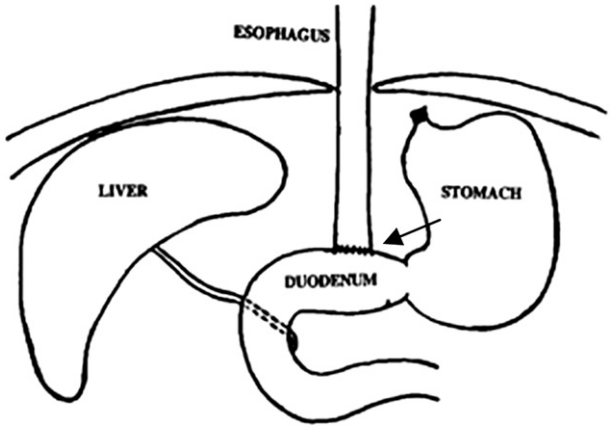

Fig. 1. Esophago duodenal anastomosis. An enterotomy was made 3 cm distal to the pylorus on the anti-mesenteric border. The distal esophagus was then anastomosed to the duodenal enterotomy with accurate mucosal to mucosal opposition.

antibody, the blots were visualized by chemiluminescence detection (Supersignal West Dura substrate Pierce). Bands were then quantitated by Scion Image (Scion Corp).

3.3. Caspase assay

Caspase 3 activity was detected in esophageal homogenates by measuring the proteolytic cleavage of the colorimetric substrate acetyl-Asp-Glu-Val-Asp (DEVD)-pNA in assay buffer [100 Mm Hepes, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.5], 1 mM phenylmethylsulfonyl fluoride, 10 mM DTT by using the absorbance of released paranitroanilide at 405 nm.

3.4. Gelatin zymography

Zymogram of protease activity was performed as described,[19] with modification. Polyacramide gels (12%) were cast containing 0.2% gelatin. Esophageal homogenate (10 μg protein) were applied on the gel in standard SDS loading buffer containing 0.1% SDS but lacking 2-mercaptoethanol. Protein denaturation was not performed by boiling the sample. The gels were run at 100 V for 2 h at 4 °C and then soaked in 2% Triton X-100 on a shaker for 1 1/2 h with three changes at 20 °C. Following this, the gels were soaked in reaction buffer (0.1 M Tris–HCl pH 7.5) overnight at 37 °C and then stained with coomassie brilliant blue.

3.5. Protein estimation

Protein was estimated by the method of Lowry[20] using bovine serum albumin as standard.

3.6. Statistical analysis

Frequencies and percentages were obtained to summarize the study variables. All categorical variables between the two study groups were compared using Fisher exact test. A *P* value of less than 0.05 was considered statistically significant.

4. Results

Peri-operative mortality was 6% and they were due to anesthetic and other surgery related complications. 25 rats in experimental and 24 rats in therapy group survived through the study period. At laparotomy, there were dense adhesions between the liver and the anastomotic site, which was seen mostly at the left sub-hepatic region. 60% of animals in the experimental group had dense adhesions and sub serosal nodules (Fig. 2). Intra operatively, rats in both groups had shown peri esophageal adhesions with the liver and diaphragm.

Esophagus was opened longitudinally along the esophago-duodenal anastomotic site and there were mucosal nodules of size from <1 mm to 0.8 mm present on the esophageal mucosal and serosal side (Fig. 3). The presence of gross mucosal nodularity was seen in 21 and 10 rats of the experimental and therapy group respectively ($p = 0.03$; Table 1). 11 rats in experimental group and 2 in therapy group had epithelial hyperplasia which was statistically significant [$p < 0.01$]. Papillomatosis, as shown in Fig. 4 was more common in the experimental group ($p = 0.02$). One rat in the experimental group showed intestinal metaplasia; however, adenocarcinoma was not seen. In both groups, there was a single case of squamous cell carcinoma each (Fig. 5).

The grossly diseased esophagus was used to study the MAPK activation in both the groups. Immuno-blots followed by densitometry analysis did not show changes in protein levels of p38 [mean−0.47, sd ± 0.64 Vs 1.07, sd ± 1.17, experimental and therapy group], though levels of ERK were significantly elevated in experimental group (Fig. 6). However, treatment with aspirin had no effect on the increased ERK protein levels (Fig. 6). Increase in protein level *per se* does not indicate activation of p38 & ERK MAP kinases, since phosphorylation of the protein is required for activation. The next series of experiments investigated the phosphorylation (Fig. 7); the phosphorylated form of p38 & ERK increased significantly in the experimental group [mean 5.9 sd ± 4.3], an effect not seen in the therapy group [*P* 0.01; mean, 2.73 sd ± 1.73].

Examination of protease activity by zymographic analysis of the esophageal samples revealed a number of gelatinolytic bands in 50% rats of the experimental group, not observed in the therapy group. The Caspase 3 activity between the groups (Normal areas − 99.74 & nodular areas − 100.34 percent of controls, $P = 0.12$) was not found to be significant. As seen in Fig. 8 and Table 1, there was no significant change in levels of VEGF protein or the phosphorylated form [mean 0.21 and 0.15, $P = 0.13$] was evident between experimental and therapy groups. The Naive control animals did not show either gross or microscopic changes.

5. Discussion

Esophageal cancer is a highly aggressive cancer with increasing incidence and mortality. Although esophageal squamous cell carcinoma and esophageal adenocarcinoma differ in their histology and epidemiologic distribution, some of their risk factors (eg. dietary deficiencies and tobacco) and underlying mechanisms of carcinogenesis are the same.[21] A number of animal models have been used to study esophageal reactive changes and the progression to carcinoma. Surgery is the most commonly used method for induction of reflux of duodenal contents into the esophagus and a number of techniques have been used.[5] Our study was carried out on rats and a duodenal reflux model was created with esophago duodenostomy, which had both pancreatic and bile juices; this model was also used by many other investigators.[18] The mortality rate in our study was similar to other studies but some studies demonstrated only 25% survival for rats through the study period.[22]

Since the esophagus is used for temporary food storage, it has been shown that animals usually had very severe inflammation across the whole esophagus, manifested by esophageal shortening, enlargement of the esophageal cavity (especially the lower and middle parts), hyperkeratinization,[23,24] not commonly seen in human patients. This was relevant in our study, as there were varying degrees of esophagitis, adhesions with the peri-esophageal tissues and adjacent organs like diaphragm and liver.

The mutagenic effects of duodenal reflux in animal models are well known to be associated with esophagitis.[25,26] In the current study, duodenal reflux associated mucosal changes were seen in both groups, however changes such as gross mucosal nodularity were significantly less in the therapy group [*P* value: 0.03].

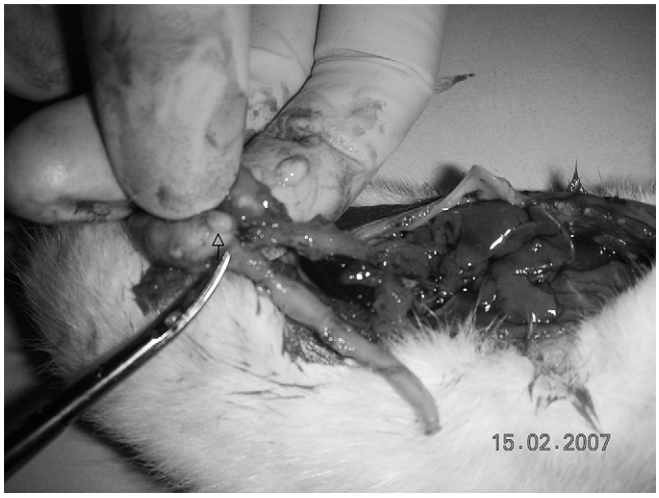

Fig. 2. Intra operative picture showing the type A esophago duodenal anastomosis. It also demonstrates peri esophageal desmoplastic reaction along the duodenum and on to the liver. The serosal nodules also can be visible at the anastomosis.

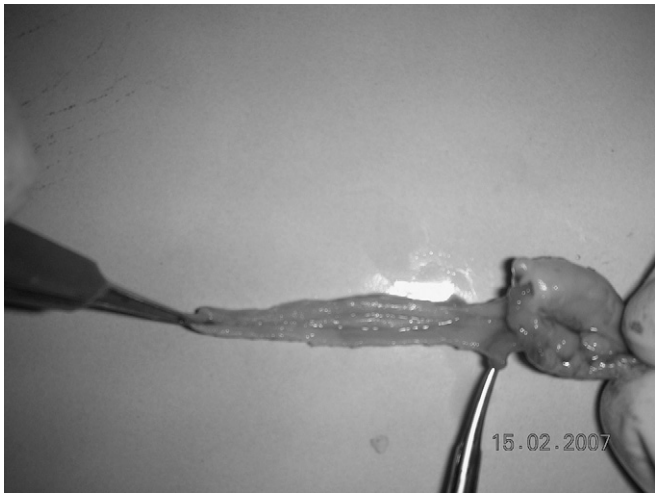

Fig. 3. The specimen of the esophagus which was cut opened longitudinally along with the duodenal anastomotic site, showing the mucosal nodules.

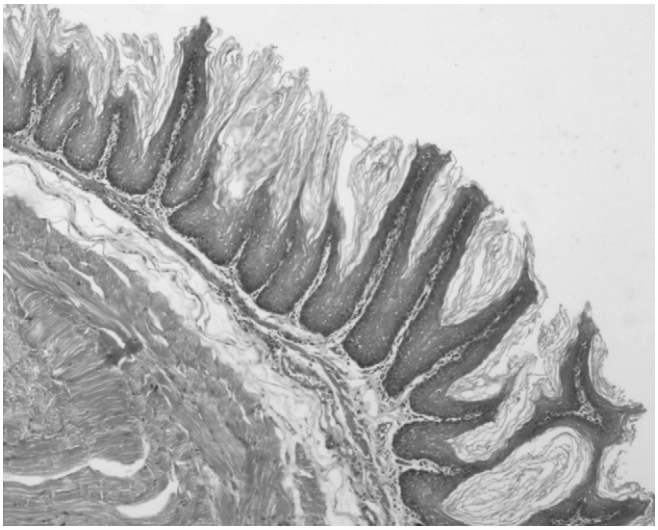

Fig. 4. Picture showing papillomatosis of the esophageal mucosa.

The striking finding in this study was that the presence of severe changes in the squamous epithelium such as basal cell hyperplasia, squamous and glandular metaplasia. Basal cell hyperplasia was more common in the experimental group, but this difference did not reach statistical significance. Epithelial hyperplasia and cysts were also significantly reduced in therapy group [*P* 0.01]. Papillomatosis was seen in 15 vs 7 rats in experimental and therapy group. Papillomatosis was characterized by basal cell hyperplasia, papillary elongation, and hyperkeratosis which were also observed by other investigators.[21] In our study, papillomatosis and keratosis were prevented significantly by aspirin [*P* 0.02].

The duodenal refluxants are typically known to produce adenocarcinomas at the anastomosis. In our experiment, there was only one case in each group with squamous cell carcinoma [SCC]. In a study, the admixture of gastric juice with duodenal juice modulated the tumorigenic effects. There was an increase in esophageal adenocarcinoma from 30 to 87% when the gasterctomy was also performed as part of the operation to reduce the amount gastric juice. Specifically, the absence of gastric juice resulted in a threefold increase in the prevalence of adenocarcinoma whereas the absence of gastric juice did not augment squamous cancers.[21] It seems that the gastric juice has a protective role against the development of adenocarcinoma, the common carcinoma seen in patients with Barrett esophagus. It is also evident that SCC is independent of the gastric juices' effect.

In our duodenal reflux model, the stomach was retained and the gastric juice was allowed to have its effect at the anastomotic site which might explain the lower incidence of carcinomas in our study, though the genetic nature of the rats might have also contributed; it was beyond the scope of this paper to study that aspect. As explained before, these refluxants can still be carcinogenic as the rats developed SCC in both the groups.

Mitogen activated protein (MAP) kinases are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. MAP kinases comprise a family of ubiquitous–proline directed, protein serine/threonine kinases, which participate in signal transduction pathways that control intracellular events like embryogenesis, cell differentiation, cell proliferation and cell death.[27] The three principal MAPK components are the extracellular regulated kinases 1 and 2 (ERK 1/2), the c- Jun N-terminal kinase (JNK) and p38. The main function of ERK 1/2 is to promote cell proliferation and epithelial cell differentiation while p38 represents a group of enzymes in the MAP kinase family that are activated and phosphorylated on a Thr-Gly-Tyr motif and hence involved in the signaling pathways.

The MAP kinase pathway has been implicated in the growth and survival of a broad spectrum of human tumors.[28] Increase in ERK 1/2 activity has been demonstrated in human epidermoid

Table 1
P value is significant for p38 and phosphorylated ERK were up regulated in the experiment group which was prevented significantly by aspirin in therapy group. Epithelial hyperplasis, papillomatosis and gross mucosal nodularity was significantly less in the therapy group [*P* value: 0.03].

	Experiment group [$n = 25$]	Therapy group [$n = 24$]	*P* value
Gross mucosal changes	21	10	0.03
Epithelial hyperplasia and cysts	11	2	0.01
Papillomatosis and keratosis	15	7	0.02
P38	0.0012	0.90	<0.05
Phosphorylated ERK	4.975	1.774	0.005
VEGF	0.213	0.155	P 0.13

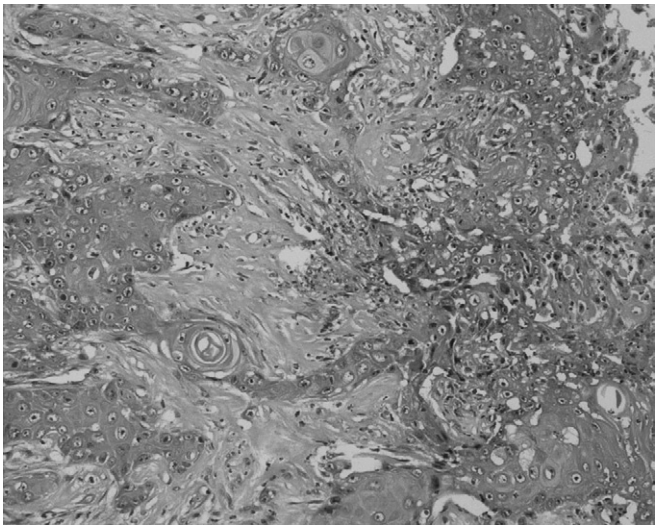

Fig. 5. Squamous cell carcinoma of the esophagus.

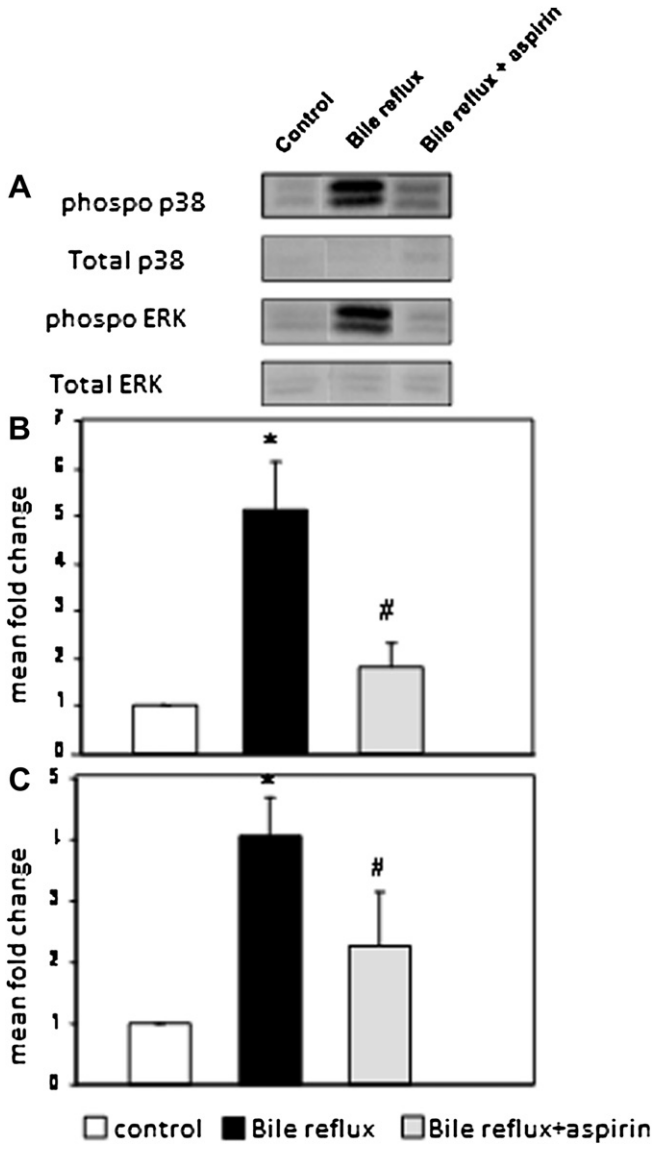

Fig. 6. 20 ug of esophageal homogenates from animals in various groups as indicated were probed for total p38 (A) and total ERK (B) protein levels. The experiments were carried out as detailed in the materials and methods section. The intensity of the bands were determined and plotted as mean fold change. *$p < 0.05$ when compared to controls.Representative blots showing levels of phosphorylated and total p38 and ERK protein in esophageal tissue from animals in various groups as indicated. (B&C) Quantification of band intensity for phosphorylated.

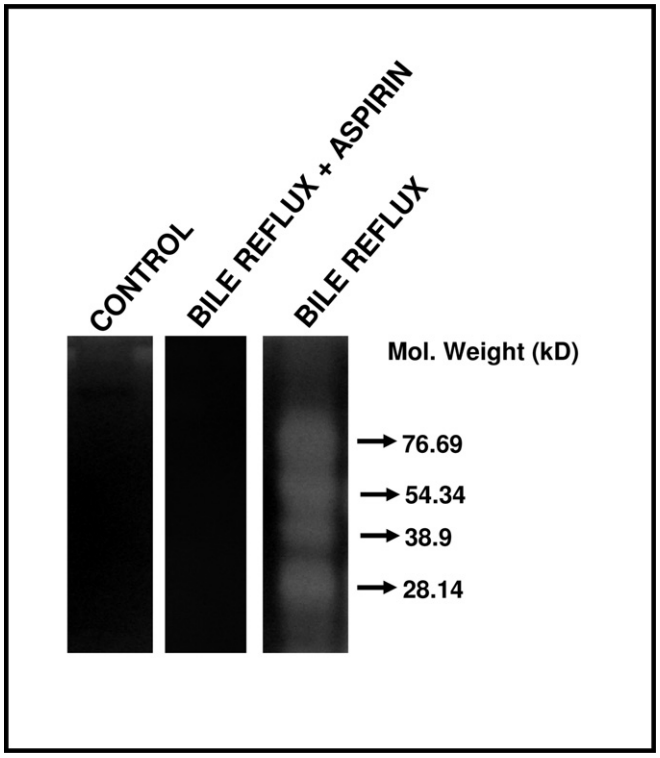

Fig. 7. Representative zymogram illustrating proteases in esophageal homogenate from animals in various groups as indicated. The experiments were carried out as detailed in the materials and methods section.

carcinoma cell lines on bile acids exposure.[29,30] It has been shown that conjugated bile salt exposure activates p38 and ERK MAP kinase pathways to produce a proliferative effect in a non-neoplastic cell line.[31] Our data supports these findings and demonstrates, for the first time to our knowledge in a surgically-induced duodenoesophageal reflux animal model, that ERK & p38 pathways are up regulated early in the process and suggest that inhibition of this pathway was probably the mechanism by which aspirin exerts its beneficial effects. However the decrease in phospho-protein levels in the aspirin treated groups may also reflect the fact that aspirin decreased inflammation and subsequent changes in phenotype. The MAP kinase findings may just be associated with the anti-inflammatory effect of aspirin and may or may not reflect a direct cause and effect relationship. But a study by Zhang et al also demonstrated differences in MAPK pathways activated by bile acid exposure in esophageal squamous cell lines and biopsies from patients with GERD[12] as well. P44 was not up regulated in our study between the groups as the mean difference was 0.93 [t test- 0.91[95% CI −1.15–2.42].

Considerable evidence exists to indicate that matrix metalloproteinases (MMPs) have an important role in tumor invasion and tumor spread.[13–16] Expression of MMPs were correlated to the progression of human esophageal squamous cell carcinoma[32,33] and increased levels of MMP-2 and MMP-9 proteins in squamous cell carcinoma as compared to normal esophageal tissues suggest their association with esophageal tumor genesis and indicated that these alterations may be early events in esophageal tumor genesis.[34] Our data in the animal model supports this as the increased protease activity was visible on zymography in the experimental ($p < 0.05$) and this is well demonstrated in Fig. 7. MMPs have been shown to be induced by MAP kinases such as ERK and p38 in fibroblasts.[35] Protection by aspirin against MMP induction in our study suggests that ERK and p38 probably act upstream of MMP induction in squamous cell carcinoma as well. In Table 2, some specimens did not show expression of either p38 or p44, which might reflect the resistant nature of the esophageal endothelium to the refluxants (Table 2). Mucosal resistance to reflux in esophagus is a well-recognized phenomenon.[41]

Caspase 3 activity was insignificant caspase 3 between the groups (Normal areas − 99.74 & nodular areas − 100.34 percent of controls) suggesting that induction of apoptosis had not occurred.

Vascular endothelial growth factor [VEGF] has been suggested to contribute to the aggressive characteristics of esophageal squamous cell carcinoma and possibly correlate with tumor stage, positive lymph nodes, and patient outcome.[36,37] However, VEGF expression can be influenced by a number of factors including type of bile acid[38] and VEGF expression in esophageal carcinomas which varies from 30 to 40%.[39,40] In our study, no change in VEGF protein level of activation was evident in the experimental group when

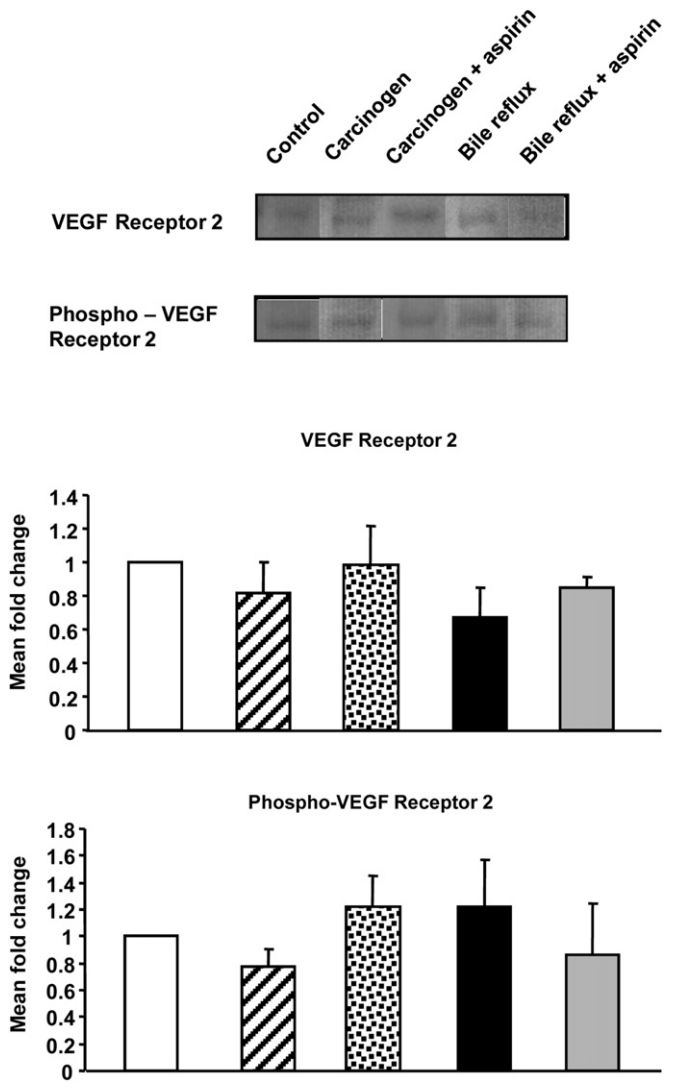

Fig. 8. (A) Representative blots showing levels of phosphorylated and total VEGF protein in esophageal tissue from animals in various groups as indicated. (B&C) Quantitation of band intensity for protein level and phosphorylated forms of VEGF plotted as mean fold change. The experiments were carried out as detailed in the materials and methods section.

compared to controls; the mean difference was 0.133[$p = 0.78$ CI-0.33–0.62] which was not significant; this could explain the low incidence of malignancy (Fig. 8).

6. Conclusion

The present study demonstrates a semi-mechanistic approximation to show that low dose of aspirin reduces the incidence of duodenoesophageal reflux induced histological changes in the esophagus by preventing activation of proliferative & anti-apoptotic MAP kinases such as p38 & ER as well as protease activity. Though Barretts' changes and adenocarcinoma have not developed, it could explain the role of duodenoesophageal reflux in the development of different histological but potential premalignant lesions and molecular level changes which are prevented by low dose aspirin.

Ethical approval

Institutional review board and animal ethics committee.

Table 2
Values are in fold change compared to controls for MAPK and VEGF protein expression. The mean of these fold changes were significant for Phophorylated p38 up regulation which was prevented by aspirin in the therapy group.

	Phospho P38	P38	Phospho P44	P44	Phospho VEGF	VEGF
Experiment group	0.91	0.00	1.06	3.29	0.00	0.00
	0.91	0.00	1.02	1.35	0.00	0.00
	0.00	0.00	0.00	4.14	0.00	0.00
	0.00	0.00	0.00	4.96	0.00	0.00
	0.00	0.00	0.00	5.61	0.00	0.00
	0.00	0.00	0.00	5.41	0.00	0.00
	0.77	4.28	0.54	1.99	1.21	0.73
	0.74	8.05	1.08	3.84	0.49	0.94
	0.00	9.83	1.47	3.03	0.89	1.85
	0.00	9.52	1.15	4.19	0.74	1.75
	0.00	16.00	0.94	2.37	1.85	0.00
	1.08	2.01	5.35	5.67	0.15	0.56
	0.00	2.98	5.83	5.96	0.20	0.69
	0.00	3.85	6.46	7.73	0.52	2.07
	0.44	3.02	0.00	5.58	0.00	0.00
	2.39	4.81	1.15	8.50	0.35	1.76
	0.77	6.29	1.01	10.46	0.00	0.57
Therapy group	0.00	0.27	0.00	0.00	0.00	0.00
	0.00	0.29	0.00	0.00	0.00	0.00
	0.00	0.51	0.00	0.00	0.00	0.00
	0.00	3.43	0.00	6.20	0.00	0.00
	1.02	1.95	2.60	5.60	0.76	0.49
	2.69	2.60	2.47	8.46	0.95	0.60
	0.00	1.13	1.40	3.73	0.82	1.48
	3.30	3.40	0.98	1.76	0.55	0.29
	2.34	3.25	1.22	1.75	0.23	0.53
	0.00	0.00	0.00	0.53	0.00	0.00
	0.99	2.12	1.02	1.65	0.10	0.59
	1.24	3.64	1.87	2.46	0.38	0.71
	2.52	5.72	1.92	2.53	0.86	2.85
	1.15	5.81	1.08	1.91	0.45	2.86
	0.00	2.35	0.00	1.01	0.00	0.00

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Conflict of interest

None declared.

Author Contribution

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