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The Resistance of Esophageal Adenocarcinoma to Bile Salt Insult is Associated with Manganese Superoxide Dismutase Expression

Suzanne C. Schiffman, MD, Yan Li, PhD, Deyi Xiao, M.D., Xuanshe Li, MS, Harini S. Aiyer, PhD, and Robert C.G. Martin, MD, PhD

Abstract

BACKGROUND—Bile acids are implicated as etiologic agents in esophageal cancer. We sought to analyze the impact of bile acid exposure on esophageal epithelial cells, Barrett’s metaplastic cells (BE), esophageal adenocarcinoma cells (EAC) and esophageal squamous carcinoma cell (ESC). We sought to determine if cellular resistance is related to manganese superoxide dismutase expression.

METHODS—Cells were exposed to sodium choleate (CA), sodium deoxycholate (DCA), sodium glycocholate (GCA), sodium taurocholate (TCA) or a 1:1 mixture (MIX) of reagents at concentrations ranging 0.2 – 0.8 mM. Cell viability was evaluated by MTT assay. MnSOD expression was analyzed by Western Blot. Statistical analysis was performed using SPSS 17.0.

RESULTS—Bile salt exposure inhibited cell viability in esophageal squamous cells in time and growth dependent manner. There was a 50% decrease in cell viability from four to 24 hours. BE, EAC and ESC cell lines were more resistant to bile insult. In untreated cell lines, MnSOD expression was significantly decreased in EAC and ESC cell lines as compared to esophageal squamous epithelial cells and BE cells ($p=0.002$). Exposure of ESC cells to bile salt increased MnSOD expression.

DISCUSSION—The confirmation of the role of ROS and bile acids in esophageal carcinogenesis has interesting implications for chemoprevention in patients with reflux esophagitis and Barrett’s esophagus. Further studies are necessary to assess the preventative role of antioxidant supplementation

INTRODUCTION

Bile acids are implicated as etiologic agents in cancer of the gastrointestinal tract, including the esophagus, stomach, small intestine, liver, biliary tract, pancreas and colon¹. The esophagus is exposed to bile acids during episodes of duodeno-gastroesophageal reflux and repetitive reflux leads to mucosal damage. Evidence indicates that gastroesophageal reflux disease (GERD) is a chronic condition of oxidative stress and is a widely accepted process to induce carcinogenetic transformation from reflux esophagitis to Barrett’s esophagus to

§Correspondence: Robert C.G. Martin, MD, PhD, Director, Division of Surgical Oncology, and Associate Professor of Surgery, Department of Surgery, 315 E. Broadway, Louisville, KY 40202. Phone-502-629-3355, Fax-502-629-3030, robert.martin@louisville.edu.

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esophageal adenocarcinoma (EAC)^{2;3}. Barrett's esophagus (BE) is a metaplastic transformation of the distal normal squamous esophageal epithelium into specialized intestinal metaplastic mucosa with goblet cells as a result of chronic exposure to bile acids^{2;4}. The presence of Barrett's esophagus is associated with an increased risk of developing adenocarcinoma (EAC)⁵⁻⁷.

Exposure of esophageal cells to bile acids generates reactive oxygen species (ROS) and induces oxidative stress, DNA damage, mutation, and apoptosis^{1;6}. Oxidative stress generates ROS (including superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite) and induces carcinogenic transformation⁸. These findings suggest that bile acids may lead to increased frequency of DNA replication and mutation. When esophageal cells are exposed to high concentrations of bile acids for a short period of time, apoptosis is induced. Over longer periods of time, bile acids cause the development of apoptosis resistant cells and eventually esophageal adenocarcinoma^{1;9}. Cells resistant to apoptosis clearly have an advantage in the presence of bile acids and will proliferate and cause further DNA damage and mutations¹⁰.

Past studies have demonstrated that tissue from patients with esophagitis and BE have elevated levels of ROS^{11;12}. The superoxide dismutase (SOD) family represents the first line of defense against oxidative stress and SOD has been shown to be decreased in esophagitis and Barrett's esophagus¹³. SOD catalyzes the reaction of the superoxide radical into water and oxygen¹⁴. ROS play a role in the cell signaling network and cellular homeostasis and must be finely regulated¹⁵. The three types of SOD are cytosolic copper/zinc-dependent SOD (CuZnSOD), iron-dependent extracellular SOD (EC-SOD), and manganese-dependent mitochondria SOD (MnSOD). Deficiency of MnSOD has been associated with carcinogenesis because MnSOD acts a tumor suppressor by inhibiting ROS and preventing cellular damage¹⁶.

In our previous studies, we determined that SOD enzymatic activity was decreased in rat esophageal tissue after esophagoduodenal anastomosis (EDA) and external bile acid perfusion. MnSOD contributes to this loss of enzymatic activity¹⁶⁻²². Supplementation with MnSOD mimetic Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) can protect rat esophageal epithelium from oxidative injury induced from increased bile acid exposure²⁰.

This study attempted to analyze the impact of bile acid exposure on immortalized esophageal squamous epithelial cells, Barrett's (BE) metaplastic cells, esophageal adenocarcinoma (EAC) cells and esophageal squamous carcinoma (ESC) cells and to determine the optimal bile salt concentration. We also sought to determine if cellular resistance to bile salts is related to MnSOD.

METHODS

Cell Culture

Human esophageal squamous epithelial cell line (Het-1A) was derived from esophageal autopsy tissue and purchased from ATCC (Manassas, VA). The cell line was maintained in Bronchial epithelial cell medium (BEBM BulletKit, Clonetics Corporation, Walkersville, MD) and supplemented with 100 U/mL penicillin and streptomycin and 10% fetal bovine serum (FBS) at 37 degrees Celsius in a humidified atmosphere of 5% CO₂. The flasks and cell culture plates were pre-coated with 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin dissolved in culture medium.

A Human hTERT-immortalized nonneoplastic Barrett's cell line (BAR-T) was utilized as a generous gift from Rhonda Souza, MD, University of Texas Southwestern. The cell line was

maintained in keratinocyte basal medium 2 (KBM-2, Clonetics Walkersville, Maryland) at 37 degrees Celsius in a humidified atmosphere of 5% CO₂.

The esophageal adenocarcinoma cell line (OE-19) was derived from human esophageal tissue from gastroesophageal junction adenocarcinoma and were a generous gift from Gerald C. O'Sullivan, MD. The cells were maintained in Roswell Park Memorial Institute – 1640 (RPMI-1640, ThermoScientific, South Logan, Utah) and supplemented with 2 mM L-Glutamine and 10% FBS and 100 U/mL penicillin and streptomycin and at 37 degrees Celsius in a humidified atmosphere of 5% CO₂.

The esophageal squamous carcinoma cell line (OC-3) purchased from ATCC (Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, CellGro Mediatech, Herndon, Virginia) and supplemented with 100 U/mL penicillin and streptomycin and 10% fetal bovine serum at 37 degrees Celsius in a humidified atmosphere of 5% CO₂.

For cell viability assay, cells were seeded at 1×10^5 cells per well on 96 well plate per standardized protocol. All cell viability experiments were performed in triplicate. One day after the cells were seeded, they were treated with bile acids sodium cholate (CA), sodium deoxycholate (DCA), sodium glycocholate (GCA), sodium taurocholate (TCA) and a mixture of reagents (MIX) at concentrations ranging from 0.2 mM to 0.8 mM. Cells were incubated with reagents for 4 to 24 hours.

Cell Viability Assay

Cell viability was evaluated by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. MTT (Sigma, St. Louis) was added into the cultured cells at 20 μ L/well (5 mg/ml in PBS) for 4 hours at the end of each bile salt treatment. The purple formazan precipitate was dissolved in DMSO (200 μ g/well) and absorbance was measured with spectrophotometry by plate-reader set at 570 nm wavelength. The mean optical density (OD) value was calculated from triplicate wells for each treatment and used as an index of cellular proliferation. OD values were normalized to reference cell control number and a growth percentage was calculated (OD value experimental group/OD value control group $\times$ 100). Statistical analysis was performed using SPSS 17.0. Multiple analyses of the variances were performed to determine difference among the reagents and among the cell lines. Tukey HSD post hoc analyses were performed when necessary. Significance was defined as $p < 0.05$.

Protein Isolation and Detection

Cells were seeded at 5×10^5 /mL into 6 well plates per standardized protocol. One day after the cells were seeded, they were treated with a mixture of bile salts (MIX) at concentrations ranging from 0.2mM to 0.8 mM for 4 hours or for 24 hours. Cells were washed with ice-cold PBS and then scraped from plates using ice-cold lysis buffer[20 mmol/L HEPES 9pH 7.4), 10 mmol/L NaCl, 1.5 mmol/L MgCl₂, 20% glycerol, 0.1% Triton X-100, 1mmol/L DTT and protease inhibitor mixture]. The cells were centrifuged at 1500 RPMs at 4 degrees Celsius for 15 minutes. BioRad protein assay stock solution was diluted with 1 part dye reagent concentrate with 4 parts distilled deionized water (DDH₂O) and 1 mL was added to each clean, dry test tube. Four dilutions of bovine serum albumin standard (BSA 1.25 mg/mL) were prepared. Samples were prepared by adding 5 μ L of sample to each tube. GeneQuant Pro was utilized to measure the absorbance at 595 nm.

Western Blot Assay

Equal number of cell equivalents were utilized across the different cell lines and loaded into the gel wells. B-actin loading control was utilized to verify equivalent amounts of protein loading. The protein samples (30 ug) were separated by sodium-dodecyl sulfate electrophoresis using a 10% gel and transferred to a nitrocellulose membrane for Western blotting. Primary and secondary antibodies were added and incubated with the membrane per protocol to evaluate MnSOD expression. The membrane was blocked with 5% milk, incubated with the primary antibody at a dilution of 1: 2000 for one hour at room temperature and detected with chemiluminescent detection using appropriate secondary antibodies (1:5,000–10,000 dilution depending on previous standardization). The specific protein bands were detected based on their molecular weight with reference to pre-labeled markers and quantified using UnScanIt software (Silk Scientific Inc., Orem, UT).

Statistical Analyses

Statistical analyses were performed on SPSS 17.0. Multiple ANOVA (analyses of the variance) were performed to determine the differences in MnSOD expression among the different cell lines. Differences among groups were regarded as statistically significant if p values were less than 0.05. In the event of a significant result, Tukey HSD post hoc multiple comparison test was performed to determine individual differences between groups.

RESULTS

Het-1A cells were exposed to four separate bile acids {(sodium choleate (CA), sodium deoxycholate (DCA), sodium glycocholate (GCA), sodium taurocholate (TCA)} and an even mixture of the four reagents (MIX) at concentrations ranging from 0.2 mM to 0.8 mM at 4, 8 and 24 hours. A significant difference in cell proliferation was found among a comparison of all five types of bile acids ($p < 0.001$) (Figure 1) (Table 1). Further analyses by Tukey HSD showed that there was not a difference in cellular viability when exposed to CA, DCA or MIX at each of the concentrations. GCA did not demonstrate any effects on cell viability and was not cytotoxic to the cells. CA, TCA, DCA and MIX inhibited viability more than GCA. The effect of MIX, CA and DCA on cell viability was equivalent. TCA did not decrease viability quite as much.

Cell viability for Het-1A cells at 4, 8 and 24 hours for the mixture of bile acids treated with 0.8 mM MIX, growth was approximately 30% (0.3 ± 0.01) at four hours and decreased to 13 % (0.13 ± 0.02) at 24 hours (Table 2). At 0.6 mM concentration MIX, viability decreased from 53% (0.53 ± 0.02) at four hours to 23 % (0.23 ± 0.04) at 24 hours. At 0.4 mM MIX, cell viability was 72% (0.72 ± 0.02) at four hours and decreased to 33% (0.33 ± 0.01) at 24 hours. Finally, when 0.2 mM MIX was utilized, cell viability was approximately 90 % (0.9 ± 0.03) at four hours and decreased to 48 % (0.48 ± 0) at 24 hours (Figure 2).

Het-1A cell line demonstrated dose dependent negative linear correlation at all time points ($R^2 > 0.99$ for all time points). At higher bile salt concentrations, the cells exhibited less viability. There was a 50% decrease in cell viability from 4 to 24 hours in the Het-1A cell line at all concentrations. There was not a large difference in cell viability from 4 hour to 8 hours. This demonstrates that the decrease in viability is a result of the cumulative effects of exposure to bile salt insult over period of time. The results of the Het-1A cell line demonstrated that higher concentrations lead to the greatest reduction in cell viability. The concentration 0.8 mM is not an optimal experimental concentration because there are not sufficient cells available after prolonged bile salt treatment. Treatment with 0.2 mM MIX does not have enough initial effect to make this a useful experimental concentration. The

optimal bile salt concentration for experimentation on Het-1A cell line is in the range of 0.4 mM – 0.6 mM MIX. These concentrations initially decrease viability at the 4 hour time point and display a predictable pattern of decreased viability as time progresses. There are sufficient cells available for experimentation at 24 hour time point (one cell cycle). At all concentrations of MIX, Het-1A showed a negative linear correlation between dosage and cell viability (increasing bile salt dosage leads to decreased cell viability). Additionally, repeated and prolonged bile acid exposure leads to a cumulative decrease in viability.

Bar-T cells were also treated with the same bile salt at concentrations ranging from 0.2 mM to 0.8 mM for 4, 8 and 24 hours. There was not a significant difference among the reagents. Cell viability index for Bar-T cells at 0.8 mM MIX, cell growth was approximately 77% (0.77 ± 0.06) at four hours and remained the same at 24 hours (0.75 ± 0.07). For 0.2 mM to 0.6 mM MIX, cell viability index was around 80% at 4 hours and increased to >95% at 24 hours (Table 2). Viability was decreased by 0.8 mM MIX at all time points. Bar-T cell viability was initially decreased at 0.2 – 0.6 mM MIX at the 4 hour time point but was not inhibited at 8 and 24 hours. We did not test bile salt concentrations greater than 0.8 mM because these are well beyond the physiologic range.

OE-19 cells did not show a statistical difference among reagents ($p=0.3$). Standardized OD values at 4, 8, and 24 hours are shown in figure 3. When cells were treated with 0.8 mM MIX, the cell viability index was 60% (0.59 ± 0.03) at four hours and increased to 83% (0.83 ± 0.09) at 24 hours. At 0.2 mM to 0.6 mM, viability was between 66–83% at four hours and increased to 100% at 24 hours (Table 2).

OC-3 cells did not show a statistical difference among reagents ($p=0.19$) and standardized OD values at 4, 8, and 24 hours are shown in Figure 3. At all MIX concentrations and time periods, OC 3 cell growth remained approximately 85–100% (Table 2).

Since a majority of the effects on cell viability were evident at the four hour evaluation, all four cell lines were compared at this time point (Figure 3). There was a significant difference in cell viability (standardized OD values) among the cell lines at all concentrations ($p<0.005$). At 0.8 mM MIX and 0.6 mM MIX, the Het-1A cells had significantly decreased viability compared to the other cell lines.

There was no difference in viability between Bar-T cells and OE-19 cells. The OC-3 cells had higher viability than the other lines. At 0.4 mM, OC-3 cell viability index was significantly greater than the other lines. Het-1A was not statistically different than the Bar-T or OE-19 cells, however, Bar-T viability was higher than OE-19 cell growth. At 0.2 mM, the OC-3 cells had significantly higher viability than the other cell lines and there were no differences detected among the other three lines. The results showed that the OC-3 cell line demonstrated significantly higher viability at all concentrations than the other cell lines. Het-1A cell line showed a negative correlation between dosage and cell viability at all concentrations.

The cell lines were compared at the eight hour time point and the 0.8 mM concentration demonstrated a significant difference among the cell lines ($p=0.032$). Het-1A cells had significantly less cell viability than the other lines. There were no viability differences detected between Bar-T cells and OE-19 cells. OC-3 cells had significantly more viability than the other lines. At 0.6 mM, there was a growth difference among the cell lines ($p<0.001$). Het-1A cells had significantly less viability than the other cell lines, however, no differences were detected among the other three cell lines. At 0.4 mM, Het-1A cells had significantly less viability than the others and no additional differences were detected ($p=0.002$). At 0.2 mM, a difference was detected among the cell lines ($p=0.032$). Het-1A cells had less viability than the other lines.

The results demonstrated that OC-3 did not show decreased viability at any concentration. Het-1A cells demonstrated decreased viability at all bile salt concentrations and displayed a negative correlation between dosage and viability. The cell growth indices (cell proliferation) for the Het-1A cells at the 8 hour point were not significantly different from the indices reported at the 4 hour time point. The indices for the OC-3 cells were slightly less (approximately 10%) at 8 hours as compared to 4 hours. The indices for Bar-T cells and OE-19 cells were increased (by between 5–20%) compared to the values reported at 4 hours at 0.2 mM MIX to 0.6 mM MIX. At 0.8 mM MIX, Bar-T cells had a slight decrease (10%) in viability at the 8 hour point compared to the 4 hour point. The OE-19 cells had no change in viability with 0.8 mM MIX between the 4 and 8 hour time points. This demonstrated that Bar-T and OE-19 cells sustained initial decrease in viability after bile salt exposure at the 4 hour time point, but that proliferation improved with exposure time except at very high concentrations of bile salt reagent (0.8 mM MIX).

The cell lines were compared at 24 hours (Figure 4), where again there was a significant viability difference detected at all concentrations ($p<0.001$). At 0.8 mM, 0.6 mM and 0.4 mM, Het-1A cells had significantly less viability than the other lines. No differences were detected among the Bar-T, OE-19 and OC-3 cell lines. At 0.2 mM, Het-1A cells had significantly less viability than the other cell lines. Bar-T and OC-3 cells had comparable viability, but significantly less than the OE-19 cells. The results showed that Het-1A demonstrated significantly less viability at all concentrations of bile salt treatments than the other cell lines.

Manganese superoxide dismutase (MnSOD) expression was determined using western blot assay. There was a significant difference in MnSOD expression among the cell lines ($p=0.002$) (Figure 5; A and B). Het-1A cells had significantly higher expression than the OE-19 and the OC-3 cells. Het-1A appeared to express a higher level of MnSOD as compared to the Bar-T cells, but this difference was not significant. Bar-T cells expressed a higher level of MnSOD than either OE-19 or OC-3 cells, but this was not statistically significant.

The MnSOD expression in each cell line after bile salt treatment with MIX at 0.2 mM to 0.8 mM was determined (Figure 5; C and D). The cell lines were exposed to the bile salt reagents at the above mentioned concentrations for 4 hours and then protein isolation and Western Blot were performed. After treatment with MIX, the treated cells were compared to untreated. OC-3 cells showed a significant increase in MnSOD expression at all concentrations of bile salt reagent MIX. The greatest increase in MnSOD expression was after OC-3 cell line exposure to 0.8 mM. OE-19 cells and Bar-T cells did not show a change in MnSOD expression after exposure to bile salt reagent MIX at any concentration (0.2 mM to 0.8 mM). There was not a significant change in MnSOD expression in the Het-1A cells between the control and cells treated with 0.2 mM to 0.8 mM MIX.

There was no change in MnSOD expression in the Bar-T and OE-19 cell lines after bile salt exposure for 24 hours. The Het-1A cells showed a decrease in MnSOD at 0.6 mM MIX. There were not enough viable cells for MnSOD expression detection at 0.8 mM MIX (Figure 6).

DISCUSSION

Exposure of cells in the gastrointestinal tract to repeated high levels of bile acids is an important risk factor for esophageal cancer¹. High levels of bile acid leads to the generation of reactive oxygen species (ROS) which cause DNA damage and mutations⁶. There is increasing evidence that oxidative stress plays a key role in esophageal carcinogenesis as a

result of increased production of ROS secondary to chronic inflammation induced by reflux¹. ROS are known to activate signal transduction pathways involved in both cellular proliferation and differentiation²³. Additionally, ROS production has been implicated in the development of reflux esophagitis, BE and EAC²⁴.

Studies have shown that bile acids cause increased production of ROS in esophageal cells. Jolly et al. treated squamous esophageal epithelial cells (Het-1A) with a bile acid cocktail and induced ROS in the cultured cells. They also demonstrated DNA strand breaks and alkali-labile sites, consistent with DNA damage stimulated by ROS²⁵. Jenkins et al. treated EAC (OE-33) cells and ESC (KYSE-30) cells with physiologic doses of bile salt reagents. Deoxycholic acid induced ROS in cultured human EAC (OE-33) and ESC cells (KYSE-30)²⁶.

Bile acids constitute about 70% of normal bile²⁷. The predominant primary bile acids present in the esophagus are cholic acid, and its taurine and glycine conjugates as well as the secondary bile salt deoxycholic acid and chenodeoxycholic acid²⁸. In our study we observed the effect of CA, DCA, GCA, TCA and MIX on normal esophageal epithelial cells, BE cells, EAC cells and ESC cells. We utilized concentrations ranging from 0.2 mM to 0.8 mM to mimic physiologically relevant conditions. Esophageal aspiration studies estimated total bile acid values to range from 0–820 μ M^{28;29}. Additionally, we treated the cells with a mixture of the bile salts to mimic in vivo gastroesophageal reflux.

Jenkins et al. suggested that the low doses of DCA (<100 μ M) will neither cause DNA damage nor activate the NF- κ B signaling molecule. At low levels of DCA exposure, there are minimal ROS generated and these low levels are effectively addressed by intrinsic antioxidant enzymes such as SOD, catalase and glutathione peroxidase. However, at higher levels of DCA, the antioxidant defenses are overwhelmed by the ROS and DNA damage is observed³⁰. Pre-treatment with vitamin C, an anti-oxidant, could reduce DCA-induced damage and decrease the amount of ROS generated by bile acid exposure²⁶.

We demonstrated that four of the bile salts and a mixture of these salts could inhibit esophageal squamous epithelial cell (Het-1A) viability in a dose and time dependent manner. CA, DCA and MIX were the most toxic to esophageal squamous mucosal cells. GCA did not appear to be toxic to the cells at any concentration. Viability was decreased by approximately 50% from four hour time point to 24 hour time point at every concentration (Figure 2). The optimal experimental bile salt concentration is 0.4–0.6 mM for Het 1A cells. Zhang et al. demonstrated that esophageal squamous epithelial cell growth appeared to be resistant to sodium glycocholate reagent but inhibited by lycochenodeoxycholate, glycodeoxycholate, taurocholate, taurochenodeoxycholate, and taurodeoxycholate salts. These bile salts caused apoptotic alterations and induced cell death²⁷.

Bar-T cells and OE-19 cells initially exhibited decreased viability at the four hour time point but had approximately 100% viability for concentrations 0.2–0.6 mM and >75% for 0.8mM at 24 hours. These cell lines appeared to be inhibited only by very high doses of bile acids. Despite experiencing initial decrease in viability, the cells appeared to overcome bile acid insult and display high viability after lag period. The OE-19 and Bar-T cells were more resistant to bile salt insult than the normal esophageal mucosal cells. The Bar-T and OE-19 cell lines were derived from a tissue environment consistently exposed to gastroesophageal reflux, whereas the Het-1A cells were not. Perhaps the Bar-T and OE-19 cells have adapted to become more resistant to bile exposure. Jolly et al compared Het-1A cell line to FLO-1 (Barrett's associated adenocarcinoma) cell line and determined that Het-1A cell consistently demonstrated more DNA damage when exposed to bile acids²⁵. This further supports the hypothesis that BE and EAC cells may have adapted to reflux environment and possess a

cytoprotective response to bile salt exposure. The OC-3 cells were resistant to the bile salt reagents at all concentrations and time points. As expected, this cell line was the most resistant to bile salt insult. ESC cells appeared to be less sensitive than EAC cells to deoxycholic acid exposure as well²⁶.

Multiple studies have demonstrated deficiencies of SOD catalytic activity in many solid tumor types^{31;32}. In our study we found that MnSOD expression was greatly reduced in EAC and ESC as compared to normal esophageal epithelial cells. MnSOD expression in the BE cells was in between that of the normal cells and the malignant cells. This further supports the hypothesis that decreased MnSOD expression may promote tumor growth.

In our previous studies, we determined that SOD enzymatic activity was decreased in rat esophageal tissue after esophagoduodenal anastomosis (EDA) and external bile acid perfusion^{16–22}. Supplementation with MnTBAP, an MnSOD mimetic, can protect rat esophageal epithelium from oxidative injury induced from increased bile acid exposure²⁰. Additionally, there was a significant decrease in MnSOD expression in patients with BE with high grade dysplasia when compared to matched controls¹³.

Initially MnSOD acts as a tumor suppressor by inhibiting ROS, however, after transformation to malignant cells occurs, MnSOD may act to protect the cells. Malignant cells with higher expression of MnSOD are more resistant to chemotherapy and radiation because these treatments employ ROS as a mechanism for cellular destruction^{33;34}. Izutani et al demonstrated an upregulation of MnSOD messenger RNA in gastric carcinoma and esophageal squamous cell carcinoma. Additionally they showed that inhibition of MnSOD increases the effectiveness of adriamycin chemotherapy^{33;35;36}.

In conclusion, sodium cholate, sodium deoxycholate, sodium taurocholate and a mixture of these bile salts inhibited the growth of cultured normal esophageal epithelial cells in a dose and time dependent manner. Bar-T and OE-19 cells were more resistant to bile salt insult than the Het-1A cells. OC-3 cells were the most resistant to bile salt exposure. MnSOD expression was significantly decreased in OE-19 and OC-3 cells compared to normal esophageal epithelial cells. Exposure of OC-3 cells to bile salt increased MnSOD expression. After exposure to bile for 24 hours, Het-1A cells showed decreased MnSOD expression at 0.6 mM MIX.

The confirmation of the role of bile acids in esophageal carcinogenesis has interesting implications for chemoprevention in patients with reflux esophagitis and Barrett's esophagus. Further studies are necessary to assess the preventative role of antioxidant supplementation. Additionally, decreasing the MnSOD expression in carcinoma cells, may increase the effectiveness of chemotherapy and radiation treatments.

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Figure 1.

Growth suppression of esophageal epithelial cell line (Het-1A) after bile salt treatments was determined by MTT assay. Esophageal epithelial cells were seeded onto 96-well plates and treated with bile acids sodium choleate (CA), sodium deoxycholate (DCA), sodium glycocholate (GCA), sodium taurocholate (TCA) and a mixture of reagents (MIX) at concentrations ranging from 0.2 mM to 0.8 mM. Spectrophotometric OD values were used as a cell growth index. Untreated cells were used as control.

Figure 2.

Het-1A cell line demonstrated a dose dependent and linear response to treatment with MIX (mixture of bile salts) at 4, 8, and 24 hours.

Figure 3.

Cell growth in esophageal epithelial cell line (Het-1A), BE cell line (BAR-T), Esophageal adenocarcinoma cell line (OE-19) and Esophageal Squamous cell line (OC-3) was determined using MTT assay at 4 hours following bile salt treatment with MIX. The spectrophotometric OD values were used to calculate a cell growth index. Cells that were not treated with bile salt were used as the control.

Figure 4.

Cell growth in esophageal epithelial cell line (Het-1A), BE cell line (BAR-T), EAC cell line (OE-19) and ESC cell line (OC-3) was determined using MTT assay at 24 hours following bile salt treatment with MIX. The spectrophotometric OD values were used to calculate a cell growth index. Cells that were not treated with bile salt were used as the control.

1) Het-1A; 2) Bar-T; 3) OE-19; 4) OC-3; 5) Het-1A/Bile; 6) Bar-T/Bile; 7) OE-19/Bile; 8) OC-3/Bile

Figure 5.

MnSOD protein expression in esophageal epithelial cell line (Het-1A), BE cell line (BAR-T), EAC cell line (OE-19) and ESC cell line (OC-3) was determined using Western Blot assay. A) Western Blot showing total MnSOD expression in each untreated cell line. B) Graphical representation of MnSOD expression in the cell lines (determined by UsanIt software). C) Western Blot showing MnSOD expression in each cell line before and after treatment with bile salt reagent. D) Graphical representation of MnSOD expression before and after treatment with bile salt reagent.

Figure 6. MnSOD protein expression in Het-1A cell line at 24 hours following bile salt insult. A) Western Blot showing total MnSOD expression B) Graphical representation of MnSOD expression in the cell lines (determined by UscanIt software).

Table 1

Post hoc analysis of bile salt reagents CA, DCA, GCA, TCA and MIX to determine significant difference among reagents when used to treat Het-1A cell line.

Reagent	Cell Inhibition	Reagent	p-value
CA	comparable to	DCA	0.056
	greater than	GCA	0
	comparable to	TCA	0.06
	comparable to	MIX	0.051
DCA	comparable to	CA	0.056
	greater than	GCA	0
	comparable to	TCA	0.085
	comparable to	MIX	1
GCA	less than	CA	0
	less than	DCA	0
	comparable to	TCA	0.055
	less than	MIX	0
TCA	comparable to	CA	0.06
	comparable to	DCA	0.085
	comparable to	GCA	0.055
	comparable to	MIX	0.093
MIX	comparable to	CA	0.051
	comparable to	DCA	1
	greater than	GCA	0
	comparable to	TCA	0.093

Table 2

Standardized OD values (mean $\pm$ standard deviation) for cell lines treated with MIX at 4, 8 and 24 hours.

Het-1A Cell Line			
CONCENTRATION	4 HOURS	8 HOURS	24 HOURS
0.8 MIX	0.3 $\pm$ 0.01	0.36 $\pm$ 0.03	0.13 $\pm$ 0.02
0.6 MIX	0.53 $\pm$ 0.02	0.54 $\pm$ 0.03	0.23 $\pm$ 0.04
0.4 MIX	0.72 $\pm$ 0.02	0.7 $\pm$ 0.06	0.33 $\pm$ 0.01
0.2 MIX	0.9 $\pm$ 0.03	0.73 $\pm$ 0.06	0.48 $\pm$ 0.00
Bar-T Cell Line			
CONCENTRATION	4 HOURS	8 HOURS	24 HOURS
0.8 MIX	0.77 $\pm$ 0.06	0.67 $\pm$ 0.03	0.75 $\pm$ 0.07
0.6 MIX	0.79 $\pm$ 0.05	0.95 $\pm$ 0.04	0.95 $\pm$ 0.03
0.4 MIX	0.81 $\pm$ 0.02	0.95 $\pm$ 0.04	0.98 $\pm$ 0.04
0.2 MIX	0.8 $\pm$ 0.03	0.85 $\pm$ 0.03	0.95 $\pm$ 0.08
OE-19 Cell Line			
CONCENTRATION	4 HOURS	8 HOURS	24 HOURS
0.8 MIX	0.59 $\pm$ 0.03	0.61 $\pm$ 0.06	0.83 $\pm$ 0.09
0.6 MIX	0.72 $\pm$ 0.05	0.95 $\pm$ 0.16	1 $\pm$ 0.08
0.4 MIX	0.66 $\pm$ 0.08	0.94 $\pm$ 0.13	1.03 $\pm$ 0.07
0.2 MIX	0.83 $\pm$ 0.15	1 $\pm$ 0.09	1.05 $\pm$ 0.1
OC-3 Cell Line			
CONCENTRATION	4 HOURS	8 HOURS	24 HOURS
0.8 MIX	1.14 $\pm$ 0.24	0.87 $\pm$ 0.23	0.86 $\pm$ 0.01
0.6 MIX	1.17 $\pm$ 0.17	0.9 $\pm$ 0.15	0.91 $\pm$ 0.02
0.4 MIX	1.2 $\pm$ 0.1	0.92 $\pm$ 0.09	0.96 $\pm$ 0.01
0.2 MIX	1.17 $\pm$ 0.2	0.87 $\pm$ 0.07	0.81 $\pm$ 0.01