Hydroferrate fluid, MRN-100 provides protection against chemical-induced gastric and esophageal cancer in Wistar rats*

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Abstract

This study examines the protective effect of a hydroferrate fluid, MRN-100, against progression of methylnitronitrosoguanidine (MNNG)-induced gastric and esophageal cancer in rats. Animals were divided into four treatment groups: (1) control, (2) MRN-100, (3) MNNG, and (4) MNNG + MRN-100. Gastric cancer was induced by administering MNNG daily for 2 weeks followed by oral NaCl once every 3 days for 4 weeks. Concomitantly with chemical induction, the rats were given MRN-100 for a total of 33 weeks. Rats were examined for histopathological changes in gastric and esophageal tissues and changes in the body and organ weight. We also examined oxidative stress and the antioxidant biomarkers as a possible mechanism through which MRN-100 exerts its oncostatic potential. Results showed that 17/20 (85 %) tissues from rats treated with MNNG alone had developed cancer/dysplasia in the stomach or the esophagus. Among these 13/20 (65 %) had developed dysplasia and 4/20 (20%) had developed cancer. Conversely, of the rats treated with MNNG + MRN-100, only 8/20 (40%), had developed cancer/dysplasia in the stomach or the esophagus. Among these 7/20 (35%) tissues showed dysplasia and 1/20 (5%) of rats developed stomach or esophageal cancer. Treatment with MRN-100 also caused dramatic modulation of γ–GT activity, a marker of neoplasm. Moreover, it protected against the loss of body and organ weight due to exposure to MNNG. In addition, the antioxidant potential of MRN-100 was shown in the blood and gastric tissues as exemplified by elevation of Glutathione (GSH) content and endogenous antioxidant enzymes including: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). This was accompanied by a reduction in total free radicals. We conclude that MRN-100 exhibited significant cancer chemopreventive activity by a mechanism that involves the protection against oxidative stress damage to tissues. Our study suggests MRN-100 may be an effective adjuvant for the treatment of gastric and esophageal cancer.

Introduction

Gastric and esophageal cancers are two leading causes of cancer-related deaths throughout the world (de Martel et al, 2013). In the United States, approximately 40,000 people will be diagnosed with esophagus or stomach cancer in 2013 and despite advancement in treatment options, the 5-year survival rates for these cancer patients remain low; 17% and 27%, respectively (Howlader et al, 2013, de Martel et al, 2013). Both cancers are thought to arise from chronic inflammation caused by *H. pylori* (Yakirevich and Resnick, 2013) or gastroesophageal reflux disease (GERD). Inflammation associated with esophageal cancer is believed to be induced by GERD, an acid reflux disease effecting 14-20% of the United States adult population (Lagergren et al, 1999, Camilleri et al, 2005). This inflammation leads to atrophy and transformation, or metaplasia, of epithelial cells in the lining of the digestive tract. As the disease progresses, the metaplasial tissue is further transformed and multiple cell types, both immature and mature, will form causing dysplasia and eventually cancerous lesions (Yakirevich and Resnick, 2013).

The most effective treatment for gastric and eshophageal cancers is surgical removal of the cancerous lesions, however, this treatment is palliative for many advanced stages and does not address the causative chronic inflammation which could lead to development of new lesions (Blakely and Miner, 2013). Several potential preventative therapies have been examined for the treatment of gastric and esophageal cancers, these include: chemoprevention, anti-inflammatory agents and eradication of *H. pylori*. However, there is still a lack of evidence that these products will be effective in humans due to an insufficient number of clinical trials (Tan and Wong, 2013, Bathaie et al, 2013); therefore, novel preventative products for treatment of esophageal and gastric cancers remain in high demand.

Hydroferrate fluid, MRN-100 (ACM Co. Ltd., Japan), is an iron based beverage composed of bivalent and trivalent ferrates isolated from phytosin. Phytosin is a plant extract that contains iron and neutral lipid compounds commonly found in rice, wheat, and radish seeds. During production of MRN-100 the lipids are removed and only iron compounds remain dispersed in water. Previous research on MRN-100 has shown its potential as a protector against age-associated oxidative stress (Badr El-Din et al, 2010) and also as a protector agent against γ–radiation (Ghoneum et al 2013) and anti-HIV activity (Ghoneum and Shaheen 2008). However,

no studies have been carried out regarding the anti-cancer activity of MRN-100. The current study was performed to examine the anti-cancer activity of MRN-100 by investigating its protective effect against chemically induced gastric/esophageal cancer in rats and elucidate the possible mechanisms behind its action. Results of this study suggests MRN-100 may be an effective adjuvant for the treatment of gastric and esophageal cancer by a mechanism that involve protection against oxidative stress damage to tissues.

Materials and Methods

1-Methylnitronitrosoguanidine (MNNG). 200mg/kg body weight of MNNG (Sigma-Chemical, St Louis, MO) was orally administered to the rats daily for two weeks.

2- MRN-100. MRN-100 is an iron based beverage composed of bivalent and trivalent ferrates isolated from phytosin. Phytosin is a plant extract that contains iron and neutral lipid compounds commonly found in rice, wheat, and radish seeds. MRN-100 was prepared in water as described previously (Ghoneum et al, 2013). The rats were permitted free access to MRN-100 through oral liquid consumption. MRN-100 was provided by ACM Co., Ltd, Japan.
The current study was carried out to examine the anti-cancer activity of MRN-100 by investigating its protective effect against chemically induced gastric/esophageal cancer in rats and elucidate the possible mechanism behind its action.

3- Animals. Male Wistar rats aged 4 months (~120 g body weight) were used in this study. The animals were obtained from the Research Institute of Ophthalmology (Giza, Egypt) and acclimatized for one week prior to the commencement of the study. Rats were caged individually in with light and temperature control (20±2˚C) and were fed standard laboratory cube pellets.

4- Experimental design. 40 rats were divided into 4 groups: (1) control untreateduntreated, (2) MRN-100 treated, (3) MNNG treated, and (4) MNNG + MRN-100 treated. Using the standard protocol for induction of gastric and esophageal cancer, rats were given carcinogen MNNG at

dose 200 mg/kg body weight once daily by oral gavage for 2 weeks, followed by oral administration of NaCl (1ml/rat) once every 3 days for 4 weeks. Concomitantly with chemical induction, the rats were given water (groups 1and 3) or MRN-100 (groups 2 and 4) for a total of 33 weeks. Animals were examined for the changes in their body weight every month. After the treatment period (33 weeks), animals were euthanized by ?????? (cervical dislocation, gas hamber…etc…). Gastric and esophageal tissues were stained with H&E and examined for histopathological changes under the light microscope. Animals were examined for changes in the weight of livers and spleens, and the antioxidant activity in the blood and in the stomach tissues.

5- Evaluation of the antioxidant activity

5.1. Sample collection. The animals were sacrificed after being fasted for 16 hours and blood was collected in heparinized tubes by heart puncture after light anesthesia using heparinized syringes, and one volume was used for measurement of total free radicals. Hemolysates were prepared and used to examine the levels of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The separated plasma from heparinized blood was used for the determination of the end product of lipid peroxidation (malondialdehyde; MDA) and nitric oxide (NO) levels. In addition, total thiols (TSH), total protein, protein carbonyls (PCO) and iron profile evaluation were assessed.

5.2. Analytical procedures. Lipid peroxidation (LPx) in the plasma was ascertained by the formation of MDA and measured as described (Yoshioka et al, 1979). Reduced GSH content in erythrocytes (Beutler et al.1963), SOD activity (Minami and Yoshikawa , 1979), CAT activity (Luck 1963), GPx activity (Lawrence et al. 1974), NO (Miranda et al. 2001), TSH (Ellman 1959), PCO (Levine et al. 1990) and total protein levels (Lowry et al. 1951).

5.3. Detection of blood total free radicals by electron spin resonance (ESR). Using lyophilization, water was removed by sublimation from the frozen tissue. Freeze drying of samples was carried out using Edwards Pirani 501 Freeze dryer Super Modulyo (Edwards Ltd., Crawley, UK). ESR signals were recorded at room temperature using a Bruker EMX spectrometer (X-band; Bruker, Rheinstetten, Germany). The operating conditions were: microwave power=1.008 mW, modulation amplitude=4 G, modulation frequency=100 kHz, sweep width=200 G, microwave frequency=9.717 GHz, time constant=327.68 ms and sweep time=41.943 s. The detection limits of the ESR technique depend on the sample material, sample

size, detector sensitivity, frequency of the incident radiation and the electronic circuits of the instrument. Samples were inserted into ESR quartz tubes and measured using suitable instrument parameters. The peak height of the radiation-induced ESR signals was determined for each sample. The reading intensities were divided by sample weight of each sample to calculate the normalization values (Heckly, 1979).

5.4. Analysis of ESR Data. For monitoring variations in the peak height of ESR signals as a function of the magnetic field, intensities were measured as the distance between top and bottom points of the first derivative recorded (Pascual et al. 2002). Quantitative assessments of free radical concentrations were, however, made according to the following equation:

$Nd=K[Ho(\Delta H2)\ A/2]/[Hm\times Ge\ \sqrt{}\ PH\ ]$

Where: Nd: number of radicals, K: factor depending on the experimental condition of spectrophotometer=103/cm, Ho: magnetic field at peak in gauss, ΔH: width peak to peak, Hm: modulation field, PH: power in mW=1.008, Ge: gain of the detector=3.17×105. Concentration=unpaired electrons/lyophilized blood (g) or spin/lyophilized blood (g), A=peak height of signals/weight.

6- Histopathological analysis. The stomach and esophagus tissues from each group were examined for histopathological changes at 33 weeks post-exposure to MNNG. Tissues were fixed in 10% formalin solution and fixed overnight in cassettes. The paraffin-embedded tissues were sectioned on a microtome to a thickness of 4 μm, stained with hematoxylin and eosin (H&E) and observed under light microscopy for signs of hyperplasia, dysplasia, and cancerous lesions.

7. Statistical analysis. Values were reported as mean±SEM (standard error of the mean) for six rats in each group, and significance of the differences between mean values was determined by one-way analysis of variance (ANOVA) coupled with the Newman-Keuls multiple comparison test. Values of $p<0.05$ were considered to be significant. Statistical significance of differences between the MNNG-treated rats and MNNG plus MRN-100 treated groups or between the control untreated and MRN-100 treated groups for body weight change and organ weight change were evaluated with the Chi-squared test or the Fisher exact test, as appropriate. $P < 0.05$ was considered statistically significant.

Results:

1- Histopathology

1. Percentages of dysplasia and cancer. Histopathological examination of carcinogen MNNG-treated rats at 33 weeks showed that 17/20 (85 %) of the gastric and esophageal (foregut) tissues with dysplasia and cancer. Among these tissues; 13/20 (65 %) showed dysplasia and 4/20 (20%) showed cancer. Conversely, rats treated with MNNG in the presence of MRN-100 showed only 8/20 (40%) showed dysplasia and cancer. Among these tissues 7/20 (35%) developed dysplasia and 1/20 (5%) of rats developed cancer (figure 1). Results depicted in Table 1 showed that treatment with MRN-100 resulted in lowering the percentages of rats bearing mild dysplasia, having small focus or many small foci. In addition, rats treated with MRN-100 showed fewer intensity of foci per each dysplastic stage: mild, moderate and severe (Table 2).

2. Histopathology examination

Esophageal tissues. Histopathological changes of H&E stained tissues of the esophageal mucosa were examined. Squamous epithelium of all control untreated rats showed esophageal mucosa with hyperkeratosis and squamous hyperplasia (Figure 2 A&B). In addition, the squamous epithelium from all rats treated with carcinogen MNNG showed hyperkeratosis and patchy areas of mild squamous dysplasia (Figure 2 C) and sever squamous dysplasia (Figure 2 D). In addition, rare small areas of invasive well-differentiated keratinizing squamous cell carcinoma were detected (Figures 2 E & F).

Gastric tissues. The gastric mucosa from the body and the antrum of all control untreated rats is within normal limits (Figures 3 A & B). Squamous hyperplasia, dysplasia or carcinoma is not observed in the control tissues. In contrast, gastric mucosa from MNNG treated rats showed hyperplastic mucinous glands and mild squamous dysplasia (Figures 3 C & D) and high grade squamous dysplasia (Figures 3 E). In addition, invasive well-differentiated keratinizing squamous cell carcinoma was also detected (Figure 3 F). Conversely, the tissues from rats that were treated with carcinogen and fed with MRN-100 showed that the patchy and small areas of mild squamous dysplasia are seen in only 7/20 tissues. Thus it appeared that MRN-100

decreased the extent of esophageal dysplasia and squamous cell carcinoma. Similar findings were also noted for gastric dysplasia and adenocarcinoma.

Figure 1. Percentages of rats bearing dysplasia. Animals with dysplasia % (65% VS 35%)

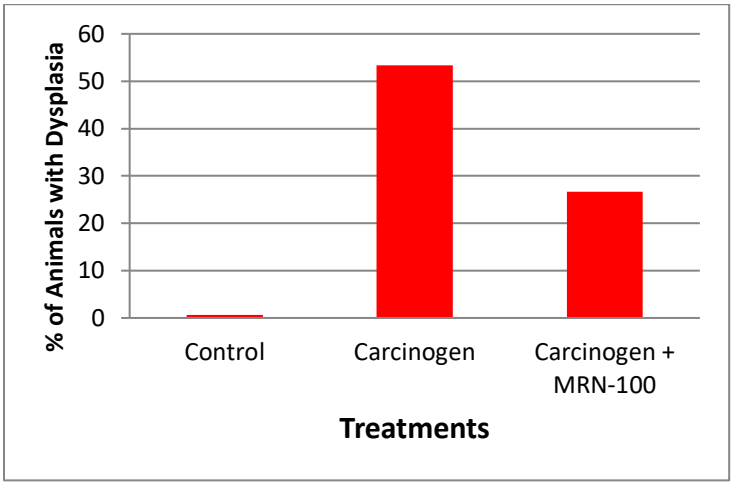


Figure 1. legend

Table 1. Intensity of foci in rats bearing mild dysplasia

	Carcinogen Treatment (9 Rats)		Carcinogen+MRN-100 Treatment (10 Rats)	
# of Foci	# Rats	% Afflicted	# Rats	% Afflicted
No Foci	0	0	4	40
1 Focus	6	66.7	5	50
≥ 2 foci	3	33.3	1	10

Table 2. Intensity of foci in rats bearing different dysplastic stages

	Carcinogen Treatment (9 Rats)		Carcinogen+MRN-100 Treatment (10 Rats)	
Dysplastic Stage	# of Foci	# Rats	# of Foci	# Rats
Mild	25	3	12	1
Moderate	9	2	1	1
Severe	6	2	0	0

Figure 2

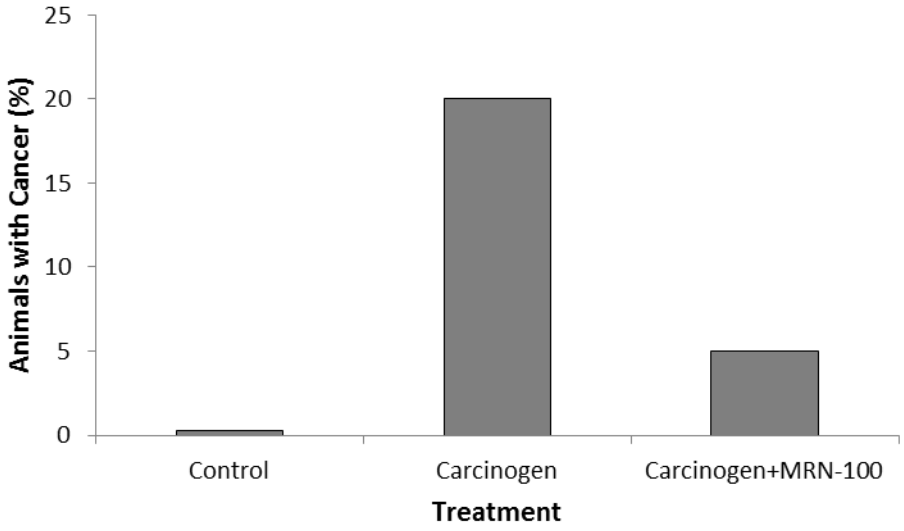


Figure 1Legend

Group	Cancer					Dysplasia				
	Pos	Neg	p-value btw con vs.carci	p-value btw con vs. carci+MRN-100	p-value btw carci vs. carci+MRN-100	Pos	Neg	p-value btw con vs.carci	p-value btw con vs. carci+MRN-100	p-value btw carci vs. carci+MRN-100
Control	0	21				0	21			
Carcinogen	5	25	0.0693			16	14	<0.0001		
Carcinogen + MRN-100	0	30		N/A	0.0522	8	22		0.0150	0.0350

Figure 2. Histopathological examination of esophageal tissues.

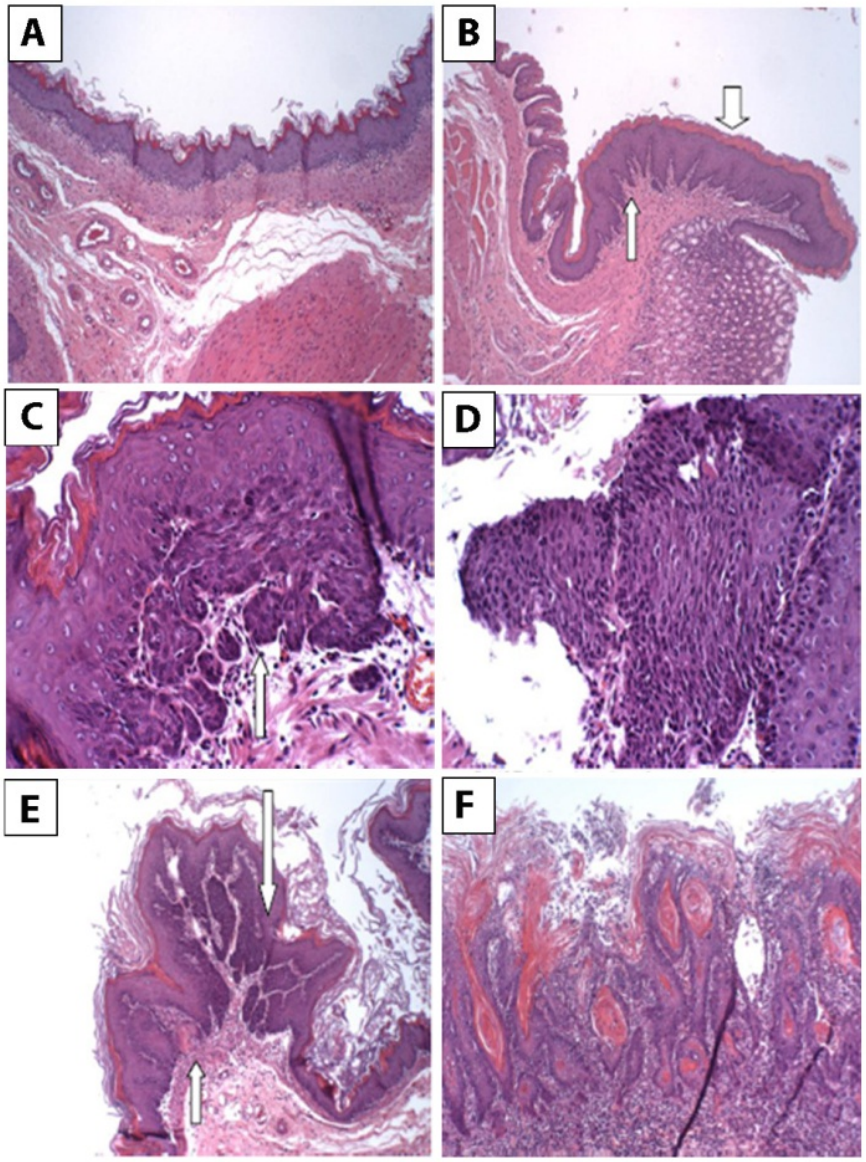


Figure 2. H&E histopathology staining from esophageal tissues in rats treated with carcinogen MNNG. . A. Section of the normal esophagus (4X). B. Section of a normal reactive esophagus-there is hyperkeratosis (down arrow) and squamous hyperplasia (up arrow): no squamous dysplasia (4X). C-F are sections from esophageal mucosa from MNNG treated rat. C. Section of esophagus showing a focus of mild squamous dysplasia (10X). D. Section of esophagus showing severe squamous dysplasia (10X). E. Section of the esophagus showing a focus of mild, moderate and severe squamous dysplasia (down arrow) and a focus of squamous cell carcinoma (up arrow) (4X). F. Section of the esophagus showing invasive well-differentiated keratinizing squamous cell carcinoma ((10X).

Figure 3. Histopathological examination of gastric tissues.

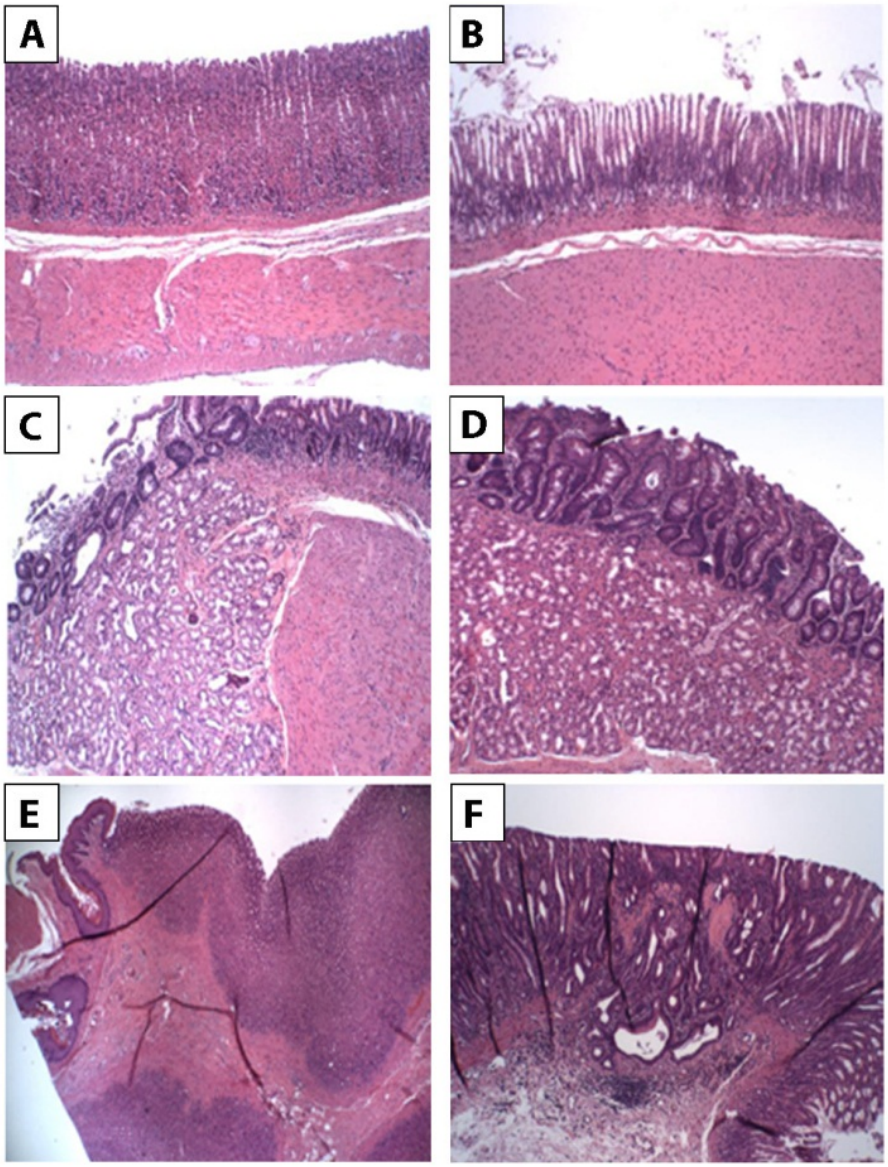
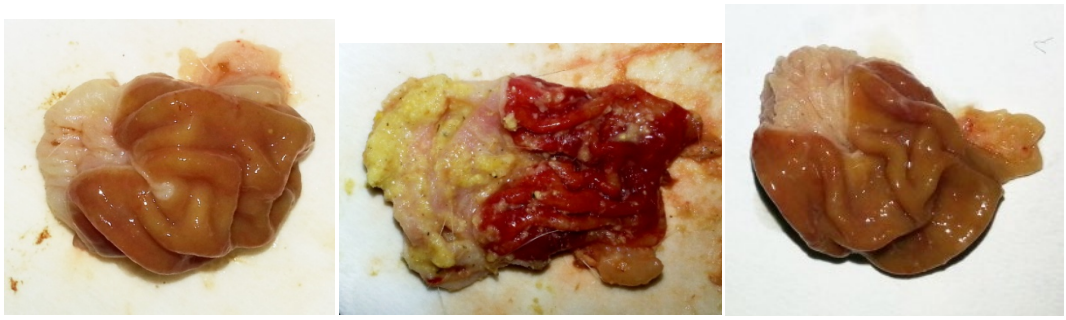


Figure 3. H&E histopathology staining from gastric tissues in rats treated with carcinogen MNNG. A. Section of a normal stomach body (4X). B. Section of a normal stomach antrum (4X). C-F are sections from esophageal mucosa from MNNG treated rat. C. Section of stomach antrum area showing mild dysplasia of glands and hyperplastic mucinous glands (4X). D. Section of the stomach antrum showing mild dysplasia of glands and hyperplastic mucinous glands (4X). E. Gastric mucosa showing hyperkeratosis, squamous hyperplasia and high grade squamous dysplasia (4X). F. Section of the stomach body showing high grade glandular dysplasia and invasive adenocarcinoma (4X).

Figure 4. Gross appearance of stomach from control rat, MNNG treated rat and MNNG plus MRN-100 treated rat. Please find clean photo for MNNG treated rat.

Control MNNG alone MNNG plus MRN-100



2-**Plasma ϒ GT.** Changes in the activities of γ–GT, a marker of neoplasm was examined. Data in Figure 5 showed that MNNG- treated rats revealed 2.5 folds increase in the activity of γ–GT as compared with control untreated rats. On the other hand, treatment with MRN-100 prevented the increase in the γ–GT activity and showed its values within the range of controls.

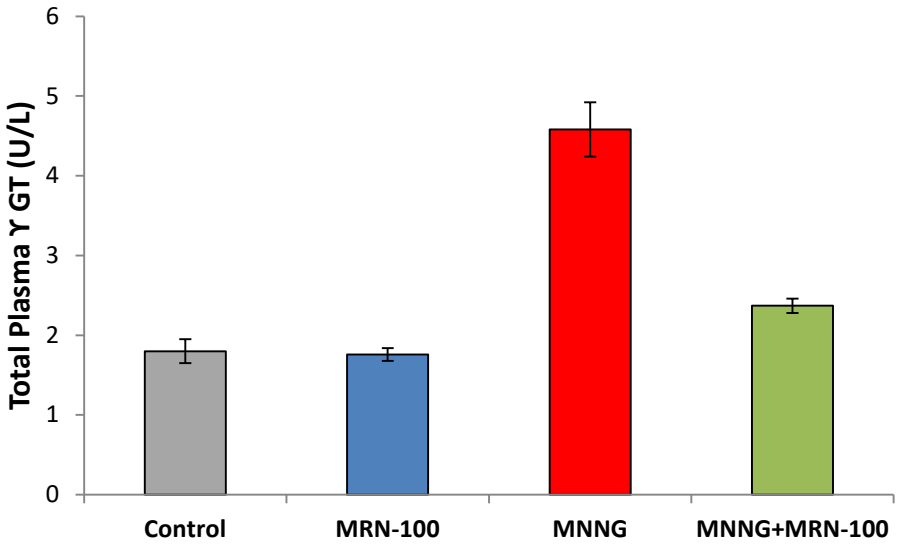


Figure 5. Activity of γ–GT post-treatment with MNNG and MRN-100. At 33 weeks, animals were examined for the changes in activity of γ–GT using…….

4- Body weight. Figure 6 shows changes in body weight of rats under different treatment conditions. Treatment with MNNG alone resulted in early weight loss that was detected at 2 months and further increased and became significant at 5 months ($p<0.01$). In contrast, treatment with MRN-100 protected the rats from weight loss. These rats maintained normal body weight throughout the 8 months treatment.

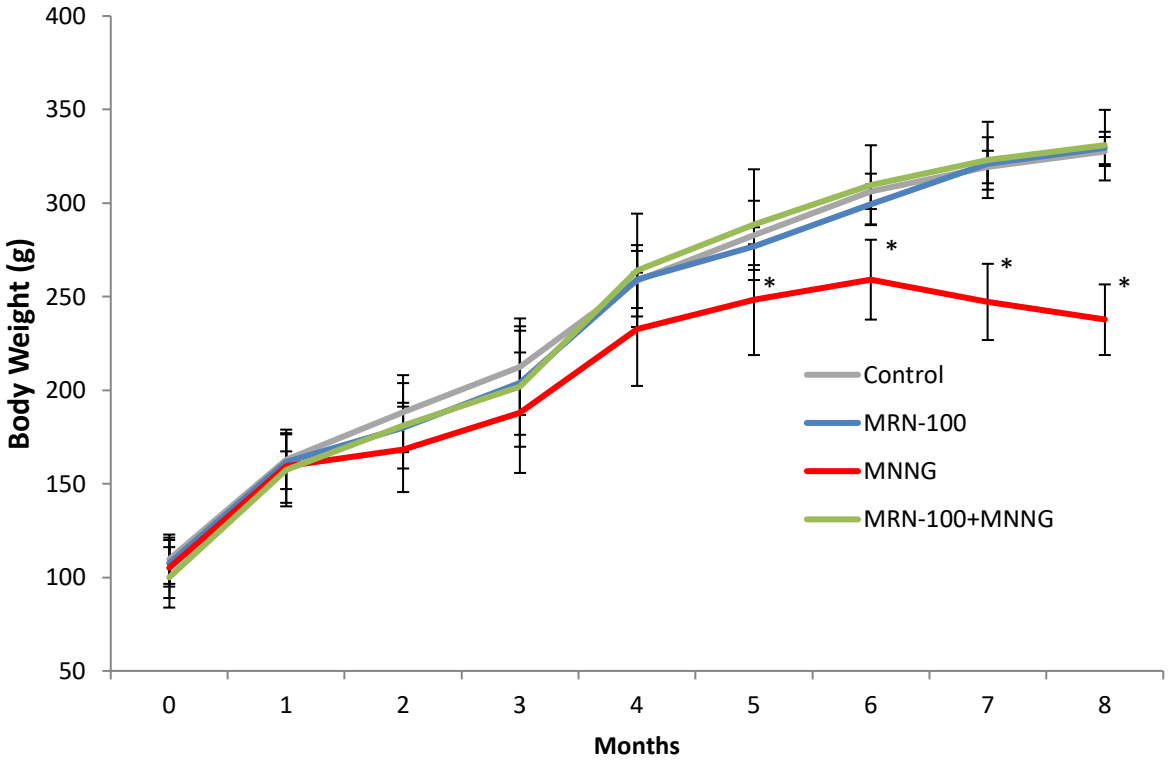


Figure 6. Changes in body weight under different treatment conditions**.** Rats were given carcinogen MNNG in the presence or absence of MRN-100. Animals in the 4 groups were examined for the changes in their body weight every month for 8 months. *$p<0.01$ compared to control and MRN-100+MNNG group. Each bar represents the mean ± SE of 10 rats/group.

5- Organ weight changes. Results of changes in the weight of the livers and spleens in rats under different treatment conditions were examined at 33 weeks. Figure 7 A&B shows that rats bearing gastric cancer have a significant decrease in the weight of liver (35%) and spleen (45%) as compared to the control untreated rats. In contrast, treatment with MRN-100 provided protection against the loss of both organs weight to be within the values of the control rats. These changes are in consistence with the organ gross macroscopic appearance shown in Figure 8 A&B. Need spleen from MNNG plus MRN-100 treated rat.

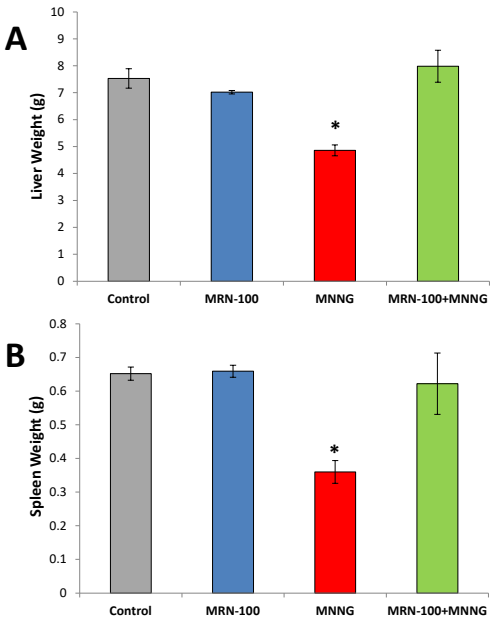


Figure 7

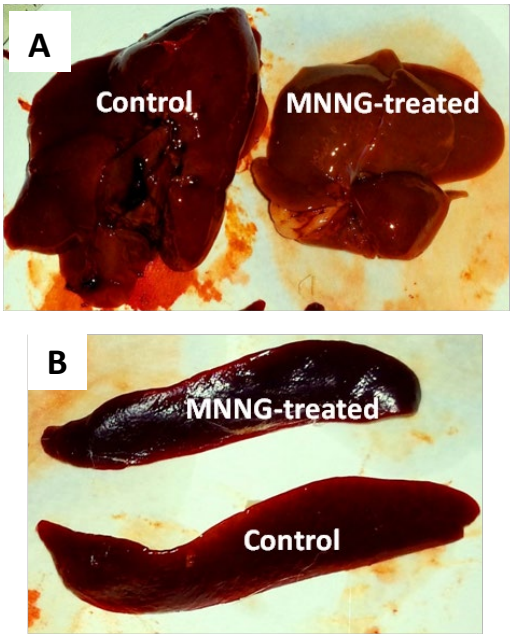


Figure 8

Figure 7 A&B. Changes in organ weight. Rats were given carcinogen MNNG in the presence or absence of MRN-100. At 33 weeks, animals were examined for the changes in the weight of livers and spleens. *Significantly different from MNNG+MRN-100 group at 0.01 level. Each bar represents the mean ± SE of 10 rats/group.

Figure 8 A&B Organ gross macroscopic appearances. Liver and spleen of control and MNNG-treated rats.

6- Anti-oxidant in the blood

Different parameters of rats under different treatment conditions were examined for anti-oxidant activity. Parameters include; total free radical levels, MDA levels, glutathione (GSH) content, endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and total antioxidant capacity .

MDA levels. Data in Figure 9A showed that treatment with MNNG alone causes marked elevation in the MDA values (40.80%, $p<0.01$) as compared to control untreated rats. However the level of MDA in rats treated with both MNNG and MRN-100 were maintained within the range of untreated controls.

GSH levels. The levels of GSH in red blood cells (RBCs) under different treatment conditions are presented in Figure 9B. MNNG-treated rats demonstrated a highly significant depletion in GSH level (-30.11%, $p<0.01$) as compared to control untreated rats. However MRN-100 supplementation prevented the decrease of the blood GSH content in MNNG-treated rats. There was also a noticeable increase in GSH content in the rats treated with MRN-100.

SOD, CAT and GPx activity. The effect of MRN-100 on the activities of the antioxidant enzymes SOD, CAT and GPx in plasma are shown in Figures 9 C-E. Treatment with MNNG resulted in a significant ($p<0.01$) decrease in the activity of the antioxidant enzymes SOD, CAT and GPx, as well as SOD activity (-45.13%), CAT (-34.04%) and GPx (-48.09%) compared to control untreated rats. On the other hand, rats treated with MNNG plus MRN-100 demonstrated significant increase in the activities of the antioxidant enzymes. The levels of decline in these enzymes were;-9.71%, -10.72% and -13.20% for SOD, CAT and GPx respectively. Daily intake of MRN-100 significantly prevented the decrease in activity of CAT and GPx to reach the control rat values.

Total antioxidant capacity. Treatment with MNNG resulted in a significantly ($p<0.01$) decreased in the total antioxidant capacity (-70.35%) as compared to control untreated rats

(Figure 9F). However, MRN-100 supplementation in MNNG-treated rats significantly s reduced the magnitude of decline to reach (-17.59%).

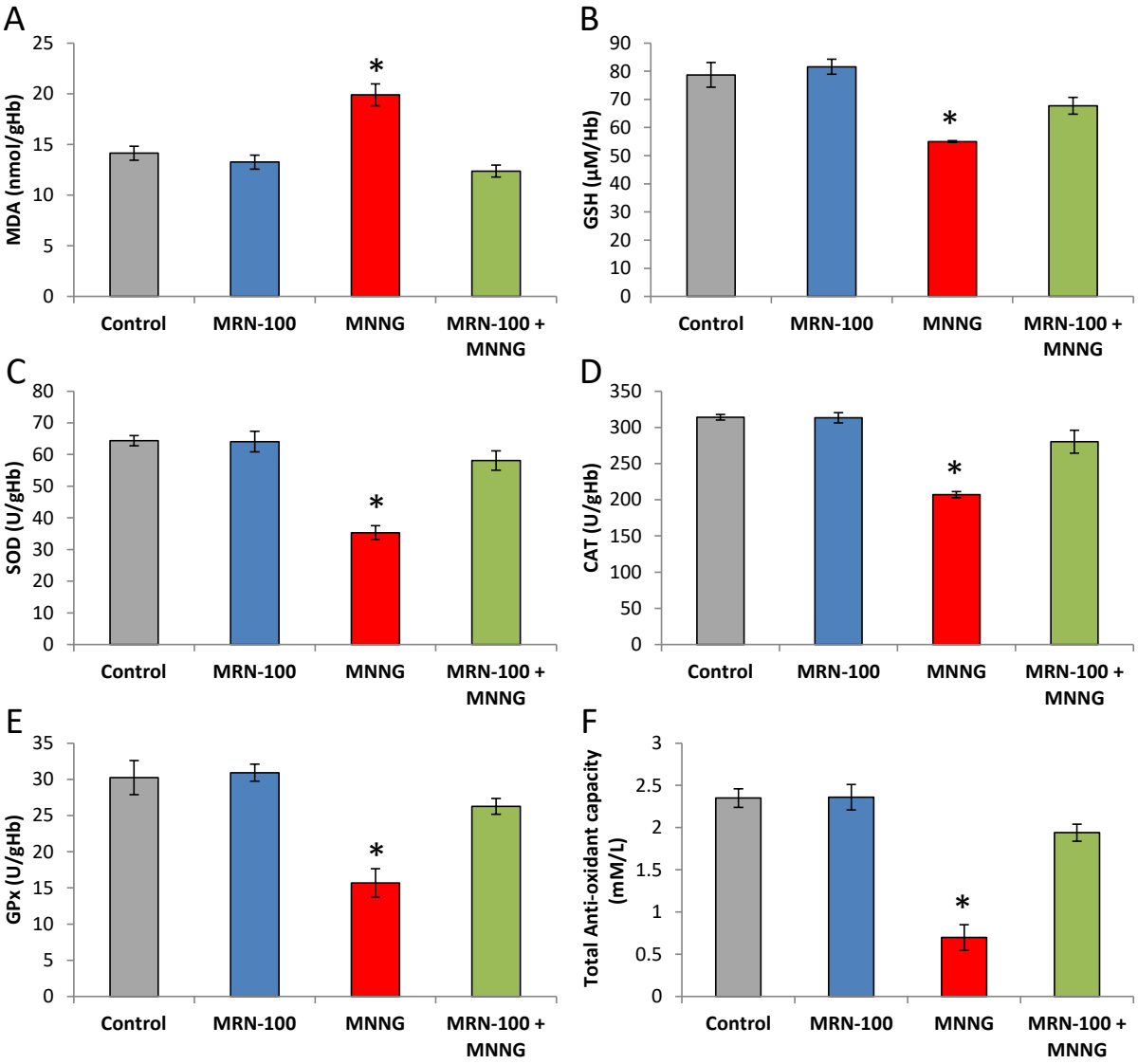


Figure 9. Effect of MNNG alone and MNNG+MRN-100 treatments for 33 weeks on blood MDA (A), GSH (B), SOD (C), CAT (D), GSH-Px (E), and total antioxidant capacity (F). * Significantly different from Control and MRN-100+MNNG group at $p<0.01$. Each bar represents the mean ± SE of 10 rats/group.

Total free radical levels. The total levels of free radicals in the blood were measured by ESR and data are presented in Figure 10. MNNG-treated rats demonstrated a significant increase in total free radicals as compared to control untreated rats (28.2 %, $p<0.01$). However, treatment with

MRN-100 prevented the decrease in MNNG-treated rats, and bringing the level to be within the range of control rats.

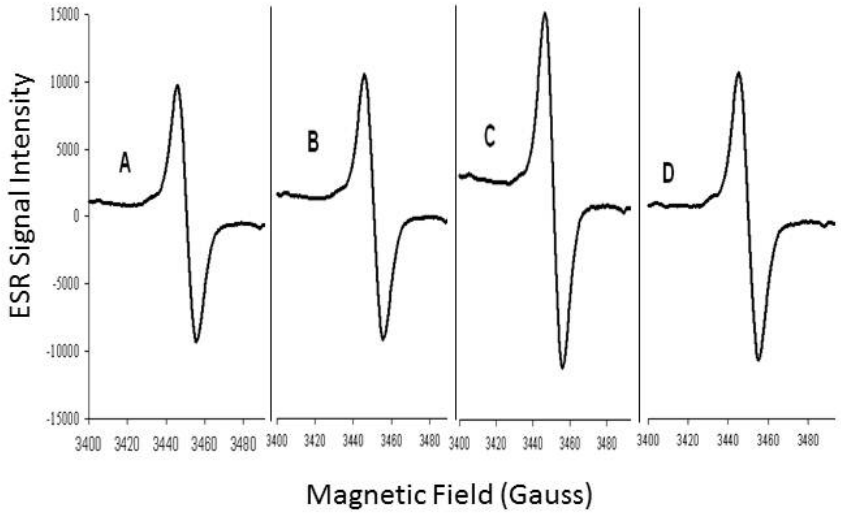


Figure 10. ESR spectra of lyophilized blood samples. Rats were treated with MRN-100 or given tap water only (control) for 33 weeks. Whole blood was collected by heart puncture after light anesthesia using heparinized syringes and was analyzed for total free radical levels by ESR. A: Controll (untreated) group; B: MRN-100; C: MNNG-treated group; D:MNNG+MRN-100-treated group.

7-Anti-oxidant in the gastric tissues

MDA levels. Table 3 shows the results of MDA levels under different treatment conditions. Rats- bearing gastric dysplasia or cancer displayed a significant increase in the MDA levels (40.80%, $p<0.01$) as compared to control untreated rats. In contrast, treatment with MRN-100 provided protection against MNNG-induced elevation of MDA values and became within the range of controls .

GSH levels. Results depicted in Table 3 show that rats- bearing gastric dysplasia or cancer revealed a significant decrease the GSH levels (40.80%, $p<0.01$) as compared to control untreated rats. On the other hand, treatment with MRN-100 in MNNG-treated rats maintained the GSH content in gastric tissues within the range of controls.

CAT and GPx activity. Table 3 shows the levels of the antioxidant enzymes: CAT and GPx ,in the gastric tissues. Rats- bearing gastric dysplasia or cancer demonstrated a significant decrease in the activity of CAT (-34.04%) and GPx (-48.09%) as compared to control untreated rats

($p<0.01$). In contrast, daily intake of MRN-100 prevented the decrease in activity of these antioxidant enzymes to reach the values of control untreated rats.

TAC. **A** significantly reduction in the TAC level ($p<0.01$) was noticed in rats- bearing gastric dysplasia or cancer (-70.35%) as compared to control untreated rats (Table 3). However, MRN-100 supplementation in MNNG-treated rats prevented the decrease in TAC levels. Please define TAC

ϒ -glutamyl transferase (ϒ GT). The total levels of ϒ GT in gastric dysplasia or cancer was significantly higher than those of control untreated gastric tissues. However, daily intake of MRN-100 defended the body against MNNG-induced elevation in the activity of ϒ GT and kept its values within the range of control rats.

Table 3: Effect of MRN-100 treatment on Stomach tissue MDA, GSH, TAC, CAT, GSH-Px , γ GT levels and total antioxidant capacity(TAC) in rats treated with MNNG.

Groups Parameter	Control	MRN-100	MNNG	MNNG+ MRN-100
MDA (µM/mg protein)	18.27±2.10	16.70±1.54 (-8.56%)	28.46±1.09**,## (55.78%)	20.76±1.50† (13.63%)
GSH (µM/mgprotein)	1.39±0.05	1.44±0.18 (3.73%)	0.83±0.03**,## (-39.90%)	1.27±0.07†† (-8.65%)
SOD(U/mg protein)	25.28±1.36	24.75±0.51 (-2.11%)	7.63±2.00**,## (-69.81%)	22.6±2.21†† (-10.61%)
CAT (U/mgprotein)	132.37±1.83	126.63±7.26 (-4.33%)	87.38±3.29**,## (-33.98%)	116.00±4.81††,* (-12.36%)
GPx (U/mg protein)	43.68±1.66	42.22±1.33 (-3.36%)	27.88±2.54**,## (-36.17%)	38.32±1.33††,* (-12.29%)
TAC (U/mg protein)	3.12±0.14	2.76±0.19 (-11.43%)	0.91±0. 10**,## (-70.73%)	2.50±0. 15††,** (-19.87%)
ϒ GT (U/mg protein)	5.9±0.28	6.1±0.41 (2.83%)	11.28±0.59**,## (91.78%)	7.5±1.25†† (26.63%)

Each value represents the mean ± SE of 6 rats/group
*& **Significantly different from control group at 0.05, 0.01 level respectively.
#& ##Significantly different from MRN-100 group at 0.05, 0.01 level respectively.
†&†† Significantly different from MNNG group at 0.05, 0.01 level respectively.
(% change of control group).

Discussion

Gastric and esophageal cancers are two leading causes of cancer-related deaths throughout the world. Preventative and protective treatment options are limited and novel products that effectively combat this disease are in demand. Results of the current study reveal effectiveness of MRN-100 in suppressing the growth of gastric and esophageal cancers. This was exemplified by histopathology study that showed significant reduction in the incidence of dysplasia as indicated by lowering the percentages of rats bearing dysplasia as well as the intensity of foci per each dysplastic stage: mild, moderate and severe. In addition data showed significant reduction in the incidence of animal bearing gastric adenocarcinoma and esophageal carcinoma. These data are associated with dramatic modulation of γ–GT activity, a marker of neoplasm.

In the current study, we observed significant loss of body weight in rats bearing dysplasia or gastric/esophageal cancer. These data are in accordance with recent findings on cancer patients that showed weight loss as characteristic of solid tumors; patients with upper gastrointestinal cancers and lung cancer have experienced a significant weight loss at diagnosis ((Huggett et al 2010 ; Spina et al 2013). Further studies also revealed that body weight is affected with the use of chemotherapy, radiotherapy, and hormonal therapy. It is of interest to note that supplementation with MRN-100 provided protection against the body weight loss at two months post-exposure to the carcinogen and continued for eight months.

Gastric and esophageal cancers are thought to arise from chronic inflammation which disrupts the epithelial lining of both tissues. Active oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical generated in inflamed tissues can cause DNA damage and contributing to tumor development (Ohshima and Bartsch ,1994). The role of ROS and oxidative stress in the production of mediators of pulmonary inflammation and initiation or promotion of the mechanisms of carcinogenesis has been well established (Valavanidis et al 2013). Other studies suggest that Helicobacter pylori increases the production of ROS and reactive nitrogen species (RNS) which elicit chronic active inflammation in the gastric mucosa that causes the gastric adenocarcinoma (Correa 2006; Farinati et al 2008; Handa et al 2011).

Results of the current study demonstrated that growth of gastric carcinoma is associated with oxygen derived free radicals accumulation, a markedly elevated MDA levels, a significant depletion in GSH content as well as the antioxidant enzymes, SOD, CAT and GSH-Px. This

observation is in accordance with other studies in animal-bearing tumor (Wong et al, 2001; Badr El-Din,2004; Gupta et al 2004). Bader Eldin et al observed a marked decrease in glutathione redox (GSH/GSSG) in the blood of Ehrlich ascites tumor–bearing mice, mainly due to an increase in blood GSSG levels as a result of oxidative stress (Badr El-Din,2004). Similar results were found in patients with gastric cancer (Bakan et al, 2002), laryngeal carcinoma (Samir and el Kholy, 1999; Seven,1999), and other types of malignancies (Cameron et al, 2003; Huang and Sheu, 1999; de Cavanagh et al, 2002).

The mechanism by which MRN-100 protects against MNNG-induces gastric and esophageal cancers could be attributed to the ability of MRN-100 to act as a potent antioxidant agent. Treatment with MRN-100 maintained different parameters of anti-oxidant activity in animal bearing tumors to be within the range of control untreated rats. These include MDA levels, GSH content and antioxidant enzymes and total antioxidant capacity. MRN-100 decreased oxidative stress biomarkers lipid peroxidation (LPx) and total free radicals. The role of MRN-100 as anti-oxidant agent was also observed in the model of age-induced ROS in rats. Treatment with MRN-100 resulted in elevating the levels of GSH and antioxidant enzymes, accompanied by reduction in lipid peroxidation and free radical levels in old rats (Badr El-Din et al, 2010).

Hydroferrate fluid, MRN-100, is an iron based composed of bivalent and trivalent ferrates isolated from phytosin. Phytosin is a plant extract that contains iron and neutral lipid compounds commonly found in rice, wheat, and radish seeds. It appeared that MRN-100 decreased the extent of gastric and esophageal dysplasia as well as squamous cell carcinoma and gastric adenocarcinoma. MRN-100 may exert its effect by a mechanism that involves the protection against oxidative stress damage to tissues via regulating the levels of free iron in the cells (Badr El-Din et al, 2010), since this metal has the ability to protect against oxidative stress (Latifi et al,2005; Udipi et al 1992;Balla et al,1992). MRN-100 may exert its effect by stimulating increased protein levels of the iron-binding compounds, such as ferritin and transferrin, thereby preventing excess iron from taking part in the Fenton reaction. This process prevents the accumulation of reactive radicals, such as protein carbonyl groups, which are a principal product of metal-catalyzed oxidation of proteins (Badr El-Din et al, 2010). GSH is known as a potent inhibitor of the neoplastic process due to mainly its important role as an endogenous antioxidant

system (Sinclair et al,1990). SOD, CAT, and GPx are involved in the clearance of superoxide and hydrogen peroxide. SOD catalyses the diminution of superoxide into H2O2, which has to be eliminated by GPx and/or CAT (Rushmore and Picket,1993).

The immune modulatory effect by MRN-100 may represent an additional mechanism by which MRN-100 suppresses the growth of gastric and esophageal cancers in MNNG –treated rats. Oral administration of MRN-100 to healthy subjects and cancer patients showed enhancement of their natural killer (NK) cell activity that was detected at 1 month post-administration of MRN100 (Ghoneum and Kijima, 1996: Ghoneum et al,1997: Ghoneum, 1998). NK cells have been shown to play an important role in the primary host defense against cancer and virally infected cells. (Whiteside and Herberman 1995; Badr El-Din et al 2008; Ames and Murphy 2013).

The biosafety of MRN-100 has been studied in rats after (33 weeks) of supplementation with no observable changes in animal behavior, body weight, or survival. Additionally, cancer patients who were orally administered MRN-100 for up to 10 months showed enhancement of NK cell activity (Ghoneum and Kijima, 1996: Ghoneum et al,1997: Ghoneum, 1998).
Conclusion: MRN-100 exhibited a significant cancer chemopreventive effect as demonstrated by the significant protection against dysplasia and gastric or esophageal cancer in rats. Our study suggests MRN-100 may be an effective adjuvant for the treatment of gastric or esophageal cancers.

Acknowledgements
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Dumping

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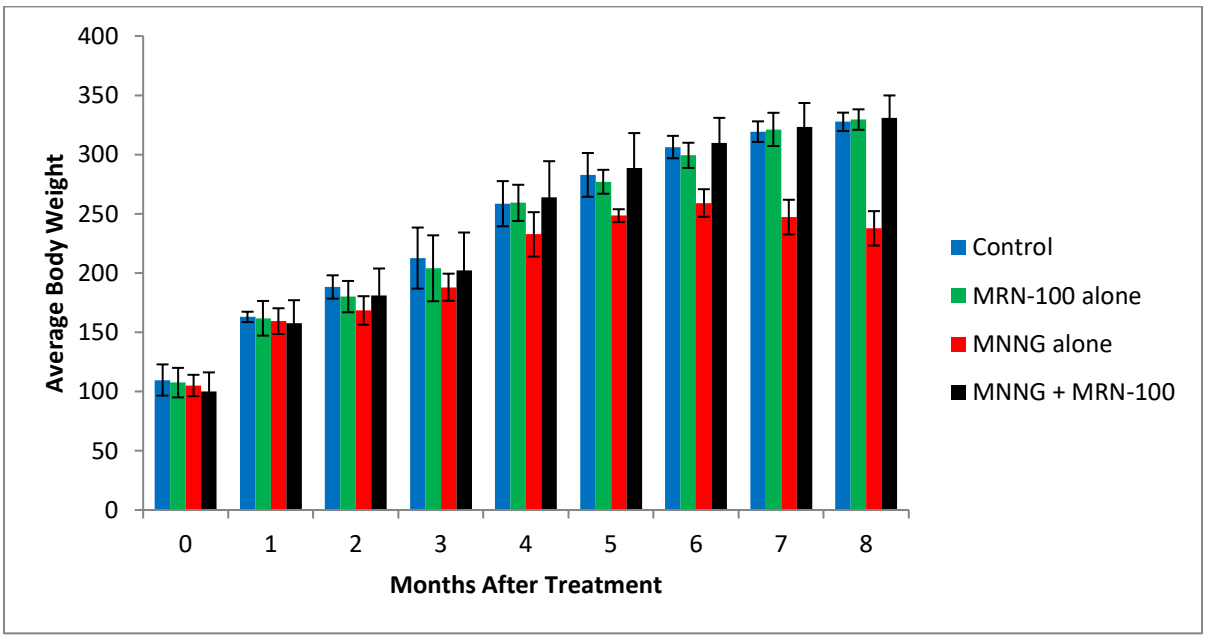


Table 1-Organs weight(g) at week 33 post MRN-100 and/or MNNG treatment

	Groups			
Organs	**Control**	**MRN-100**	**MNNG**	**MNNG+ MRN-100**
Stomach	**1.73±0.042**	**1.71±0.043 (-0.81%)**	**1.69±0.117 (-2.03%)**	**1.76±0.052 (2.03%)**
Liver	**7.53±0.360**	**7.02±0.065 (-6.81%)**	**4.86±0.202**$^{*,\#,\dagger}$ **(-35.48%)**	**7.98±0.594 (5.93%)**
Spleen	**0.652±0.196**	**0.659±0.018 (1.07%)**	**0.360±0.339**$^{*,\#,\dagger}$ **(-44.79%)**	**0.622±0.911 (-4.60%)**

Each value represents the mean ± SE of 10 rats/group

*Significantly different from control, MRN-100 at 0.01 level.
#Significantly different from MRN-100 at 0.01 level.
†Significantly different from MNNG+MRN-100 group at 0.01 level
(% change of control group).

Table 2: Effect of MRN-100 treatment on blood MDA, total free radicals, GSH, SOD, CAT, GSH-Px , γ GTlevels and total antioxidant capacity in rats treated with MNNG for 33 weeks.

Parameters	Control	MRN-100	MNNG	MNNG+ MRN-100
MDA(nmol/gHb)	14.13±0.69	13.25±0.69 (-6.25%)	19.90±1.08**,##,†† (40.80%)	12.37±0.59 (-12.5%)
Total Free radicals(gx10^{17})	3.14±0.27	3.35±0.81 (6.94%)	3.35±0.719**,##,†† (101.96%)	3.73±0.38 (23.67%)
GSH(µM/Hb)	78.70±4.35	81.59±2.66 (3.67%)	55.00±0.30**,##,†† (-30.11%)	67.71±2.95*,## (-13.96%)
SOD(U/gHb)	64.37±1.58	64.08±3.23 (-0.45%)	35.32±2.21**,##,†† (-45.13%)	58.12±3.04 (-9.71%)
CAT(U/gHb)	314.00±3.80	313.20±6.95 **(-0.25%)**	207.12±4.38**,##,†† (-34.04%)	280.34±15.77*,# (-10.72%)
GPx(U/gHb)	30.24±2.34	30.92±1.18 (2.23%)	15.70±1.96**,##,†† (-48.09%)	26.25±1.09 (-13.20%)
Total antioxidant capacity (mM/L)	2.35±0.11	2.36±0.15 (0.25%)	0.70±0.15**,##,†† (-70.35%)	1.94±0.10*,# (-17.59%)
γGT				

Each value represents the mean ± SE of 6 rats/group
*& **Significantly different from control group at 0.05, 0.01 level respectively.
#& ##Significantly different from MRN-100 group at 0.05, 0.01 level respectively.
†† Significantly different from MNNG+MRN-100 group at 0.01 level.
(% change of control group).

Table - Change in body weight (g) during MRN-3 and/or MNNG treatment

Time (Month)	GI	GII	GIII	GIV
0	109.7±13.18	107.5±12.46 (-2.01%)	105.1±9.01 (-4.19%)	100±16.15 (-8.84%)
1	163.0±4.34	161.8±14.62 (-0.74%)	159.4±10.86 (-2.21%)	157.5±19.56 (-3.37%)
2	188.2±19.87	180.1±13.20 (-4.3%)	168.4±12.04* (-10.52%)	181±22.82 (-3.83%)
3	212.6±25.79	204.0±27.81 (-4.05%)	188.0±11.46* (-11. 57%)	202.0±32.19 (-5.93%)

4	258.5±19.06	259.2±15.27 (0.27%)	232.6±18.76$^{*,\#\#,\dagger\dagger}$ (-10.02%)	264.1±30.28 (2.17%)
5	282.8±18.48	277.0±10.10 (-2.05%)	248.4±5.44$^{**,\#\#,\dagger\dagger}$ (-12.16%)	288.5±29.59 (2.02%)
6	306.3±9.46	299.3±10.64 (-2.29%)	259.1±11.63$^{**,\#\#,\dagger\dagger}$ (-15.41%)	309.6±21.34 (1.08%)
7	319.3±8.69	321.2±14.02 (0.60%)	247.2±14.62$^{**,\#\#,\dagger\dagger}$ (-22.58%)	323.1±20.37 (1.19%)
8	327.6±7.76	329.5±8.64420 (0.58%)	237.7±14.53$^{**,\#\#,\dagger\dagger}$ (-27.44%)	331±18.87 (1.04%)

Each value represents the mean ± SD of 10 rats/group

*Significantly different from control, MRN-100 at 0.05 level

**Significantly different from control, MRN-100 at 0.01 level.

$^{\#\#}$Significantly different from MRN-100 at 0.01 level.

††Significantly different from MNNG+MRN-100 group at 0.01 level

(% change of control group).

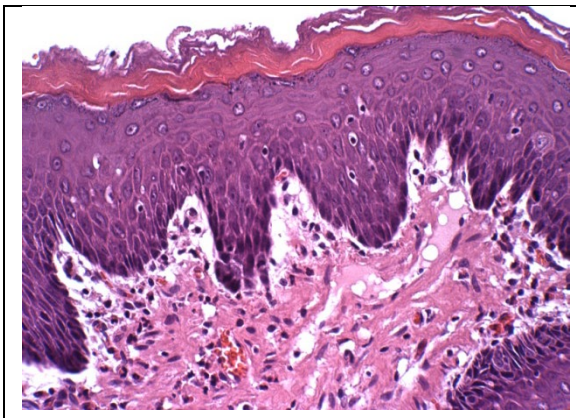
Discussion

In the current study, we observed 4 main characteristics in rats bearing dysplasia or gastric/esophageal cancer: significant loss of body weight, anemia, lymphopenia, and thrombocytosis. These data are in accordance with similar data gathered on cancer patients. Weight loss is characteristic of solid tumors; patients who have upper gastrointestinal cancers (Huggett et al 2010) and lung cancer have experienced a significant weight loss at diagnosis (Spina et al 2013). Anemia is highly prevalent in cancer patients (Levin and Conley 1994) and it is associated with different types of malignancies, including gastric cancer (Vannella et al 2013), prostate cancer (Varlotto and Stevenson 2005) and lung cancer (Crawford et al 2006). Lymphopenia has been reported to be a possible prognostic factor for survival of patients in different types of cancer malignancies, including carcinoma, sarcoma, and lymphoma (Ray-Coquard et al 2009). Further studies have showed that increased platelet count is correlated with tumor progression and suggest that thrombocytosis may be a useful indicator in patients with malignant disease of the gastrointestinal tract (Sasaki et al 2012), ovary (Haddad and Laufer 2008; Stone et al 2012) and lung (Maráz et al 2013).

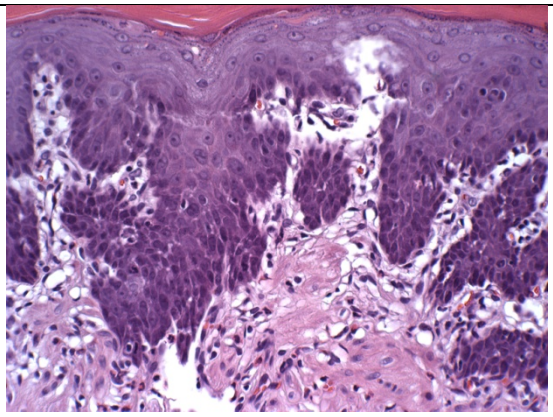
In addition to being indicators of malignancies in cancer patients, the rates of anemia, thrombocytosis, and body weight also rise with the use of chemotherapy, radiotherapy, and hormonal therapy. Therefore, it is necessary to find an alternative way to correct for these

effects in cancer patients. It is of interest to note that supplementation with MRN-100 provided protection against the above-mentioned changes. Protection of body weight loss was noted within two months post-exposure to the carcinogen and became more significant as time proceeded to eight months for animals bearing tumors. MRN-100-treated rats also showed to be non-anemic, with lymphocytes and blood platelets being maintained within the range of control (untreated) rats. In a recent study, we also observed that treatment with MRN-100 provides protection of the RBCs series against γ–radiation in Nile tilapia (Ghoneum et al 2013).

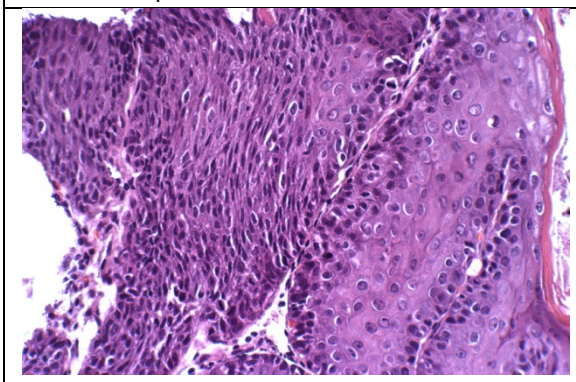
Figure 2. Histopathological examination of esophageal tissues.



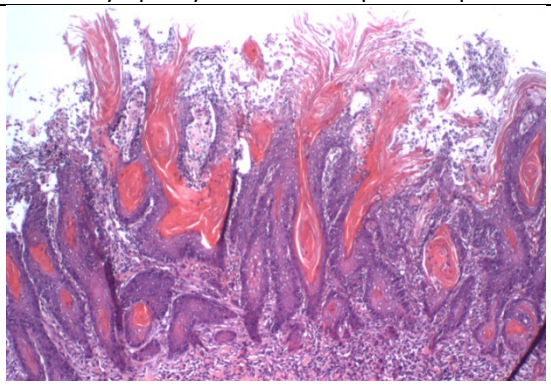
A. Esophageal mucosa (foregut) from control rat showing hyperkeratosis and squamous hyperplasia. Intraepithelial lymphocytes and stromal lymphocytes and eosinophils are present.



B. Esophageal mucosa from MNNG treated rat showing hyperkeratosis, squamous hyperplasia and mild squamous dysplasia. Intraepithelial lymphocytes and stromal lymphocytes and eosinophils are present.

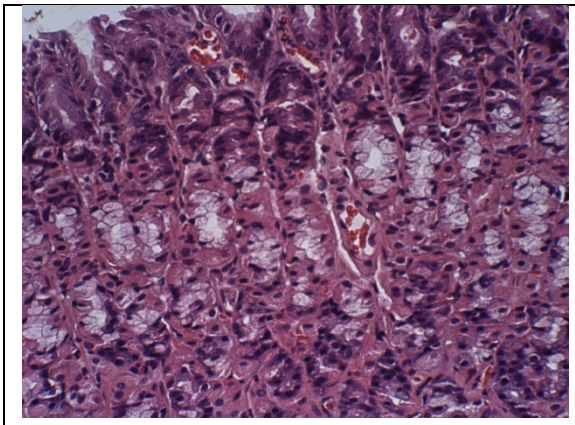


C. Esophageal mucosa from MNNG treated rat showing hyperkeratosis, squamous hyperplasia and high grade squamous dysplasia.

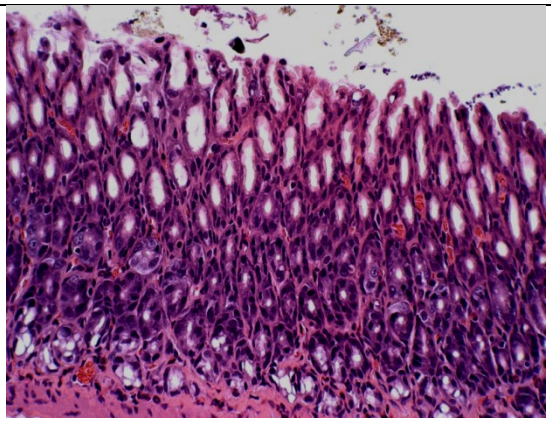


D. Esophageal mucosa from MNNG treated rat showing invasive well-differentiated keratinizing squamous cell carcinoma.

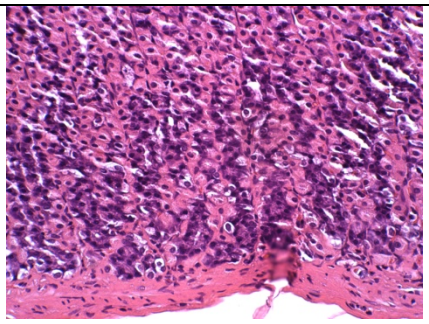
Figure 3. Histopathological examination of gastric tissues.



A. Mucosa from the body of the control stomach is within normal limits.



B. Mucosa from the antrum of the control stomach is within normal limits.



D. Gastric mucosa from MNNG treated rat showing invasive well-differentiated keratinizing squamous cell carcinoma.

MRN-100. MRN-100 was prepared in distilled water (DW) with the concentration of Fe^{2+} and Fe^{3+} ions at about 2×10^{-12} mol/l.MRN-100 is obtained from phytosin, a plant extract that contains iron and neutral lipid compounds and can be found in rice, wheat, or radish seeds. To start, 1 unit of phytosin is dispersed in 100 ml DW, and ferric chloride as FeCl3•6H2O is added. The lipid compounds are removed through a liquid–

liquid extraction using a separation funnel. The remaining liquid is filtered with No. 5 filter paper and the filtrate is evaporated and condensed in a water bath. The iron compound obtained is subjected to fractional determination with respect to bivalent ferrate and trivalent ferrate in order to generate MRN-100 (Ghoneum et al 2013) [13, 21]. Briefly, the sample's Fe (II) quantity is determined using the o-phenanthrolin method. Hydroxylamine-HCl 10% solution is added to the sample liquid to reduce Fe (III) to Fe (II) beforehand. Subsequently, all of the ferrate quantities are determined, followed by the determination of the quantity of Fe (III). As a result, the iron compounds thus obtained turned out to be bivalent and tervalent ferrates. MRN-100 was provided by ACM Co., Ltd, Japan.