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ORGANIC SELENIUM DELAY LIVER TUMOR GROWTH INDUCED BY DEN THROUGH MAINTAIN C-MET SIGNALING ON BALB-C MICE

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ABSTRACT

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Notably, it was found that DEN accelerate liver tumor growth through enhanced c-met signaling loss while organic selenium delayed chemical carcinogenesis through maintaining hepatocyte growth factor receptor and improving antioxidant status of treated mice. Moreover selenium treated groups showed fewer and smaller foci and nodules than the DEN group. At the end of the experiment no mice showed hepatocellular carcinoma (HCC) in the DEN-Se group but showed only advanced dysplasia. While acontrol positive group which administered DEN showed hepatocellular carcinoma in all mice. Additionally, selenium maintained liver enzymes and antioxidant liver status to basal level while DEN enhanced significant increase of liver enzymes and significant decrease of super-oxide dismutase and glutathione levels to basal level. We conclude that selenium had an inhibitory effect on the initiation and promotion stages of DEN-induced preneoplastic foci and nodules. Selenium also prevented progression of these nodules to HCC.

Key words: *HCC-selenium-HGF (Hepatocyte growth factor)-c-met (Hepatocyte growth factor receptor)-DEN (diethynitrosamine) –SOD1 (superoxide dismutase enzymes)*

INTRODUCTION

The importance of hepatocellular carcinoma in Egypt is increased with elevation of the major risk factors of chronic liver infection with hepatitis B and hepatitis C viruses (Ezat *et al.,* 2005) or aflatoxin (Anwar *et al.,* 2008) while pesticides and schistosemiasis may enhanced occurrence of liver cancer (Badawi and Michael, 1999 and Ezat *et al.,* 2005).

Based on data collected from international agency for research on cancer (*IARC*) (Ferlay *et al.,* 2004) has found that cancer incidence and mortality in the world. The worldwide liver cancer deaths in 2002 was estimated as 598, 412 which constitute 9% of all cancer deaths and a third in site -specific cancer deaths, followed by lung and stomach cancer. Distribution of liver cancer was more serious among less developing countries. Notably, Liver cancer is the fifth most common cancer in men and the ninth in women. A detected 782,500 new liver cancer cases occurred in the world during 2012, with China alone accounting for about 50% of the total. Rates are more than twice as high in men as in women. Liver cancer rates are the highest in central america, west and central africa, and east and southeast asia (London WT, McGlynn KA., 2006).

It was reported that selenium could have a role in cancer treatment of different tissue like mammary gland (Helen *et al.,* 1984), Colon (Kim *et al.,* 2011) pancreatic (Aichler *et al.,* 2007) prostate (Venkateswarah *et al.,* 2004) and lung cancer (Fritz *et al.,* 2011). Moreover, selenium may enhance the therapeutics efficacy of anti-cancer drugs like doxorubicin (Cao *et al.,* 2004).

loss of c-met accelerate early stage of liver cancer in c-met conditional knockout transgenic mice treated by DENe (Takami *et al.,* 2007), Loss of c-met accelerates development of liver fibrosis in response to CCl4 exposure through deregulation of multiple molecular pathways (Marquardt *et al.,* 2012), deletion of the met tyrosine kinase in liver progenitor oval cells increased sensitivity to apoptosis *in vitro* (Xiao *et al.,* 2001, Castillo *et al.,* 2008) and lack of hepatocyte growth factor receptor (c-met) gene expression in fulminant hepatic failure livers before transplantation explained no response to exogenous hepatocyte growth factor (HGF) that could be

maintain normal liver growth and regeneration (Trusolino *et al.,* 2010).

C-met truncated transgenic mouse didn't show any neoplastic lesions along whole life span of mice (Amicone *et al.,* 1995, 1997) while loss of c-metmade alteration of gene expression need for g2/m phase progression during liver regeneration in mice (Factor *et al.,* 2010), In contrast, c-met accelerate tumor growth in c-myc/ c-met double transgenic mouse model of liver cancer (Amicone *et al.,* 2002). In consistent role of c-met in cancer, HGF/c-met mediates a proliferative advantage and promotes tumor invasion and metastasis (Navab *et al.,* 2009).

Notably, N-acetyl-cysteine administration slowed tumor growth initiated by n-DEN in c-met deficient liver. At 3 months, the averages size of focal lesions in Metliv KO mice was reduced by 3 fold and reaches level found in Cre-ctrl mice when treated with N-acetyl-cysteine. More over N-acetyl-cysteine retain c-met receptor in treated mice (Takami *et al.,* 2007).

The rational of this study to test if mechanism of selenium chemoprevention of liver tumor could be through maintaining c-met expression or not.

MATERIALS and METHODS

Experimental animals:
Male balb-c mice of 20g and 8 weeks of age were obtained from the animal house of the medical research centre, faculty of medicine, Mansoura, Egypt. They were kept for 32 weeks under good ventilation and illumination conditions and allowed standard diet and water ad libitum. Mice were acclimatized for 2 weeks before experimental use.

Treatment:
DENe was purchased from Sigma Chemical Co. (St Louis, MO. USA). It was in the form of solution packaged in a 100 ml serum bottle with butyl rubber stopper and aluminum tear steal. The bottle content was dissolved in 100 ml saline to make a 1% solution. Tumor initiation was achieved by 6 times injection with DEN at a dose of 100 mg/kg b.w., twice dose every one weeks for 6 weeks according to (Shiota *et al.,* 1999).

Experimental design:
The experimental animals were divided into four groups, each of ten rats as follows:

1st group: 6 Male Balb-c received diethyl nitrosamine at dose of 100 mg/kg twice per week for 6 weeks and kept on maintenance ration all long the experiment for 32 weeks.

2nd group: 6 Male Balb-c received diethyl nitrosamine at dose of 100 mg/kg twice per week for 6 weeks but kept on ration supported with selenium –l-methionine* at dose of 3ppm all long the experiment for 32 weeks.

3rd group: 6 male balb-c kept on ration supported with selenium –l-methionine at dose of 3ppm.

4th group: 6 male balb-c kept on maintenance ration all long the experiment for 32 weeks.

After 6 week all groups kept for 32 weeks then mice weighted and sacrificed. Liver was examine grossly, weighted and divided into 2 parts: one preserved in buffered formalin saline 10% for histopathology, other was frozen -80 for biochemical and other molecular tests.

*Selenium –l-methionine: is brownish commercial substance add to concentrated ration at dose of 3ppm and mixed with ration in agriculture faculty research unit

Histopathological study:
Specimens from liver were fixed immediately in 10% neutral buffered formalin, dehydrated in different grades of alcohol, cleared in xylol, embedded in paraffin wax, sectioned at 4-6µ thick and stained with Haematoxylin and Eosin (Bancroft *et al.,* 1996) and examined microscopically.

Biochemicalanalysis
Blood was collected from each mice in a centrifuge tube and placed at room temperature for 20 min. Serum was then separated by centrifugation at 3,000 rpm for 10 min. Serum sample was divided into aliquots, one for determination of serum alanine transaminase (ALT), serum aspartate transaminase (AST) (Reitman and Frankel (1957).

Total glutathione content in liver samples was previously described by method of Anderson (1985) while SOD1 was determined according methods of Minami and Yoshikawa (1979).

RNA isolation, reverse transcription and RT-PCR
The trizol reagent kit was used for total RNA isolation from cells. Reverse transcription was carried out using Superscript II reverse transcriptase. Primers that were used: c-met, 5-GACTTCAGCCATCCCAATGT-3,3-GGTGAACTTCTGCGTTTGC-5 (Gordin *et al.,* 2010) and b-actin 5-ggcattgttaccaactgggacg-3, 3-ctctttgatgtcacgcacgatttc-5. Moreover, Conditions for RT-PCR were as follows: 10 min at 95˚C followed by 40–50 cycles of 15 s at 95˚C, 15 s at 60˚C, and 15 s at 72˚C. RT-PCR was performed as previously described (Luger *et al.,* 2004).

Statistical analysis:
The results were statistically evaluated using Student's "t" test. (SPSS 2013).

RESULTS

DEN slightly reduced the body weight while selenium maintains body weight similar to control group. Notably, DEN significantly increased liver weight and liver to body weight ratio when compared to other groups treated with selenium only or with DEN. Fig 1.

Notably selenium prevent progression of dysplasia into ultimate liver tumor which appeared grossly in DEN treated group (d) when compared with other groups treated with both selenium and DEN (c), selenium treated group (b) or control group which appeared normally (a). Figure 2.

Moreover, selenium appeared to maintain liver enzymes (AST, ALT) at basal level similar to control group while DEN treated group shown significantly increased of both AST and ALT when compared with control group. Additionally, selenium maintains antioxidant enzymes (SOD1 and total glutathione) while DEN treated group showed significantly reduced of both total glutathione and SOD1 when compared with control group. Figure 3.

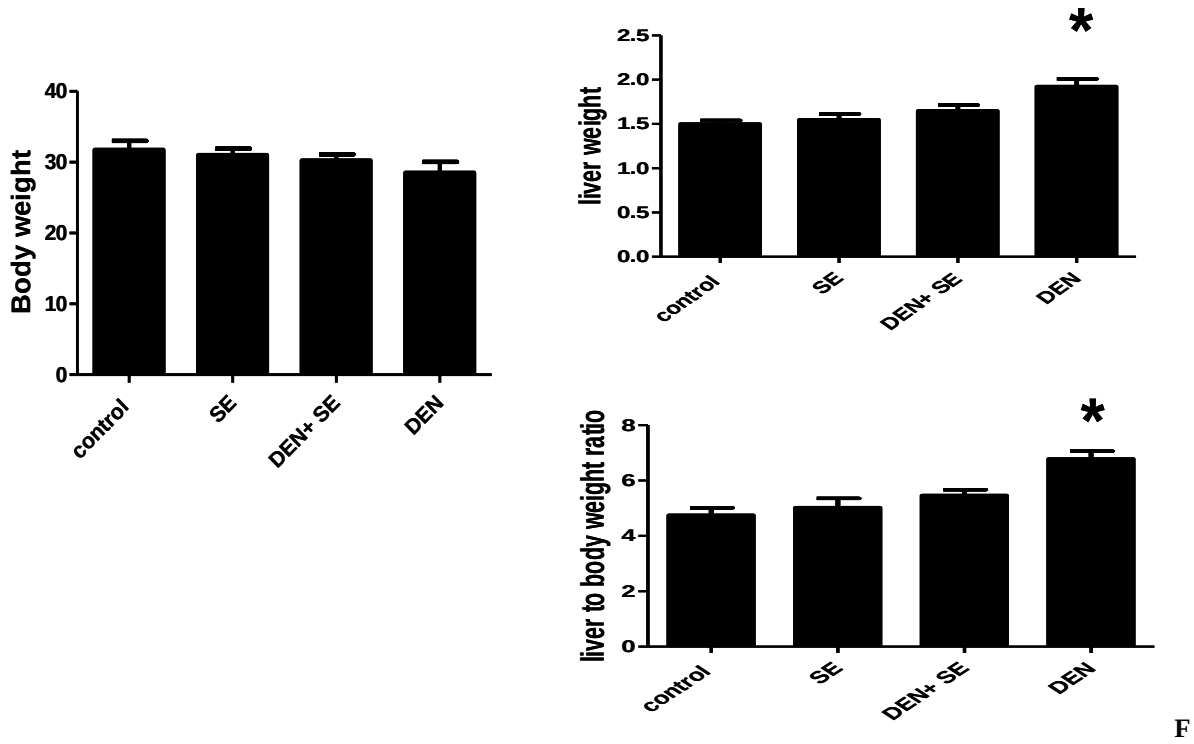

Fig. 1: Showed that DEN slightly reduced the body weight while selenium retained body weight similar to control group. Notably, DEN significantly increased liver weight and liver to body weight ration

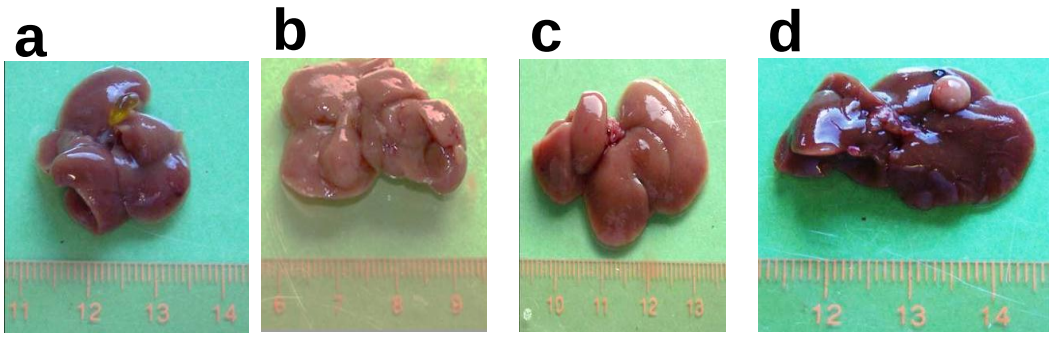

Fig. 2: Showed that liver treated with DEN show grossly liver tumor (d) while other group treated with both selenium and DEN when compared with other groups treated with both selenium and DEN (c), selenium treated group (b) or control group which appeared normally (a).

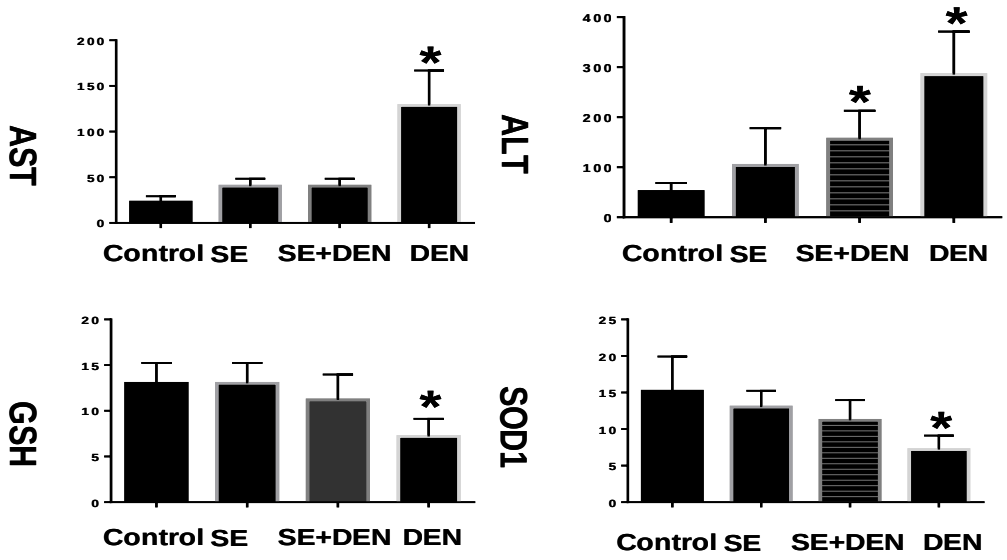

Fig. 3: Show that DEN treated group show significant increase of transaminase enzymes while reduced significantly both of antioxidant parameters. Selenium appears to slight increase of transaminase enzymes when compared with control. More over selenium maintain both SOD1 and total glutathione to basal level similar to control when treated with DEN.

Histopathology of liver treated with DEN showed increased activity of hepatocytes and progression to hepatocellular carcinoma with typical malignant criteria after 32 weeks of observation from DEN treatment. In contrast selenium maintain liver architecture with minimal hepatic dysplasia but only one mice out of 6 show dysplastic nodules and one small HCC. Group treated with selenium only show showing normal hepatocytes with normal hepatic architecture. While control group showed normal liver. Figure4

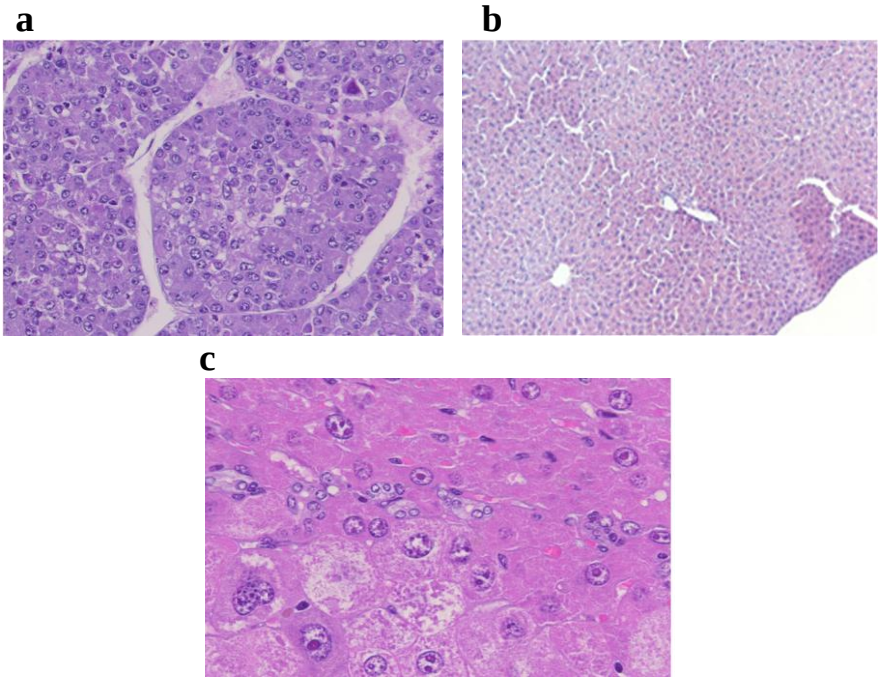

Fig. 4: (a) Group treated with DEN, Liver show progressive hepatocellular carcinoma when compared with control group which show normal liver architecture (b).While group treated with DEN +se show macrocytic dysplasia of hepatocytes indicated by enlarged nuclei with eosinophilic inclusions (c).

Notably DEN enhanced c-metm RNA expression loss while selenium and DEN+ SE groups maintain hepatocyte growth factor receptor (c-met) expression similar to control group. While B-actin show no change in all groups as b-actin consider house-keeping gene figure (5).

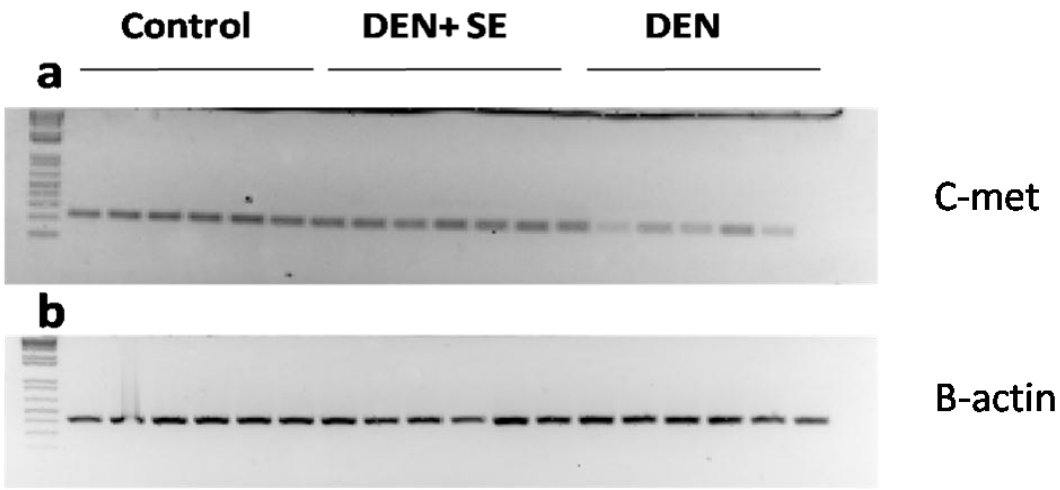

Fig. 5: DEN enhanced c-met gene expression loss (a) while selenium and DEN+ SE groups maintain c-met gene expression similar to control group. While B-actin show no change in all groups as b-actin consider house-keeping gene (b)

DISCUSSION

As shown by this study, selenium maintain, SOD1 and total glutathione was similar to control, redox microenvironment when treated with DEN. In other word selenium made its tumor suppressive power through modulation of the tumor redox microenvironment. SOD1 is the most important antioxidant enzyme that protects cells from ROS such as hydrogen peroxide and singlet oxygen species (Mates *et al.,* 1999 and Sinha and Mimnaugh, 1990). ROS have the potential to create oxidative stress within cells that causes DNA damage, protein degradation, peroxidation of lipids, and finally leads to cell transformation or death based on ROS concentration. It is a well-documented fact that cancer cells are under high levels of oxidative stress compared with normal cells (Simon *et al.,* 2000, Schumacker 2006 and Mizutani H., 2007).

Notably, selenium maintain hepatocyte growth factor rector (c-met) in mice treated with both selenium and DEN while DEN treated mice shown loss of c-met signaling. C-met reported to maintain liver body mass constant in c-met truncated transgenic mice (Amicone *et al.,* 2002). Moreover, Takami *et al.* (2007) found that loss of c-met enhanced liver tumor growth in conditional knockout transgenic mice while these mice when treated with N-acetyl-cysteine, slowed tumor growth initiated by n-nitrosodiethylamine in c-met deficient liver. At 3 months, the averages size of focal lesions in Metliv KO mice was reduced by 3 fold and reach level found in Cre-ctrl mice when treated with N-acetyl-cysteine. More over N-acetyl-cysteine retain c-metreceptor in treated mice.

In other words, there are correlations between c-met, redox microenvironment and anti-tumor effect of selenium. As most of the anticancer agents kill tumor cells by generating ROS or amplifying oxidative stress. We concluded that c-met expression and maintain redox microenvironment activity could elevate tumor chemo-sensitivity.

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السيلنيوم العضوي له دور فعال في منع وتاخير سرطان الكبد الناتج عن ماده الداي اثيل نيتروزامين في الجرزان البيضاء

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ان ماده الداي اثيل نيتروزامين لها دور مساعد في سرعه نمو سرطان الكبد من خلال فقدان عنصر النمو الكبدي ولكن السيلنيوم العضوي له دور فعال في تاخير ومنع تحول الخلايا الكبديه الي خلايا سرطانيه من خلال المحافظه علي عنصر النمو الكبدي. وايضا لوحظ ان السيلنيوم العضوي له تاثير فعال في الحفاظ علي انزيمات الأكسده والتي تظهر فقدان سريع في المجموعه المعامله بماده الداي اثيل نيتروزامين وايضا حافظ السيلنيوم العضوى علي انزيمات الكبد بمستويات طبيعيه بالمقارنه بالمجموعه المعالجه ماده الداي اثيل نيتروزامين بصوره مفرده. اما من ناحيه الفحص الهسيتوباثولوجي قد وجد ان المجموعه المعالجه لداي اثيل نيتروزامين شوهد بها نمو سرطاني علي سطح الكبد ولمن المجموعه المعالجه بماده لداي اثيل نيتروزامين مع لسيلنيوم العضوي لم تظهر اي تغيرات باثولوجيه علي سطح الكبد وتم التأكد من ذلك بالفحص الباثولوجي. من هنا نستنتج ان السيلنيوم العضوي له دور فعال في تاخير و منع تحول الخلايا الكبديه الي خلايا سرطانيه.