

## **CASPASE-3 and FAS-L expression and their roles as signal transduction in hepatocellular carcinoma**

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### **ABSTRACT**

**H**epatocellular carcinoma (HCC) is a common malignant affecting approximately-one million of people around the world every year. This study aimed to early recognition of the carcinogenic effect of *N*-nitrosodiethylamine (NDEA) on hepatocytes and expression of Fas-L and Caspase-3. The study was done using tissue homogenate of 60 male albino rats divided into 2 groups: group 1, control group (30 rats) and Group 2, *N*-nitrosodiethylamine (NDEA) treated group (NDEA, in a single dose 200 mg/Kg wt and; Carbon tetrachloride (CCL<sub>4</sub>), twice per week 3 ml/Kg wt) (30 rats). 10 rats from each control and treated group were sacrificed at the 1<sup>st</sup>, 2<sup>n</sup>, and 3<sup>rd</sup> months of the treatment for evaluation (1) of glutathione s transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GRase), glutathione reduced form (GSH), superoxide dismutase (SOD) levels, (2) studying expression of Fas-L and Caspase-3 and (3) for histopathological examination. The results revealed that the treated group antioxidant enzymes levels (Glutathione family and SOD) showed a significant decrease with gradual rates through the three months of the treated compared to the control group, whereas the GRase level was significantly increased compared to the control group. Caspase-3 and Fas-L in liver tissue homogenate in the treated group was significantly decreased in comparison with the control group. The histopathological results showed pronounced changes with evident fatty degeneration (steatosis), marked microvascular steatosis with malignant hepatocytes amounting grade 1. Finally, study can conclude that investigation of liver tissue markers induced by the chemical carcinogen NDEA at different stages of the development of cancer could help in getting more information about the development of HCC and more investigated studies on Caspase-3 and Fas-L, could be a promising way for the treatment of HCC.

**Key words:** CASPASE-3 - FAS-L expression- hepatocellular carcinoma

## **INTRODUCTION**

Hepatocellular carcinoma (HCC) is a common malignancy affecting approximately one million of people around the world every year. It represents the fifth most common cancer worldwide with an incidence equal to the death rate **(Marrero and Pelletier, 2006)**. The incidence of HCC is low in the occidental world and high in Southeast Asia and sub-Saharan Africa. However, it has been rising during the last two decades in Europe, United States and Japan **(Motola, et al, 2006)**. HCC primarily affects old people, reaching its highest prevalence among those aged 65 to 69 years old. Chronic infection by viral B hepatitis HBV is the most common cause of this neoplasm. Other important causes are cirrhosis, chronic viral hepatitis (hepatitis C virus, and hepatitis B plus D viruses), alcohol abuse, obesity and toxins. The nitrosamine and various other N-nitroso compounds, form a large group of typically genotoxic carcinogens **(Magee and Barnes 1967; Preussman et al, 1984)**. Nitrosamines have been

found to be carcinogenic in over 40 animal species and one or more of the compounds has induced tumors in almost every organ in rodents. The carcinogenic N-nitrosodiethylamine (NDEA), one of the previous compounds, is one of the main point of interest in this study. The second point of the interest is the expression of Fas-L and caspase-3. The Fas-Fas ligand (Fas-L) system is recognized as a major pathway for the induction of apoptosis in cells and tissues **(Nagata, 1997)**. Fas-L has recently been detected in many types of cancer, including astrocytoma's **(Saaa et al., 1997)**, colon cancer **(Oconnell et al, 1996)**, basal cell carcinomas **(Arai et al, 1997)**, melanoma **(Hahne et al, 1996)**, and lung cancer **(Nehans et al, 1997)**. Tumor necrosis factor (TNF) - related apoptosis - inducing ligand (TRAIL) is known as a major mediator of acquired immune tumor surveillance, and is currently being tested in clinical trials as a novel cancer therapy **(Mucha et al, 2009)**.

Many caspase substrates have been identified, only a few have been clearly demonstrated to play defined roles in the apoptotic process. Fas-L ligates to its receptor, Fas, resulting in upstream activation of caspase-8, which may intern mediate cytochrome-c release. Cytochrome-c release may also function as an amplification loop to potentiate the effects of caspase-3.

One approach that has been promising results in many tumors is immunotherapy (**Greten and Jaffee, 1999**). Also in HCC, several different studies suggest that immunotherapeutic approaches will be successful for the treatment of this disease. So, aim of work was to study expression of Fas-L and caspase-3 and their roles with antioxidant enzymes.

## **Materials and Methods**

### **Materials:**

- **Chemicals.** N-nitrosoamine, carbon tetra chloride, 5, 5 – dithiobis (2-nitrobenzoic acid) (DTNB), phenazine methosulphate (PMS),

Nicotineamide adenine dinucleotid phosphate hydrogen (NADPH), glutathione oxidized form (GSSG), glutathione reduced (GSH), ethylene diamine tetraacetic acid (EDTA), formalin, potassium phosphate, sulfosulcylic acid were obtained from Sigma, Aldrich.

- **Kits,** for caspase provided from BioSourceinternational, Ine, USA, caspase3/Cpp32, and for Fas-L ligand from Diaclone, USR, UKAS (producer No .043-A).
- **The animals** were obtained from National Cancer Institute, Cairo University.
- **Diet,** The standard diet prepared as described by **Reeves et al. (1993),**

### **Methods:**

The experiment was designed on 60 waster albino male rats weighing 165 ±15g. The rats were divided into 2 groups (30 rats each). Group 1- normal healthy control (30

rats), and group 2- N-nitrosodiethylamine (NDEA) treated group (30 rats), 200 mg/Kg wt NDEA was injected intraperitonean as a single dose then after two weeks the rats received 3 ml / Kg wt (CCI4) in oil twice per week until the end. The animals were housed in cages. All were fed with a normal standard, diet and tap water during the total period of the experiment.

***Liver tissues preparation,***

A small piece of each liver (normal and treated) was stored in 10% neutral formaline for 24 hours, for histopathological examination. Tissue specimens were washed in running tap water for 15 hours, dehydrated in standard alcoholic series 50, 70, and 95%, and then cleared in xylene before embedding in paraffin wax. Samples were then sectioned 4-6 micron thickness and stained with the usual haematoxylin and eosin method (Scheuer and Chalk, 1986). Half of gram of each liver (normal and treated) was dissected, washed with saline and homogenates in 2 ml of 0.1M phosphate buffer pH7.4

of the experiment. 10 rats from each control and treated group were sacrificed under anesthesia after the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> months of the treatment for the biochemical, molecular and histopathological studies.

for all biochemical assays except assays for reduced glutathione, the liver tissues were homogenated in 1ml 0.1M phosphate buffer pH 7.4 plus 1ml (4%) sulfosalicylic acid to precipitate the proteins. The prepared homogenates for all assays, were kept at -20C° until used.

**Glutathione reduced (GSH)** and **GST** were measured according to (Jakoby, 1985), **Glutathione peroxidase (GPx)** was estimated according to (Paglia and Valentine, 1976), the Specific activity of GPx was expressed in units per milligram of protein and the standard curve protein concentration was determined as described by Bradford (1976), **Glutathione Reductase (GRase)** was measured as described by Carberg and Mannervick (1985), **Superoxide dismutase**

(SOD) was assayed as **Dwivedi and Partap, (1986)**.

#### **Caspase assay**

Caspase -3 was made using the DEVD Asparate- glutamate- valine- aspartate (substrate of

caspase) kit were determined by **Casciolo et al, (1996)**, Fas/ Fas ligand was determined by Fas-L kit according to **Talanía et al, (1997)**.

### **Results & discussion**

#### ***The antioxidant enzymes***

**GSH, GST, and Gpx mean  $\pm$  S.D** levels showed a significant decrease in all treated group through period compared with their control mean values, (table .1). GSH showed a nonsignificant decrease in first month, but the second and third months showed decrease in its levels table .1.

As it was known, the first phase of chemical carcinogenesis are often characterized by intracellular production of superoxide anion radicals or peroxides ( **Little et al , 1983;Oberly et al ,1984**), which are free radicals or generate of free radicals. It was known that these free radicals were responsible of neoplastic phenotype damage DNA (**Emerit and Cerutti, 1981; Birnbiom, 1982**). The nitrosoamines, the carcinogen

agent, in this study has a great effect on super family microtonal mixed function oxidizes (**Swenberg et al, 1991**), and this was observed in most antioxidant enzymes measured. GSH depletion results was confirmed by **Dhanasekaran et al (2008)**, who concluded that the animals treated with NDEA showed a decrease in activity of detoxication enzyme as GSH and other liver markers. These results reflected the disturbance in function of hepatocytes was duo to decrease in GSH especially in the last stages of treatment. This agreed with a recent study by **Jakovljevic et al, (2009)**.

The reduced level of GST using NEDA as hepatocarcinogen and CCL<sub>4</sub> as a promoter agreed with results study of **Nishimura et al, (2008)**, who used fenofibrate to induce hepatocarcinogenesis in rats after NDEA initiation.

Also **Subramanian et al, (2008)** confirmed the decrease in GST level. On the other hand, study by **Wang et al, (2009)** who used high fat -diet as promoter for NDEA in hepatocarcinogen in rats ,found that GST level was elevated ,this may indicate a specific pathway for high fat diet to increase GST level . However this point must be discussed in depth to get full information about GST level and initiation of hepatocarcinogenesis.

G Px activity showed a significant decrease by nearly constant rate through all the three months period of treatment, this was supported by finding of previous studies on NDEA on hepatocarcinogenesis made by **Ramakrishnan et al, (2006)**. Also, **Dhanansekaran et al, (2008)**, and **Jakovljevic, et al (2009)**, confirmed GPx ranged level from promoter or inhibition for NDEA and study its effect on liver tumor will be promising as new biomarker .

In contrast to other glutathione family GRase mean  $\pm$ S.D level showed a significant increase in all treated group

(table .2.), this was supported by **Jakovljevic et al, (2009)** who concluded that GRase activity increased in NDEA induced hepatomas, this significant increase of GRase may be directly correlated to high GRase protein content of cancer cell as bovine leukemia virus transformed fibroblast, there for disequilibrium of delicate oxidant versus antioxidant balance moved towards an oxidative side as partly demonstrated by dramatic loss of glutathione synthesize (GS) activity.

Liver SOD mean level showed a non-significant change, only a slight decrease observed in last stage of experiment table.2.

The correlation study between antioxidant enzymes, showed a negative correlation between glutathione -S transferase and SOD( $r = -0.646$ ,  $p = 0.05$ ) Fig.1. Also, a negative correlation between glutathione -S-transferase and glutathione reductase ( $r = -0.792$   $P = 0.01$ ) Fig.2., also SOD was correlated negatively with GPx with  $P = 0.05$ .

***Fas- L and caspase expression:***

Fas-L mean level, which was determined in the three month varied between a nonsignificant change in early stage of treatment to a significant decrease in liver Fas-L in last stages of treatment (P values 0.7, 0.03 and 0.009 respectively) table.2.

Caspase activity was determined using three different concentration of liver tissue homogenate of each animal (25 ug, 50 ug, 100ug protein) to determine caspase activity during the period of the treatment using the average OD values of caspase -3 at three different protein concentration, data curve in Fig.3. ,represented the expression of caspase -3 during the three months of treatment , the curve showed down expression of caspase -3

Furthermore, it was proved that the increased serum soluble Fas (s Fas) in HCV patients is accompanied by down — regulation of Fas / Fas L expression resulting in inhibition of apoptosis in liver cells (Xia et al, 2001). However, other study emphasized that Fas in

when compared to the normal control group.

Human hepatocellular carcinoma (HCC) appeared to be strongly associated with apoptosis and its breakdown may be involved in the occurrence of HCC. Like Fas /Fas L, the tumor necrosis factor (TNF) -related apoptosis -inducing ligand (TRAIL) transduces apoptosis in a number of cancers; it is also candidate for cancer therapy (Yano et al, 2003). In this study the dramatically decreased level in both Fas / Fas L and caspase -3 at the end of the treatment, were confirmed by the previous study of Lee, et al (2001), who demonstrated that all HCCs showed one or more alterations of the Fas pathway molecules known to inhibit Fas mediated apoptosis.

hepatocytes showed strong expression and Fas-L also showed intensive expression in infiltrating lymphocytes and scattering hepatocytes, so the apoptosis caused by Fas might be one of the important pathogenesis of hepatitis gravis. Also, the decrease in caspase -3 expression was

confirmed by **Yano et al (2003)**, who concluded that caspase -3 activity and TRAIL- R1, R2 expression in tumor tissues were significantly lower than those in non-tumor tissue in HCB — related HCC, however, some HCV-related HCC cases, demonstrated elevated caspase -3 activity and TRAIL — R1, R2 expression in tumor tissues. In this study a significant positive correlation between Fas-L and both GST and SOD enzymes mean level was found ( $r = 0.912$ , P values 0.001 and 0.01 respectively) Fig.4

The decrease in caspase-3, may contribute to cirrhotic nodules related to HCC that appeared at the third month of the treatment. This was in agreement with **Caillot, et al (2009)**, they reported that tumor suppression activated pathway-6 (TSAP6) that transcript codes for a transmembrane molecule which is an inducer of a caspase -3 dependent apoptotic pathways, could reflect a decrease in the apoptotic process. Whereas **Lu, et al (2008)** found that the increased expression of caspase-3 in the transfected Hep G2 cells provided

feasible evolution of the treatment of the primary liver cancer. Moreover, activation of caspase -3 and subsequent apoptosis by 5-fluorouracil (5 -FU) and Andrographolide (ANDRO) which is a natural bicyclic diterpenoid lactone that was isolated from *Andrographis paniculata*, and has been shown to suppress the growth of HCC cells and to trigger apoptosis *in vitro*. This might be effective in the treatment of HCC cells SMMC-7721. In present study the combination of CC1<sub>4</sub> and NDEA induced HCC *via* the decrease level of the antioxidant enzymes and inhibition of caspase -3. Also **Takashima, et al (2008)**, had studied the mechanism of di-(2-ethylhexyl)- phthalate (DEHP) for inducing hepatocarcinogenes is *via* suppression "of G2/ M arrest that was regulated by Gadd45a and caspase 3- dependent apoptosis in peroxisome proliferator activated receptor alpha (Ppara) null mice. However these genes may not be involved in tumorigenesis in the wild type mice.

#### ***Histopathological results:***

The histopathological results for the control group (C) showed



normal pattern of hepatocytic liver architecture (Fig 5).The histopathological results for treated groups showed Cirrhotic nodules and moderate portal chronic inflammatory infiltrate with interface hepatitis was shown after first and second treatment months (Fig.6). After the third month the histopathology further showed pronounced changes with evident fatty degeneration (steatosis), marked microvascular steatosis with malignant hepatocyte amounting grade 1 HCC, (Fig.7).

## CONCLUSION:

Finally, we can conclude that investigation of liver tissue markers by chemical carcinogen NDEA at different stages of the development of cancer could help in getting more information about the development of HCC. More immunotherapy of studies on Caspase-3 and Fas-L, could be a promising way for the treatment of HCC.

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**Table 1:the mean ± S.D. values for G SH, GST, in all period of the treatment**

Group N (10)	Mean ± S.D , ± S.E , P values								
	1 <sup>st</sup> month			2 <sup>nd</sup> month			3 <sup>rd</sup> month		
	Control Group C <sub>1</sub>	Treated Group T <sub>1</sub>	P value	Contro 1 Group C <sub>2</sub>	Treated Group T <sub>2</sub>	P value	Control Group C <sub>3</sub>	Treated Group T <sub>3</sub>	P value
GSH mg/g tissue	2.377± 0.79	2.426± 1.16	0.913	2.506± 0.71	1.574± 0.67	0.008	2.36± 0.89	1.53± 0.44	0.016
*P, **P					*p =0.048			*p =0.034	
GST Nmol/min/mg	48.8± 11.87	37± 15.68	0.07	42.8± 13.0	14.2± 9.22	P<0.001	42.2± 12.1	15.4± 11.3	<0.001
*P, **P					*p<0.001		*p=0.002	**p =0.7	
GPX Nmol/min/mg	67.16± 37.2	50.81± 17.1	0.06	83.92± 26.2	51.53± 15.1	0.003	77.87± 30.8	51.5± 17.4	0.03
*P, **P					*p =0.9		*p =0.9	**p =0.9	

C:control animals, T: treated animals,1,2,3 are the period of scarifying animals /months

P≤0.05 was considering significant,P for value versus control, \*p for versus between T1,T2,

\*\*p for versus between T2,T3.

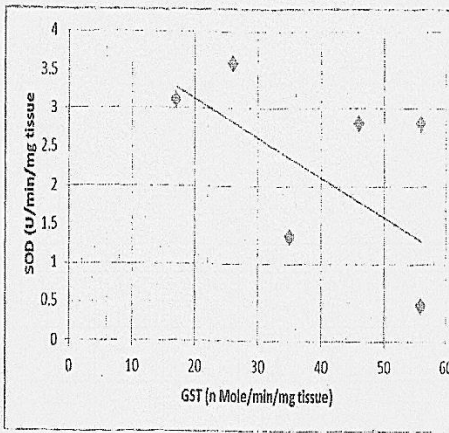
**Table 2: the mean ± S.D. values for GRase,SOD, Fas-L after all the three months treated period**

Group N (10)	Mean ± S.D , ± S.E , P values								
	1 <sup>st</sup> month			2 <sup>nd</sup> month			3 <sup>rd</sup> month		
	Control Group C <sub>1</sub>	Treated Group T <sub>1</sub>	P value	Control Group C <sub>2</sub>	Treated Group T <sub>2</sub>	P value	Control Group C <sub>3</sub>	Treated Group T <sub>3</sub>	P value
GRase Nmol/min/mg	22.05± 9.52	67.86± 35.64	P<0.001	21.55± 9.22	85.38± 24.5	P<0.001	24.61± 9.8	44.78± 19.4	P= 0.008
<i>*P, **P</i>					<i>*p</i> =0.2		<i>*p</i> =0.08	<i>**p</i> <0.001	
SOD Nmol/min/mg	2.606± 1.8	2.264± 1.23	P=0.6	3.529± 1.45	2.826± 1.53	P=0.3	3.064± 1.88	1.504± 0.96	P= 0.03
<i>*P, **P</i>					<i>*p</i> =0.3		<i>*p</i> =0.1	<i>**p</i> =0.03	
Fas-L	1.147±0. 52	1.08±0. 47	P=0.7	1.214± 0.52	0.728± 0.33	P=0.01	1.075± 0.33	0.609± 0.195	P= 0.0011
<i>*P, **P</i>					<i>*p</i> =0.039		<i>*p</i> =0.0091	<i>**p</i> =0.158	

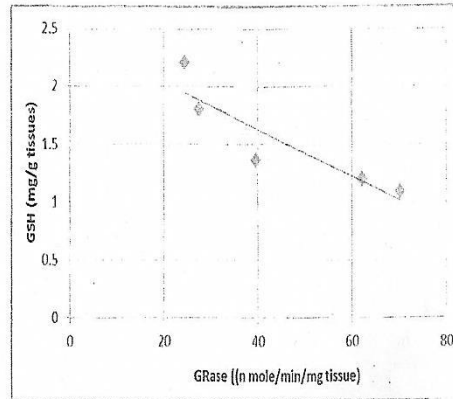
C:control animals, T: treated animals,1,2,3 are the period of scarifying animals /months

P≤0.05 was considering significant,P for value versus control, *\*p* for versus between T1,T2,

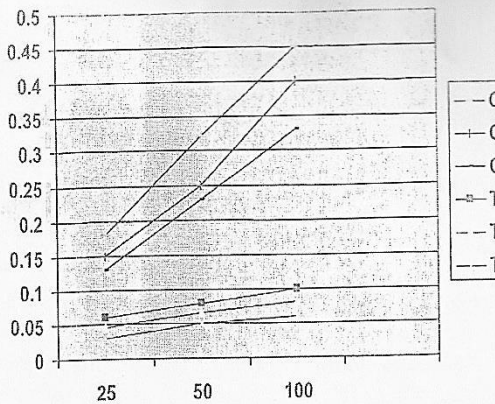
*\*\*p* for versus between T2,T3.



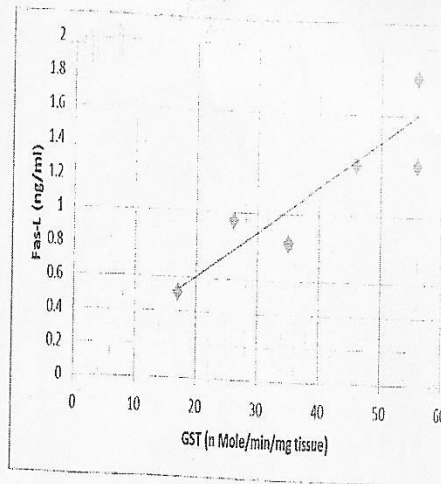
**Fig (1) :** negative correlation between mean value of rate liver tissue homogenate SOD and GST in the treated group after the first month



**Fig (2) :** negative correlation between mean value of rate liver tissue homogenate GRase and GSH after third month



**Fig (3) :** shows the inhibited caspase in all treated groups C1,C2,C3 control gps of the three months; T1,T2,T3 treated gps of the three months



**Fig.4:** positive correlation between mean value of rat liver tissue homogenate Fas-L and GST after first month treated.



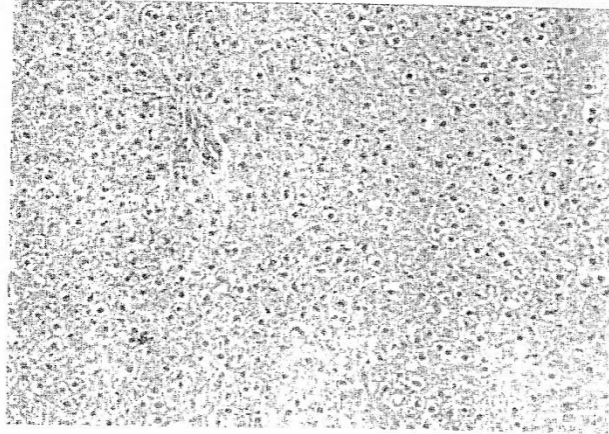


Fig .5: a photograph of control group of liver (H& E 100). Showed normal architecture pattern of liver cells

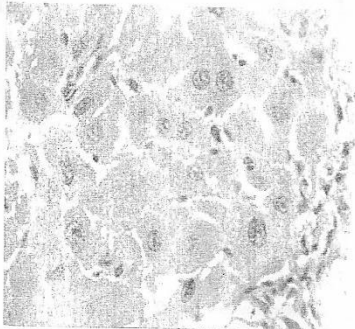


Fig.6: a photograph of treated group at second month represented by fatty degenerate hepatitis grade 11 no evidence of malignancy or cirrhosis.

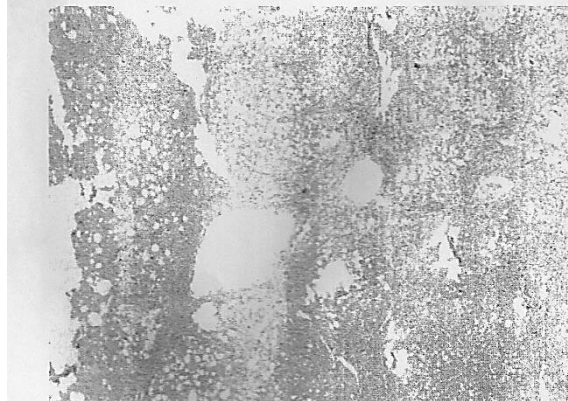


Fig.7: a photograph of the third month treated of rat liver homogenate (H&E 100). The normal hepatic architecture is replaced totally by cirrhotic nodules with intervening chronic inflammatory infiltrate grade 111, some foci at the peripheral show invasive trabecula of moderately differentiating hepatocytes amounting grade 1 HCC.

## تقدير بروتينات الفاس و الكسباز ودورهما في نقل الاشارة المتسلسلة في خلايا الكبد المسرطنة باستخدام حيوانات التجارب

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### الملخص العربي

يعتبر سرطان الكبد من اكثر السرطانات انتشارا تبلغ نسبة انتشاره بين السرطانات الخامس. تهدف الدراسة الي ابراز و توضيح تاثير المركب الكيميائي النترازامين (NDEA) علي خلايا الكبد و مستمي البروتينات المسؤولة عن عملية الانتحار المبرمج-FAS-L. CASPASE-3. هذه الدراسة اجريت باستخدام ذكور حيوانات التجارب الجرذان البيضاء و قد قسمت هذه الجرذان الي مجموعتين: المجموعة الاولى حققت ب NDEA مرة واحدة ٢٠٠جم|كجم وبعد اسبوعين من الحقن كل مجموعة اخذت جرعتين اسبوعيا من CCL4. ذبحت ١٠ جرذان بعد الشهر الاول من العلاج. ذبحت ١٠ جرذان بعد الشهر الثاني من العلاج ذبحت. ١٠ جرذان بعد الشهر الثالث. المجموعة الثانية . مجموعة ضابطة تضم حيوانات تجارب صحيحة و تناولت هذه المجموعة زيت فقط وذبحت في نفس الوقت من المجموعات المعالجة . تم دراسة العوامل الاتية: الجلوتاثيون بجمع صوره جلوتاثيون بيروواكسيديز, اس ترانسفيراز, سوير اكسيدديز ميوتيز), بالاضافة الي تقدير بروتينات الفاس و الكسباز في خلايا الكبد و فحص شرائح كبد الجرذان الضابطة و المعالجة. ووضحت نتائج هذه الدراسة نقص في مستوي الانزيمات المضادة للاكسدة خلال الثلاث شهور فترة اجراء التجربة في كل المجموعات المعالجة بالمقارنة مع مجموعات الضابطة علي العكس من ذلك اوجدت هذه الدراسة زيادة في مستوي انزيم جلوتاثيون المختزل في كل من المجموعة المعالجة مقارنة مع مجموعة الضابطة. نتائج مستوي 3- CASPASE أظهرت نقص في مستوي البروتين في كل من المجموعة المعالجة بالنسبة إلي مجموعة الضابطة. مستوي FAS-L اظهر نقص في كل المجموعة المعالجة بالمقارنة مع مجموعة الضابطة. النقص في كل من بروتينات الفاس و الكسباز يعكس اهمية دور كل منهم في نمو سرطان من خلال نقص مستوي الانزيمات المضادة للاكسدة و بروتينات عملية الانتحار المبرمج. نتائج فحص شرائح الكبد أظهرت تغير ملحوظ في خلايا الكبد مع ظهور سرطان الكبد من الدرجة الاولى. وقد استنتجنا من هذه الدراسة ان دراسة العوامل المؤثرة في نمو سرطان الكبد نتيجة تاثير المركب الكيميائي NDEA في مراحل مختلفة من نمو تساعد في دراسة تطور سرطان الكبد وايضا الدراسة المستقبضية علي بروتينات عملية الانتحار المبرمج وخاصة الفاس و الكسباز مما قد يؤدي الي طرق جديدة في العلاج.

**الكلمات المفتاحية:** بروتينات الفاس و الكسباز- سرطان الكبد