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Effect of gasoline inhalation on tumor necrosis factor-alpha (TNF- α) expression in liver of rats

Bushra Jasim Mohammed

Institute of Genetic Engineering and Biotechnology -University of Baghdad, Iraq

*Correspondence: bbushra880@gmail.com Accepted: 13July 2017 Published online: 30 Sep. 2017

Tumor Necrosis Factor-alpha (TNF- α) is essential proinflammatory cytokines which contributes to wide range of vital activities in the body. Current study was planned to investigate the harm effects of gasoline on TNF- α expression in liver of rats. Subjects and Methods: Rats were employed as an experimental model, twenty rats divided randomly and equally into four groups; each contains five. Rats of work groups were exposed to gasoline vapor 3 hours a day for two, three and four weeks by using inhalation chamber. TNF- α expression was estimated in liver of rats by immunohistochemistry and real-time RT-PCR. Results: The results revealed that, the expression of TNF- α was elevated in all work groups comparing with control group, moreover the highest value was observed at group with exposure period for 4 weeks as compared with others work groups. Regarding to the immunohistochemical results, it showed a graded intensity of liver tissue staining, beginning from negative staining in the control group, through weak, medium and strong staining depending on the inhalation intervals two, three and four weeks, respectively in work groups. Furthermore the results of real-time RT-PCR. showed increasing of the TNF mRNA expression in livers of all work's groups in comparison with control group and the highest level was noticed in livers of group 4 with four weeks gasoline inhalation (14.68 fold) with significant difference ($P \leq 0.01$) as compared with others work groups. The data revealed that gasoline inhalation had a significant effect on TNF- α expression in rat's livers either in immunohistochemistry or real-time RT-PCR assays.

Keywords: Immunohistochemistry, gasoline, RT-PCR. TNF- α ., liver.

INTRODUCTION

Gasoline is one of the main world environmental and occupational pollutants which causes a serious health threat (Tunsaringkarn et al. 2012). Its mixture of several hundred of hydrocarbons and aromatic compounds as benzene, toluene, ethyl benzene and xylene (The Agency for Toxic Substances and Disease Registry -ATSDR, 1995). Given its health importance many previous studies have been investigated its toxicological effects (Poklis, 1976 and Boeckx et al. 1977). Exposure to gasoline leads to damage a numerous cells, body organs especially the liver (Domej, 2007), its can affects innate and adaptive

immunity (Reese and Kimbrough,1993), as well as it activates macrophages and some other immune cells to secrete inflammatory cytokines such as Tumor necrosis factor-alpha (TNF- α) (Bradham et al. 1998), which is an important multifunctional cytokine produced by several immune cells as macrophages, T-lymphocytes and some other cells like fibroblasts and keratinocytes (Parameswaran and Patial,2010), TNF- α has a broad range of biological activates to protect from infection and tumors (Wang et al. 2008). Also it contributes in various essential and vital events which are implicated in many critical physiological and pathological states in many

organs, especially liver, such as proliferation, inflammation and programmed cell death or so-called apoptosis (McGill et al. 2010 and Mohammed et al. 2016). The role of TNF- α in liver damage has been investigated in several workers (Wang, 2014 and Wang, 2015), furthermore many of these works have been done to study the inflammatory responses of gasoline inhalation in laboratory animals and humans worldwide (ATSDR,1995), that's what encouraged us to conduct such a study, which examined the effect of gasoline inhalation on TNF- α expression in livers by immunohistochemistry and real-time RT-PCR in rats as an experimental model.

MATERIALS AND METHODS

Twenty albino male rats of eighty to twelve weeks age at weights ranged between (250-300 g) were purchased from controlled and Pharmaceutical Research center/ Baghdad, hosted and dieted according to (Vodopich and Moore,1992). They were fed a basal diet (standard rodent pellets) and drunk a sterile water. The rats were placed in plastic cages of (90 x 50 x 30 cm) size, in groups of five rats per cage, with floors covered by soft sawdust. The cages were kept clean at temperature (20-25°C).

Rats were divided randomly into four groups each contain 5 rats as following:-

Group 1: Control group.

Group 2: Rats were exposed to gasoline vapor 3hours/day for 2 week.

Group 3: Rats were exposed to gasoline vapor 3hours/day for 3 weeks.

Group 4: Rats were exposed to gasoline vapor 3hours/day for 4 weeks.

Iraqi leaded gasoline (Octane 80-83) containing tetraethyl lead was purchased from a filling station in Baghdad.

Rats were exposed to gasoline vapor according to (Elsayed, 2015) by placed them in gasoline inhalation chamber of (100 x 100 x 100 cm) size which locally made and designed specifically to fit this experience, the chamber provided with wire network to separate the rats from the containers contain 200 ml of gasoline which evaporate 50 ml /hour. At the end of last day of the experiment, rats were anesthetized with an IM injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) (Sigma/USA) according to (Rozaini et al. 2004). Then the rats were sacrificed, livers removed and divided into two parts the first to estimate TNF- α expressions by real-time RT-PCR, the second fixed in (10%) formaldehyde (Sigma/USA) for (24 hr), then

subjected to immunohistochemistry examination according to (Lynch et al.1969).

Immunohistochemistry examination

Livers were fixed in (10%) formaldehyde. The preparation of paraffin blocks and deparaffinize carried out according to (Lillie, 1965). Immunohistochemistry examination were done by using anti-TNF - α antibody and DAB Chromogen (abcam/USA) depending on (Cuello,1993) to detect TNF- α expression in liver's tissues.

Real-time RT-PCR

Total RNA was isolated with TRIzol reagent (Thermo fisher/USA) from rest liver tissue, using RNA purification kit (Bioneer/ korea) according to instructions of manufacturer company, RNA pellet was dissolved in 25 μ l of molecular biology grade water (Sigma/USA), then the tubes put in ice. The RNA concentration and purity were carried out by using Nanodrop(BioNeer /Korea), according to (Sambrook et al. 1989). The RNA/primer mixture (Thermo fisher/USA) was done according to (Wang and Seed,2003) by adding RNA to random hexamers, dNTP and DEPC H₂O, then incubated at 65°C for 5 min and on ice for 1 min, after that mix briefly with reaction mixture (Thermo fisher/USA) contains MgCl₂, DTT, RNAase and RT buffer, put at (18-25°C) for 2 min, mixed with SuperScript II Reverse Transcriptase (Thermo fisher/USA) and incubated at 25°C for 10 min then the tubes were incubate for 50 min at 42°C and at 70°C for 15 min to heat inactivate, then on ice to chill, added RNase H and incubated at 37°C for 20 min, cDNA was kept at -20°C until use. Real-time PCR was done using SYBR Green Mix (2x) (Thermo fisher/USA) and primers were supplied by Alpha DNA company/ Canada, depending on NCBI and as mentioned by (Khan et al. 2013) (Table 1). Twenty five μ l of Real-time PCR mixture reactions containing components were illustrated in table (2) .The cycling parameters (Wang and Seed,2003) were one cycle of 50°C for 2 min , 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s and one cycle of 72°C 10 min, in real time PCR instrument (Thermo fisher/USA), using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene according to (Khan et al. 2013) A melting curve analysis was then performed

Table 1: Primer's sequences

Primers		Forward 5' - 3'
TNF- α	F primer	ACT GAA CTT CGG GGT GATTG
	R primer	GCT TGG TGG TTT GCT ACGAC
GAPDH	F primer	GACATGCCGCCTGGAGAAAC
	R primer	AGCCCAGGATGCCCTTTAGT

Table 2: PCR components

Component	Quantity (μ l)
cDNA	0.2 μ l
H ₂ O	11.3 μ l
Primer pair mix (5 pmol/ μ l)	1 μ l
SYBR Green Mix (2x)	12.5 μ l
Final volume	25 μ l

Statistical analysis

The Statistical Analysis System- SAS (SAS,2012) program was used to effect of difference factors in study parameters Least significant difference – LSD test (ANOVA) was used to significant compare between mean

RESULTS

The results showed significant changes in TNF- α expression among all gasoline exposure rats' groups when compared with control, and these levels varied association with the period of exposure. Regarding to immunohistochemical examination, the result revealed that TNF- α

protein was observed in the cytoplasm of hepatocytes and inflammatory cells, TNF- α protein accumulation was scored as follows: if staining was seen in only (0–1 %) of the cells the case was graded as negative staining, in (2–33 %) as weak or low-expression, in (33–79 %) as moderate and over (80 %) as strong according to (Zhang et al. 2014). Current results found that the TNF- α expression was seen in all rat's groups which exposure to gasoline and there was clear associations between TNF- α protein accumulation and the duration of gasoline exposure. The microscopic appearance of liver tissue sections which obtained from rats didn't exposed to gasoline inhalation as group (1) (control) had normal hepatocytes, central vein, kupffer cells, sinusoids and negative staining with DAB against counterstain H & E, which mean no detectable TNF- α protein expression as shown in figure (1).

Sections of liver tissues obtained from rats exposure to gasoline vapor (3hours/day) for two weeks as group (2), appeared with a weak staining which means low-expression of TNF- α as shown in figure (2).

A median staining shown in the liver sections of rats exposed to gasoline inhalation (3hours/day) for three weeks as group (3), indicated a moderate expression of TNF- α as shown in figure (3)

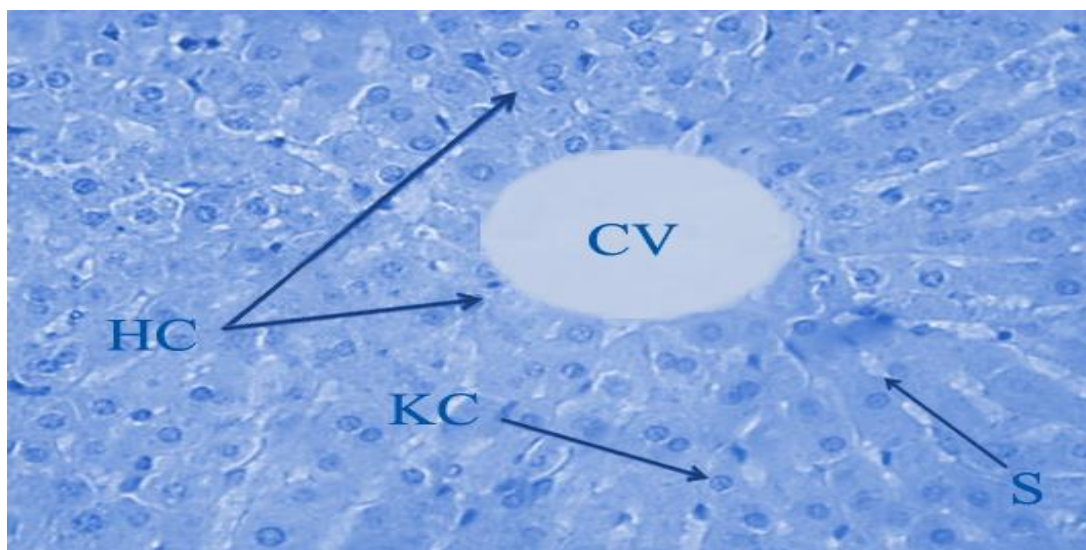


Figure 1: Rat liver tissue of group 1 (control) showing normal central vein (CV), hepatocytes (HC), kupffer cells (KC), sinusoids (S) and negative immunohistochemical localisation of TNF- α staining with DAB against counterstain H & E. (X400).

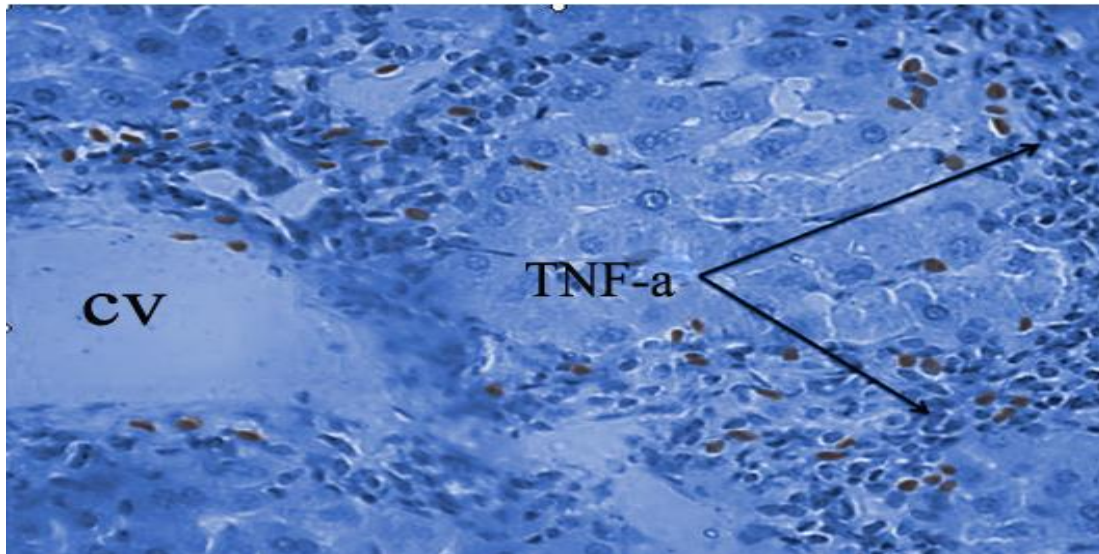


Figure 2: Rat liver tissue of group 2 (Exposure to gasoline inhalation 3 hours a day for 2 weeks) showing weak immunohistochemical localisation of TNF- α , staining with DAB against counterstain H & E. (X400).

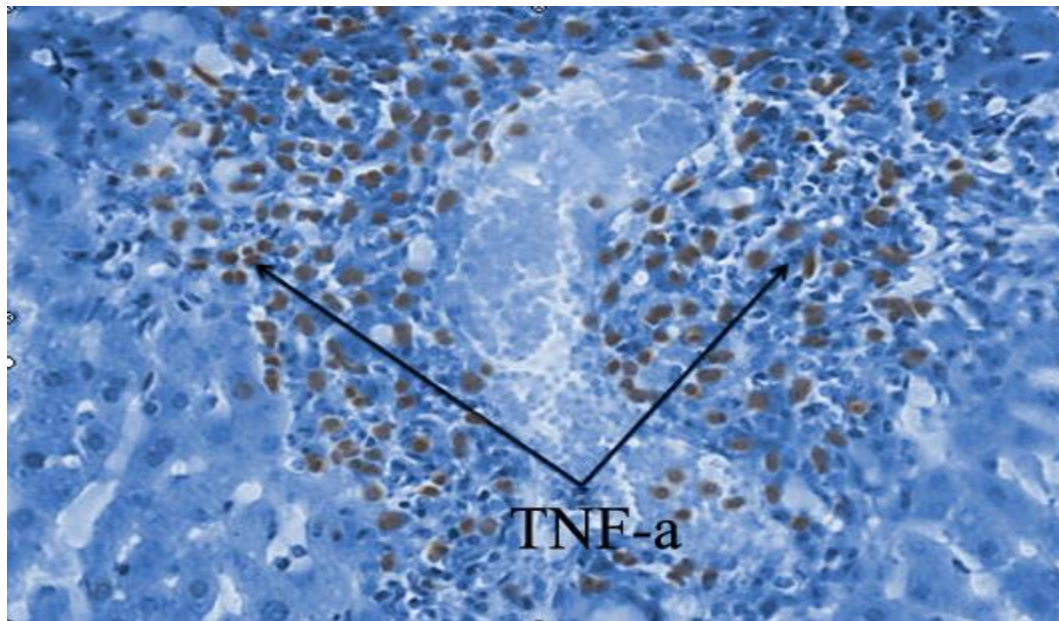


Figure 3: Rat liver tissue of group 3 (Exposure to gasoline inhalation 3 hours a day for 3 weeks) showing moderate immunohistochemical localisation of TNF- α , staining with DAB against counterstain H & E. (X400)..

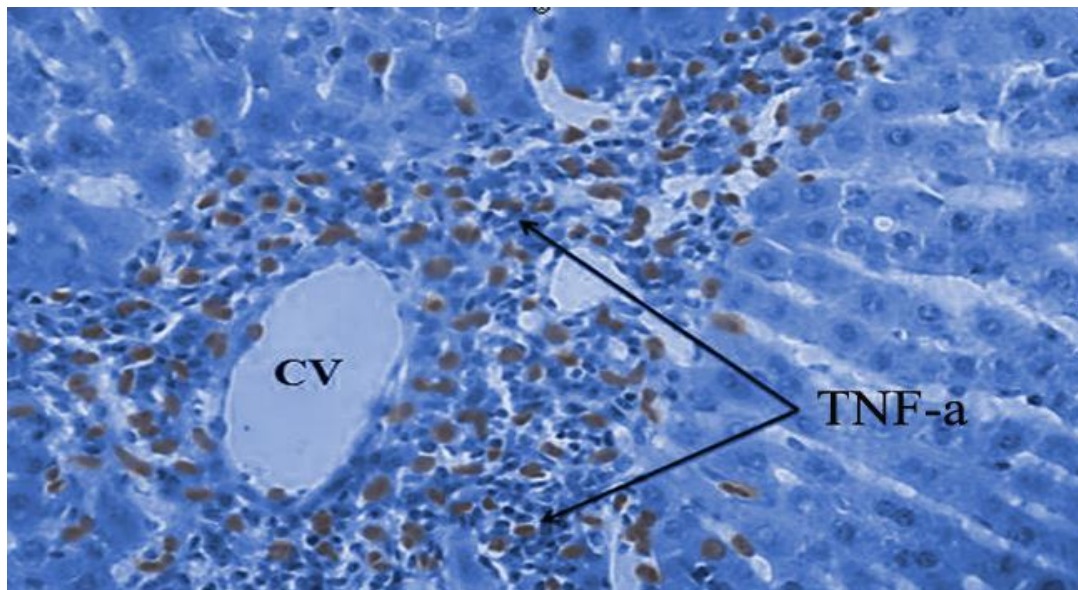
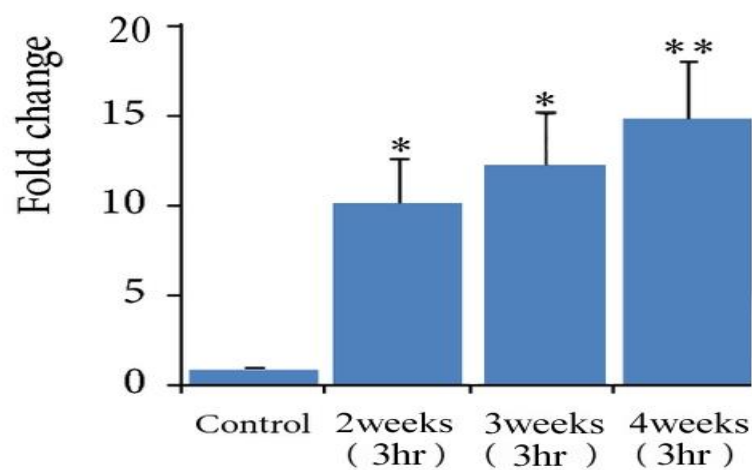


Figure 4: Rat liver tissue of group 4 (Exposure to gasoline inhalation for 3 hours a day for 4 weeks) showing strong immunohistochemical localisation of TNF- α , staining with DAB against counterstain H & E. (X400)



* ($P < 0.05$), ** ($P < 0.01$).

Figure 5: The effect of gasoline inhalation (3 hours a day for 2, 3 and 4 weeks) on TNF- α expression in liver of rats measured by real-time RT-PCR.

A strong staining was noticed in liver sections of rats exposure to gasoline inhalation (3hours/day) for four weeks as group (4), represented in over-expression of TNF- α (Figure 4). About to molecular study, result of real-time RT-PCR showed there were significant and time-dependent increases in TNF- α mRNA expression in rat livers of work groups even in the least exposed period (10fold) and medium exposed period (12 fold) with a significant difference ($P \leq 0.005$) when the rats exposed to gasoline vapor (3hours/day) for (2) and (3) weeks respectively. While the highest TNF- α expression (14.68 fold) with a high significant difference ($P \leq 0.01$) had obtained from rats of group (4) who exposed to gasoline vapor (3hours/day) for (4) weeks as compared with others work groups, otherwise, all work groups showed significant increase when it compared with control group (0.937 fold) as shown in figure (5).

DISCUSSION

The results indicated that the gasoline exposure was behind TNF- α expression elevated whether in immunohistochemical examination or Real-time RT-PCR, that lead to liver damage which characterized by hepatic inflammation and hepatocyte apoptosis (Beggs et al. 2014) Tumor necrosis factor TNF- α , consider as mediator factor in several liver pathogenesis, toxicity and injures (Chakraborty et al. 2012). The initial hepatocyte injury stimulates more inflammatory responses, leading to further TNF- α production, subsequent accumulate in liver tissue (Schwabe and Brenner, 2006). In fact, gasoline provokes wide range of immune responses (ATSDR, 1995). As the results showed that gasoline of all exposure periods causes significantly increase in TNF- α gene expression in comparison to that in control, especially in rats exposure to gasoline (3hours/day) for (4) weeks as group (4) which noticed elevate TNF- α expression, a strong staining in liver tissues and over-expression in real time PCR. The gasoline which was used in this study contains one of the important heavy metal- lead- which consider by in vivo and in vitro approaches as affecter and stimulator macrophages, neutrophils, T-lymphocytes, B-lymphocytes (Bergeret et al. 1990), TNF- α is mainly produced by macrophages in inflammatory tissues subsequently in blood during injury healing and development of cancers (Aggarwal, 2003). It was found that the significant increase of TNF- α expression in both immunohistochemistry and real time as compared

with control, it was a time-dependent raising with grades expression according the period of inhalation which has been shown to be directly proportional to increase with body damage, TNF- α expression was noticed after (2) weeks of gasoline inhalation whereas overexpression noticed after (4) weeks treatment which clarified exacerbated liver toxicity induced by gasoline (ATSDR, 1995). TNF- α is produced as result to tissue damage via huge amount TNF- α that producing by monocytes which is relating with a raise of progression of cell-cycle and occurrence the oxidative stress by formation of 8- oxo-deoxy guanosine, as marker of DNA injury related with chronic hepatitis (Wheelhouse et al. 2003). Tumor necrosis factor- α (TNF- α) has two functions in the liver, first it is necessary for normal hepatocyte propagation during liver regeneration, the second it is the mediator of hepatotoxicity in numerous animal models, also has been concerned as an significant pathogenic mediator in patients with liver disease (Bradham et al. 2008) The liver is the main organ in the body that responsible for detoxification the toxins, in the case of gasoline exposure the liver tend to remove hydrocarbons from gasoline, by adding oxygens using specific enzymes which oxygenate toxins making them soluble in blood and less toxic, easily remove from the body (Guicciardi et al. 2013). In accordance with current results Wang et al. (1998) showed elevated of TNF- α level in patients with liver cirrhosis when compared with chronic hepatitis and suggested that TNF- α may be related to liver fibrosis and might promote liver fibrosis and study of Simeonova et al. (2001) when study the role of tumor necrosis factor- α in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride also Mohamad (2016) who investigated TNF- α expression in three organs lung, liver kidney of mice infected with *Aspergillus Fumigatus* using immunohistochemical technique and found positive results in all the studied organs, but different in score and intensity, depending of period of infection.

CONCLUSION

In conclusion, the current study suggest that the inhalation of gasoline can significantly effect on expression of TNF- α in liver of rats in both immunohistochemistry and real-time RT-PCR examinations, which necessitates finding ways to protect workers in this field in order to maintain public health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Mohammed BJ designed the study, performed the laboratory work and animal treatment and data analysis. Also, the author reviewed the manuscript, read and approved the final version.

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