ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Study of the Effects of Melatonin on Experimentally Induced Hepatic Fibrogenesis in Rats

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Manuscript Info

Manuscript History:

Received: 14 November 2015
Final Accepted: 22 December 2015
Published Online: January 2016

Key words:
Melatonin, Liver fibrosis and Oxidative stress.

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Abstract

Background: liver fibrosis is considered one of the most common consequences of liver damage caused by variety of liver diseases. Melatonin (n-acetyl-5-methoxy-tryptamine) is produced and secreted in circadian fashion mainly by the pineal gland. **Aim:** the aim of the present work was to study the effect of melatonin on hepatic fibrosis induced by thioacetamide (TAA) in rats. **Materials and methods:** this study was carried on 30 male waster rats, the animal were divided into 3 groups (each 10 rats). Control vehicle treated group, thioacetamide (TAA) group and thioacetamide - melatonin treated group. Blood and liver tissue were taken for estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (alp), TNF-α and bilirubin in the serum. Caspase-3, hydroxyproline (HP), malondialdehyde (MDA). Ascorbic acid and glutathione (GSH) were determined in the liver tissue**. Results**: serum markers of liver damage were significantly elevated after TAA toxicity .melatonin administration nearly reversed these changes. Liver HP, MDA were increased in TAA but inhibited by melatonin. On the other hand there was significant reduction of the concentration of liver antioxidant GSH and ascorbic acid in TAA group compared with the control group. Melatonin significantly increased GSH and ascorbic acid. Biochemical changes were corroborated by histolopathological findings.

Conclusion: liver fibrosis and liver damage caused by TAA were nearly reversed by melatonin treatment therefore, it can be concluded that melatonin could be considered antifibrotic therapy.

Introduction:-

Advanced liver fibrosis is one of the major causes of morbidity and mortality worldwide especially in Egypt. The long-held dogma that liver fibrosis is irreversible and progressive has been challenged by the increasing evidences that liver fibrosis is a highly dynamic process [1,2,3]. Progress in elucidation of the cellular and molecular mechanisms of hepatic fibrosis has brought us to a juncture where translation of these discoveries into treatments is remaining relatively obscure [4].

Melatonin (N-acetyl-5-methoxytryptamine, melatonin), the major product of the pineal gland in circadian fashion with peak level during the dark phase of the Light/Dark cycle, plays a fundamental role in the neuro-immuno-endocrine system [5,6].

Oxidative stress has been accused in the genesis of liver damage in many conditions such as toxin exposures, liver ischemia, and viral infection [7]. Overproduction of reactive oxygen species (ROS) and nitrogen species, together with a significant decrease of antioxidant protection in these pathological

conditions, disturbs various cellular functions through the process of lipid peroxidation [8]. Cirrhosis results from induction of oxidative stress, mitochondrial dysfunction and depletion of antioxidant status [9].

Thioacetamide TAA was originally used as a fungicide. TAA is a weak carcinogen that mainly affects liver and kidney [10] . TAA has been considered to be an inducer of liver fibrosis and cirrhosis [11].

Liver cirrhosis induced by thioacetamide is associated with excessive lipid peroxidation and the exhaustion of antioxidant state [12].

Materials & methods:-

1. **Experimental animals:** 30 Male Wister Albino rats weighing (200-225 g) were obtained from the Animal Breeding Center of the Faculty of Science Tanta University. All animal experiments were undertaken with the approval of Ethical Animal Research Committee of Tanta University. The animals were housed at temperature 22-24 ◦C and were exposed to alternate cycles of 12 h dark/light throughout the study. Animals were kept for 2 weeks for acclimatization.
2. **Experimental design**: Animals were randomly divided into 3 groups 10 per each.

Group	**Treatment**
Group I (Control Group)	Vehicle treated for 8 weeks.
Group II (Thioacetamide TAA Group).	I.P. injection with TAA, 150 mg/Kg, twice weekly, for 8 weeks to induce hepatic fibrosis.
Group III Thioacetamide (TAA) + Melatonin	I.P. injection with Melatonin (3mg/Kg/daily dissolved in 0.9% NaCl once daily for 8 weeks plus TAA in the same dose and time as in group II [13].

3. **Chemicals:** Melatonin and Thioacetamide (TAA) were obtained from Sigma-Aldrich Egypt.
4. **Sample collection and biochemical assay:** At the end of the study, the animals were fasted overnight and were then anesthetized with 45 mg/kg of sodium pentobarbital and sacrificed in the next day. Blood was collected by cardiac puncture, serum was separated by centrifugation at 4000 rpm (4 ◦C) for 15 min and serum was frozen at – 70^{o}C in aliquots until biochemical analysis were performed. The liver was excised quickly. One lobe of the liver homogenized with buffer and liver homogenate used for biochemical analysis and the other lobe was used for histopathological examination.
5. **METHODS:** Aspartate amino transferase (AST) and Alanine aminotransferase (ALT) were estimated with a spectrophotometric technique by the Olympus AU 2700 auto analyzer using commercial kits (India) according to the manufacturer's instructions and presented as IU/L [14]. Bilirubin was measured according to the method used by **Young 1990** [15].

Alkaline phosphatase was measured according to the method used by **King and Armstrong;1988** [16].
Serum TNF –α concentration was measured by enzyme linked immunosorbant assay following the manufacturer's instructions [17].
Tissue homogenate was used for the estimation of Hydroxyproline in the liver tissues by the method as described by **Jamall et al; 1981** [18].
MDA levels were assayed and expressed as nmol MDA/ mg liver tissue [19].
Glutathione (GSH) was determined as a level of endogenous antioxidant [20].
Determination of Ascorbic Acid: according to the method of **Tavazzi et al; 1992** [21].
The hepatic caspase-3 activity was determined by a colourimetric method [22]. Other liver pieces were fixed in buffered 4% formalin and embedded in paraffin. Sections of about 4 u m thickness were stained with hematoxylin and eosin (H&E) for the evaluation of histopathological changes according to method described by ***Dashti et al;1997*** [23] . Histpathological findings were expressed quantitatively according the method recorded by **Adler , and Schaffner** [24].
Statistical analysis: All values were expressed as mean ± SD. SPSS version 16.0 was used for statistical analysis. Data were statistically analyzed using one-way ANOVA for multiple group comparison. Significance was set at $p \leq 0.05$ [25].

Results:-

1. Serum biochemical values

TAA caused significant increase in the biochemical parameters ALT, AST, A-P as well as bilirubin compared to the control group ($p < 0.001$. Table 1) . Administration of Melatonin significantly reduced the elevated parameters ($p < 0.001$.Table 1). No significant changes were observed when comparing TAA+ Melatonin group with the control group ($p > 0.05$). (Tab.1-Fig.1&3).

2. Effects of melatonin on hepatic caspase-3 activities

There was significant increase in hepatic caspase-3 activities of TAA treated group compared to the control group ($p<0.001$). Melatonin reduced significantly this elevation ($p<0.001$) but still significantly higher than control group ($p<0.001$). (Tab.1-Fig. 3).

3. Liver Lipid Per oxidative Products:

There was a significant increase in the concentration of MDA in TAA group compared to the control group ($p<0.001$). Melatonin administration significantly reduced this elevated parameter ($p<0.001$). No significant change was observed when comparing TAA with the control group ($p > 0.05$). (Tab.1-Fig. 2).

4. Liver enzymatic antioxidants:

There was significant decrease in the concentration of GSH and Ascorbic acid in TAA compared with the control group ($p<0.001$). Significant increase in the TAA+ Melatonin group compared with TAA group ($p<0.001$). There was significant decrease of GSH and Ascorbic acid in the TAA+ Melatonin group compared to the control group ($p<0.001$). (Tab.1-Fig.2).

5. Effect of Melatonin on Liver fibrosis parameter:

As regard hydroxyproline content, there was significant increase in TAA group compared to the control group ($p<0.001$). Melatonin significantly reduced hydroxyproline content to TAA group ($p<0.001$). No significant changes were observed when comparing TAA+ Melatonin to the control groups ($p > 0.05$). (Tab.1-Fig. 3).

6. Effect of Melatonin on serum TNF-α:

As regard TNF-α, there was significant increase in TAA group compared to the control group ($p<0.001$). Melatonin significantly reduced TNF-α ($p<0.001$). Significant change was observed when comparing TAA+ Melatonin to the control groups ($p > 0.05$). (Tab.1-Fig.4).

Table 1: Serum ALT, AST, Alkaline –P and Bilirubin. Hepatic Caspase-3 MDA, GSH, Ascorbic acid and Hydroxyproline in the studied groups.

Parameter	**Groups**			
	Control	**TAA**	**TAA + Melatonin**	**F value**
ALT (IU/L)	51.6±4.35	105.0± 10.29 **	64.80±4.21 ##	162.681
AST (IU/L)	103.1±16.77	263.90± 26.66 **	109.0±20.71 ##	175.436
A P (IU/L)	86.65 ±11.20	488.60±23.12 **	82.00±16.44 ##	152.622
Bilirubin (mg/dl)	1.02±0.28	3.32±0.58 **	0.98±0.12 ##	156.184
Serum (TNF-α)	18.50 ±3.865	43.10±7.030 **	26.50±4.836 *##	9.325
Caspase-3 (nmol/mg liver tissue)	1.32± 0.20	9.60±1.83 **	3.34±0.86 **##	176.234
MDA (n mol/mg liver tissue)	0.010±0.002	0.027±0.003 **	0.011±0.003 ##	121.709
GSH (µ/g wt. tissue)	0.69±0.04	0.24±0.05 **	0.62±0.04 **##	256.794
Ascorbic Acid (mg/g weight liver tissue)	0.153±0.025	0.013±0.002 **	0.061±0.005 **##	214.071
Hydroxyproline (µg/100mg liver tissue)	5.46±0.72	8.94±1.49 **	5.00±0.80 ##	52.932

** p<0.001 Versus Control Group.
p<0.001 Versus TAA Group .

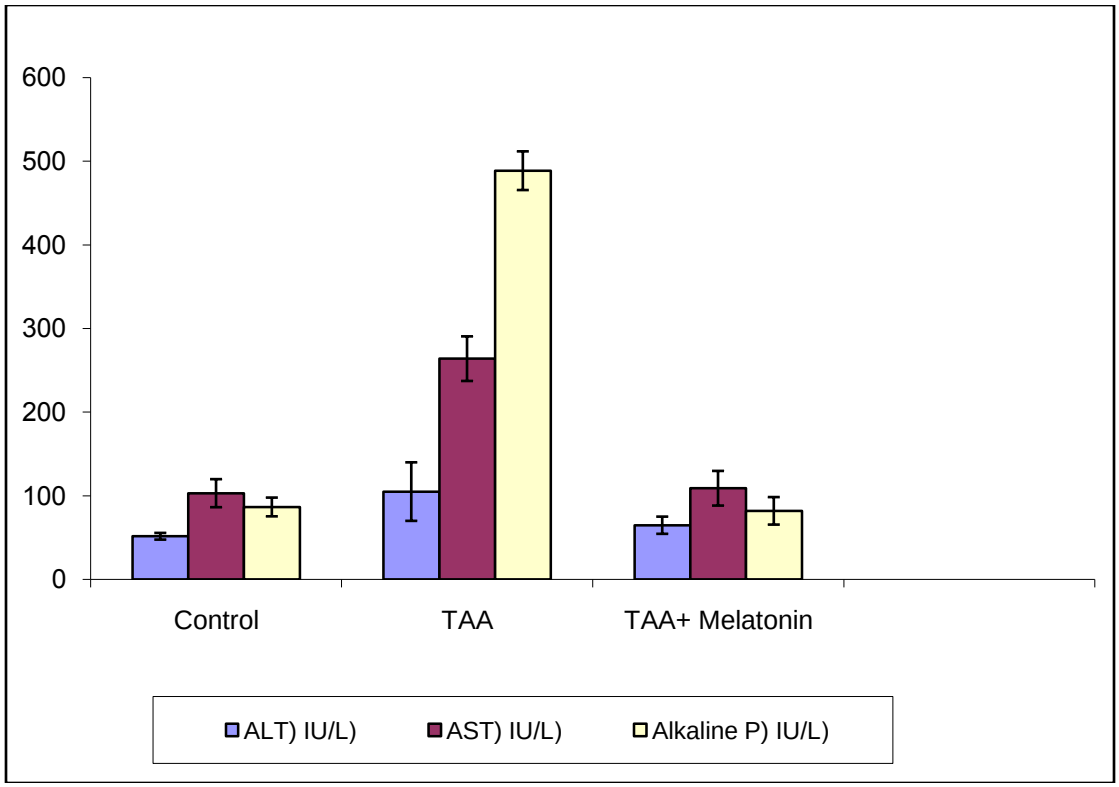

Fig. 1 : ALT (IU/L), AST (IU/L) and Alkaline P (IU/L) in the studied groups

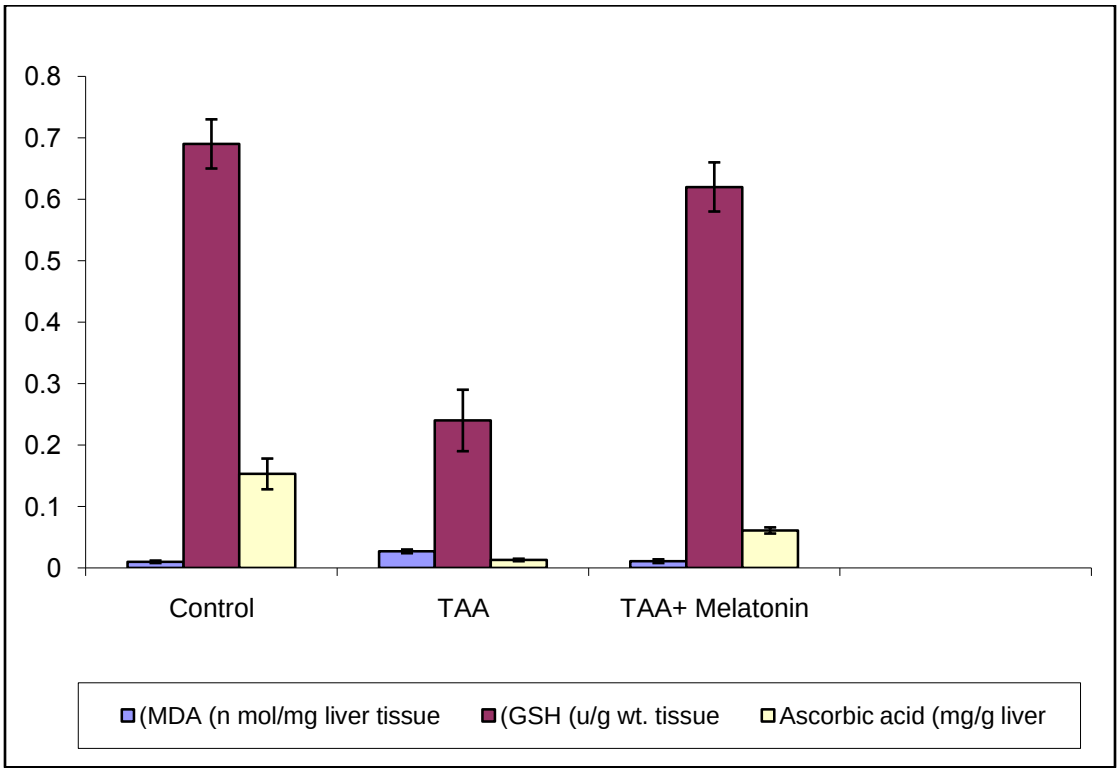

Fig.2 : MDA (n mol/mg liver tissue), GSH (u/g wt. liver tissue and Ascorbic acid (mg/g liver tissue.

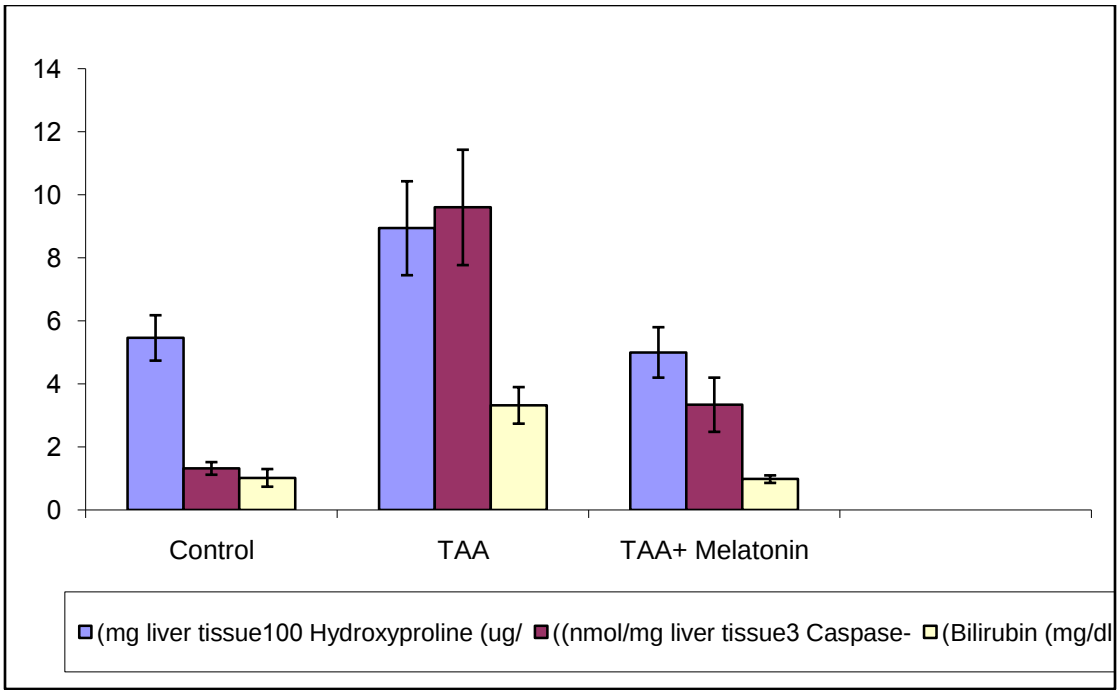

Fig. 3: Hydroxyproline, Caspase-3 and Bilirubin level.

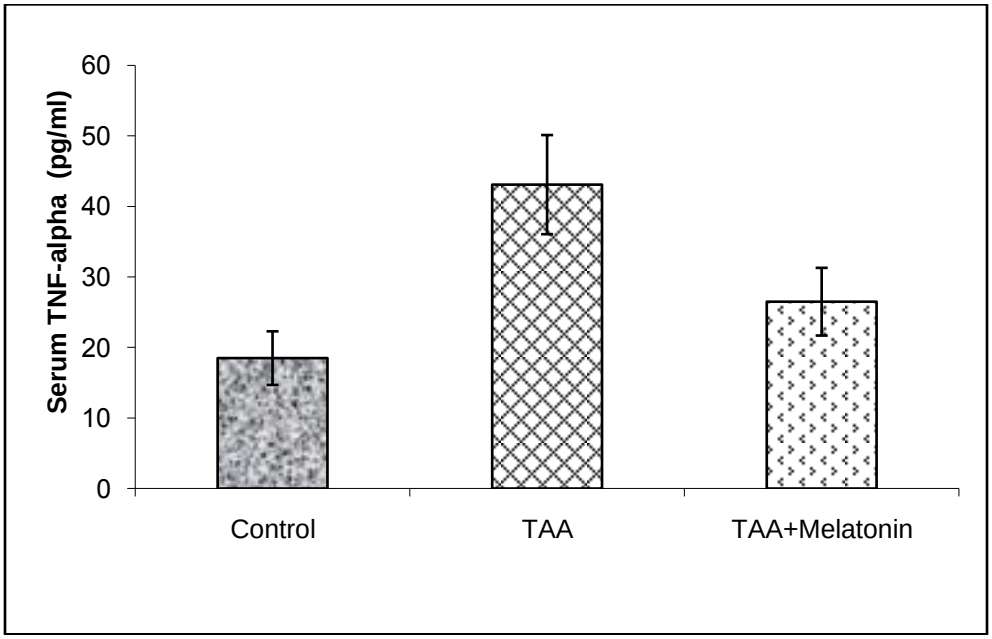

Fig.4: Serum TNF-α (pg/ml)

Histopathological Findings:-

Table 2: Histopathological changes in the liver tissue of the studied groups .

	Control		TAA		TAA+ Melatonin	
	+	%	+	%	+	%
MNC infiltration						
▪ **Slight**	-	-	**0**	-	**7**	**70%**
▪ **Moderate**	-	-	**7**	**70%**	**2**	**20%**
▪ **Severe**	-	-	**3**	**30%**	**1**	**10%**
Fibrosis						
▪ **Slight+**	-	-	-		**5**	**50%**
▪ **Moderate++ ****	-	-	**8**	**80%**	**4**	**40%**
▪ **Severe+++**	-	-	**2**	**20%**	**1**	**10%**
Fatty Degeneration						
▪ **Slight+**	-	-	-		**4**	**40%**
▪ **Moderate++ ****	-	-	**7**	**70%**	**5**	**50%**
▪ **Severe+++ ***	-	-	**3**	**30%**	**1**	**10%**
Focal Necrosis						
▪ **Slight+ ***	-	-	**3**	**30%**	**6**	**60%**
▪ **Moderate++ ***	-	-	**7**	**70%**	**4**	**40%**
▪ **Severe+++**	-	-	-	-	-	-

* TAA+ Melatonin versus TAA $p<0.05$. **TAA+ Melatonin versus TAA $p<0.01$. Fischer's Exact Chi-square test was used for histopathological parameters (mononuclear cell infiltration (MNC), fibrosis, focal necrosis and fatty degenaration). [(24)]

A **B**

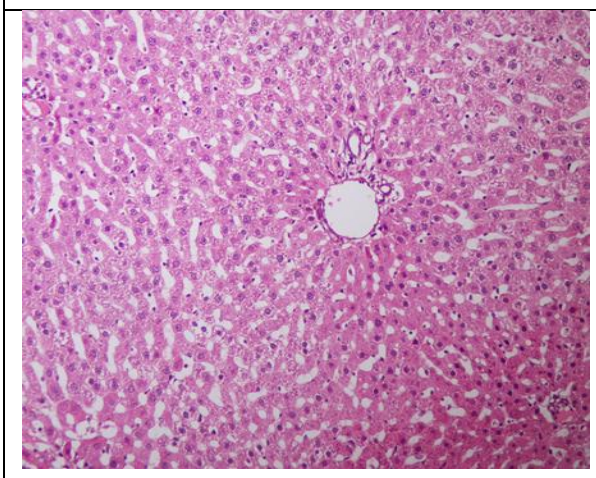
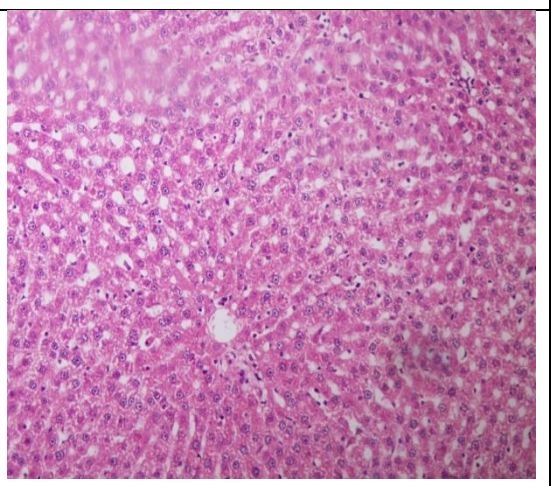

Fig. 5 (A&B) : The control group showing normal hepatocytes and normal liver lobular architecture.

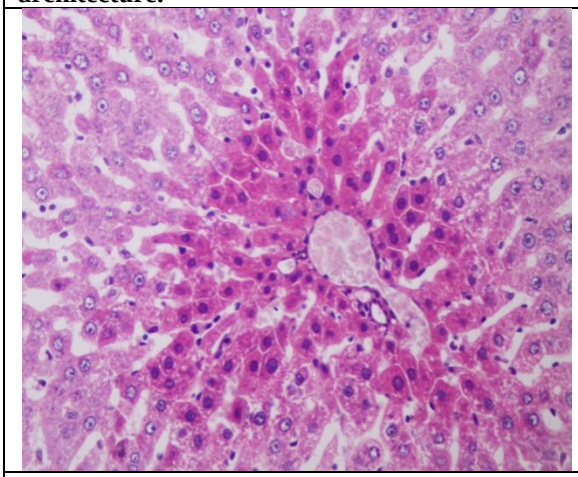
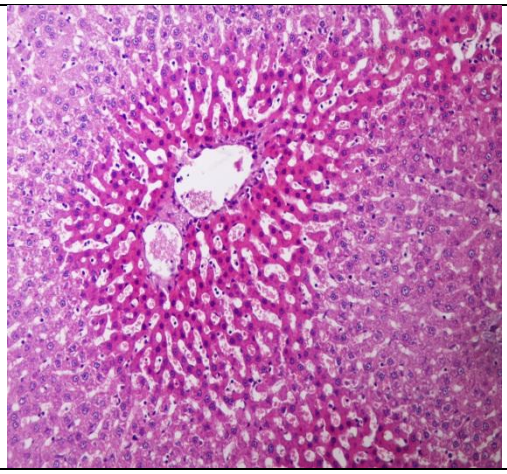

Fig. 6 **(A&B)** :H& E stain after 8 weeks TAA administration revealed degeneration, focal necrosis, vacuolization, inflammatory cellular infiltrations and fibrosis.

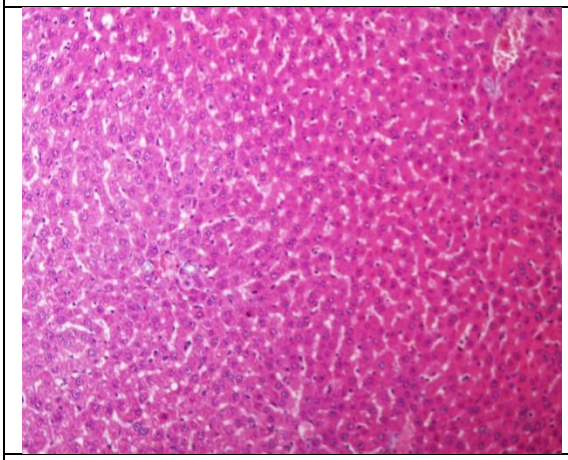
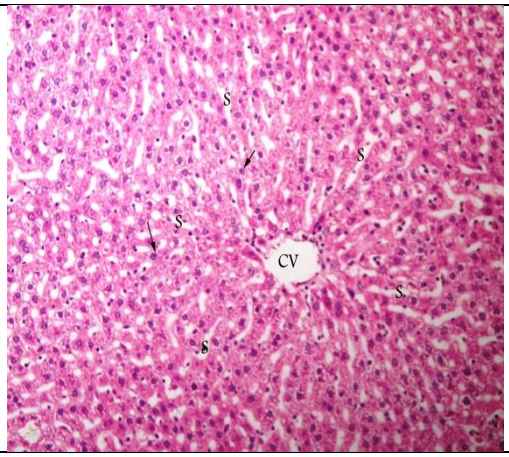

Fig.7 **(A&B)** :H& E Melatonin administration revealed normal lobular appearance of the hepatocytes in the area near to the central vein and reduced signs of swelling degeneration and necrosis.

Discussion:-

Liver is vulnerable to a vast variety of harmful endogenous and exogenous agents so, it is considered one of the most frequently injured organ in the body . There has been a growing interest about understanding the pathophysiology of hepatic fibrosis. This has contributed to the development of new medications that could inhibit or reverse hepatic fibrosis in the near future [26].

The hepatotoxin thioacetamide (TAA) has subsequently used as a fungicide. Although TAA itself is not toxic to the liver, its intermediates are able to covalently bind to hepatic macromolecules and eventually initiate necrosis of liver cells. In this study TAA increased liver enzymes significantly. The increase of liver enzymes could be due to liver damage or injury as supported by the work of **Wang et al & Chen et al.** [27,28]. They also, stated that hepatic enzymes increase during liver dysfunction indicating severe inflammation or liver injury and these liver enzymes in the hepatocytes cannot diffuse out of the cells in the physiological condition .When these hepatocytes are injured, plasma membrane can be disrupted and the leakage through extracellular fluid of the enzyme occurs where they can be detected at abnormal levels in the serum [29].

Serum liver enzymes (ALT, AST and AP) are important items for evaluation of liver damage. Since these enzymes are normally located in the cytoplasm and released in the circulation after cellular injury. The amount of enzymes that leak into the blood indicate the severity of liver damage [30,31]. Melatonin significantly reduced the elevated enzymes (AST,ALT and ALP) compared with TAA group. Melatonin tends to prevent liver damage, suppresses the leakage of enzymes through cellular membranes, preserves the integrity of the plasma membranes and hence restores these enzymes levels [30] .

Oxidative stress is not only considered as marker of tissue damage, but is involved in the pathogenesis as well as modulation of extracellular matrix (ECM) resulting in liver fibrosis. It is a key event in the inflammatory process of hepatic diseases [26].

It was postulated that hepatotoxins induce liver damage by forming free radicals that reacts with cellular lipids to promote lipid peroxidation [32]. In this study, higher MDA in TAA group supports this suggestion. Lipid peroxidation is taken as indirect in vivo reliable index for oxidative stress. The overproduction of ROS would break down the balance of the oxidative/ antioxidative system in the liver, resulting in the lipid peroxidation via ROS and hepatocyte apoptosis, Once formed, free radicals trigger a cascade of reactions that culminate in lipoperoxidation which may be closely related to the reduction of antioxidative enzymes [33].

Excessive liver damage and oxidative stress caused by TAA depleted the level of GSH and Ascorbic acid as reported in this study by significant reduction of GSH and ascorbic acid levels after TAA administration. Reduced glutathione is an important endogenous antioxidant system that is found in particularly high concentration in liver, and it is known to have key functions in protective processes. The reduced form of GSH becomes readily oxidized to glutathione disulfide (GSSG) on interacting with free radicals. Excessive production of free radicals leads to damage of macromolecules, e.g. lipids and can induce lipid peroxidation in vivo [(34)]. Melatonin attenuates oxidative stress by acting as scavenger of free radicals and providing antioxidant protection of biomolecules [34].

TAA induced liver damage is characterized by massive apoptosis of hepatocytes and inflammation as evidenced in this study by significant elevation of hepatic caspase-3 activity and TNF-α . Melatonin significantly reduced this elevation. Melatonin is an anti-apoptotic mediator [35,36,37,38]. Moreover, melatonin significantly attenuated D-galactosamine -induced hepatic DNA fragmentation [17].

Melatonin may be useful for the treatment of inflammatory disease, as it reduces inflammatory injury by blocking transcription factors and NFB [39], thereby decreasing further ROS formation within cells. In the same way, melatonin seems able to inhibit the activation of cyclooxygenase 2 (COX-2) and of the inducible NO synthase (iNOS), both activated in chronic inflammation disorders [40].

Melatonin treatment caused a significant and pronounced decrease in the hydroxyproline level compared to TAA. Melatonin exhibited a protective effect on the development of liver fibrosis may be attributed to suppressing TAA-mediated induction of MMP-2, MMP-13, TIMP-1,TIMP-2 and TGF-β1 [34].

Results of the effects of TAA is evidenced by the histopathology of the liver of this group that showed hepatocellular focal necrosis, thickening of blood vessels with cellular infiltrations and fibrosis. Melatonin improved these histopathological findings.

Conclusion:-

Liver fibrosis and liver damage caused by TAA was nearly reversed by melatonin treatment. Therefore, it can be concluded that melatonin could be considered as antifibrotic therapeutic agent.

Acknowledgment:-

This study was supported by Prof. Mohamed Alsharef Assistant Prof. of Pathology, Faculty of Medicine, Tanta University.

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