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THE PROTECTIVE ROLE OF MELATONIN ON OVARIAN FUNCTION IN ADULT FEMALE RATS EXPOSED TO CARBON TETRACHLORIDE (CCL_4)

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ABSTRACT
This study was carried out to investigate the protective role of melatonin against the passive effect of CCL_4 on ovarian function in adult female rats by studying the ovarian weight to body weight ratio, serum gonadotropin hormones (LH and FSH) concentration, fertility and viability index and histological study of ovary. Thirty adult rats (24 females and 6 male for mating) were randomly divided into four groups. The first group received distilled water 3 CC and olive oil 0.5 ml/ kg of body weight twice a week for six weeks and considered as control group, animals of second group (T1 group) received melatonin 10 mg/Kg of body weight with olive oil intraperitonally twice a week for six weeks, rats in third group received CCL_4 0.5 ml/kg (500mg/kg of body weight) with olive oil twice a week for six weeks intraperitonally considered (T2 group), animal in fourth group received melatonin with CCL_4 also for the same period. Blood samples were collected in (zero, 7, 14, 21, 28, 35, 42) days of experiment for measuring serum hormones (LH, FSH). At the end of experiment two male adult rats allowed to mating with each group for the study of fertility and viability indexes. Also four animals of each group were scarified to examine the histological study of the ovary. The result revealed a significant ($P<0.05$) decrease in ovarian weight to body weight ratio and LH and FSH concentrations in CCL_4 (T2 group) treated group compared with other groups, while animals received melatonin (T1 group) showed a significant increasing ($P<0.05$) in these parameters mentioned above, while melatonin caused a significant increasing in the ovarian weight to body weight ratio and gonadotropin hormones concentrations. Carbon tetrachloride treated rats showed a significant decreasing in the fertility and viability index, but melatonin caused improvement of these index . Histological study of CCL4 treated rats (T2 group) indicated that the atretic follicles with patchy of blood with trophoblastic cells in the ovary. On other hand, treatment of rats with melatonin caused disappear of these histopathological changes with normal growth and development of ovarian follicles especially graffian follicles. it seems that dosage of rats with 0.5ml/kg (500mg/kg of body weight) of CCL_4 caused a significant harmful effect on the function of ovary and the productive dose (10 mg / kg of body weight) of melatonin showed obvious improvement of this function.

KEYWORDS: CCL_4, graffian follicles, melatonin, function.

INTRODUCTION

Several antioxidants have been described to have beneficial effects in oxidative stress-associated diseases (Jang *et al.*, 2000). In recent studies melatonin is an indolamine secreted by pineal gland in a circardian manner (stehle *et al.*, 2011) which is used as free radicals scavenger (Tan *et al*., 1993; Rieter *et al*., 2003 and Galano *et al*., 2011). Melatonine has ability to protect all cells and organs from oxidative and nitrosative damage has been confirmed in more than a thousand publications (Leon *et al.*, 2005 and Gitto *et al*., 2009). Carbon tetrachloride (CCl_4) is a haloalkane used in variety of industrial and chemicals application. It has been widely used for its solvents properties, as an intermediate in the synthesis of chorofluorocarbon (ATSDR, 1994). CCl_4 leads to generation of free radicals caused cell injury and apoptosis to cells (Kamel et *al*., 2010). It's known to be hepatotoxic as well as nephrotoxic to experimental animals (Karadeniz *et al.*, 2009). Long exposure to CCl_4 cause reduction in fertility, ovarian size, and histopathological ovarian atrophy with increase in weight of uterus, pancreas and adrenal gland. In additional CCl_4 can effect central nervous system (lieu *et al.*, 1993). The study of antioxidant effect of melatonin on female reproductive when exposed to carbon tetracholoride designed to demonstrate the role of melatonin in suppression of oxidative stress and passive effect of CCl_4 in adult female rats, the toxicity of CCl_4 on female reproductive system and the effect of melatonin as antioxidant on fertility studied by using the following parameters:

1- ovarian weight to body weight ratio
2- Estimation of female reproduction hormones
a- FSH.
b- LH.
3- fertility and Viability index
4- Histological study of Ovaries

MATERIALS & METHODS

Experimental animals

Thirty adult rats (24 female+6male for mating); weighted 180-400 gram were housed in the animal in the College of Veterinary Medicine, Baghdad University / Department of Physiology and Pharmacology. The animals were adapted

for ten days before experiment beginning and kept in plastic cages 50×35×15 under uniform environmental conditions, at temperature between 21-25 C°, air of the room changed by using ventilation vacuum and with light / dark cycle was (12-12 hr.) rat were fed on pellets diet and water. This experiment was performed at 27/11/2016 last to 1/4/2017.

Experimental design

Twenty four adult female rats were divided into four groups randomly, each group consist of eight animals as below:

1- Control group: they were received 3 cc of distilled water mixed with olive oil 0.5mg/Kg intraperitonally twice a week for six weeks.
2- Melatonin treated group: they were received 10mg/Kg of body weight mixed with 0.5 mg/kg of body weight from olive oil intraperitonally (Kanter *et al*., 2006 and sudnikovich *et al* ., 2007)
3- CCL_4 treated group: they were received (0.5ml) 500mg/kg body weight with an equal volume of olive oil o.5 mg/kg of body weight intraperitonally twice a week for six week (Yin *et al*., 2006)
4- CCl_4– melatonin treated group: they were received (10mg /kg body weight) of melatonin with0.5 ml (500mg/kg of body weight) of CCl_4 and olive oil 0.5 mg/kg twice a week for six week

Blood samples collection

The blood samples were collected at zero time, 7, 14, 21, 28, 35, 42 days of experiment animal were anesthetized by intra muscular injection of (ketamine 90 mg/kg B.W & xylazine 40mg/kg B.w). Blood samples were obtained from retro-orbital artery, samples were centrifuged at 2500 rpm for 15 minute, and then serum samples were stored in freezer at -18 C till use.

Hormonal assay

Hormonal assay was conducted for the samples (FsH) using kits purchased from immunotech (Marseille –France), while LH assay was done using kits obtained assay Dia Sorine (veselli-Italy).

Ovarian weight to body weight ration

Ovarian weight to body weight ratio was calculated as in the following equation:

Ovarian weight (gm)/body weight (gm) ratio=wt. of ovaries (gm)/ wt. of animal (gm) ×100.

Fertility and viability indexes:

Fertility and viability were calculated as follows:

Fertility index = (No. of pregnant rats / No. of mated rats successfully) ×100

Viability index = (No. of alive babies / No. of total babies) × 100

Histological study of ovaries and uterus:

After the end of treatment, four female rats from each group were sacrificed and by anesthesia, ovaries and uterus were excised and cleared off the attach fat and connective tissue. Histological sections were prepared according to Luna (1968) for histological study.

Statistical analysis

Statistical analysis of data was performed on the basis of two way analysis or variance (ANOVA) using a significant level of ($P< 0.05$) depending on the experimental design. Specific group differences were determined using least significant difference (LSD) test (Steel and Torrie, 1980).

RESULTS

Ovarian weight to body weight ratio

The data recorded in table (1), illustrated that melatonin treated rats showed non-significant increase in ovarian weight to body weight ratio, while CCL_4 caused significant ($P<0.05$) decrease in this ratio compare with control group and other treated groups . On other hand the ratio of ovarian weight in animals treated with melatonin and CCL_4 significantly ($P<0.05$) increase as compare with CCL_4 group.

TABLE 1: Effect of melatonin, CCL4 and melatonin with CCL4 on ovarian weight to body weight ratio (gm.) in female rats

Control group	Mean of animal weight /g	222
	Mean of ovary weight	0.379
	Mean of ratio %	0.17o A
T1 group (melatonin)	Mean of animal weight /g	238
	Mean of ovary weight	0.511
	Mean of ratio %	0.214 A
T2 group (CCL4)	Mean of animal weight /g	206
	Mean of ovary weight	0.075
	Mean of ratio %	0.036 C
T3 (mel. With CCL4)	Mean of animal weight /g	233
	Mean of ovary weight	0.263
	Mean of ratio %	0.112B

Values are expressed as percentage n = 6 each group.
Capital letters denote difference between groups, $P<0.05$ vs . control

Serum LH concentration

Serum LH concentration shows a significant ($P<o.o5$) increase in melatonin group (T1 group) as a compared with control group in all periods of experiment except the first week while CCL_4 (T2 group) cause a significant ($P<0.05$) decrease in LH concentration at all periods , while the treatment of animals with both melatonin and CCL4(T3 group) lead to a significant($P< 0.05$) increase in serum LH concentration compared with CCL4 group at 3^{rd} - 6^{th} week and become closed to LH value in control group table(4-2). Within group, there are no significant difference in all treatment groups for all period.

TABLE 2: Effect of melatonin, CCl4 and melatonin with CCL4 on serum LH concentration (u/ml) in female rats

Groups / Period	Control group	T1group (melatonin)	T2group (CCL4)	T3group (Mel+CCl4)
1st week	3.25±0.10	3.98±0.41	2.56±1.16	2.62±0.35
	A	A	B	B
2nd week	3.10±0.36	3.92±0.32	1.94±0.36	2.03±0.29
	B	A	C	C
3rd week	2.90±0.40	3.96±0.26	1.63±0.61	2.53 ±0.05
	B	A	C	B
4th week	2.30±0.23	3.32±0.85	1.34±0.48	2.60±0.70
	B	A	C	B
5th week	1.88±0.36	3.76±0.18	1.29±0.08	2.29±0.15
	B	A	C	B
6th week	2.90±0.41	3.41±0.29	0.85±0.21	2.81±0.13
	B	A	C	B

L.S.D=0.81

Values are expressed as mean ± SE, n= 6 rats / each group

Capital letters denote difference between groups , P<0.05 vs control

Serum FSH concentration:

The data which have referred to a significant (P<0.05) increase in FSH concentration was recorded in melatonin treated rats (T1 group) compared with control group at all treatment periods table (4-3) except the first week on other hand CCL4 treated group showed a significant (P<0.05) decrease in FSH concentration especially at all period compared with control group and melatonin group. Also melatonin and CCl4 treated (T3 group) showed a significant increase of FSH concentration at the last two periods compared with CCl4 treated group. During the different periods of experiment there are a significant difference within (T2 and T3 groups) between the first three weeks and the last week, and within (T1 group)between the first week and other weeks .

TABLE 3: Effect of melatonin , CCl4 and melatonin with CCL4 on serum FSH concentration (u/ml) in female rats.

Groups / Period	Control Group	T1 group (Melatonin)	T2 group (CCL_4)	T3 group (Mel+CCL_4)
1st week	3.58 ±0.05	3.31 ±0.08	2.98 ±0.31	3.48 ±0.31
	A	A b	B a	A a
2nd week	3.25 ±0.32	4.14 ±0.05	2.62 ±0.24	3.04 ±0.27
	B	A a	C a	B a
3rd week	3.50 ±0.26	4.37 ±0.02	2.61 ±0.07	2.79 ±0.40
	B	A a	C a	C ab
4th week	3.28 ±0.14	3.22 ±0.13	2.10 ±0.16	2.42 ±0.08
	B	A a	C b	C bc
5th week	3.40 ±0.07	4.06 ±0.21	1.77 ±0.24	2.17 ±0.22
	B	A a	D c	C c
6th week	3.22 ±0.18	4.79 ±0.08	1.16 ±0.33	2.45 ±0.04
	B	A a	D d	C bc

L.S.D=0.41

Values are expressed as mean ± SE, n= 6 rats / each group

Capital letters denote difference between groups, P<0.05 vs control

Small letters denote difference within groups, P<0.05

Fertility and viability indexes (%)

The effect of melatonin and CCL_4 on fertility and viability indexes is mentioned in table (4) , and (5) respectively, the results showed that melatonin caused increase in percentage of the above parameters as compared with the group that exposed to CCL_4, also the treatment of animals with melatonin and CCL_4 showed improvement of these parameters comparing to CCL_4 treated group.

TABLE 4: Effect of melatonin, CCL_4 and melatonin with CCL_4 on fertility index (%) in female rats

Group / Parameter	Control group	T1 group (mel)	T2 group (CCL_4)	T3 group (mel. + CCL_4)
No. of mated successfully	6	6	6	6
N0. of pregnant rats	6	6	4	5
Fertility index	100%	100%	66%	83%

TABLE 5: Effect of melatonin, CCL_4 and melatonin with CCL_4 on viability index (%) in female rats

Group / Parameter	Control group	T1 group (mel)	T2 group (CCL_4)	T3 group (mel+ CCL_4)
No. of total babies	30	36	28	33
N0. Of alive babies	30	36	20	22
No. of dead babies	0	0	8	11
Viability index	100%	100%	71%	66%

The histological changes in ovary

The histological structure of ovary has revealed that the ovary of melatonin treated rats has showed an increase in number of ovarian follicular .The histological picture of ovary showed normal structure with many degenerated follicles in T1 group (melatonin) (figure1). While CCL_4 caused atrertic follicles and patchy of blood with necrotic trophoblastic cells and development follicles in tunica albugina (figure 2). On other hand treatment of rats with melatonin with CCL_4 (T3 group) showed development graffian follicles.

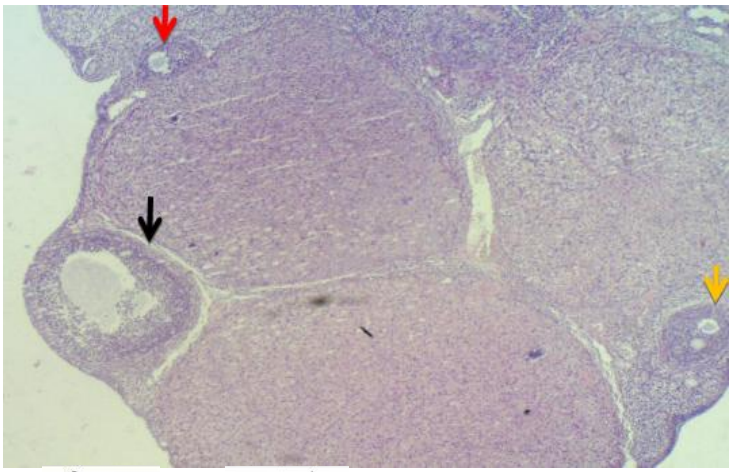

FIGURE 1: Histological section of ovary of rat in control group showed different developed follicles (primary secondary and graffian follicles) 10X (H&E stain)

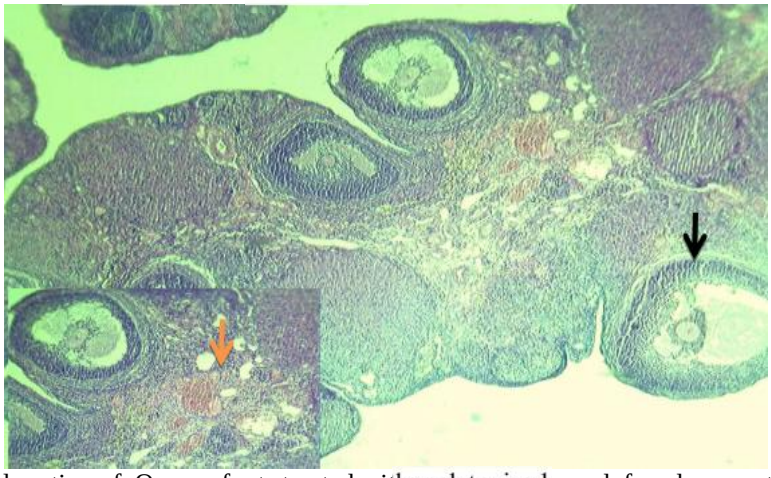

FIGURE 2: Histological section of Ovary of rats treated with melatonin showed few degenerated follicles with mature graffian foolicles with oriented multiple layers of graffian follicles ( ) 10 X H&E stain) and congested blood vessels as seen in inserted portion 40 X ( )

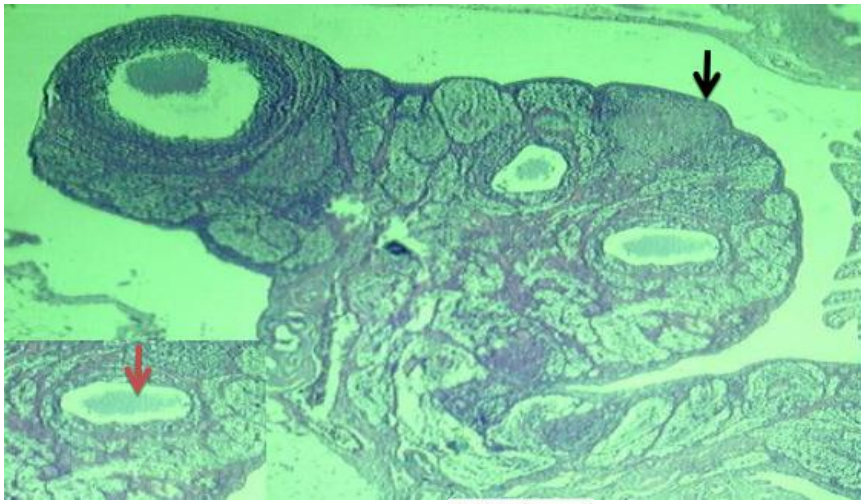

FIGURE (4-2):Histological section of ovary of rat treated with CCl4 showed; hypolpastic tunica albugenia (⟶) (H&E stain, 10x) and severe degenerated trophoplastic cells (⟶) as appeared in inserted portion (40x)

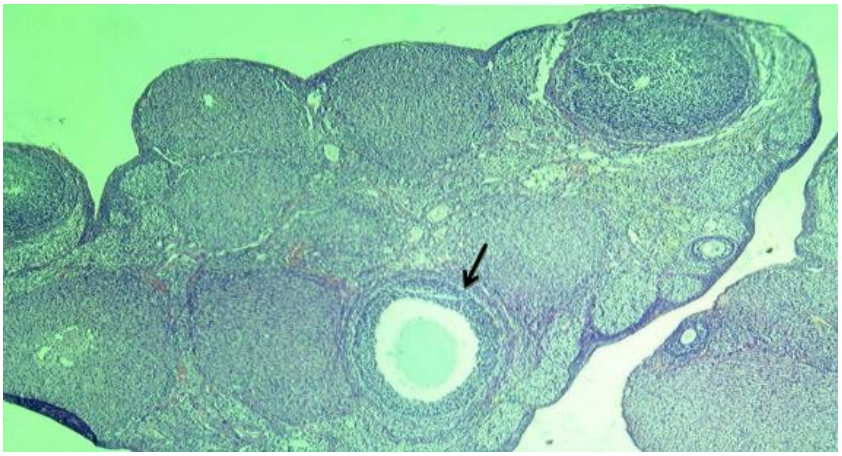

FIGURE 4: Histological section of ovary of rats treated with melatonin and CCL_4 which showed developed graffian follicles (arrow) 10 x (H& E Stain)

DISCUSSION

Effect of melatonin and CCL_4 on ovarian weight to body weight ratio

The increase of Ovarian weight to body weight ratio in the rats treated with melatonin T1 group might be due to increase of gonadotropin hormones especially FSH which lead to the growth and development of ovarian follicles and cause increase of diameters of them (McDonald *et al*., 1989 and Solkoff *et al*., 1999). Also melatonin considered as antioxidant act as scavenger of free radicals lead to increase the number of primary and graffian follicles, while CCL_4 caused significant decrease of ovarian weight to body weight ratio (Yoshida *et al*., 2005) thus CCL_4 as a toxic material lead to decrement of gonadotropin hormones and so that development of ovarian follicles decrease beside to decrease of number and diameters of graffian follicles. In T3 group the antioxidant nature of melatonin play important role in decreasing the toxicity of CCL4 and protected the ovary from free radicals and improved the number and development of ovarian follicles (Tan *et al.*, 2010).

Effect of melatonin and CCL4 on LH and FSH:

The increment of LH level in melatonin treated group (T1 group) may be due to antioxidant effect of melatonin which improve and stimulate hypothalamus and / or pituitary gland to increase synthesis and release of this hormone from pituitary gland (El. Desoky *et al*., 1995). Also melatonin administration , can imitate short days and there for activate reproductive activity and increase gonadotropin hormones (Soliman and Soliman, 1958; Dostal *et al*.,1996), on other hand , melatonin act as pro gonadotropic factors (FSH and LH) will be increase, also the stimulation of the hypothalamus to produce GN-RH, which in turn, signals the anterior pituitary to produce FSH and LH hormones. Carbon tetrachloride (CCL_4) cause significant decrease in gonadotropin hormones (LH and FSH) this decrement may be attributed to CCL_4 effect on pituitary gland leading to decrease these hormones also the reduction of gonadotropin concentration due to oxidation stress induced by CCl_4 (Khan and Ahmed, 2009).

On other hand the elevation in LH and FSH levels in T3 group as compared to T2 group may be due to protective

role of melatonin against CCL_4 because of its free radical scavenger activity, so that gonadotropin hormones return ne ar to normal values (Tan *et al* ., 2007 and 2010).

Effect of melatonin and CCL_4 on fertility and viability indices

Melatonin could be indicated to act as progonadotrophic in some species of animals and showed that melatonin increase the capacity of protection to the gametes from oxidative stress (Bustos-obregon *et al*., 2005 and sarabia *et al*., 2009). Melatonin administration can imitate short days and there for activate reproductive activity (Arendt *et al*., 1983 and Chemineau *et al*., 2008). On other hand melatonin caused increase the activity of pituitary gland and increment of FSH and LH hormones , these hormones are essential for the completion of follicular maturation , development of mature follicles more over stimulating gonadal ovogenesis and steroidogenesis as a result fertility and viability indexes increase. CCL_4 treated animals showed significant decrease in these indexes; these effect may be related to passive role of CCL_4 as atoxic material, Caused reduction in gonadotropins hormones concentration so that disturbance of maturation , development and decrease in ovarian follicles occur and these effects attributed to depression in fertility and viability indexes (Hardy *et al*., 2005), while the protective role of melatonin (T3 group) against CCL_4 revealed the antioxidant role of this hormones and improve the fertility and viability indexes.

Effect of melatonin and CCL_4 on histological changes in ovary

The most histological changes in the ovary of rats treated with CCL_4 may be due to oxidative effect and toxicity of CCL_4 which caused atretic follicles with patchy of blood with trophoblastic cells and decrease of number and diameter of graffian follicles because of the disturbance in gonadotropin hormones (LH and FSH) (Gyton, 2006). On other hand; melatonin caused increased of number and diameters of follicles with normal structure of these follicles and increase of LH and FSH hormones. The increment of gonadotropin hormones (LH and FSH) lead to enhancement of diameters and number of follicles (Tan *et al*., 1993).

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