

Expression of PCNA and P16^{INK4a} in Rat Kidney Intoxicated with CCl₄ and the Antagonistic Effect of Silymarin

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Abstract: The present study was undertaken to investigate the antagonistic effect of silymarin on kidney cortex damage induced by carbon tetrachloride (CCl₄) in male Sprague Dawley rats. Rats were randomly divided into three groups; control group (n=10), group II (n=30) was injected IP with CCl₄ (1ml /Kg) for 3 doses weekly, each other, for 12 weeks and group III (n=30) was injected with CCl₄ and treated with silymarin in a daily oral dose (50 mg/ Kg). Results revealed that CCl₄ induced prominent damage in kidney cortex; congestion, hyalinization of glomerular tuft, hyaline casts in proximal convoluted tubules and pyknotic nuclei in widened distal tubules ended with fatty degeneration. Silymarin treatment inhibited the deleterious toxicity of CCl₄ and returned to normal architecture after 12 weeks. PAS stain showed morphologic criteria for acute tubular necrosis; focal loss of the luminal brush border in proximal tubules and PAS positively revealed thickened basement membranes in CCl₄ group as compared with other groups. Collagen fibrils accumulated in renal corpuscle around larger vessels and Bowman's capsule and in renal interstitial space with more significant increase ($p > 0.001$) in CCl₄ group especially after 8 weeks and returned to normal content after silymarin treatment. Immunohistochemically; numbers of proliferative cell nuclear antigen (PCNA) showed significant increase ($p > 0.05$) and the numbers of cell cycle inhibitor p16^{INK4a} showed significant decrease ($p > 0.001$) in group III after silymarin treatment as compared with their counterparts. In conclusion: Silymarin antagonized nephrotoxicity induced by CCl₄ by its anti-inflammatory and anti-fibrogenic effect along with enhanced PCNA and inhibited p16^{INK4a}.

Key words: Kidney • CCl₄ • Silymarin • Collagen • PAS • PCNA and P16^{INK4a}

INTRODUCTION

Carbon tetrachloride (CCl₄) is one of such widely used environmental toxicant to experimentally induce animal models of acute nephrotoxicity and hepatic damages. It induced oxidative stress and resultant dysfunction of rat liver and kidney [1]. Oxidative stress may be a major mechanism for the toxicity of CCl₄ which induced oxidative damage in the liver, brain, kidney of male Wistar rats [2, 3] as well as renal oxidative damage in mice [4, 5]. Oral administration of CCl₄ to rats increased the oxidative stress markers and decreased the levels of enzymatic antioxidants in both liver and kidney [6]. Jayakumar *et al.* [7] as well as Khan *et al.* [8] investigated,

histopathologically, the toxicity effects of CCl₄ on some organs of Wistar and Sprague- Dawley rats respectively including the kidneys and they confirmed kidney injuries.

Silymarin, a standardized extract obtained from seeds of *Silybum marianum*, is widely used in treatment of liver diseases of varying origins [9]. It protects liver and kidney cells from toxic effects of drugs, including chemotherapy [10]. Silymarin has potentially beneficial antioxidant, anti-inflammatory and antiproliferative activities against chemically induced renal cancer [11]. It is one of the candidate chemopreventive agents for renal cell carcinoma therapy through its role in inhibiting cell growth and inducing apoptosis [12].

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Therefore, the purpose of this study was to clarify the beneficial mechanism of silymarin on the alterations induced by CCl₄ on kidney cortex of male rats through histopathological, histochemical and immunohistochemical studies.

MATERIALS AND METHODS

The current study was applied on 70 adult male Sprague-Dawley rats weighing 130-150 g. The rats were housed in a temperature controlled room (24± 1°C) on a 12 hours light and dark cycle, with free access to food and water. This study was carried out in accordance with the protocol of Laboratory Animal Unit of Medical Research Institute, Alexandria University concerning with guiding principles for biomedical research involving animals.

Rats were randomly divided into three groups. Group I: ten rats were served as controls. Group II: 30 rats were injected IP with CCl₄ (Sigma Chemicals Co.) in a dose of 1.0 ml/kg b.w. for 3 doses, every other day, per week for 12 weeks [13]. Group III: 30 rats were injected with CCl₄ in the same dose and treated with silymarin (SEDICO Pharmaceutical Co., Egypt) in a daily oral dose of 50 mg/kg b.w. half an hour after CCl₄ injection [14].

Ten rats of each experimental group and three rats of control group were sacrificed after 4, 8 and 12 weeks from the beginning of the experiment. Kidney was immediately removed and either fixed in 10% neutral phosphate-buffered formalin or in Carnoy's fixative, embedded in paraffin and processed for the following studies:

Histopathological Studies

Hematoxylin and Eosin (H&E): For histopathological investigations, 4 µm thick formalin fixed paraffin sections were deparaffinized and processed down to distilled water, then stained with H&E stains, dehydrated, cleared and mounted [15].

Masson's Trichrome Stain: For demonstration of collagen content; 4 µm thick formalin fixed paraffin sections were processed down to distilled water, mordant in Bouin's solution, stained with Weigert's hematoxylin and rinsed in running tap water. Sections were stained in Biebrich scarlet-acid fuchsin solution, washed in distilled water, differentiated in phosphomolybdic- phosphotungstic acid solutions, then stained with fast green FCF and processed to be mounted [16].

The degree of renal collagen content by Masson's trichrome stain was analyzed with a semi-quantitative scoring system. The area of blue-stained interstitial fibrosis was detected using the Q Win V3 software package and related to total tubule-interstitial area.

Histochemical Study: Periodic acid Schiff's reagent (PAS) was used for identification of basement membrane changes and glycogen deposition. Carnoy's fixative paraffin kidney sections of 4µm thick from different groups were prepared, deparaffinized, hydrated and oxidized in periodic acid, stained with Schiff's reagent, counterstained with hematoxylin and then processed to be mounted. Control reaction was done using diastase technique [17].

Immunohistochemical Studies: 10% neutral buffered formalin fixed sections 5 µm thick were deparaffinized, hydrated in a descending series of ethanol to phosphate-buffered saline (PBS; pH 7.5). Immunostaining of proliferating cell nuclear antigen (PCNA) and cell cycle inhibitor p16^{INK4a} were carried out with the use of the streptavidin/biotin immunoperoxidase method (LSAB kit, Dako Corp). The sections were immersed in 3% hydrogen peroxide to quench the endogenous peroxidase activity and then incubated with PBS containing 1% bovine serum albumin to reduce the nonspecific background staining. The sections were washed with PBS and incubated overnight at 4°C with primary antibodies; monoclonal mouse anti-proliferating cell nuclear antigen (PCNA; clone PC 10; Dako Corporation, Carpinteria CA, Denmark) [18] and a monoclonal mouse antibody against p16^{INK4a} (F-12 antibody, sc-1661; Santa Cruz Biotechnology, Santa Cruz, CA) [19]. Slides were then washed in two changes of PBS (each 5 min). The primary antibodies were then linked with biotinylated goat anti-mouse IgG antibody (Dako, LASB Universal) for 35 min at room temperature, washed in two changes of PBS (each 5 min), then the sections were incubated with streptavidin-conjugated peroxidase for 35 min. After being washed with PBS, the sections were stained with 3, 3'-diaminobenzidine solution (DAB, Sigma) for 10 min, then washed in distilled water and counterstained with hematoxylin stain. Negative control samples were carried out by omitting the primary antibodies for both PCNA and p16^{INK4a}.

Analysis for PCNA as well as P16^{INK4a} was done by counting 5 randomly photographed high-power fields (× 400 magnifications) within the cortex by a blinded

observer. PCNA positive cells were counted in tissue as a whole in the field. P16^{INK4a} positive cells were attributed to distal tubules and the percentage of positively stained nuclei was related to the total number of nuclei of the same tubules. Data were analyzed using IBM SPSS software package version 20.

RESULTS

Histopathological Results

Hematoxylin and Eosin Stain (H&E): Kidney sections of control group stained with H&E revealed normal histological structure of kidney cortex; well developed glomeruli, distal and proximal convoluted tubules and macula densa as shown in Figure (1).

After CCl₄ induction in group II, mononuclear cell infiltration and congestion in interstitial and in branches of blood vessels were observed after 4 weeks (Figure 2A&B). Congestion and inflammation around degenerated glomerulus, degeneration and hyalinization of glomerular tuft as well as hyaline casts in proximal convoluted tubule lumen were observed after 8 weeks (Figure 3 A&B). Fatty degeneration, glomeruli with detached basement membrane and deformed proximal convoluted tubules that had detached brush border and pyknotic figures were observed after 12 weeks (Figure 4).

Silymarin treatment in group III showed increased number of proximal convoluted tubules with pyknotic nuclei and interstitial congestion after 4 weeks of treatment (Figure 5). Distal convoluted tubules with wide lumen, glomerulus with wide space and pyknosis in little number of proximal convoluted tubules were observed after 8 weeks (Figure 6). After 12 weeks of silymarin treatment, the kidney cortex revealed more or less normal architecture of glomerulus, proximal and distal convoluted tubules (Figure 7).

Masson's Trichrome Stain: Kidney sections of control group stained with Masson's trichrome stain for demonstrating collagen deposition revealed homogeneously distributed collagen in parietal layer of glomerular capsule, in basement membrane of the glomerular capillaries of the glomerulus and in basal membrane of distal and proximal convoluted tubules (Figure 8).

Examination of kidney sections of group II after CCl₄ induction showed increased collagen deposition in parietal layer of glomerular capsule of the glomerulus

and around distal and proximal convoluted tubules after 4 weeks, more increased in its content after 8 weeks and ended with short collagen filament around proximal tubules after 12 weeks as compared with control group (Figure 9 A, B&C).

Silymarin treatment in group III diminished the effect of CCl₄ on collagen deposition and showed reduction in collagen content with time onwards where was more or less normal homogenous distribution of collagen content at last interval after 12 weeks as compared with control group (Figure 10 A, B&C).

The degree of kidney collagen content by Masson's trichrome stain was analyzed with a semi-quantitative scoring system. Comparison between different groups was analyzed using F-test (ANOVA) and Post Hoc test (Scheffe) for pair wise comparison.

Comparison between the three studied groups according to collagen distribution after 4, 8 and 12 weeks were summarized in Table (1) and Figure (11). Significant increase ($p < 0.05$) in group II (50.95 ± 6.15) as compared with group I (34.70 ± 6.55) and no significant difference was observed between group II (50.95 ± 6.15) and group III (48.99 ± 13.16) after 4 weeks of CCl₄ injection. More significant increase ($p > 0.001$) in group II (56.92 ± 2.71) as compared with each of group I (34.70 ± 6.55) and group III (32.66 ± 4.54) after 8 weeks. No significant difference was observed after 12 weeks between group I and each of the two other groups. Comparison between averages of the three studied groups revealed that collagen content returned to more or less normal distribution after silymarin treatment (37.03 ± 7.40) as compared with control group (34.70 ± 6.55) and CCl₄ group (43.0 ± 4.21).

Histochemical Results: Normal kidney sections stained with PAS showed strong glycogen content in glomeruli and in basal membrane of proximal and distal convoluted tubules. Positive strong PAS reaction was also observed in intact brush border of proximal convoluted tubules (Figure 12).

Sections of group II after CCl₄ injection for 4 and 8 weeks showed strong PAS reaction in thickened basal membrane and moderate content in brush border of proximal convoluted tubules as well as in parietal layer of glomerular capsule (Figure 13 A, B). Weak PAS reaction in loosed brush border as well as in parietal layer of glomerular capsule and moderate in basal membrane of deformed proximal tubules were observed after 12 weeks of CCl₄ injection (Figure 13 C).

Table 1: Comparison between all studied groups according to collagen distribution after 4, 8 and 12 weeks

	Group I (n=5)	Group II (n=5)	Group III (n=5)	F	p
4 Weeks	29.26 - 44.90	43.13 - 57.0	28.99 - 63.31	4.650*	0.032*
Min. – Max.	34.70 ± 6.55	50.95 ± 6.15	48.99 ± 13.16		
Mean ± SD.	32	53.17	50.54		
Median					
p ₁		0.049*	0.087		
p ₂		0.945			
Pair wise comp.	I-II**				
8 weeks	29.26 - 44.90	54.09 - 60.80	27.70 - 39.26	38.354*	<0.001*
Min. – Max.	34.70 ± 6.55	56.92 ± 2.71	32.66 ± 4.54		
Mean ± SD.	32	57.25	33.13		
Median					
p ₁		<0.001***	0.807		
p ₂		<0.001***			
Pair wise comp.	I-II***, II***-III				
12 weeks	29.26 - 44.0	13.43 - 30.29	15.92 - 43.92	3.593	
Min. – Max.	34.70 ± 6.55	21.12 ± 7.32	29.45 ± 9.96		
Mean ± SD.	32	22.21	29.27		0.06
Median					
Average	29.26 - 44.90	37.27 - 47.73	29.49 - 46.82	2.382	0.134
Min. – Max.	34.70 ± 6.55	43.0 ± 4.21	37.03 ± 7.40		
Mean ± SD.	32	41.95	35.8		
Median					

F: F test (ANOVA)

Pair wise comparison was done using Post Hoc Test (Scheffe)

p₁ : p value for comparing between group I and each other group p₂ : p value for comparing between group II and group III

*: Statistically significant at p ≤ 0.05 **: Statistically significant at p ≤ 0.01 ***: Statistically significant at p ≤ 0.001

Table 2: Comparison between the different groups according to PCNA positive nuclei number after 4, 8 and 12 weeks

PCNA	Group I (n=5)	Group II (n=5)	Group III (n=5)	F	p
4 Weeks	2.0 - 4.0	0.0 - 3.0	1.0 - 5.0	1.442	0.275
Min. – Max.	2.80 ± 0.84	1.60 ± 1.14	2.60 ± 1.52		
Mean ± SD.	3	2	2		
Median					
8 weeks	1.0 - 4.0	0.0 - 2.0	1.0 - 5.0	3.65	0.058
Min. – Max.	2.60 ± 1.14	1.0 ± 0.71	2.80 ± 1.48		
Mean ± SD.	3	1	3		
Median					
12 weeks	1.0 - 3.0	0.0 - 2.0	2.0 - 6.0	5.333*	
Min. – Max.	2.40 ± 0.89	0.80 ± 0.84	3.20 ± 1.64		
Mean ± SD.	3	1	3		0.022*
Median					
p ₁		0.144	0.579		
p ₂		0.024*			
Pair wise comp.	II - III*				
Average					
Min. – Max.	2.0 - 3.33	0.33 - 1.67	2.0 - 4.33		
Mean ± SD.	2.60 ± 0.49	1.13 ± 0.61	2.87 ± 0.90	9.187*	0.004*
Median	2.67	1.33	2.67		
p ₁		0.018*	0.831		
p ₂		0.006**			
Pair wise comp.		I' - II, II - III **			

F: F test (ANOVA)

Pair wise comparison was done using Post Hoc Test (Scheffe)

p₁ : p value for comparing between group I and each other group p₂ : p value for comparing between group II and group III

*: Statistically significant at p ≤ 0.05 **: Statistically significant at p → 0.01 ***: Statistically significant at p ≤ 0.001

Table 3: Relative number of P16^{INK4a} positive cells in distal tubules in kidney sections of all studied groups after 4, 8 and 12 weeks

P16 ^{INK4a} (×10 ⁻²)	Group I (n=5)	Group II (n=5)	Group III (n=5)	F	p
4 Weeks				10.404*	0.002*
Min. – Max.	20.2 – 26.4	6.8 – 37.7	25.2 – 33.4		
Mean ± SD.	22.75 ± 2.31	32.03 ± 4.25	29.80 ± 3.23		
Median	22.5	31.65	29.82		
p ₁		0.003**	0.020*		
p ₂		0.59			
Pair wise comp.	I – II**, I – III*				
8 weeks				12.759*	0.001*
Min. – Max.	22.58 ± 2.85	35.06 ± 6.07	25.41 ± 2.33		
Mean ± SD.	22.5	37.16	25.93		
Median					
p ₁		0.002**	0.566		
p ₂		0.010**			
Pair wise comp.	I – II**, II**-III				
12 weeks				41.302*	<0.001*
Min. – Max.	18.0 – 24.8	38.6 – 61.9	19.4 – 25.9		
Mean ± SD.	22.07 ± 2.92	49.43 ± 8.65	22.14 ± 2.66		
Median	23.6	47	21.16		
p ₁		<0.001***	1		
p ₂		<0.001***			
Pair wise comp.	I – II***, II***-III				
Average					
Min. – Max.	21.6 – 23.8	33.0 – 46.6	24.2 – 27.6		
Mean ± SD.	22.47 ± 1.12	38.84 ± 5.04	25.79 ± 1.38	39.339*	<0.001*
Median	21.69	37.89	25.36		
p ₁		<0.001***	0.274		
p ₂		<0.001***			
Pair wise comp.	I – II***, II***-III				

F: F test (ANOVA)

Pair wise comparison was done using Post Hoc Test (Scheffe)

p₁ : p value for comparing between group I and each other group p₂ : p value for comparing between group II and group III

*: Statistically significant at p ≤ 0.05 **: Statistically significant at p ≤ 0.01 ***: Statistically significant at p ≤ 0.001

Table 4: Correlation between PCNA with P16^{INK4a}

P16 ^{INK4a}	PCNA	
	r	p
4 Weeks	-0.055	0.846
8 weeks	-0.421	0.118
12 weeks	-0.592*	0.020
Average	-0.684*	0.005

r: Pearson coefficient

*: Statistically significant at p = 0.05

Kidney sections of group III after 4 weeks of CCl₄ and silymarin treatment showed moderate to strong PAS reaction in parietal layer of glomerular capsule, in basal membrane of tubules and in brush border of proximal convoluted tubules (Figure 14 A). Decrease in PAS reaction to be moderate content in most structures of kidney section after 8 weeks (Figure 14 B) as well as homogenous moderate distribution of PAS reaction was observed after 12 weeks in the same group (Figure 14 C).

Immunohistochemical Results

Immunostaining of Proliferating Cell Nuclear Antigen (PCNA): Immunostaining reaction of PCNA was confined to the nuclei and only the nuclei with moderate to strong stained reaction were considered positive.

Normal control kidney sections showed a few number of PCNA positive nuclei in some kidney tubules (Figure 15).

Sections of group II showed slight and moderate decrease in PCNA positive nuclei after 4 and 8 weeks of CCl₄ injection respectively and more reduction in PCNA positive nuclei after 12 weeks of CCl₄ injection as compared with control group at the same intervals (Figure 16 A, B&C).

Immunostaining reaction of PCNA in kidney sections of group III after CCl₄ and silymarin treatment after 4 and 8 weeks revealed more or less the same positivity of PCNA as in control group after the same periods (Figure 17 A, B) and slight increase in PCNA positive nuclei was observed after 12 weeks of treatment in the same group (Figure 17 C).

Comparison between the different groups according to the number of PCNA positive nuclei after 4, 8 and 12 weeks was summarized in Table (2) and Figure (18).

Table (2) indicated that the number of PCNA positive nuclei showed no significant difference between groups II and III comparing with group I after 4 and 8 weeks. After 12 weeks, the number of PCNA positive nuclei was significantly ($p \leq 0.05$) higher in group III (3.20 ± 1.64) than in group II (0.80 ± 0.84).

Comparison between averages of the three studied groups indicated that the number of PCNA positive nuclei was significant ($p \leq 0.05$) in group I (2.60 ± 0.49) than group II (1.13 ± 0.61) as well as more significant difference between group III (2.87 ± 0.90) and group II (1.13 ± 0.61) at $p \leq 0.01$.

Immunostaining of Cell Cycle Inhibitor P16^{INK4a}:

Immunostaining reaction of p16^{INK4a} was confined to the nuclei and its expression was assessed as positive reaction attributed to nuclei of distal tubules.

Examination of kidney sections of control group showed P16^{INK4a} positive nuclei with weak to moderate expression in distal tubules (Figure 19).

Sections of group II after CCl₄ injection showed P16^{INK4a} positive nuclei in distal tubules with moderate to strong expression after 4 weeks, strong expression after 8 weeks and intense expression after 12 weeks as compared with their counterparts in control group as shown in Figure (20) A, B and C respectively.

The positivity of P16^{INK4a} in sections of group III after CCl₄ and silymarin treatment showed gradual decline to be more or less similar to that of the control group at 12 weeks' interval (Figure 21A, B& C).

Comparison between relative number of P16^{INK4a} positive cells in distal tubules in kidney sections of all studied groups after 4, 8 and 12 weeks was summarized in Table (2) and Figure (22). Table (2) revealed that the relative number of P16^{INK4a} after 4 weeks was significantly higher ($p \leq 0.01$) in group II (32.03 ± 4.25) and significantly higher ($p \leq 0.05$) in group III (29.80 ± 3.23) as compared with group I (22.75 ± 2.31). After 8 weeks, the relative number of P16^{INK4a} was highly significant ($p \leq 0.01$) in group II (35.06 ± 6.07) as compared with group I (22.58 ± 2.85) and group III (25.41 ± 2.33). After 12 weeks, the relative number of P16^{INK4a} was more highly significant ($p \leq 0.001$) in group II (49.43 ± 8.65) as compared with group I (22.07 ± 2.92) and group III (22.14 ± 2.66).

Comparison between averages of the three studied groups indicated that the relative number of P16^{INK4a} was more highly significant ($p \leq 0.001$) in group II (38.84 ± 5.04) as compared with group I (22.47 ± 1.12) and group III (25.79 ± 1.38). No significant difference ($p > 0.05$) was observed between group I (22.47 ± 1.12) and group III (25.79 ± 1.38).

Correlation between PCNA with P16^{INK4a} was illustrated in Table (4) and Figure (23) and indicated that there was a significant ($p \leq 0.05$) negative correlation ($r=0.592$) between PCNA with P16^{INK4a} after 12 weeks as well as significant ($p \leq 0.05$) negative correlation ($r=0.684$) between average of PCNA with P16^{INK4a}.

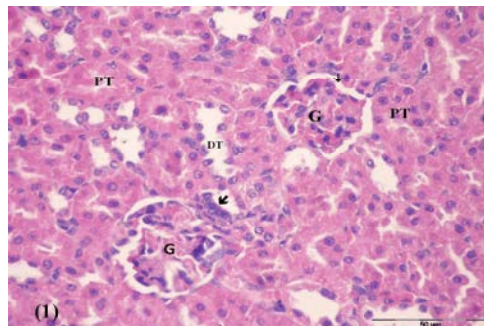


Fig. 1: A photomicrograph of the kidney section of control rat showing normal histological structure of glomerulus (G), glomerular space (↓), proximal convoluted tubule (PT), distal convoluted tubule (DT) and macula densa (thick arrow). (H&E, bar=50 μ m)

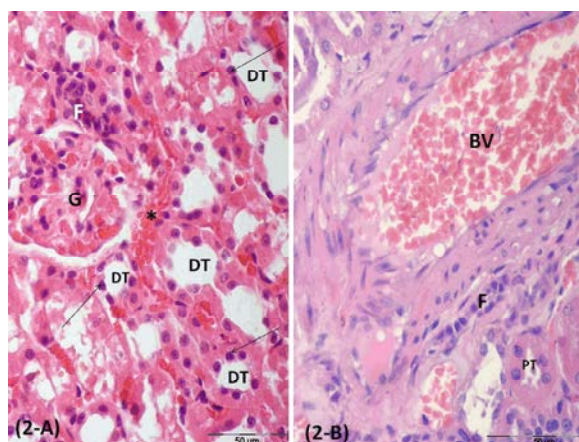


Fig. 2: Photomicrographs of the kidney sections of group II after 4 weeks of CCl_4 injection
 A. Mononuclear cell infiltration (F) and interstitial congestion (*) around glomerulus (G) Note distal convoluted tubule (DT) with wide lumens and pyknotic nuclei (†) B. High dilatation in inter tubular blood vessel (BV) impacted with haemolysed blood. Note cellular infiltration (F) and proximal convoluted tubule (PT). (H&E, bar=50 μm)

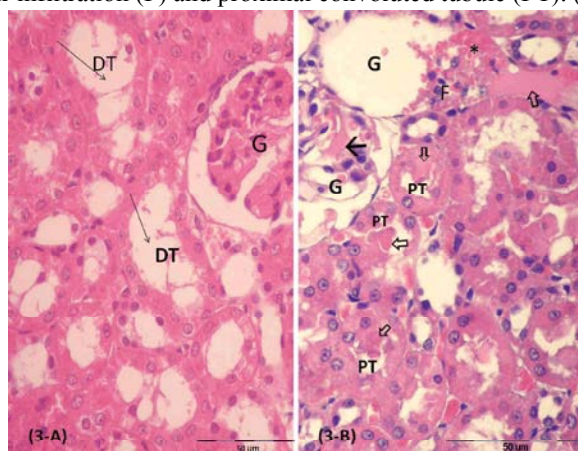


Fig. 3: Photomicrographs of the kidney sections of group II after 8 weeks of CCl_4 injection
 A. Distal convoluted tubule (DT) with wide lumen (†) B. Congestion (*) and inflammation (F) around degenerated glomerulus (G), degeneration and hyalinization of glomerular tuft (-) as well as hyaline casts (‡) in proximal convoluted tubule lumen (PT). (H&E, bar=50 μm)

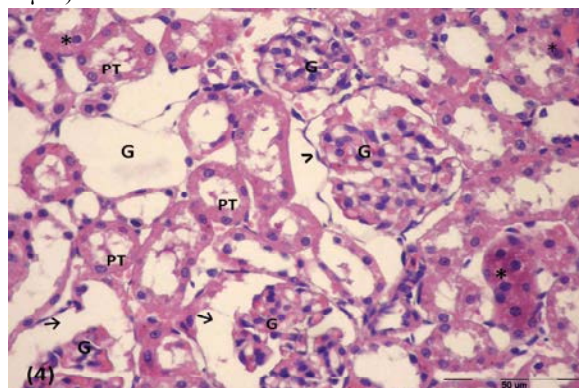


Fig. 4: A photomicrograph of the kidney section of group II after 12 weeks of CCl_4 injection showing fatty degeneration; degenerated glomerulus (G), other glomeruli with wide space (†) and detached basement membrane (arrow head) and deformed proximal convoluted tubules (PT) with detached brush border. Note: pyknotic figures (*). (H&E, Bar = 50 μm)

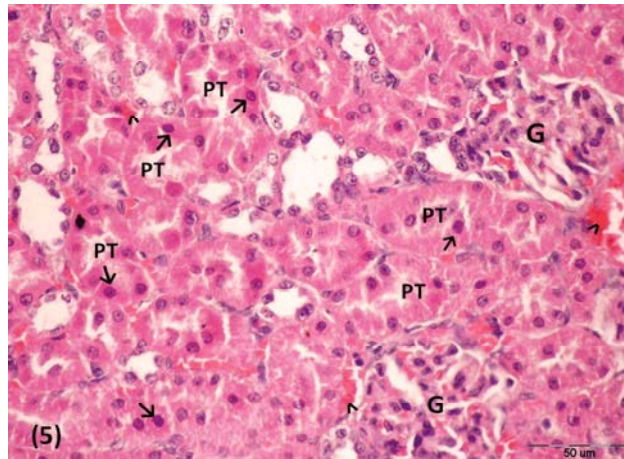


Fig. 5: A photomicrograph of the kidney section of group III after 4 weeks of CCl₄ and silymarin treatment showing glomerulus (G), increased number of proximal convoluted tubules (PT) with pyknotic nuclei (↑) and interstitial congestion (arrow head). (H&E, bar=50 μm)

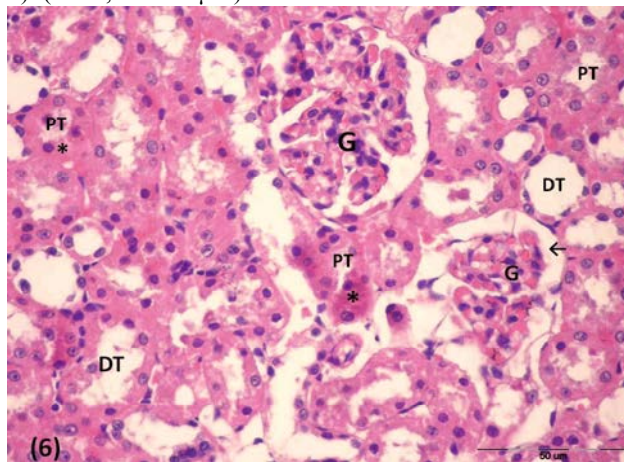


Fig. 6: A photomicrograph of the kidney section of group III after 8 weeks of silymarin treatment showing distal convoluted tubules (DT) with wide lumen, glomerulus (G) with wide space (↑). Note pyknosis in proximal convoluted tubule (*). (H&E, Bar=50 μm)

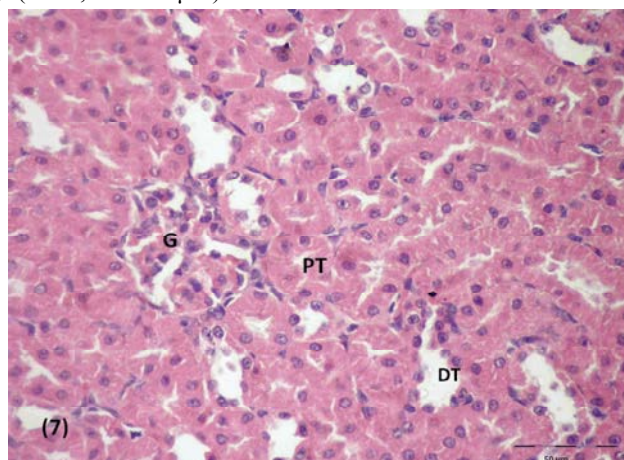


Fig. 7: A photomicrograph of the kidney section of group III after 12 weeks of silymarin treatment showing more or less normal architecture of glomerulus (G), proximal convoluted tubule (PT) and distal convoluted tubule (DT). (H&E, bar=50 μm)

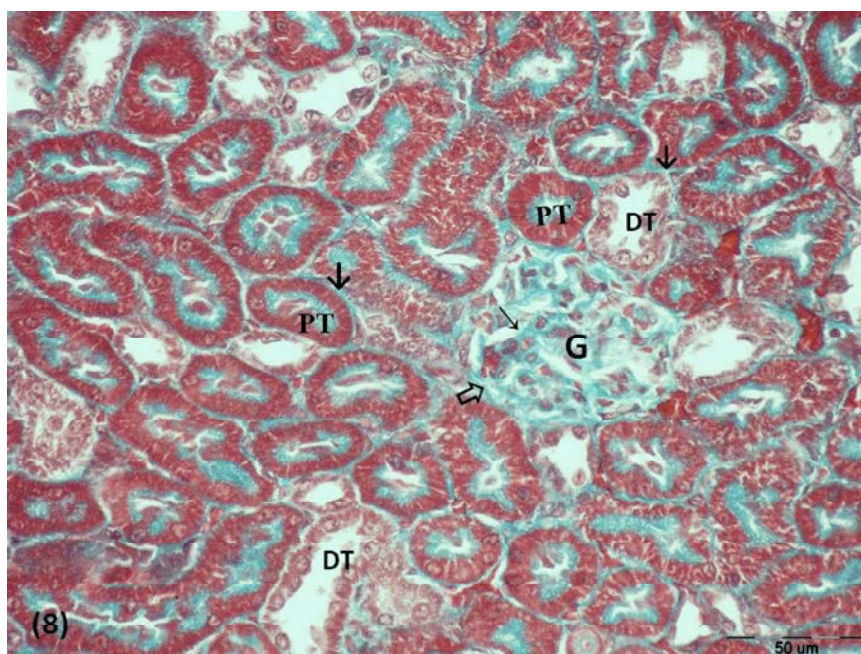


Fig. 8: A photomicrograph of the kidney section of control group showing collagen deposition distributed homogenously in parietal layer (↑) of glomerular capsule, in basement membrane of the glomerular capillaries (⬆) of the glomerulus (G) and in basal membrane (thick arrow) of distal (DT) and proximal (PT) convoluted tubules. (Masson's trichrome, bar= 50 μm)

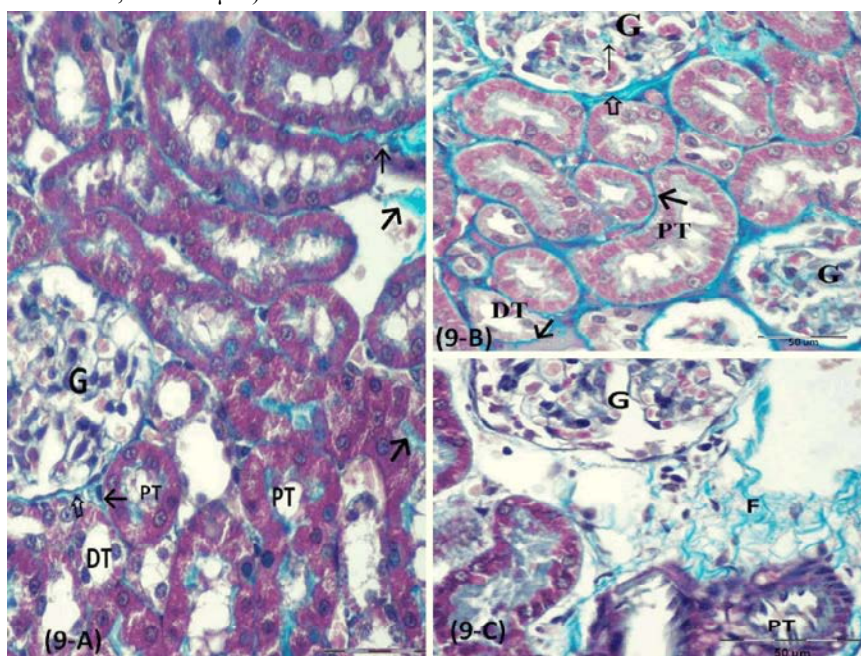


Fig. 9: Photomicrographs of the kidney sections of group II after CCl_4 injection
 A. After 4 weeks, increased collagen deposition in parietal layer of glomerular capsule (↑) of the glomerulus (G) and collagen deposition (⬆) around proximal (PT) and distal (DT) convoluted tubules.
 B. After 8 weeks, more increased collagen deposition in parietal layer of glomerular capsule (↑), in basement membrane of the glomerular capillaries (⬆) of the glomerulus (G) and in basal membrane (⬆) of distal (DT) and proximal (PT) convoluted tubules.
 C. After 12 weeks, short collagen filament (F) around proximal tubule (PT). (Masson's trichrome, bar= 50 μm).

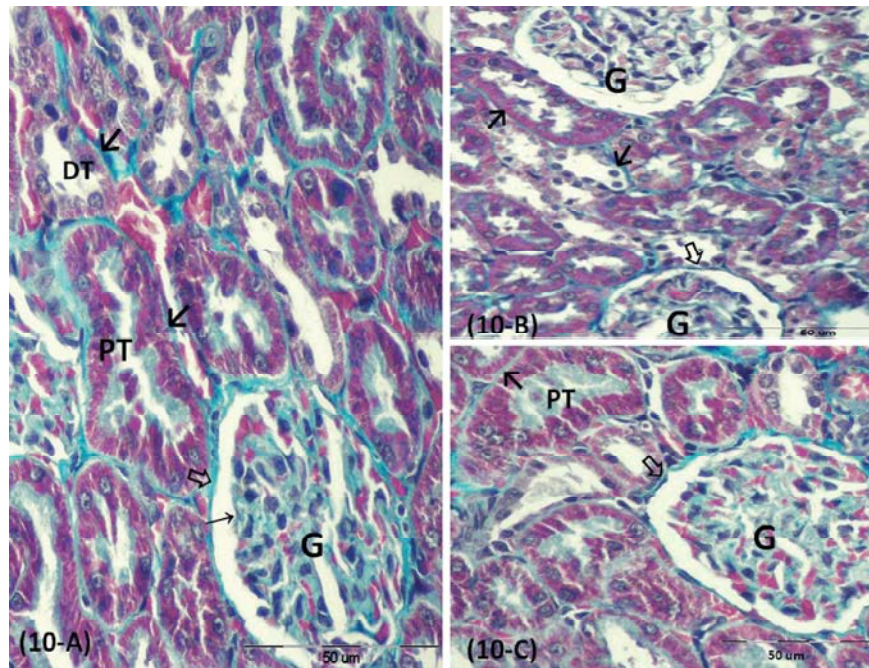


Fig. 10: Photomicrographs of the kidney sections of group III after CCl_4 and silymarin treatment.

- A. After 4 weeks, collagen deposition in parietal layer of glomerular capsule (\parallel) and in basement membrane (\parallel) of the glomerular capillaries (G) as well as in basal membrane (\parallel) of proximal (PT) and distal (DT) convoluted tubules.
- B. After 8 weeks, a thin rim of collagen deposition (\parallel) in parietal layer of glomerular capsule (G) and in basal membrane (\parallel) of distal (DT) and proximal (PT) convoluted tubules.
- C. After 12 weeks, more or less normal homogenous distribution of collagen deposition (\parallel) in parietal layer of glomerular capsule (G) and in basal membrane (\parallel) of proximal convoluted tubules (PT). (Masson's trichrome, bar= 50 μm).

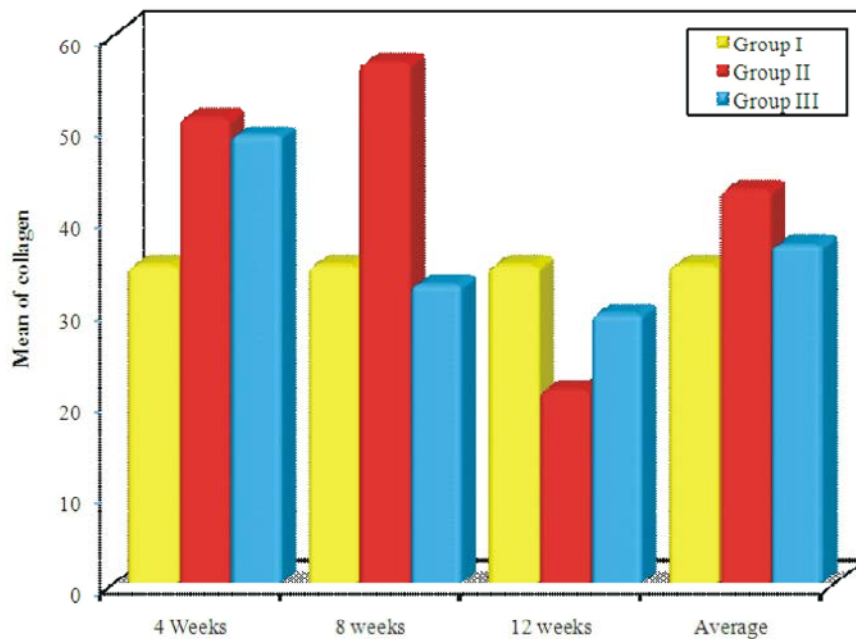


Fig. 11: Collagen distribution in groups I, II & III and the average of the 3 groups

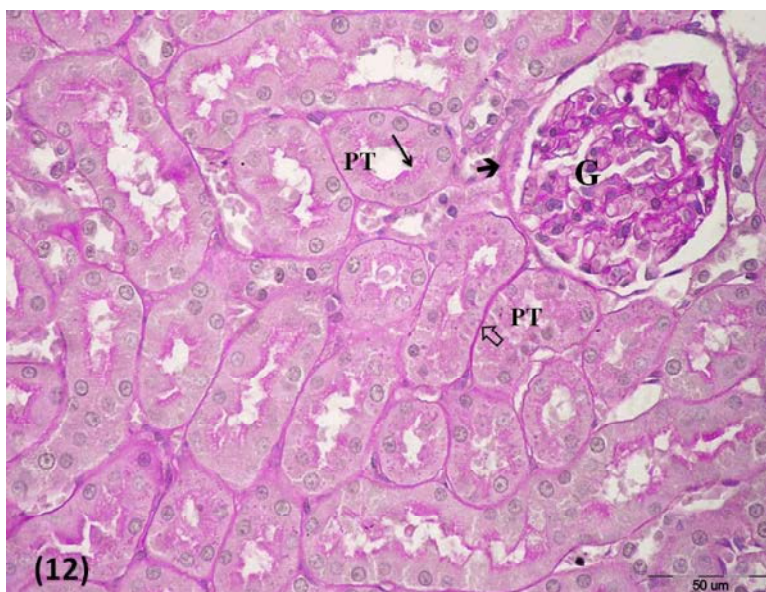


Fig. 12: A photomicrograph of the kidney section of normal control group showing strong glycogen content in glomerulus (G), moderate content in parietal layer of glomerular capsule (↯) and in basal membrane (↯) of proximal convoluted tubules (PT). Note strong PAS reaction (↯) in intact brush border of proximal convoluted tubules (PT). (PAS, bar= 50 μm).

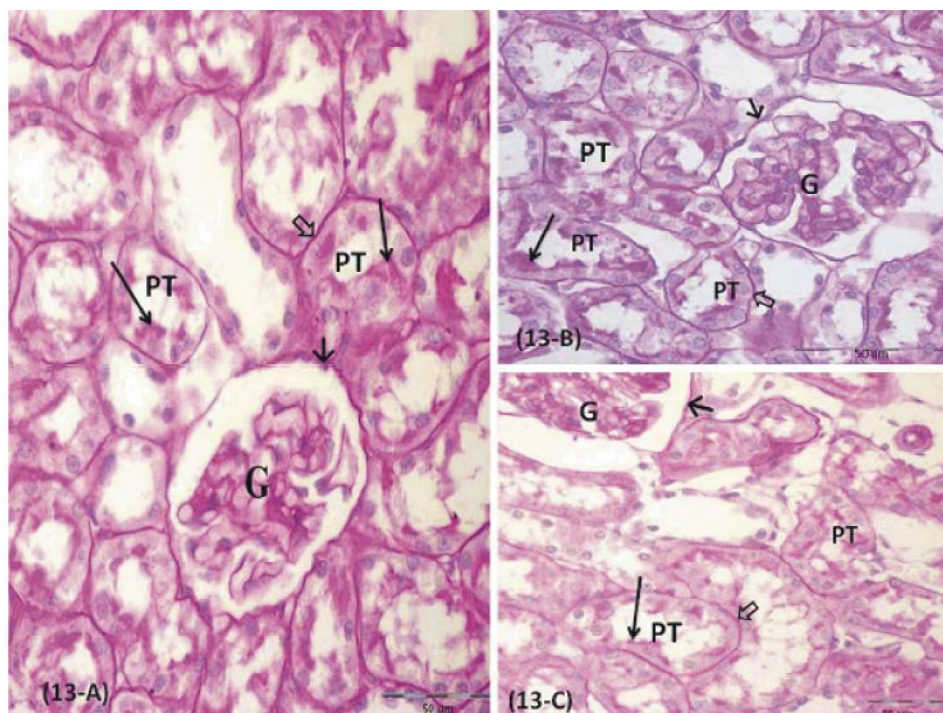


Fig. 13: Photomicrographs of the kidney sections of group II after CCl_4 injection. (A & B). Strong PAS reaction (↯) in thickened basal membrane and moderate in brush border (↯) of proximal convoluted tubules (PT) as well as in parietal layer of glomerular capsule (↯) after 4 and 8 weeks. C. Weak PAS reaction in loosened brush border (↯) as well as in parietal layer (↯) of glomerular capsule of the glomerulus (G) and moderate in basal membrane (↯) of deformed proximal tubules (PT) after 12 weeks. (PAS, bar= 50 μm).

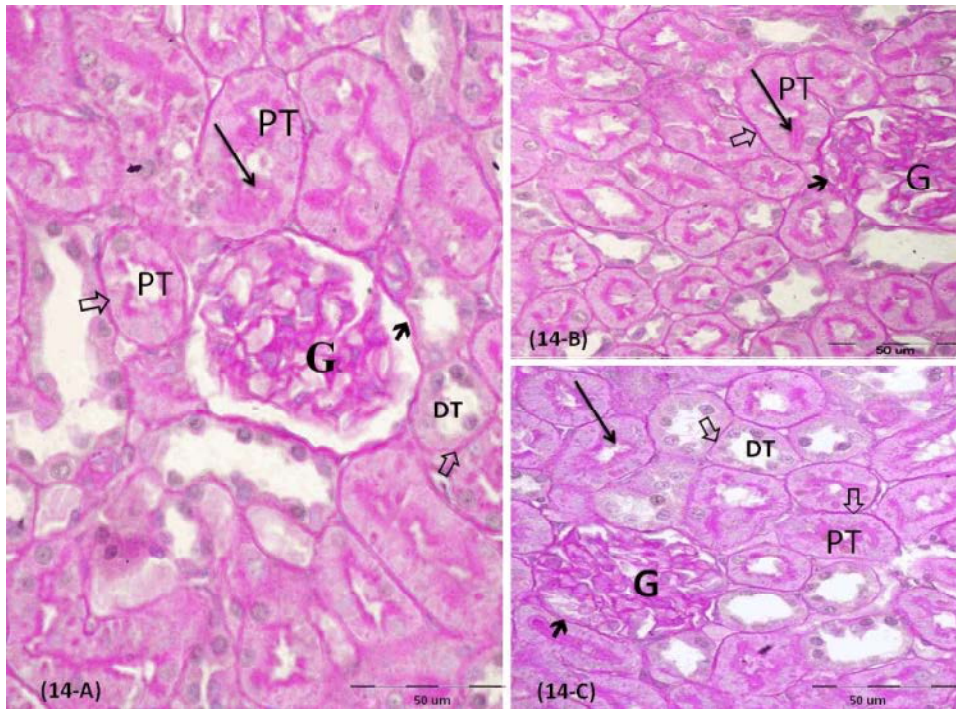


Fig. 14: Photomicrographs of the kidney sections of group III after CCl_4 and silymarin treatment.

- A. After 4 weeks, moderate to strong PAS reaction (¶) in parietal layer of glomerular capsule of the glomerulus (G), in basal membrane (¶) of proximal (PT) and distal (DT) tubules and in brush border (¶) of proximal convoluted tubules (PT)
- B. After 8 weeks, moderate distribution of PAS reaction in basal membrane (¶) and in intact brush border (¶) of all proximal convoluted tubules (PT) and in parietal layer (¶) of glomerular capsule of the glomerulus (G)
- C. After 12 weeks, homogenous moderate distribution of PAS reaction (¶) in basal membrane of all convoluted tubules (PT & DT) and parietal layer of glomerular capsule of the glomerulus (G) as well as in brush border (¶) of proximal tubules (PT). (PAS, bar= 50 µm)

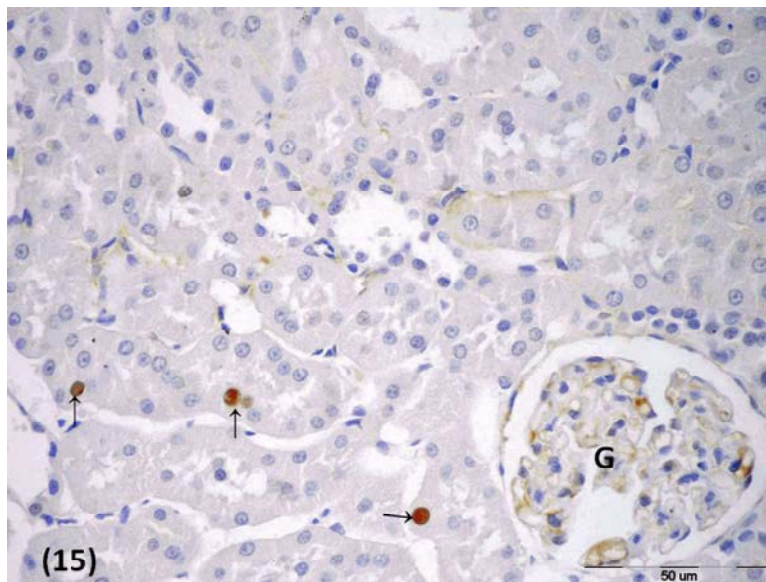


Fig. 15: A photomicrograph of kidney section of control group showing PCNA positive nuclei in kidney tubules (¶) with a limited number. (Avidin-biotin complex system / bar = 50 µm)

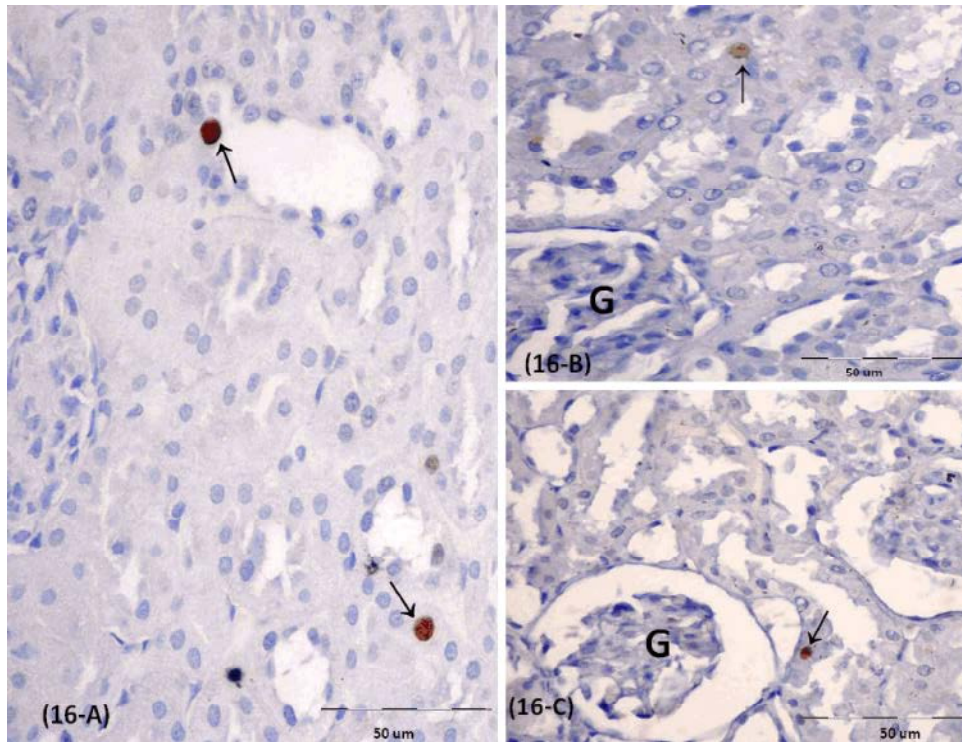


Fig. 16: Photomicrographs of kidney sections of group II after CCl_4 injection showing PCNA positive nuclei in kidney tubules (↑); (A) a slight decrease after 4 weeks, (B) moderate decrease after 8 weeks and (C) more reduction after 12 weeks as compared with control group. (Avidin-biotin complex system / bar= 50 μm)

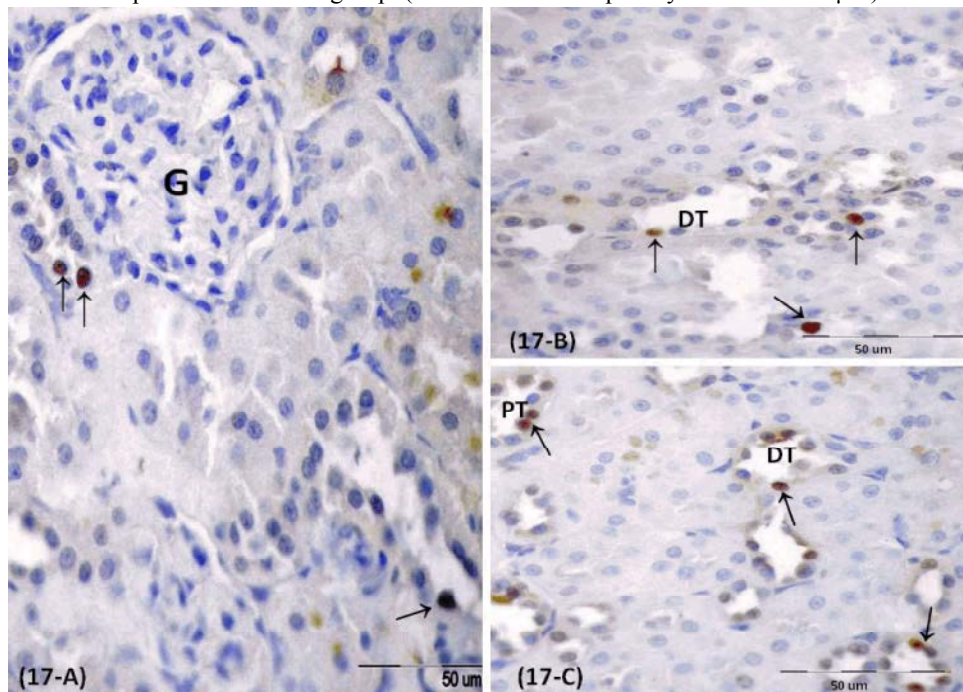


Fig. 17: Photomicrographs of the kidney sections of group III after CCl_4 and silymarin treatment (A, B): PCNA positive nuclei (↑) in distal tubules (DT) more or less as normal after 4 and 8 weeks respectively. (C): Slight increase in PCNA positive nuclei (↑) in proximal (PT) and distal (DT) tubules after 12 weeks as compared with counterparts. (Avidin-biotin complex system / bar= 50 μm)

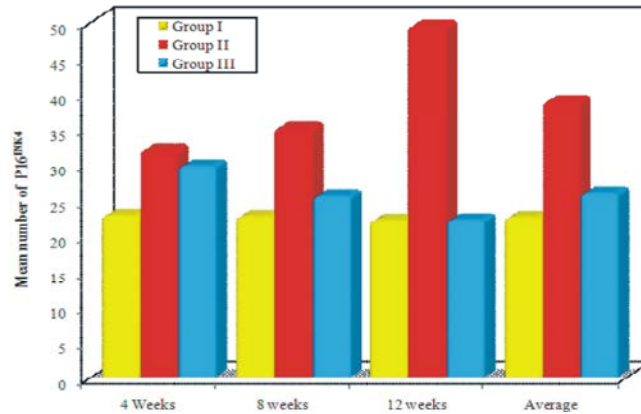


Fig. 18: Expression of PCNA in all studied groups after 4, 8 and 12 weeks and the average of the 3 groups

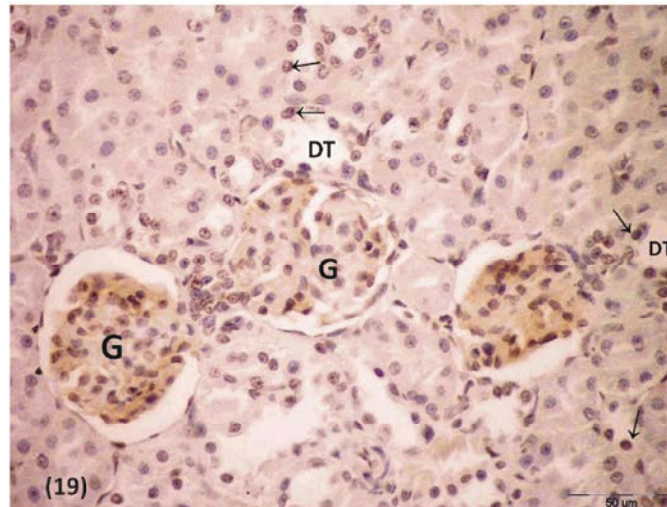


Fig. 19: A photomicrograph of the kidney section of control group showing P16^{INK4a} positive nuclei with moderate expression in distal tubules (↑). (Streptavidin-biotin complex system / bar= 50 µm)

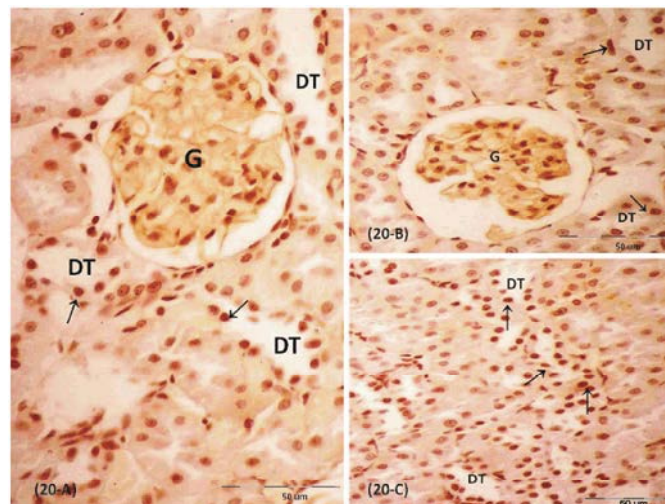


Fig. 20: Photomicrographs of the kidney sections of group II after CCl₄ injection showing P16^{INK4a} positive nuclei (↑) in distal tubules (DT); (A) moderate to strong expression after 4 weeks, (B) strong expression after 8 weeks and (C) intense expression after 12 weeks. (Streptavidin- biotin complex system/ bar= 50 µm)

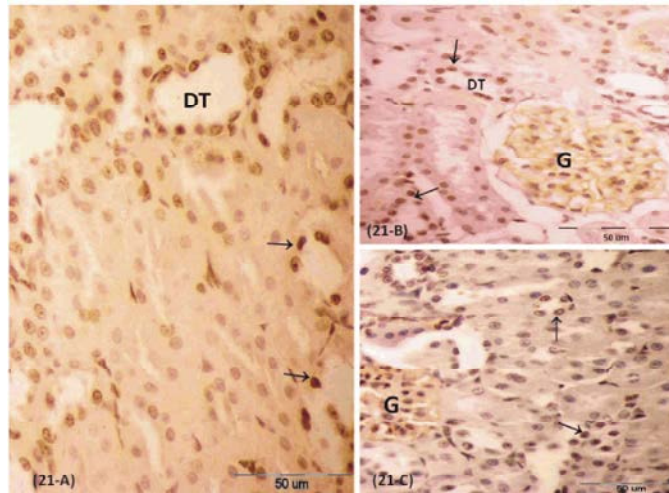


Fig. 21: Photomicrographs of the kidney sections of group III after CCl₄ and silymarin treatment showing P16^{INK4a} positive nuclei (Ⓛ) in distal tubules (DT); (A) high moderate increase in P16^{INK4a} positive nuclei after 4 weeks, (B) moderate in P16^{INK4a} positive nuclei after 8 and (C) more or less normal expression of P16^{INK4a} positive number after 12 weeks as compared with controls (Streptavidin-biotin complex system/ bar= 50 μm)

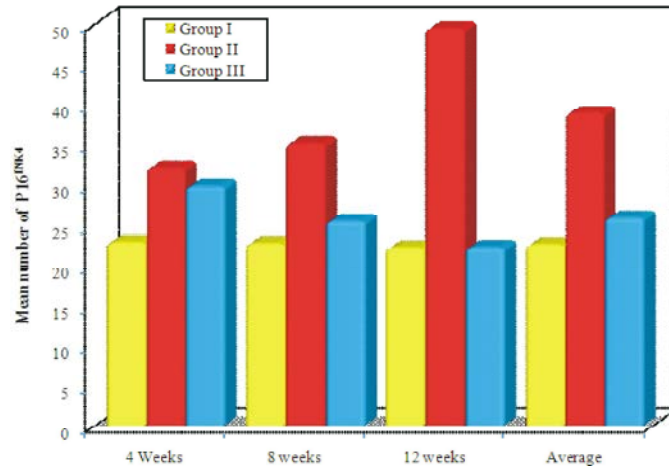


Fig. 22: Relative number of P16^{INK4a} positive cells in distal tubules in kidney sections of all studied groups after 4, 8 and 12 weeks

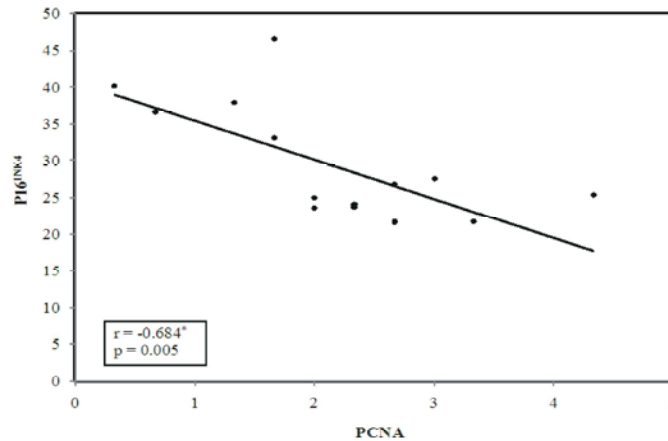


Fig. 23: Correlation between PCNA with P16^{INK4}

DISCUSSION

Many previous studies on CCl₄ intoxication indicated that it was distributed at higher concentrations in the kidney than in the liver [20] and it showed a high affinity to the kidney cortex which contains cytochrome P-450 predominantly [21]. CCl₄ was used in many studies for inducing renal damage and has a role in contributing an assortment to nephrotoxicity [22, 23, 24]. It was established that the nephrotoxicity induced by chemical agents is one of the consequences of the accumulation of certain metabolites in kidneys [25].

It is well known that CCl₄ is metabolized by cytochrome P450 to trichloromethyl radical (CCl₃^{*}). The nephrotoxic effects of CCl₄ were associated with free radical production [26] and the damage induced by CCl₄ was also able to alter the antioxidant state of the tissues which was manifested by abnormal histopathological changes [27]. Free radicals that induced lipid peroxidation caused oxidative damage in DNA, proteins and lipids [28]. The cell membrane damage leading to a number of pathological changes in acute and chronic renal injuries were reported in many studies [8, 29].

It was evident from the current results that the kidney damage induced by CCl₄ manifested histopathologically by degenerative changes in cortex from 4 weeks after CCl₄ injection and onward. Mono-cellular infiltrations, congestion in interstitial and in blood vessels, hyalization in glomerular tuft as well as hyaline casts in lumen of some proximal tubules. Deformed tubules with detaching brush border, pyknotic figures and degenerated glomeruli ended with fatty degeneration were more obvious after 12 weeks of CCl₄ injection. The degenerative changes observed in the present study would indicate the action of CCl₄ toxicity on kidney cortex reflecting oxidative stress condition, a major mechanism for the toxicity of CCl₄, as a result of free radicals production.

The current results were in consistency with many studies and confirmed their obtained results. Those studies revealed that CCl₄ caused histopathologically prominent damage in kidney; monocellular infiltration, interstitial hemorrhage, vascular and glomerular congestion with vacuolization of the glomerular tuft, tubular degeneration and necrosis [22- 24, 30-33].

The present results showed that silymarin treatment exerted its beneficial effect on kidney tissue and antagonized the toxic effect of CCl₄ gradually from the 4th week till the 12th week of treatment. Tubular pyknosis and interstitial congestion were still observed after 4

weeks, distal convoluted tubules with wide lumen and decreased pyknotic figures encountered after 8 weeks. The kidney cortex revealed more or less normal architecture after 12 weeks of silymarin treatment; glomeruli, proximal and distal convoluted tubules. These current results confirmed the antioxidant role of silymarin in preventing the oxidative damage and alleviating the oxidative stress induced with CCl₄ in rat kidney.

In accordance to the present results; El-Shitany *et al.* [34] reported that silymarin inhibited renal tubular damage in rat induced by adriamycin and they attributed that result to inhibition of lipid peroxidation. Moreover, Soto *et al.* [35] established that silymarin treatment prevented tissue damage and considered as a potentially free radical scavenger in diabetic nephropathy. Silymarin exacerbated the renal impairment and tubular apoptosis in glycerol-induced acute kidney injury in rats [36]. Similarly, karimi *et al.* [37] and Abdelmeguid *et al.* [38] reported that silymarin has anti-nephrotoxic activity and attenuated glomerular and tubular atrophy in rats induced by cisplatin. Agarwal *et al.* [39] investigated that silymarin was a potent anti-inflammatory agent and it inhibited aflatoxin B (1)-induced lipid peroxidation in rat liver and kidney [40].

Renal fibrosis is an excessive accumulation of extracellular matrices such as collagen and fibronectins and considered the principal process involved in the progression of chronic kidney disease [41].

The normal glomerular basement membrane, composed of type IV collagen, has an important function in the process of filtration [42]. Therefore, increased collagen production by mesangial cells plays a key role in the development and progression of glomerular sclerosis [43].

The current results showed, in addition to histopathological damage in kidney, that CCl₄ induction also resulted in increased collagen deposition. Our data clearly revealed that silymarin treatment diminished the collagen level and showed reduction in collagen deposition with time onwards till reaching to more or less normal distribution after 12 weeks. Average content of collagen in the three studied groups revealed that its content returned to more or less normal distribution after silymarin treatment (37.03 ± 7.40) as compared with control group (34.70 ± 6.55) and CCl₄ group (43.0 ± 4.21). This current result was most probably attributed to anti-fibrogenic effect of silymarin.

The present result confirmed the result of the previous study of Ohyama *et al.* [44], who concluded that the fibrogenetic factor from damaged rat kidney after CCl₄ treatment was considered as a common endogenous collagen synthesis stimulator, thus providing a driving force for collagen over-production.

The same explanation was reported by Yang *et al.* [45], who stated that the development of interstitial fibrosis was a secondary process that resulted from defective epithelial repair and could be regarded as a default mechanism of inadequate regeneration as well as tubular cells that were arrested in G2/M after acute injury secrete profibrogenic factors such as TGFβ1 and connective tissue growth factor.

Similarly, Cohen [46] reported that the presence of kidney fibrosis seems mostly to be viewed as an endpoint or marker of tissue or organ failure and loss of function. It was also stated by Pradère *et al.* [41] that the renal fibrosis is the principal process involved in the progression of chronic kidney disease.

Many previous studies were in compatible with our results; Dođukan *et al.* [22] and Ogeturk *et al.* [24] demonstrated interstitial fibrosis induced by CCl₄ in rat's kidney. Those results were confirmed by Hamed *et al.* [31] who reported increased collagen deposition after CCl₄ induction. They attributed their results to the nephrotoxicity induced by CCl₄ enhancing inflammation and fibrosis.

Several studies established the anti-fibrotic role of silymarin. The previous studies of Mourelle *et al.* [47] and Favari and Perez-Alvarez [48] reported that silymarin reduced collagen content in liver of rat as compared to CCl₄ treated rats. The same results were estimated by George *et al.* [49] and Zhao *et al.* [50] and reported reduced collagen level after silymarin treatment in liver fibrosis induced by dimethylnitroamine reflecting the role of silymarin as a non inducer of collagen deposition or fibrosis.

Concerning the histochemical result of glycogen, sections of kidneys were stained with PAS highlighted basement membranes as well as demonstrated the prominent brush border projecting into lumen of proximal convoluted tubules.

The current result revealed that induction of CCl₄ revealed reduction in glycogen content with time dependent to be weak in loosed brush border and in parietal layer of glomerular capsule and moderate in basal membrane of deformed proximal tubules after 12 weeks as compared with counterparts.

The focal loss of luminal brush border on the PAS stain in the present result was considered another morphologic criterion for acute tubular necrosis. The thickened basement membrane in the present result was an indication of tubular degeneration which stained strongly with PAS after 4 weeks of CCl₄ induction then finally showed moderate reaction as a result of deformed tubules which attributed to toxic effect of CCl₄ on kidney structure and function.

In agreement of this result, Jiang *et al.* [51] indicated that CCl₄, beside its oxidative stresses to multiple rat organs, caused alterations of their functions including renal osmotic regulations, accelerated glycolysis and protein and nucleotide catabolism. In this context, Condelario-Jalil *et al.* [52] indicated that CCl₄ caused depletion in glycogen content in liver cells. Muriel *et al.* [53] attributed the decrease in hepatic glycogen content to decreased gluconeogenesis by the liver cells and explained that sufficient glycogen level was essential to provide glucose to drive glycolysis.

The findings of the present study indicated that silymarin treatment upset the effect of CCl₄ on glycogen content and revealed homogenous moderate distribution of PAS granules in intact brush border as well as in basal membranes after 12 weeks. The present result was attributed to the positive effect of silymarin on tubules which came back to normal architecture and hence returned their function.

Coincided with the present results, Favari and Perez-Alvarez [48] found that CCl₄ injection reduced glycogen content in liver tissues and this reduction was completely prevented by silymarin treatment. Abouzeinab [54] estimated that silymarin ameliorated the principal functions of the liver related to the regulation of carbohydrate metabolism and blood glucose homeostasis and confirmed that result which was reported by Abdel-Salam *et al.* [55], who demonstrated the protective effect of silymarin and ribavirin on CCl₄-induced liver toxicity in male and female rats.

Proliferating cell nuclear antigen (PCNA) is a stable cell cycle-related nuclear protein and represents a reliable marker for the determination of proliferative activity [56]. It is expressed in late G1 and expressed maximally during S- phase of the cell cycle and its rate of synthesis correlated with the proliferative rate of cells [57, 58].

Immunohistochemical results of PCNA in the present study demonstrated PCNA expression in the nuclei which were closely associated with renal tubular cells. The current results demonstrated that the number of PCNA

positive nuclei was decreased after CCl₄ injection in group II (1.13 ± 0.61) with less significant than group I (2.60 ± 0.49) at ($p \leq 0.05$). This present finding may be attributed to increased damage in renal tubules that had a defect in their function.

In contradiction to the current results, study of Kim *et al.* [59] showed increasing in PCNA expression in dimethylnitrosamine –treated group. The same result was observed by Amin *et al.* [60] who revealed increased PCNA positive cells in liver of CCl₄ treated rats as compared with control and attributed to the response to liver damage where the liver undergo a compensatory regeneration.

In the present study, CCl₄ and silymarin treatment in group (III) showed increased in expression of PCNA positive nuclei to become more or less as normal number when compared with control. Also, the expression of PCNA was increased in group III (2.87 ± 0.90) with more significant difference at $p \leq 0.01$ as compared with group II (1.13 ± 0.61). This result established the effective role of silymarin in increasing the proliferative activity of renal tubular cells as cells regenerated after silymarin treatment.

In supporting to the present results, a significant increase in expression of PCNA after silymarin treatment in acute liver injury induced by dimethylnitrosamine [59] and in liver injury induced by CCl₄ [60]. They attributed the increment in PCNA expression to the stronger replicative activity of liver cells after silymarin treatment.

P16^{INK4a} is a cyclin-dependent kinase inhibitor act upstream of retinoblastoma (Rb) in the 'p16^{INK4a}/Rb pathway. It has been associated with non-replication-dependent growth arrest [61, 62, 63] as well as associated with cyclin-dependent kinases, CDKs, (cdk4/6), thereby inactivating cdk4/6 [64, 65], predominantly cdk4 [66]. It has been shown that phosphorylation of Rb, in the proximal tubules and mesangial cells of the kidney, is critical for the passage of cells from G1-to-S phase [67, 68].

The result of the present work revealed that P16^{INK4a} (a cell cycle inhibitor) was activated in group II after CCl₄ injection and it was time dependent; showed positivity with more significant in the number of cells expressing p16^{INK4a} after 12 weeks.

Group III after CCl₄ and silymarin treatment showed gradually decreased in expression of P16^{INK4a} positive number till reached to more or less normal expression after 12 weeks of treatment as compared with controls in the same intervals. Statistically, comparison between averages of the three studied groups indicated that the relative number of P16^{INK4a} was more highly significant ($p \leq 0.001$) in group II (38.84 ± 5.04) as compared with

group I (22.47 ± 1.12) and group III (25.79 ± 1.38). No significant difference ($p > 0.05$) was observed between group I (22.47 ± 1.12) and group III (25.79 ± 1.38).

Clarification of these current results was based on the increment of free radicals production (ROS) as a result of CCl₄ intoxication which most probably caused disturbance in cell cycle as a reactive process of kidney injury. In accordance, Jayakumar *et al.* [7] demonstrated that CCl₄ caused generation of reactive oxygen species (ROS) in many tissues other than the liver including the kidney, heart, lung, testis, brain and blood. Also, free radicals induced lipid peroxidation and caused oxidative damage in DNA, proteins and lipids as demonstrated by Melin *et al.* [28].

In this context, Finkel [69] stated that although ROS are required for the physiological function of the cells, excessive ROS caused anti-proliferative effects such as apoptosis and/or cellular senescence. Also, Chkhotua *et al.* [70] studied the influence of renal ischemia / reperfusion on the expression of cyclin-dependent kinase inhibitor genes (CDKIGs) and showed over-expression of p16, p21 and p27, positively related with the reperfusion time, indicating on substantial DNA damage and/or accelerated tissue senescence. Tanaka *et al.* [71] demonstrated that the cell cycle progression and arrest of renal tubular cells after acute injury is a reactive process of renal regeneration.

Melk [72] in his study on senescence of renal cells showed that in aging kidney, there was a progressive increase in the number of cells expressing p16^{INK4a} and exiting the cell cycle and that high numbers of cells expressing p16^{INK4a} could limit repair in the ageing kidney. The author also stated that p16^{INK4a} expression and/or telomere shortening could be causal in the development of tubular atrophy and interstitial fibrosis.

The normalization of p16^{INK4a} expression of positive nuclei after silymarin treatment especially after 12 weeks in group III in the present results revealed the antioxidant role of silymarin on kidney injury as reducing increased ROS production and alleviated the oxidative stress induced by CCl₄ thus reduced the positive number of p16^{INK4a}. This result confirmed the results which obtained from the current histopathological study in which silymarin reduced the toxic effect of CCl₄ on kidney cortex and returned to normal structure and function. These present findings supported the results of Soto *et al.* [35] and Homsy *et al.* [36] who established that silymarin treatment prevented tissue damage and considered as a potentially free radical scavenger in kidney injury induced in rats.

The correlation of PCNA with P16^{INK4a} in the present result indicated that there was a significant ($p \leq 0.05$) negative correlation ($r=0.592$) between PCNA with P16^{INK4a} especially after 12 weeks of experiment as well as significant ($p \leq 0.05$) negative correlation ($r=0.684$) between average of PCNA with P16^{INK4a}. This result confirmed the important antioxidant role of silymarin in antagonizing the deleterious effects of CCl₄ on rat's kidney cortex and revealed that as PCNA expression gradually increased, the expression of P16^{INK4a} decreased, where the renal tubular enhanced proliferation and decreased the cell cycle inhibitor after silymarin treatment.

In conclusion, from the current study, increasing in ROS production during CCl₄ toxicity resulted in damage to kidney tubules, increasing in collagen as well as decreasing in PAS. Thereby, all cumulative changes inhibited the expression of proliferating cell nuclear antigen (PCNA) and activated the expression of cell cycle inhibitor P16^{INK4a}. After silymarin treatment, the kidney tissues reversed to normal architecture and antagonized the toxic effect of CCl₄ which enhanced PCNA and inhibited P16^{INK4a}.

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