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Detection of *cnf1* gene in the local human uropathogenic *E. coli* isolate and study some immunological and histopathological effects *in vivo*

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Abstract: Uropathogenic *Escherichia coli* produce cytotoxic necrotizing factor-1 (CNF-1). Which is mediates its effects on epithelial cells, and acts as one of uropathogenic *E. coli* virulence factors . Therefore this study was aimed to know the behavior of peripheral blood lymphocytes during the intravenous injection of CNF-1toxin , which has been poorly investigated *in vivo* .By using flow cytometery and light microscope, the effect of CNF1 was explored in rabbits, after certified the chromosomal *cnf1* gene is found in the bacterial isolate. PCR analysis confirmed the foundation of the chromosomal *cnf1* gene in the selected uropathogenic isolate. Whereas the assays of lymphocytes apoptosis and necrosis, revealed different effects, which were correlated with the investigated doses (1.82,3.65,5.47, and 7.3 µg/kg).The last two doses shown severe effects on some vital organs (liver, kidneys, and lungs)of rabbits. Histological evaluation of rabbits treated with the dose 3.65 µg/kg revealed different changes occurred grossly due to the cytotoxic effect of CNF1. They ranged between severe congestion in liver and lung tissues to paleness of kidney with changing in their textures. Microscopic examination showed, infiltration of inflammatory cells with fibrin network and cellular necrosis were observed. A conclusion of study revealed that,CNF1of uropathogenic *E. coli* , coded by chromosomal *cnf1* gene with 498bp ,has cytotoxic effects on immune system represented by lymphocytes apoptosis and necrosis. As well as, different histopathological effects in some vital organs have been observed.

Keywords: Cytotoxic necrotizing factor-1 , Apoptosis , Necrosis , Uropathogenic *E. coli*.

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Introduction:

Bacterial toxin is one of the pathogenic bacterial virulence factors, represents one of the main bacterial strategies that interact with mammalian cells. Toxins manipulate host cell functions in a way that can favor the survival and spread of the microbes (1). The first toxin described to dominantly activate Rho GTPase (is a cohort of small (~21 kDa) signalling G proteins, and is a sub cohort of the Ras superfamily) was the cytotoxic necrotizing factor-1 (CNF-1), which is produced by pathogenic *E.coli* strains. It obtained its name from its necrotizing effect on rabbit skin when it was isolated and tested (2). CNF1 is the best-analyzed member of the growing toxin family with identical (CNFs) or similar (Dermonecrotic Toxin) activities. Uropathogenic *E.coli* (UPEC) is one of the most prevalent strains in infections of the urinary tract (3,4). It is generally accepted, that most of the UPEC strains live in the intestine and enter the urinary tract via the urethra. CNF1 is a major virulence factor of UPEC strains (5). It was reported, that

98% of cnf1+ strains were also positive for hemolysin gene (hly) (6). Meanwhile,four CNF isoforms are known: CNF1, CNF2 (85% sequence identity with CNF1) and CNF3 (70% sequence identity with CNF1) from *E. coli* and CNFY (61% sequence identity with CNF1) from Yersinia pseudotuberculosis. Present study was conducted to detect *cnf1* gene in local uropathogenic *E.coli* isolates,whether peripheral blood lymphocytes can be affected by CNF-1. In addition, the effect of CNF-1on several vital normal rabbits was also evaluated.

Materials and Methods:

This study was conducted during November 2013 to February 2014. Fourty uropathogenic *E. coli* were isolated from several hospitals of Baghdad city in case of urinary tract infections(7), and characterized according to (8,9). The suitable local isolate which was resistant to most usable antibiotics, and has a necrotizing effect on mice footpad was selected.

Detection of the *cnf1* gene by PCR technique:

According to (10), a standard ′boiling method′ was used to extract DNA from 1.5 ml of anovernight bacterial growth culture (Luria-Bertani broth /himedia) was harvested, suspended in sterile distilled water, and incubated at 95ºC for 10 min. then centrifugation of the lysate (8000xg) for 10 minutes was done. The supernatant was stored at -20 ºC as a template DNA stock. Detection of *cnf1* gene was performed by amplifying the gene by PCR. Nonpathogenic standard *E. coli* (ATCC 25922) was used as negative control. The primer sequence for detection of *cnf1* gene (ALPHA DNA), was designed according to (11) with a size of 498 bp. The primers sequence for detection *cnf1* gene were

5'-AAGATGGAGTTTCCTATGCAGGAG-3'
5'-CATTCAGAGTCCTGCCCTCATTATT-3'

Amplification was performed in a thermal cycler (Table 1), according to a method described by (12).

Table (1): Thermocycling conditions of *cnf1* amplification.

Steps	temperature	time
Initial denaturation.	95° C	5 min.
denaturation.	95° C	30 sec.
Annealing	(42-45-47-49-52)° C	45 sec.
Extension	72° C	45 sec.
Final extension	72° C	7 min.
No. of cycle	35	

In vivo test:

The stock suspension of partial purified CNF1 with protein concentration 73 µg/ml, prepared previously **(7)** was tested on 15 healthy male New Zealand white rabbits, weighing 1kg. Animals were randomly divided into five groups (3 animals/ group), by injecting the laboratory animals intravenously with four different concentrations. Group 1, represented as a control, was treated

with normal saline. Whereas the rest groups (2, 3, 4, 5) were treated with 1.82, 3.65, 5.47, and 7.3 µg/Kg respectively. All groups were monitored for 24 hrs. Blood samples were obtained from the heart; animals were sacrificed at the end of experimental period. Blood samples were transferred into heparinized tubes, and some vital organs were preserved with 10% formalin (BDH).

Apoptosis assay:

By using Flow cytometer technique, the procedure was done according to the kit instructions (EXBIO Praha, a.s. Storage in dark at 2-8°C).

Tissue sectioning technique:

The tissue of collected organs (liver, lung, kidney , and peritoneum) were prepared and sectioned in Specialized Medical Laboratory (Babylon city).

Statistical analysis:

Statistical analyses were performed by using SPSS 19.0. Differences between the groups were statistically analyzed by 2-way analysis of variance (ANOVA). Data are expressed as the mean ± standard deviation (SD). A P value of ≤0.05 was regarded as statistically significant.

Results and discussion:

In order to confirm the presence of CNF1 in the isolate, an amplification of *cnf1* gene was done using specific primers, and after comparison with the non-pathogenic standard *E.coli* (ATCC 25922).

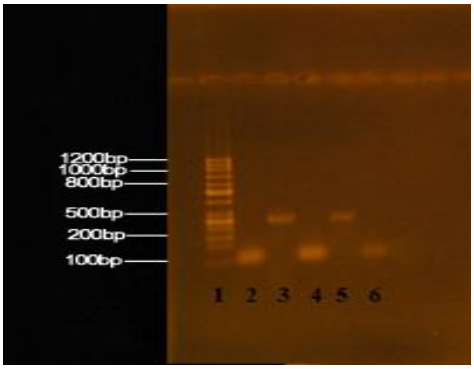


Figure (1): Agarose gel electrophoresis of the PCR products lane 1: DNA marker (Ladder); lane 2, 4, 6: Standard *E.coli;* and lane 3, 5: PCR product of pathogenic isolates.

The results in (figure 1) showed, the appearance of a band with a molecular size of 498bp in the amplified PCR products (DNA) of the pathogenic isolates, while the standard *E. coli* did not, hence this gene may be acquired as transposon or insertion sequence from other bacterial strain especially among enterobacteriaceae members(13). This result is confirmed with that reported by (11),thus we suggest that, there was a similarity between the local strain and that documented in Iran.

In vivo test: The results of using different doses (1.82, 3.65, 5.47 and 7.3 µg/ml) showed, different effects, which led to succumb the treated animals with a period varied according to the doses (26, 8, and 7 hrs.) for 3.65, 5.47 and 7.3 µg/ml respectively. Whereas rabbits

treated with 1.82 µg/ml remained live beyond 72 hours with illness throughout this period. These results suggest, the high toxicity of extracted CNF1, and may be due to the threshold of activation of Rho proteins by CNF1. However, because of a concomitant decrease of their cellular levels, due to the depletion of activated-Rho GTPases by ubiquitin-mediated proteasomal degradation (14,15).

Apoptosis assay :The detection of the apoptosis and necrosis effects which occurred on lymphocytes due to using different concentrations of the extracted CNF1 showed, there was significant variation (P≤ 0. 05) in the percentage of early apoptosis, necrosis, and late apoptosis among treated groups. These elevations were dependent on dose, with a significant variation in comparison with the control group, (Table 2 and Figures 2A, 2B, 2C, 2D), These effect may be due to CNF-1 that induces lymphocyte phenotypic changes by activation of GTP-binding protein Rho furthermore, CNF-1 induces an important F-actin reorganization and distribution in various models of the early, late apoptosis and necrosis which were occurred (14).

Table (2): Flow cytometer analysis of lymphocytes after treatment with partial purified CNF1.

Dose(µg/kg)	A live cells(%)	Early apoptosis (%)	Necrosis (%)	Late apoptosis (%)
1.82	80.12± 2.04*	4.95± 1.05*	9.05± 0.05*	5.88± 2.08*
3.65	58.29± 1.06*	10.23± 2.03*	16.35± 2.05*	15.13± 2.03*
5.47	49.50± 0.1*	13.31± 1.04*	20.41±1.04*	16.78±1.04*
7.3	30.47± 1.05*	24.11± 1.04*	25.91± 1.04*	19.51± 1.04*
Control	98.68± 1.02	1.01± 1.0	0.07± 0.05	0.24± 0.06

***Significant at P≤0.05 in comparison with control group**

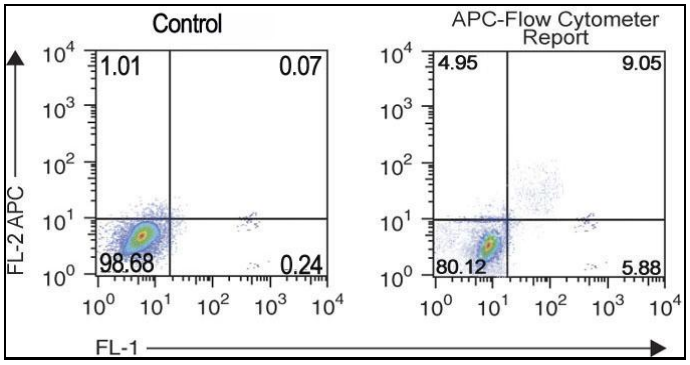


Figure (2A): The effect of 1.82µg/kg in rabbit lymphocytes in comparison with the control group.

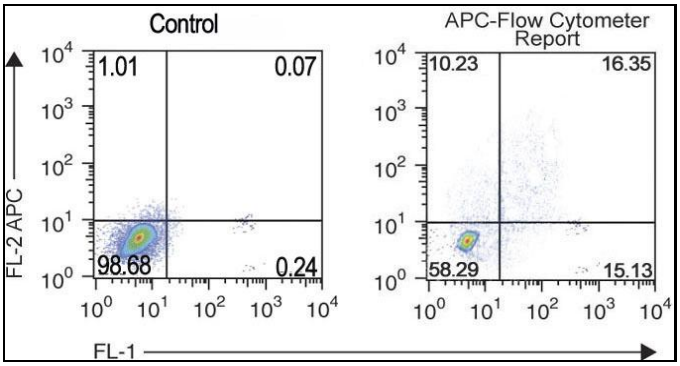


Figure (2B): The effect of 3.65µg/kg in rabbit lymphocytes in comparison with the control group.

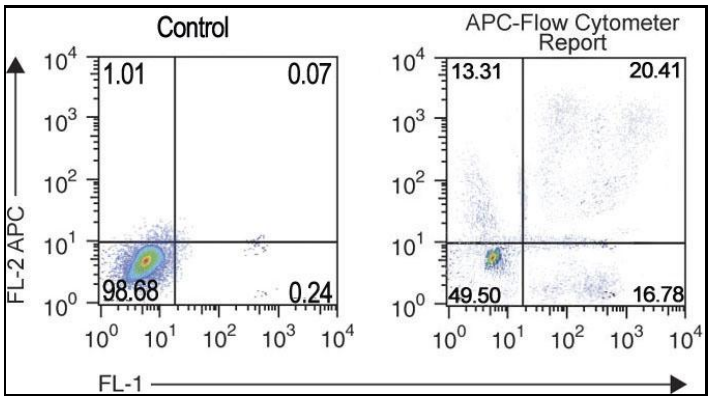


Figure (2C): The effect of 5.47µg/kg in rabbit lymphocytes in comparison with the control group.

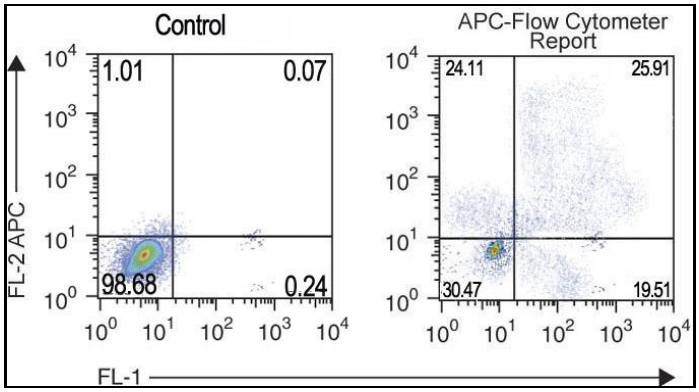


Figure (2D): The effect of 7.3µg/kg in rabbit lymphocytes in comparison with the control group.

Histological test:

The intra-venous injection of rabbits with different doses of partial purified CNF1 was led to produce abnormal lesions (depended on the conc. of dose) in the tested organs.

Liver:

Different changes were occurred grossly and microscopically in the liver which grossly showed severe congestion and intermediate trophy as well as variation in its texture (Figure3). Whereas, the microscopic examination revealed a congestion and infiltration of inflammatory cells particularly neutrophils and mononuclear cells around blood vessels in the portal areas. In addition dilated sinusoidal blood vessels with fibrin network (pink color) were observed in liver parenchyma, with atrophy of hepatic cord, and necrotic areas of hepatocytes. Moreover, there was a proteinaceous material in the lumen of dilated central vein (Figure 4 B, in comparison with the normal one (Figure 4 A). Hence, CNF-1 provoked an increase in the content of actin stress fiber. These results suggest that the CNF-1 effect, by acting on the liver, may increase in an important fashion the virulence of certain strains of *E. coli* against the liver cells as well as because the liver is considered a rich organ with blood vessels, and functions that made the effect was intensive.

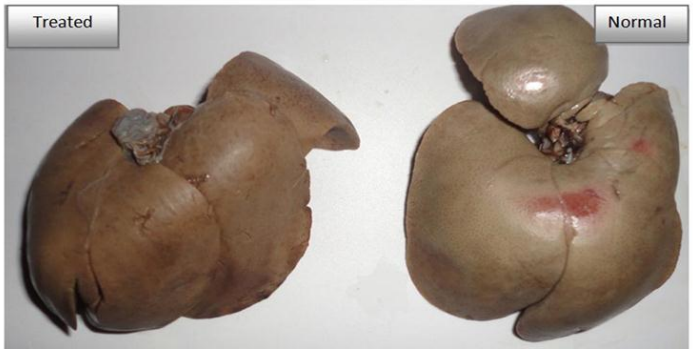


Figure (3): Gross appearance of normal and treated animal liver.

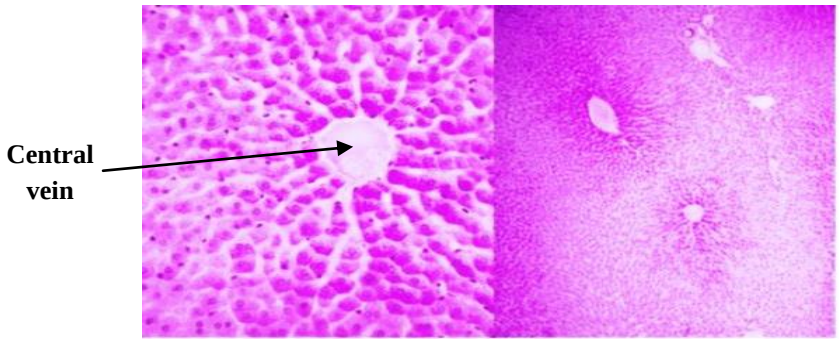


Figure (4A): Tissue section for the normal rabbit liver. (H&E stain) (40x and 10x).

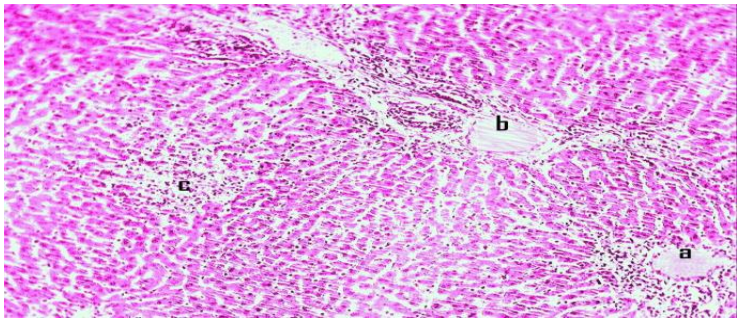
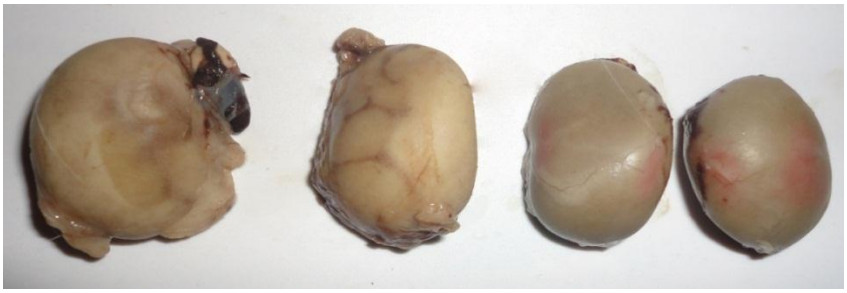


Figure (4 B): Histological section of rabbit liver treated with CNF-1 at dose 3.65mg/50µl for18hr. (a): congestion and dilation of central vein with infiltration of inflammation cells, (b): congestion and dilation of blood vessel of portal area with fibrin network (c): focal aggregation of inflammatory cells in the hepatic parenchyma area.(H&E stain) (20x).

Kidney:

The results showed a grossly intermediate trophy with paleness color in both kidneys especially the left one, (Figure 5). Furthermore, the microscopic view for the cross section of the cortex and medulla showed a fibrin deposition and inflammatory cells in capsular region, dilation in the renal tubules as well as aggregation mononuclear cells infiltration in interstitial tissue with distension of bowman's space. Moreover, it was found a cystic dilatation with contact proteinaceous material and atrophy of glomerular tuft (Figure 5 B). In comparison with normal (Figure 5 A) the consequences of these changes may be mainly characterized by an intense actin cytoskeleton polymerization into the intoxicated cell that is led to the paleness of kidneys, in comparison with normal one. Szemiako and coworkers (16) tested some bacterial factors that identified as

facilitators of UPEC translocation through renal epithelial cells. Thus, based on the results obtained in this study, we can suggest that CNF1 may acts as invasive agent for uropathogenic *E. coli* facilitated bacterial distribution and colonization through the infected organs. Yarery *et al.*, (17) reported the effect of CNF1, such as sub mucosal edema and therefore, they speculated that Hly and CNF1 may be mainly responsible for the signs and symptoms of cystitis in humans infected with toxigenic UPEC.



Treated **Normal**

Figure 5: Grosse appearance of normal and treated animal kidney.

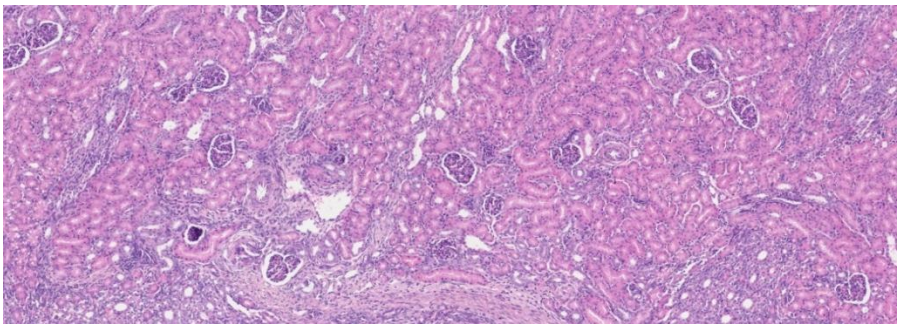


Figure (5A): Tissue section for the normal rabbit kidney(H & E stain), (20x).

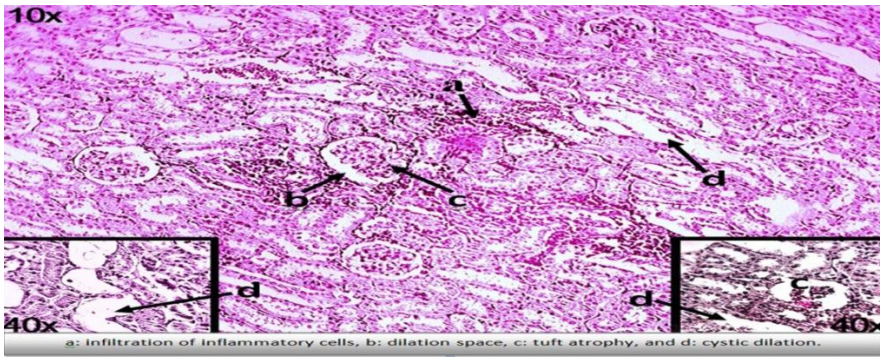


Figure (5 B): Histological section of rabbit kidney cortex treated with CNF1 3.65mg/50µlfor 18hr.,showed increased Bowman's space, with infiltration of inflammatory cells in interstitial tissue (H & E stain), (10x). The small figure (40x) showed the cystic dilation of renal tubule's with cellular debris in their Lumen.

Lung:

The gross appearance showed there were severe congestion, trophy and abnormality in its texture (Figure 6). The microscopic examination revealed collapse of alveoli, congestion of alveolar capillaries, pulmonary blood vessels, hemorrhage, infiltration of

inflammatory cells and macrophages in the interstitial tissue as well as in alveolar wall to their lumen (Figure 6 B). In addition, emphysema was also seen in comparison with the normal one (Figure 6 A). Thus, it can be said that the effect of CNF1 on WBC is similar to that of bacterial infection, which is characterized by an acute inflammation with polymorpho -nuclear leukocyte (PMNL) which usually migrate into the lung in response to inflammatory cytokines. These results was agreed with Honda *et al.*,(18) who established the relationship between left shift and WBC count over the course of a bacterial infection, and proved that the left shift was principally depended on the response of the bone marrow to neutrophil depletion from the blood. In spite of this effect, CNF1 decreased the antimicrobial activities of poly-morphonuclear leukocytes as mentioned by Szemiako *et al.*,(16).

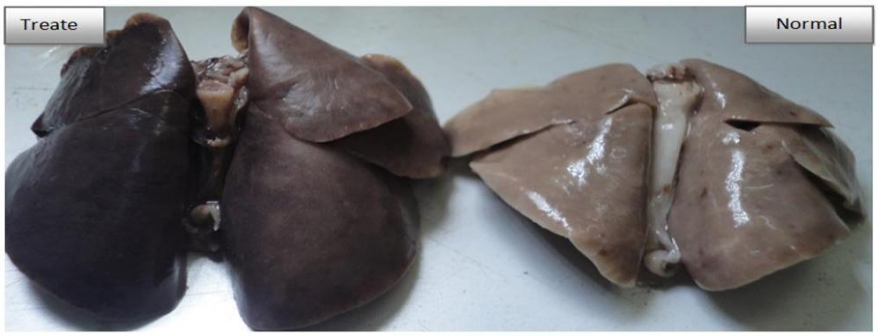


Figure (6): Grosse appearance of normal and treated animal lung.

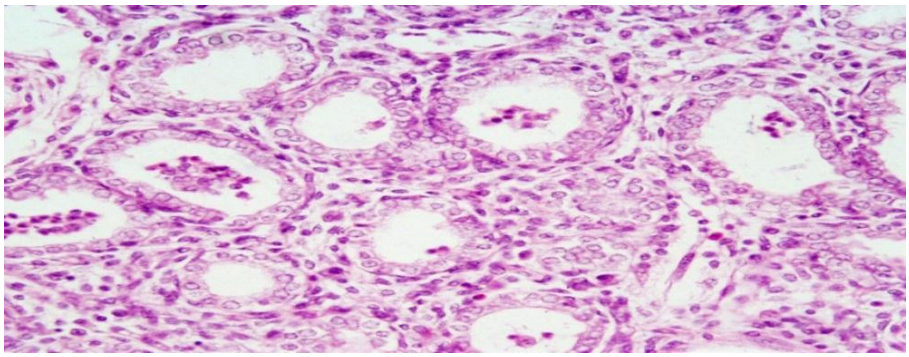


Figure (6A): Tissue section for the normal rabbit lung (H&E stain) (40x).

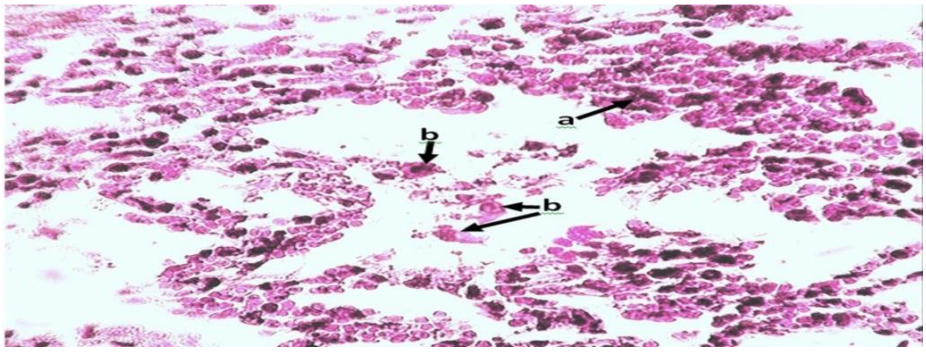


Figure (6B): Histological section in lung of rabbit treated with CNF-1 at dose 3.65 mg/50µl at 18hr. (a): thickening of alveolar walls due to congestion of alveolar capillaries, (b): presence of pulmonary macrophages in lumen of alveolar. (H&E stain) (40x).

Peritoneum:

The Microscopic view showed a great thickening of peritoneum due to congestion of blood vessel with edema, hemorreghe and fibrous network, infiltration, as well as aggregation of inflammatory particularly mononuclear cells in comparison with the normal one (Figure 7, and 7A).

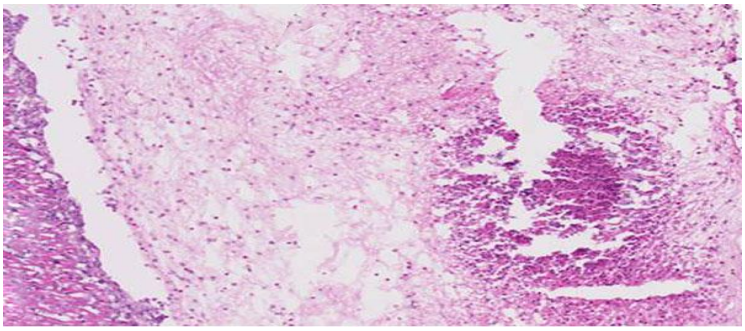


Figure (7): Tissue section for the normal rabbit Peritoneum (H&E stain) (40x).

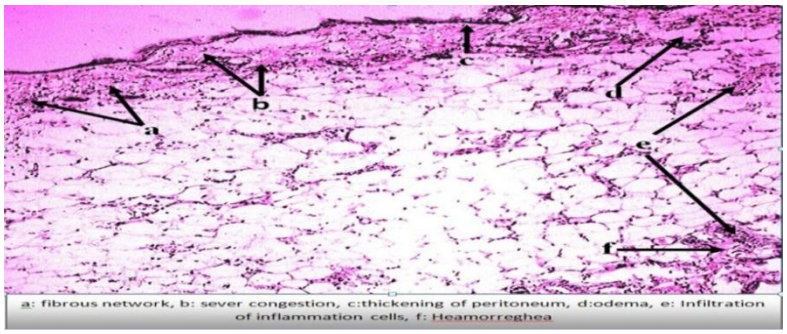


Figure (7A): Histological section of rabbit peritonea treated with CNF-1 at dose 3.65 mg/50µl for 18hr, elucidate increasing in peritoneum thickness, edema, and severe congestion.

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