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Evaluation Of Meropenem-Resistance Genes Expression In Acinetobacter Baumannii Isolates Using Nanobiopolymer Composite (Histidine:Meropenem:Nanochitosan) And Its Cytotoxicity And Bioassay

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Abstract

The aim of the study is to evaluate the effect of the nanobiopolymer composite Hisidine:Memropenem:Nanachitosan (His:Mem:NC)) on the efficiency of expressionMem-resistance genes in Mem-resistant Acinetobacter baumannii isolates and their cytoxicity on cell lines in addition to its bioassay on lab. Animals. Sixty-five isolates of multidrug resistant of A. baumannii were selected out of 240 isolates collected from variousinfection sources includedthe wounds, sputum, urine, blood, andmedical device.The selected isolates have minimum inhibitory concentration (MIC) of Mem more than 16µg/ml. Nineteen isolates were selected that have MICof Mem ranged between 64 -256 µg/ml and MBC 128-512 µg/ml. The genes most associated with Mem-resistance were selected in A. baumannii bacterium, which are the OXA genes (blaOXA23, ISAbablaoxa23, ISAbablaOXA51). Allisolates were identified as Acinetobacter baumanniiusing 16srRNA gene amplification technique. The results showed that 16 (84.21%)harbor the blaOXA23gene; ISAbablaoxa23,14(73.6%) and ISAbablaOXA51,13(68.4%) with the molecular size of 501,321and 227 bp, respectively. Nanobiopolymer composite of His:Mem:NCwas prepared andcharacterization (as mentioned in research published in Turkish Journal of Physiotherapy and Rehabilitation; 32(3), 2021). It was foundthat the MIC of Mem in nanobiopolymer compositereduced to ≤8µg/ml.Gene expression level of blaOXA23,ISAbablaOXA23 and ISAbablaOXA51genes was measured by real time quantitative polymerase chain reaction (RT-qPCR) before and after 4 and 8 hr of treatment the isolates withnanobiopolymer. The Ct value of the blaOXA$_{23}$, ISAbablaOXA$_{23}$ and ISAbablaOXA51 genes isolates after treated with nanobiopolymer, at the selected MIC, It failed to express after 4 hours of treatment, and all isolates did not express sequentially after 8 hours of treatment, depending on the values of ct and 2 folds..It was found thatthe inhibition percent of cancer cell line (HepG2) ranged between 5.1% - 57.4% after treating with nanobiopolymer at concentration 6.25- 400 µg/ml, while the percentage of inhibition fornormal cell line (WRL68) ranged between 5.1% - 26.1% at the same concentrations. The bioassay of nanobiopolymer composite on rabbits infected with bacteria that suffering from septicemia and wound inflammation, has played a major role in inhibiting the growth of bacteria and they recovered and healing the wounds in comparison with untreated rabbits which appeared swelling and suppuration in

the wound area and they died after 6, 7 and 11 days after infection.Also, theblood analysis,liver, and renal functiontests results of infected and treated rabbits by nanobiopolymer were like those of the control rabbits.

Keywords: Acinetobacter baumannii,OXAgenesexpression, Nanobiopolymer composite, MIC, Meropenem, cytotoxicity, bioassay.

Introduction

Acinetobacter baumannii isobligate aerobic bacteria, not fermenting sugars, Gram-negative, bacillus. It is classified as opportunistic (it takes advantage of a weak immune system and often does not cause diseases in healthy people), it has the property of adapting to temperature, pH, and humidity over a wide range as it can survive on dry surfaces for five months, also characterized by its widespread in diverse environments such as soil, water, vegetables, animals, and insects facilitate their transmission to humans[1]. It has a high ability to acquire resistance to many diverse antibiotics due to the possession of many virulence factors, including endotoxin represented by polysaccharides, lipopolysaccharides, siderophores, cytotoxic necrotizing factor, colicin V production, capsule production, biofilm formation, pili formation and the production of the protease enzyme .This bacteriumis characterized by their high resistance to the beta-lactum, aminoglycoside, fluoroquinolones,and Sulphamethoxazole-Trimethoprim antagonists groups due to the production of broad-spectrum of enzymes thatplay a major role in its resistance which makes getting rid of them very difficult [2].

The study aims to know the effect of the nanobiopolymer composite (His:Mem:NC) on the efficiency of expression of resistance genes for Mem in A. baumannii resistant to this antibiotic (blaOXA23, ISAbablaOXA23 and ISAbablaOXA51) .Also to evaluate the toxicity of the nanocomposite in rats and its bioassay on the healing of rabbits wounds and recovery from septicemia caused by a bacterial infection with A. baumannii.

Materials and Methods

Materials

Blood agar base (BAB), MacConkey agar (MAC), Muller hinton agar (MHA), Muller hinton broth (MHB) All media were supplied by (Oxoid, England and Hi-media, Indian). DNA Extraction kit (Intron;Korea); cDNA kit; Wizpure ™ qPCR master mix syper (Wizbio, Korea); General RNA extraction kit (Dongsheng Biotech, Korea) in addition to the marker 100-3000 bp DNA Ladder (Bioneer, Korea); Meropenem powder (Meronem IV, UK); Chitosan (Xi an lyphar Biotech, China) and L-Histidine (Direvo industrial Biotechnology, Germany);Gavage (fisher scientific,USA).

Preparation of nanobiopolymer composite (His:Mem:NC)

The nanobiopolymer was prepared and characterized and the effective concentrations were studied according to AL-Naymi and Ahmed, (2021) [3].

Molecular studying

The genomic DNA of A.baumannii was extracted according by procedure of INTRON Biotechnology Kit for gram negative bacteria. The amplification of 16srRNA,ISAba1blaOXA51,blaOXA23 andISAba1blaOxa23genes was conducted by polymerase chain reaction (PCR) according to the manufacturer instructions (Bioneer, Korea) using a set of primers forward and reverse as shown in table (1), PCR master mix, DNA template and deionized distal water in an thermocycler PCR (BIORAD,Singapore).PCR were executed in 20 µl reaction volume consist of 4 µl master mix ,1µl of each primer,5µl DNA template and 9µl ddH2O,adjusting conditions for PCR were initial denaturation at 95 ℃ for 5 min,denaturation at 95 ℃ for 40 sec,an annealing temperature for 16srRNA is 55℃,ISAba1blaOxa51, blaOxa23 are 58 ℃ and ISAba1blaOxa23 is53 for 30 sec. followed by an extension at 72c for one min, finally extension at 72 ℃ for five min. After amplification of PCR products,they are migrated on agarose gel electrophoresis using electric current of 100 volt and 70 miliamper for 1hr.DNA bands were detected under UV-Trans-illuminator at a wavelength of 320nm. The molecular size of the bands was determined in comparison with the DNA ladder.

Table (1): The primers used in study

Gene name	Primer sequence 5ˈ-3	Product size(bp)	Reference
16srRNA	27FAGAGTTTGATCMTGGCTCAG 1492R TACGGYTACCTTGTTACGACTT	1500	[4]
blaOXA23	F-GATCGGATTGGAGAACCAGA R-ATTTCTGACCGCATTTCCAT	501	[5]
ISAbablaOXA23	F-TGAGATGTGTCATAGTATTC R-AGAGCATTACCATATAGATT	321	[6]
ISAbablaOXA51	F-AAGCATGATGAGCGCAAAG R- GGTGAGCAGGCTGAAATAAAA	227	[6]

*M=A or C **Y=C or T

Gene expression analysis using qRT-pcr technique

The estimated of gene expression by qRT-pcr for 4 genes 16srRNA ISAba1blaOXA51, ,blaOXA23 and ISAba1blaOXA23 for 19 A.baumannii isolates was carried out at 4 and 8 hr after addition of Nanobiopolymer composite using prefer concentration equal to MIC,RNA was extracted according to general RNA extraction kit (DONSHENG,biotech), after that mRNA samples were reverse transcribed to cDNA by using the Wizscript ™ RT FD mix kit (wizbiosolution), qPCR- Master mix Syber kit (wizbio) korea for qRT-PCR , Housekeeping gene 16SrRNA amplification was used as internal control to the used incalculating threshold cycle between the target gene and reference gene relative method was used to calculate the fold changes of gene expression level [7,8].

Cytotoxic effect of nanobiopolymer (HIS:Mem:NC)

Normal cell line WRL 68 and human liver cell line carcinoma HepG2 were used for cytotoxicity studying of the nanobiopolymer (His:Mem:NC) at concentrations 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml by using MTT Kit [9,10].

Bioassay of nanobiopolymer (His:Mem:NC) on laboratory animals

Rabbits were divided into 5 groups, 1st group is the control; 2nd group was wounded and without treatment with nanobiopolymer, 3rd group was wounded and treated by injected 0.5 ml intramuscularly (IM) of nanobiopolymer at concentration 48 µg/ml by femoral muscle twice a day; 4th group of rabbits had wounded and treated IV with nanobiopolymer by ear vein twice a day and the fifth (5th) group of rabbits was wounded and treated locally twice a day.

Blood analysis

Blood samples collection was done according to Dyer, (2008) [11].Blood analysis, liver and kidney function were done according to Simek, et al., (2017) [12].

Lethal dose (LD50) for nanobiopolymer composite

According to Krus and wilcox, (1970) [13].18-inch stomach tube (gavage) usedto dose30 rats in weight a ranged between 250 to 275 gm in aserial concentration of nanobiopolymer composite 1000, 2000, 3000, 4000 and 5000 mg/kg from body weight in addition it was dosed a group with ddH_2O alone as control for 14 days, then the rats was monitored and anatomical characterized was performed.Statistical analysis of the results was performed by using Least significant difference (LSD) test [14].

Result and discussion

Molecular studies

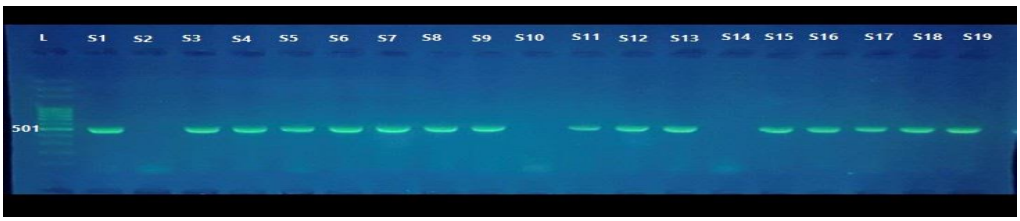
Detection of blaOXAgenes

Table (2) and figure (1 a, b, and c) show the results of gel electrophoresing the specific OXA genes amplification products by multiplex PCR assay reaction using the specific primers for each gene in addition to 16srRNA for the selected A. baumannii isolates. The molecular size of blaOXA23 was501bp;ISAaba1blaOXA23, 321bpand ISAaba1blaOXA51227bp. It was found that 84.21% of selected isolates contain the blaOXA23; ISAba1blaOXA23, 73.6% and ISAba1blaOXA51was68.4%. All isolates contain 16srRNA specific gene with molecular size 1500 bp.

Table (2): Genetic identification of A.baumannii and their resistance genes

Approxi. Size (bp) / Isolate	Gene detection			
	blaOXA 23	ISAbablaOXA51	ISAbablaOXA23	16srRNA
	501	227	321	1500
S1	+	+	+	

S4	+	+	+	
S6	+	+	+	
S8	+	+	+	
S9	+	+	+	
S11	+	+	+	
S18	+	+	+	
S19	+	+	+	
S13	+	+	+	+
S3	+	+	-	
S16	+	+	-	
S7	+	-	+	
S12	+	-	+	
S5	+	-	-	
S17	+	-	+	
S15	+	-	+	
S2	-	+	+	
S14	-	+	-	
S10	-	-	-	



a

b

c

Figure (1): Molecular detection of Mem- resistant genes in A. baumaniiisolates.

a:blaOXA23; b: ISAba1blaOXA51; c: ISAaba1blaOXA23.

The table showsthat several isolates contain all Mem-resistant genes and other isolates have variable number of these genes and this explains the difference in Mem-resistance between the isolates through the MIC values [3]. Most antimicrobial susceptibility goes back to work OXA-type especially resistant to Mem. The results of current study agreed with Tafreshi, (2019) [15], Who had found that the OXA B-lactamase is responsible for carbapenem resistance and the location of ISAba1 insertion sequence in blaOXA23 and blaOXA51genes was the major mechanism in risk of increasing the diversity in Oxa B-lactamases and the most trouble in carbapenem resistance because the ISAba1 encoded to the transposases upstream of blaOXA genes and provide an effective promoter for the gene as mentioned by [16].

Effect of nanobiopolymer composite on expression of blaOXA genes

Expression of the blaOXA23, ISAbablaOXA23 and ISAbablaOXA51 genes was decreased in all isolates after treated with nanobiopolymer at the selected MIC(48µg/ml) after 4 hr of treatment and some isolates were failed to express after 8 hr. The evident was from the increasing in the value of the Ct in all isolates when compared with control treatment. The results of 2foldedvalue($2^{-\Delta\Delta c+}$) for each gene after treating with nanobiopolymer composite are shown in table (3).statics analysis showsto the non-significant differences in genes expression among all isolates treated with nanobiopolymer after 4hr and 8hr of incubation period.

Table (3): Folded values ofMem-resistant genes in A. baumannii isolates treated with nanobiopolymer composite (NC: Mem: His)

Isolate	2folded value of gene expression at time (h)					
	blaOXA23		ISAbabla OXA23		ISAbabla OXA51	
	(4h)	(8h)	(4h)	(8h)	(4h)	(8h)
S1	0.00022	- (1)	0.015	0.000104	0.00055	0.0000340
S2	-	-	0.00089	0.0000105	0.00011	0
S3	0.00073	0.0000658	-	-	0.00052	0.0000106
S4	0.000068		0.00027	0.00000711	0.000048	0
S5	0.000020		-	-	-	-
S6	0.0000081		0.000322	0	0.00048	8.594E-7
S7	0.000028		0.00111	0	-	-
S8	0.00333	0.0000214	0.026	0.0000425	0.0020	0.0000095
S9	0.000181	0.00000258	0.00035	0	0.00052	0.0000133
S10	-	-	-	-	-	-
S11	0.00103	0	0.00034	0	0.00011	0
S12	0.0000043	0	0.00079	0	-	-
S13	0.0000075	0	0.0039	0.0000531	0.000053	0.00000499
S14	-	-	-	-	0.000055	0

S15	0.000119	0.00000314	0.00096	0.00000469	-	-
S16	0.00026	0	-	-	0.000099	0.00000392
S17	0.0000081	0	0.00037	0.00000547	-	-
S18	0.00022	0	0.00146	0	0.00418	0.0000114
S19	0.0000037	0	0.00032	0	0.000075	0
p-value	0.238NS*	0.097 NS*	0.216 NS*	0.442 NS*	0.297 NS*	0.438 NS*

(1) Undetermined; (0), gene failed to expression at 8 hr; NS*, non-significant.

It was found that the nanobiopolymer composite had a high inhibitoryactivity on the growth of A.baumannii and on the expression level of Mem- resistant genes. There are no studies found on this nano therapeutic composition yet. The growth inhibition might be returned to the effective of amino acid histidine in the mixture which increasing the permeability of membrane and lead to rupture and death of A.baumannii by electrical interference contact between the G(-ve) lipopolysaccharides bacteria and positive charge of histidine [17].As well as the chitosan is highly efficient and versatile polymeric materialand widely applied for drug delivery and antimicrobial activity because of their properties of like deacetylation, molecular weight, and chemical structure. The functionalization and the mechanism of antimicrobial is depending on electrostatic attraction between inionic surface of A. baumannii and chitosan material lead to changing in the permeability of bacterial membrane and to the leaking out of bacterial compounds that result in cell death [18]. So, the mixing of His, Mem and NC in nanostructure may be led to increasing in the effectiveness of this nanocomposite to attractthe surface of A. baumannii and collected on the cell membrane then creating holes that facilitated introducing into the cytoplasm and interacted with DNA of bacterial.

In vitro cytotoxic effect of nanobiopolymer

Table (4) and figure (2) show the results of the effect of serial concentrations of nanobiopolymer composite on the viability of normal hepatic cell line WRL68 and carcinoma cell line HepG2 after 24 hr of treating.The percentage of inhibition the cancer cells was ranged between 5.1%-57.4% using the nanobiopolymer at concentrations 6.25-400 µg/ml, while the percentage of inhibition from normal cells ranged between 5.1-26.1% at the same concentration. There are no studies found on the effect of nanobiopolymer on normal and cancerous cells yet. These results indicated the potent cytotoxicity and anticancer activity of the nanobiopolymer composite that can be used as apotential anticancer agent.

Table (4): Effect of serial concentations of nanobiopolymer composite on the viability of normal hepatic cell line WRL68 and carcinoma cell line HepG2

Conc.(µg/ml)	(Mean ± SD) n=3 cell viability HepG2 (%)	(Mean ± SD) n=3 cell viability WRL68 (%)
6.25	95.911 ± 0.291	94.946 ± 1.542
12.5	93.326 ± 0.869	94.522 ± 1.772
25	85.378 ± 1.753	96.181 ± 2.084
50	74.923 ± 5.178	96.180 ± 1.252
100	62.847 ± 1.518	87.886 ± 2.560

200	51.929 +¯2.589	84.105+¯0.928
400	42.593 +¯4.928	73.804 +¯2.215

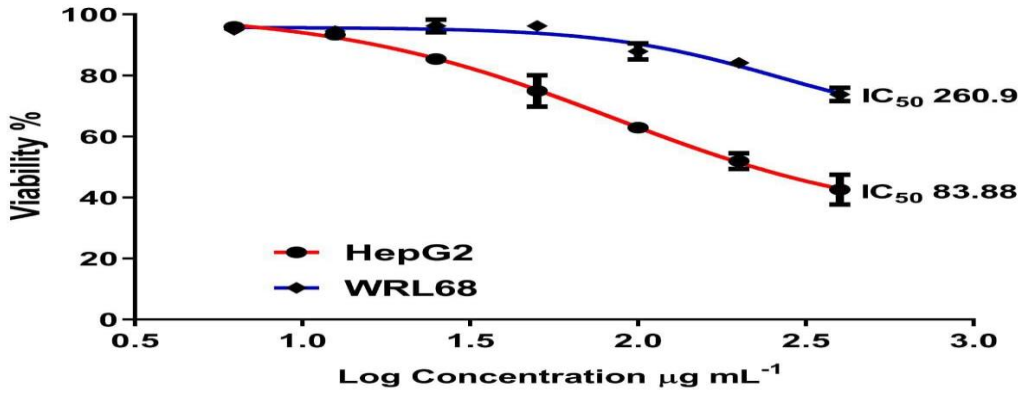
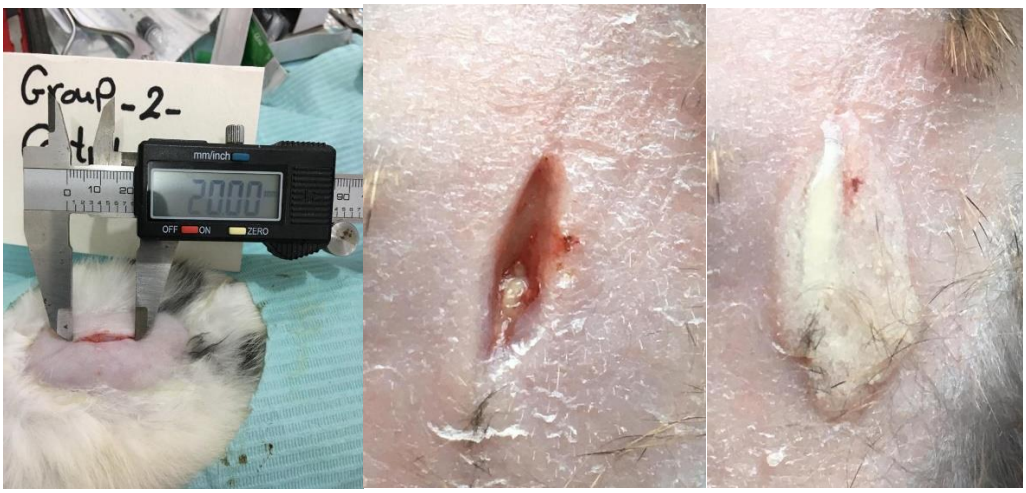


Figure (2): Effect of nanobiopolymer on normal cell WRL68 and hepatic carcinoma cell line HepG2.

Bioassay of nanobiopolymer (His:Mem:NC) on laboratory animals.

The wounds infected with A.baumannii showed the presence of pus and inflammatory exaudation at the site of the wound with tissue necrosis after three days of infection. The infected wounds were healed with nanobiopolymer through the formation of exaudate granulation tissue after ten days of local treatment, seven days by intramuscular injection and three days by intravenous injection as shown in figure (3a, b, c,d, and e,f respectively).The nanobiopolymer composite played a major role in inhibiting the growth of bacteria isolated from the blood of infected rabbitsthat suffering from septicemia, and the wounds healed and the rabbits recovered, but untreated rabbits were appeared swelling with the suppuration in the wound area as shown in figure (4a, andb) . It was found the presence of A. baumannii bacterium in blood culture of infected and untreated rabbits then dead during 6, 7 and 11 days after infection.The healing and recovery of infected and treated rabbits with nanobiopolymers may be due to the synergistic interaction among the components of nanocomposite,as the meropenemin preventing the formation of cell wall, and the action of histidine which increasing the keratinocyte migration and proliferation and increased the collagen that contributes in skin wound healing [19], in addition to the effect of nanochitosan in their ability to regulate of re-epithelization process and fibroplasia [20].

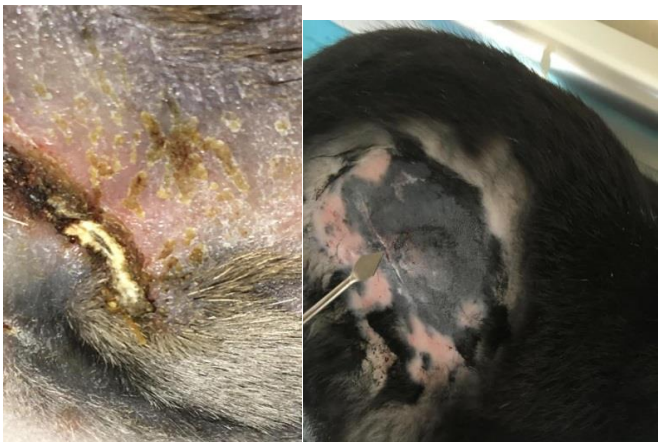


a b c

def

Figure (3): Rabbit infectedwound and treated with nanobiopolymer.

a- Ruler to measure wound distance; b- Infected wound; c- Treated wound locally; d- Healing of wound in rabbit treated locally; e- Treatedi/v; f- Healing of woundin rabbit treated i/v;



a **b**

Figure (4) : Rabbit infected wound and untreated with nanobiopolymer composite

a- Infected wound after 3days from infection. b- Another rabbit didn't treatment suffered pus and swelling of wound before the death.

Blood analysis

Hematological analysis

Table (5) shows the blood analysis of the infected and untreated rabbits. It was found an increasing in white blood cell, lymph, mid, HGB, RBC, RDW-CV, PLT and PCT in infected animals. The change in the parameters refer to the presence of infection and septicaemia, but in the group of infected and treated rabbits, there was avery slight rise in lymph and PLT when comparison with erference range but near control rabbits (table 4).

Table (5): Hematological analysis of rabbit's treatment

Parameter	Reference range	Control	Infected and untreated rabbits	Infected and treated rabbits
WBC	4.0-11.0	6.3*10g/l	17.3*10 g/l	10.4*10 g/l
Lymph	0.8-4.0	2.3*10 g/l	8.6*10 g/l	4.4*10 g/l
Mid	0.1-1.5	0.7*10 g/l	2.4 *10 g/l	1.4 *10 g/l
Gran	2.0-7.0	3.3*10 g/l	6.3 *10 g/l	3.6 *10 g/l
Lymph%	20.0-40.0	37.2 %	49.4 %	45.8 %
Mid %	3.0-15.0	11.4 %	14.0 %	13.2 %
Gran %	50.0-70.0	51.4 %	36.6%	45.0 %
HGB	11.0-16.0	10.3	19.5 g/d	10.6 g/dl
RBC	3.50-5.50	4.87*10	8.88*10	4.61* 10
HCT	37.0-54.0	34.5 %	65.4 %	34.5 %
MCV	80.0-100.0	71.0 fl	73.7 fl	75.0 %
MCH	27.0-34.0	21.1 pg	21.9 pg	22.9 %

MCHC	32.0-36.0	29.8 g/dl	29.8 g/dl	30.7 %
RDW-CV	11.0-16.0	16.9 %	16.3 %	15.6 %
RDW-SD	35.0-56.0	43.2 fl	44.1 fl	43.2 fl
PLT	100-400	478*10 g/l	591*10 g/l	475 *10 g/l
MPV	6.5-12.0	5.5 fl	6.0 fl	5.5 fl
PDW	9.0-17.0	14.9	15.6	15.3
PCT	0.108-0.282	0.227%	0.354	0.261 %

Liver function

Table (6) shows the results of liver function test. It was found that an increasing in the parameters of GPT, GOT and T.S.B while there were no differences between the treated rabbits when compared with the control, and this is an indication of the safety of the nanobiopolymer on liver enzyme and liver function.

Table (6): liver function testof rabbits

Test name	Reference range	Control	Infected and untreated rabbit	Infected and treated rabbit
Alk.phos.	10-270 U/L	127	210	183
GPT	2-44 U/L	20	45	42
GOT	2-40 U/L	24	76	24
T.S. B	0-1.2 mg/dl	0.18	0.20	0.25

Melillo, (2007) [21] was found as diagnosed when increased AST enzyme cause damage of liver, T.S.B levels reflect hempatocyte or bile tract infection and slightly increased of ALT and ALP caused lipidosis or torsion of liver lobe.

Renal function

The results of the renal functiontests ofrabbit's groupsshowed that an increasing in blood urea in infected untreated group and normal values in control and infected treated groups rabbits. These results were indicatedto the safety of nanobiopolymer on kidney functions.

Lethal dose (LD50) of nanobiopolymer

The concentrations used in the determination the LD50 of nanobiopolymer by dosage stomachtube were showed a noticeable toxic effect after administration, asthey didn't result in any fatalities and no signs of hemorrhage or changes appeared on rats after performing the anatomical characterization of them as show in figure(5a, b). when comparing it with the findings of the Kohda et al., (1992) [22], it was found that the LD50 values of meropenem were 2850mg/kg in male and 3200mg/kg in female rats intravenous and more than 5000 mg/kg when treated orally or subcutaneous.

ab

Figure(5):a-Dosing rat with nanobiopolymer by stomach tube, b-Anatomy of rat dosing with nanobiopolymer.

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