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Antibiotics Immobilized Gelatin Nano Carrier Influences on *Brucella* melitensis Filed Strain

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Abstract

Drug delivery system refers to combination between nanotechnology and drugs to improve the ability of targeting specific cell or tissue. Intracellular Pathogens like Brucella hard to eradicate by using conventional antimicrobial agents resulting in increasing of relapse *Brucella* infection cases. so, we propose an ideal drug delivery system for brucellosis scaling back using gelatin nano carrier loaded with Doxycycline and streptomycin in four shoots doses in comparison with traditional treatment. About 90 male guinea pig animals were allocated into two main groups the first group (n=45) was infected artificially with *Brucella melitensis b.v3* and the second group remained as a normal control group. Infected animals were subdivided to tow treated groups. The first one was treated with immobilized antibiotics Doxycycline and Streptomycin in a gelatin nano particles carrier and the other group with a combination of the antibiotics in the traditional form. Three and fourteen days post the end of each treatment course (Three days after every treatment to exclude the effect of used antibiotics on bacterial count and fourteen days after the end of the treatment to study the relapse infection rate). the animals from each group under investigation were weighted and slaughtered .We used the weight of spleen and liver , *Brucella* C.F.U counts from these organs furthermore liver and kidney functions to evaluate success of treatments .Gelatin drug delivery system improve its capabilities against Brucella by reducing colonies forming units number $p< 0.05$ in infected treated animals if compared with non-infected non-treated group and $p< 0.01$ in infected treated animals by traditional treatment if compared with infected non treated animals control groups. None of the two treatments could completely eliminate *Brucella* infection from infected treated animals. So, we recommend to apply more other trials for *Brucella* infection scaling back.

Keywords: Nanomedicine, Drug delivery system, Gelatin nanoparticle carrier, *Brucella melitensis*

1. Introduction

Brucellosis is a contagious disease that can infect both of animals and transmitted human caused by several species of the genus *Brucella* [1]. The painstaking nature of these infections is mainly due to the ability of these bacteria to keep in existence in host macrophages. So, *Brucella* species create a virulence and chronic infections due to their ability to escape lysis mechanisms within macrophages, such as lysosomal enzymes and products of the oxidative burst [2]. *B. melitensis* causes no abortion storms in pregnant cattle. Moreover, brucellosis is renowned for its latent infection which impede any control programs [3]. After invasion of the reticuloendothelial system, the bacteria develop within lymphocytes and monocytes cell, and the infected cells play a fatal role in the spreading of the bacteria to specific body organs, such as; spleen, brain, heart, and bones [2] The general mode of infection transport are mainly via direct contact with brucellosis infected animals or their products, self-inoculation with animal vaccine strains, or as a result of laboratory accidents [3]. The combined therapies are more effective in the case of brucellosis than individual due to high rates of relapse, World Health Organization (WHO) recommend doxycycline with Rifampicin for six weeks, but later recommendations also suggest the use of Doxycycline for six weeks with the aminoglycosides Streptomycin for two-three weeks or Gentamicin for one week [4]. However, despite the reasonable efficacy of current treatment regimes, they often fail to eradicate the infection with relapse rates of about 10% [5]. Nanotechnology has emerged as a promising approach for the treatment of intracellular infections by providing intracellular targeting and sustained release of vehiculized drugs inside the infected cells [6,7]. Nano-antibiotics are a promise approach to hold both microbial infections and the increased bacterial resistance to antibiotics and considered a potential treatment option to control infectious diseases and to reduce the massive microbial resistance prevalent nowadays [8, 9]. Doxycycline, a broad-spectrum

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antibiotic, is the most commonly prescribed antibiotic worldwide for treating infectious diseases So, WHO recommended using of Doxycycline in treating adult human with acute *Brucella* infection, combined with Rifampicin for a six-week duration or Doxycycline for 45 days and Streptomycin for15 days [4]. Drug delivery systems are developed to carry these drugs and deliver them specifically to the pathological sites. Patient's healthiness has been expressively improved with the discovery of novel therapeutic drugs which help us to treat some of the most challenging illnesses [10]. Gelatin, is a non-expensive protein polymer that is derived from the thermal hydrolysis of collagen [11], could be readily dissolved in aqueous solutions, does not express antigenicity in vivo, biocompatible and biodegradable. So, gelatin is a good choice for biomedical and pharmaceutical applications. Gelatin Microspheres (GMs) have been used for delivery of different drugs [12].

The facility of forming complexes with different drugs by gelatin has been studied for controlled release applications. Optimizing gelatin degradation and drug delivery kinetics have been tuned by modified gelatin parameters, such as crosslinking density and isoelectric point [13]. The current study is mainly a design to investigate in vivo the ability of gelatin nano particles as a carrier for delivering Doxycycline and Streptomycin combination against *Brucella* pathogen.

2. Experimental:

2.1. Tissue samples:

Lymph nodes, supramammary, fetal liver and fetal stomach contents were collected from slaughtered serologically positive animals from Giza, Beni-Suef, Kafr El-Sheikh governorates for the isolation and typing of *Brucella* microorganisms.

2.2. Milk samples:

About 145 raw milk samples were collected from different corrals in Giza and Menofia governorates

2.3. Bacteriological and molecular typing of the *Brucella* isolates: according to [14,15].

Bacteriological isolation using Trypticase soy agar (blood agar base) (catalogue no. BB11043, BBL, Becton Dickinson Company, USA) and identification and typing of *Brucella* (colonial, morphology, microscopic appearance, catalase, oxidase and urease). DNA extraction from *Brucella* culture suspended in phosphate buffer saline was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit (AMOS-PCR) Primers used were supplied from biobasic (Canada) and (CO_2 requirement, H_2S production, growth in the presence of thionine and basic fuchsin and agglutination with monospecific antisera).

2.4. Drugs:

2. 4.1. Doxycycline

Doxycycline prepared with nano-technology (Doxyral 80%) was obtained as a water-soluble powder. Each gram contains 923.32 mg Doxycycline Hyclate (Equv. 800 mg/gm Doxycycline base) from LILY PHARMA BV. BELGIUM.

2.4.2. Streptomycin:

Streptomycin prepared with nano-technology was obtained as a water-soluble powder (MW 1457.3, purity > 99.9%) from LILY PHARMA BV. BELGIUM

2.4.3. Gelatin:

It was obtained from OXFORD LAB CHEM company to prepare the nanoparticles, gelatin (80 mg) was dissolved in distilled water (5 ml) under constant heating at 40 ± 1°C. pH was either adjusted to pH 6 or 9 for Doxycycline or Streptomycin, respectively.

2. 5. "Preparation of gelatin nanoparticles loaded with drugs".

Gelatin in nanoparticles loaded with Doxycycline and Streptomycin was prepared for intraperitoneal injection. Target injection volume (0.1 ml) should contain 2.5 mg Doxycycline and 3.5 mg Streptomycin. (i.e., each 1 ml contain 25 mg Doxycycline and 35 mg Streptomycin).

Method of choice: one-step desolation gelatin nanoparticles loaded with Doxycycline or Streptomycin was prepared and delivered in separate vials ready to be mixed directly before injection. To prepare the nanoparticles, gelatin (80 mg) was dissolved in distilled water (5 ml) for each drug with constant heating at 40 ± 1°C. pH was either adjusted to pH 6 or 9 for Doxycycline or Streptomycin, respectively. 125 mg of Doxycycline or 175 mg Streptomycin were added then 2 ml acetone was added dropwise in both cases under continuous stirring for more 10 min. after that 15 µl glutaraldehyde solution (25% v/v in distilled water) was added as a cross-linking agent, and the solution was stirred again for 30 min. Finally, the particles suspension was evaporated on rotavap and the volume was adjusted to 2.5 ml, thus achieving double the needed concentration. Just before administration, solutions are mixed and thus each 1 ml will contain 25 mg Doxycycline and 35 mg Streptomycin which is the target concentration needed for injection and the pH gets closer to neutral [16].

2.6. Analysis of encapsulation efficiency

Analysis of encapsulation efficiency for Doxycycline and Streptomycin loaded on gelatin nanoparticles,1 ml sample of each preparation of gelatin nanoparticle suspension was centrifuged for 10 min at 10,000 rpm, 1/2 ml supernatant from each was aspired and measured at 345 nm for doxycycline and 237nm for streptomycin to estimate the concentration of

unentrapped drug on gelatin particles. The following equation was applied:

Entrapment Efficiency % (EE%) = (Total drug – unentrapped drug / total drug) x 100[17].

Measurements were repeated in triplicate. Average and standard deviations are reported.

2.7. Measurement of particle size

Transmission electron microscope TEM High resolution-transmission electron microscope (HR-TEM, JEM-1230, Japan) operated at 120 kV images are taken using negative staining technique. To confirm the preparation of nanoparticles, particle size was measured via dynamic light scattering (DLS) using a Malvern Nano Sizer (MALVERN, UK Ver .7.13).

2. 8. Guinea pigs

A total of 90 male *Brucella*-free adult guinea pigs each weighing (250-350 gm) were collected from commercial sources. The animals were left for one week before the commencement of the experiment for adaptation and fed with well-nourished food.

2. 9. Experimental design:

The lab animals (n=90guinea pigs) in this study were allocated into two main groups, the first group (n=45) was inoculated with *Brucella melitensis* field strain isolated from infected caw milk with a dose of $1X10^5$ CFU/ guinea pig. After 14 days of inoculation, guinea pigs (n = 5) from the infected group were euthanized and subjected to bacteriological examination to ensure the attainment of artificial infection. Body, spleen and liver weights were also recorded**.** The treatment regime, started 14 days post the infection date on the remaining infected animals were divided into subgroups as follow:

-1st sub group (n=10) was treated with the combination of Doxyral (2.5mg/animal) and Streptomycin (3.5mg/animal) intraperitoneally single dose per day for 10consecutive days.

-2nd sub group (n=10) was treated with gelatin nanocarrier loaded with Doxycycline and Streptomycin in a single dose per day 0.1 ml /animal contain (2.5mg Doxycycline &3.5 mg Streptomycin) intraperitoneally for 4 shoots with one day apart between doses.

-3rd sub group(n=10) was treated with gelatin nano carrier in the same manner as the second sup group.,

-4th sub group(n=10) left without treatment as infected non-treated control group.

The second group of guinea pigs (n=45) were left without inoculations and subdivided into:

-1st subgroup (n=15) served as a non-infected non-treated control group.

-2ndsubgroup (n=10) non-infected treated with combination of Doxyral and streptomycin with the recommended dose.

-3rd sub group (n=10) non-infected treated with gelatin nanoparticles.

-4th sub group(n=10) non-infected treated with gelatin nanoparticles carrier loaded with Doxycycline and Streptomycin. Members of animals in subgroups of the second group were treated as the first group.

N.B: Few animals were died through the experiment and were omitted from counting of animals served in the experiment. Three days after the end of the treatment regime guinea pig (n=5) from each sub group were weighed then euthanized and subjected to bacteriological examination and blood chemical testes as liver Aspartate transferase and Alanine transferase (AST and ALT) and kidney (Creatinine and Urea) function tests .To ensure the effectiveness of the treatment's regimen fourteen days later the remaining animals of the subgroups were left and delt as previously stated considering bacteriological and chemical tests.

2.10. Organs body weight ratio:

After recording animals body weight their livers and spleen were aseptically removed for measuring their weight and estimating organs body weight ratio [15].

organs body weight ratio = Organ weight x 100/Body weig.ht (2).

2.11. Bacteriological examination:

Liver, spleen as well as lymph node at the inoculation sites from each scarified animal were removed and homogenized with double its volume in saline. Tissue homogenate were serially diluted and plated on trypticase soy agar (3 plates for each dilution) to determine the viable *Brucella* CFU/ organ **[**15].

2.12. Blood parameter:

Blood of the sacrificed guinea pigs was subjected to chemicals tests as liver function (Aspartate transferase AST, Alanine transferase ALT) and kidney function (Creatinine and Urea) function tests.

2.13. Statistical analysis:

Two-way ANOVA was used to study the significant difference in the means between types of treatments and control groups.

3. Result and Discussion:

Brucellosis is a zoonotic disease caused by the species of *Brucella* genus. Infection of livestock animals causes a big economic loss. Moreover, human brucellosis is an incapacitating acute infection that can be turned into chronic with many complications [18]. *Brucella* microorganisms are an intracellular pathogen, there for control of this infection is very difficult by using traditional treatment which always associated with harmful side effect. From this point of view, it is become necessary to find new antimicrobial agents against brucellosis. Nanotechnology has already participated significantly to antimicrobial therapy through drug delivery systems targeting cells that are infected by intracellular pathogens [19]. In our study we have introduced a new approach to step down brucellosis using a gelatin drug delivery system. Gelatin is a derivative from natural protein collagen so, it's a safe and good choice for nanocarrier and drug release applications as well as its properties as being biocompatible, degradable, non-toxic, cheap and

readily available [20] rendering it an excellent choice as a drug delivery system.

3.1. Bacteriological isolation and identification:

Bacteriological trials to isolate *Brucella* from infected animal species resulted in the recovery of 12 field isolates including 2from milk, 3from Fetal stomach contents, 4 from Suppra-mammary lymph node and 3 from spleen and fetus liver. Phenotypic bacteriological identification result at the genus (colonial morphology, microscopic appearance, biochemical tests were 12 isolates of *Brucella* sp. Multiplex PCR (AMOS) has been applied for molecular typing of *Brucella* at the species level. Bacterial strains showed positive AMOS PCR results characterized by a specific band for *B. melitensis* yielded a band of 730 bp fig (1). Bacteriological identification at the biovar levels (CO_2 requirement, H_2S production, growth in the presence of the dyes thionine and fuchsin, agglutination with monospecific antisera) recognized **12** isolates as *Brucella melitensis* biovar 3. The primary hosts for *Brucella melitensis* are small ruminants the almost sole biovar reported over the last 15 years in Egypt [22.23].

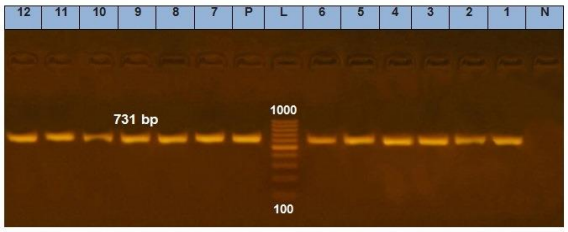


Fig (1): Identification of 12 *Brucella* isolates at species level using AMOS multiplex PCR, Lane L pcr marker; lane (1-12) B. *melitensis* field isolates.

3.2. Characterization of particles size:

3.2.1."Dynamic light scattering (DLS) analysis":

To confirm the preparation of nanoparticles, particle size was measured via dynamic light scattering (DLS) using a Malvern Nano Sizer. Table (1) below shows the particle size of blank gelatin NPs as well as loaded NPs (average of 3 trials +/- standard deviation). The particles size of drugs load on gelatin matrix is well noted in Table (1) are 521±5nm for blank gelatin nano particles ,690±27nm, and507.5±33nm for Doxycycline gelatin loaded NPs and Streptomycin gelatin loaded NPs respectively.

Table(1): particle size of blank gelatin NPs as well as loaded NPs with antibiotics.

Formula	Particle size (nm)
Blank gelatin NPs	521.2 +/- 5
Doxycycline gelatin loaded NPs	690.0 +/- 27
Streptomycin gelatin loaded NPs	507.5 +/- 33

± standard deviation

Doxycycline caused the matrix to expand and absorb more water. On the contrary, Streptomycin caused the gelatin matrix to slightly shrink and get smaller in size. Yet, all prepared particles remained in the nano range. Preparation of both anti-biotics separate was preferred because both Doxycycline and Streptomycin- loaded gelatin nanoparticles were found to exhibit incompatibility in the form of aggregate formation after few hours if they are prepared together. This is mostly due to electrostatic interaction between oppositely charged drugs at a pH close to neutral.

Blank gelatin nanoparticles (NPs) average size was 521.2nm, 114.8 nm, 5382nm at peaks 1,2 and 3 with %Intensity about of 84.8 ,8.6and 6,6 respectively as illustrated in Fig (2).

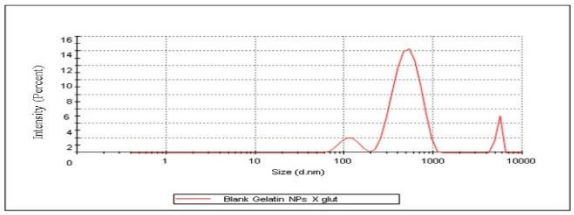


Fig (2): Blank GNPs size distribution by Malvern Nano sizer Peak (1) show particles size average about 521.2±164.2, peak (2) 114.8±25.5 and peak (3) 5382±321 with %Intensity about 84.8, 8.6and 6,6 respectively.

Gelatin nanoparticles carrier loaded with Doxycycline average size provided in fig (3) is 690nm with % intensity 87.7 at peak (1),130,8 nm % intensity 8.8 at peak (2) and 3.5% intensity for particles size nm 7560nm at peak (3). While, Fig (4) illustrates size of the gelatin nanoparticles carrier loaded with streptomycin which is 507nm with % intensity79.7 at peak (1), 63.70 nm % intensity10.4 at peak (2) and 4617 nm % intensity 9.9 at peak (3). Dynamic light scattering (DLS) or photon spectroscopy (PCS) are the same technique that used to measure the hydrodynamic size (the diameter of a hard sphere that diffuses at the same speed) during their Brownian motion in solution. The intensity of scattering light mentions to the particles which in the same speed [24].

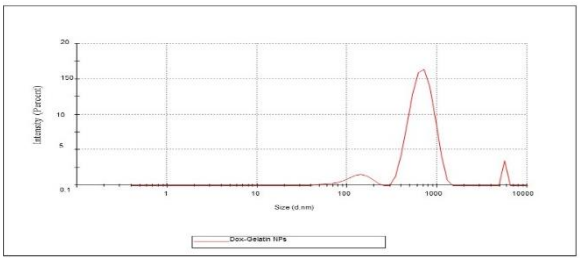


Fig (3): GNPS loaded with Doxycycline distribution by Malvern Nano sizer Peak (1) show particles size average about 690±194nm, peak (2)130±8.54nm and peak (3)7560±3.3nm with %Intensity about 87.7 ,8.8 and 3,5 respectively.

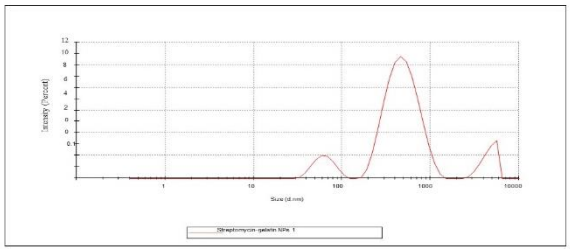


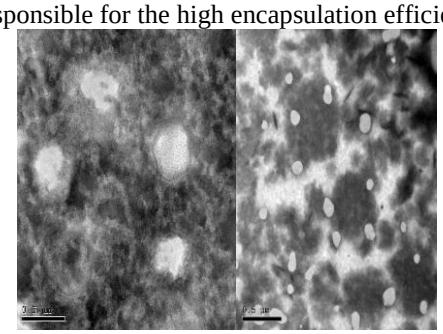
Fig (4): GNPS loaded with Streptomycin distribution by Malvern Nano sizer Peak (1) show particles size average about 507± 20.6nm, peak (2) 63.70±16.4nm and peak (3)4617±323nm with %Intensity about 79.7, 10.4 and 9.9 respectively.

3.2.2. Electron microscope Characterization:

These Transmission Electron microscopes (TEM) images were taken using the negative staining technique where the background becomes dark and particles are white in color. The particles in white are the gelatin nanoparticles loaded with Doxycycline as provided in Fig (5) and loaded with streptomycin as Fig (5) illustrates. Doxycycline caused the matrix to expand and absorb more water. On the contrary, streptomycin caused the gelatin matrix to slightly shrink and get smaller in size.

3.2.3. Encapsulation efficiency:

The encapsulation efficiency of gelatin nanoparticle carrier loaded with Doxycycline and Streptomycin was 89.32 ± 3.22% and 81.74 ± 1.15%. respectively. The method of preparation of microspheres and the choice of glutaraldehyde as a crosslinker could be responsible for the high encapsulation efficiency.



Fig(5): Gelatin nano particle carrier loaded with Doxycycline in spherical shape that larger than gelatin nanocarrier loaded with Streptomycin in the spherical shape too.

3.3. The attainment of infection:

Guinea pig (n=5) from each group (first and second group) were slaughtered 14-days post- inoculation with 105 CFU/ gm of organ infected with *Brucella* melitensis filed strain before the commence of treatment to ensure the attainment of infection. The spleen, livers and lymph nodes from euthanized animals yielded high numbers of *Brucella* colonies in occurrence Table (2).

Table(2): Results of sacrefied animals 14 days post inoculaition to ensure the attainment of *Brucella* infection.

Organs	Log C.F.U	Slaughtered animals/infected organs	Control animals/ infected organs
Spleen	2.8±1.9	5/5	5/0
Liver	4.56±1.5	5/5	5/0
L.N	5.69±2.3	5/5	5/0

Values = mean± standard deviation

3.4. statistical results:

A combination of antibiotics is preferred for brucellosis treatment to prevent relapse infection cases. Using of Tetracyclines as a monotherapy has been considered inconvenient due to unapproved relapse rates, ranging from 2% to 39% [25]. *Brucella* sp. is intracellular pathogens that infect host macrophages [26]. Hence delete, the antibiotics to be used for treatment should have the capability to penetrate the cell and be there at the therapeutic level inside the cell. Doxycycline and Streptomycin combination in the traditional form displayed a highly significant reducing estimated marginal means of *Brucella* CFU/gm (log10) at $p < 0.01$ as illustrated in Table (3) three- and fourteen-days post the end of 10 days of consecutive treatment for the infected group.

Doxycycline displays excellent action in the acidic phagolysosome atmosphere where the mediator of Brucellae, and its antibacterial activity have been continually proven but using this drug as a single treatment is always accompanied by a higher relapse rate at the same time Streptomycin confirm its ability as an antibacterial agent against *Brucella* pathogen. Coupling Doxycycline and Streptomycin in a long-term treatment will reduce relapse of infection moreover Streptomycin has a synergistic effect on Doxycycline influences [26]. Doxycycline binding to the 30S bacterial ribosome causing inhibition of bacterial protein synthesis, Streptomycin binds to the 30S ribosomal subunit and interfere with t-RNA causing misreading resulting in irregular proteins.

The ability of a polymer-based gelatin nano particles as a carrier in a combination therapy (Doxycycline and Streptomycin), and the efficacy of treatment compared with the traditional form of the same combination of drugs against *Brucella melitensis* b.v3 were attempted and the outcomes of this trail are recorded in statistical data illustrated in Table(4).Results displayed a significant reduction of the log of *Brucella* C.F.U/gm in tissue at $p < 0.05$ three days after the end of the treatment regium which consisted of four doses with one day a part between doses for infected treated group if compared by the infected non-treated control group **.** We observed that gelatin nano particles have no effect or are nearly neglected

as an antibacterial agent against *Brucella* and the deleterious effect against *Brucella* was mainly due to Doxycycline and Streptomycin either 3- or 14-days post treatment as displayed in Table (4). Both of the traditional and drug delivery system treatment regimens exhibited a significant deleterious effect against *Brucella mlitensis* b.v3 in infected animals if compared with infected non- treated animals the result coincided with [27] for traditional prolonged treatment with Doxycycline and for nano-treatment as they used the same antibiotics but with another carrier [28].Looking at the results gained from these experiments it is clearly unmistakable evidence that both the treatments regimes achieved excellent consequence against the studied strain. Being the traditional was more effective which could be attributed to low number of shoots for the nano technique (4 doses only) and the prolonged more doses in the traditional one (ten doses). In the general treatment of human brucellosis require prolonged treatment (Doxycycline 200 mg daily for 6 weeks coupled with Streptomycin1gm for two to three weeks or Rifampicin. [3,4]. In the current study this could be explain why the traditional technique appeared more worthy than the nano one in combating the infected strain. The drug delivery system has been examined as an alternative to free drugs. Unfortunately, free drugs reach the target cells or tissues in very small amounts or do not stay long enough in the circulatory to induce the demanded therapeutic effect. On the contrary, the Nano-form stay longer with slower clearance from the body and with extended circulatory time. At the same time, the drug carrier should ensure sustained release of the drug in therapeutic level at the target site or tissue.

The task of our study is to design a drug delivery carrier system that can be engulfed by the phagocytes, releasing the carried drug sustainedly inside the macrophage which holding the intracellular pathogen, this mechanism will improve the efficiency of drug and reduce the side effect of antibiotics [16] Moreover using the drug delivery system based on nano particles carrier enables us to reduce the number of doses and to incriminate a new material previously banned for its toxicity. Our study proved that gelatin nano particles carrier is a good new approach to drug loading and delivery system convention with [27], the drug delivery systems used for caring materials to defeat intracellular bacterial infections, the proposed system improves bioavailability, and sustained release related with reduced frequency of administration and enhanced antimicrobial activity and decreased toxicity [29].Gelatin nano particles carrier can be used as a release controlling system for different drug delivery drives and increase drug loading efficiency [30].To explore the effect of fabricated nano formula and the anti-biotics used in each treatment on animals' tissue. Liver (A.S.T& A.L.T) and kidney (Creatinine& Urea) function tests were estimated as well as spleen, liver body weight ratio for each animal under investigation were taken. Table(5) represents the end result of the previously aforementioned parameter for traditional Doxycycline and Streptomycin combination treatment after 3and 14 days post the end of the treatment which indicate no significant changes in spleen & liver weight between the normal group, normally treated group, infected non treated group and infected treated group 3 days post ten days of consecutive treatment however, enlargement at $p < 0.05$ in liver and spleen were observed in infected non-treated group after 14 days post the end of the treatment. Likewise, the results of gelatin nanoparticles carrier loaded with Doxycycline and Streptomycin treatment after four doses with one day a part illustrated in Table (6). A significant increase at $p < 0.05$ was observed in liver weight in infected non-treated and infected treated after 3 and 14 days of treatment and both of liver and spleen weight for infected non-treated group after 14 days post the end of the regime if compared with normal control group. The increase in liver and spleen weight in animals belonged to the infected non-treated groups and infected treated groups resulting from *Brucella* infection creating spleen hepatomegaly [3]. *Brucella melitensis* infection cause a spectrum of hepatic lesion including scattering foci of inflammation like viral hepatitis. Inflammatory cells aggregate in liver parenchyma areas of liver necrosis [31]. Liver function are serum blood tests measure many enzymes include Aspartate aminotransferase (AST) and Alanine aminotransaminase (ALT). AST is found in kidney, muscles, heart and ALT is found in kidney. Both of AST and ALT are found with highest concentration in liver [32]. Aminase transferase enzymes catalyze the transfer of α-amino groups from aspartate amino acid and alanine to the α-keto group of ketoglutaric acid to produce oxalacetic then pyruvic acids, which are important to the Krebs cycle [33]. Injury of kidney, heart, skeletal muscles cause AST alternation. while, ALT elevation in serum is mor specific to liver injury [34]. Kidney serum blood tests as Creatinine and Urea are measure kidney function. Creatinine is produced at a constant rate during muscle contractions from creatine phosphate. Urea is nitrogenous wastes resulted by catabolism of proteins. Both of Creatinine and Urea are filtered from the blood by the kidney. So, increasing level of Creatinine and Urea in blood serum related with renal injury [35]. There was minor deference in the results of liver ALT and kidney Creatinine and Urea tests between infected treated group and the infected non-treated control infection group 3 days post the end of treatment compared to the non-infected non-treated normal control group. On the otherwise, a significant difference at $p < 0.05$ in the traditional treatment was noted in results of AST test in infected non- treated group, non-infected treated group post 3 days of

traditional treatment and infected non-treated group post 14 days of consecutive regime if compared with normal group as Table (7) displays. Also, there was no significant change in outcomes data of ALT, Creatinine and Urea tests between groups under investigation but there was a significant elevation at $p < 0.05$ in AST enzyme level in the case of animals belonging to infected non-treated and infected treated groups if compared with the normal group as observed in Table (8). The results after 14 days post the end of the treatment which illustrated in Table (8) were similar to the result after 3 days post four shoots.

Examination of hepatic lesion due to *Brucella melitensis* included dispersed small foci of inflammation like viral hepatitis [3] that lead to AST significant elevation in these animals [36]. Also, Doxycycline hyclate has correlated with hepatic injury in some cases receiving treatment program of the antibiotic and resulted markable elevtion [37].

Table (3): Log CFU of Brucella viable count 3and14 days post 10 consecutive daily treatment with traditional Doxycycline and Streptomycin.

Time	Groups of Treatment	Log C.F.U /Organs					
		Log C.F.U/Spleen	Spleen reduction rate	Log C.F.U/Liver	Liver reduction Rate	Log C.F.U/L. N	L. N reduction Rate
After 3days	Infected non-treated	5.79±0.06	N. A	3.49±0.22	N. A	5.80±0.06	N. A
	Infected treated	0.16±0.07*	5.63	0.14±0.03*	3.35	0.62±0.09*	5.18
After14 days	Infected non-treated	4.98±0.06	N. A	3.8±0.145	N. A	5.7±1.797	N. A
	Infected treated	0.10±0.174*	4.88	0.11±0.08*	3.69	0.55±0.06*	5.15

Values = mean± standard deviation * = $P < 0.01$ N. A: not applicable.

Table (4): Log CFU of Brucella viable count 3- and 14-days post 4 shoots of gelatin nano carrier loaded with Doxycycline and Streptomycin.

Time	Groups of treatment	Log C.F.U /Organs					
		Log C.F.U/Spleen	Spleen reduction rate	Log C.F.U/Liver	Liver reduction Rate	Log C.F.U/L. N	L. N reduction Rate
Treatment after 3 days post 4 shoots of drug	Infected non-treated	4.78±0.06	N. A	3.49±0.145	N. A	4.70±1.79	N. A
	gelatin nano carrier loaded with anti-biotics	1.92 ±0.72*	2.86	1.33±1.5*	2.16	2.66±1.60*	2.04
	gelatin nano carrier	5.09±0 .05	N. A	6.12± 0.69	N. A	5.16 ±0.140	N. A
Treatment after 14 days post 4 shoots of drug	Infected non-treated	6.2±0.02	N. A	3.5±0.14	N. A	6 ±0.3	N. A
	gelatin nano carrier loaded with anti-biotics	2.02 ±0.27*	4.18	1.6±1.15*	1.9	3 ± 1.83*	3
	gelatin nano carrier	5.87±0.58	N. A	4.75±0.67	N. A	5.86±0.07	0.54

Values = mean± standard deviation *= $P < 0.5$ N. A: not applicable.

Table (5): Organs body weight ratio 3- and 14-days post 10 of consecutive treatment with traditional Doxycycline and Streptomycin.

Organ's parameter	Groups of treatment						
	3days post 10 days 0f end of the treatment				14 post the end of the treatment		
	Non-infected non-treated	Infected non-treated	Non-infected treated	Infected treated	Infected non-treated	Non-infected treated	Infected treated
spleen body weight ratio(gm)	0.23±0.26	0.3±0.00	0.17±0.0	0.20±0.01	0.5±0.10*	0.2 ±0.0	0.24±0.06
Liver body weight ratio(gm)	4±0.15	4.5±0.04	4.6±0.26	4.4±0.55	5.2±0.20*	4.5±0.30	3.9±0.2

Values = mean± standard deviation *= $P < 0.05$

Table (6): Organs body weight ratio 3,14 days post 4 shoots of gelatin nano carrier loaded with Doxycycline and streptomycin treatment.

Organ's parameter	Groups of treatment						
	3days post 10 days 0f end of the treatment				14 post the end of the treatment		
	Non-infected non-treated	Infected non-treated	Non-infected treated	Infected treated	Infected non-treated	Non-infected treated	Infected treated
spleen body weight ratio(gm)	0.23±0.26	0.3±0.0	0.21±0.03	0.4±0.17	0.5±0.10*	0.20±0.00	0.40±0.17
Liver body weight ratio(gm)	4±0.15	4.5±0.04*	4.3±0.26	6.3±0.9*	5.2±0.20*	4.2±.06	6.03±0.17*

Values = mean± standard deviation *= P < 0.05

Table (7): liver and kidney functions 3- and 14-days after 10 of consecutive treatment with traditional Doxycycline and Streptomycin.

Blood parameter		Groups of treatments						
		3days post 10 days of end of the treatment				14 post the end of the treatment		
		Non-infected non-treated	Infected non-treated	Non-infected treated	Infected treated	Non-infected treated	Infected non-treated	Infected treated
Liver function U/l	AS. T (SGo.t)	87±6.5	114±19.9*	102±17.5*	73±8	85±5.5	122±10.7*	80±1
	AL. T (SGp.t)	72±15	80±0.8	87±16	75±14	67±7	60±19.5	63±13
Kidney function mg/dl	Creatinine	0.76±0.11	1.3±0.10	0.83±0.3	0.7±0.06	0.85±.05	1.2±0.6	0.7±.05
	Urea	64±6.4	60±2.8	57.2±7.7	53±7.7	64.6±4	71±7	50±1.3

Values = mean± standard deviation *= P < 0.5

Table (8): liver and kidney function 3,14 days post 4 shoots of gelatin nano particles carrier loaded with Doxycycline and streptomycin treatment.

Blood parameter		Groups of treatment						
			After three days			After fourteen days		
		Non-infected non-treated	Infected non-treated	Non-infected treated	Infected treated	Infected non-treated	Non-infected treated	Infected treated
Liver function U/l	AL. T (SGp.t)	72±15	80±0.8	77.8±7.39	68.4±8.00	60.3±19.5	69.4±4.4	63.7±10
	AS. T (SGo.t)	87±6.5	114 ±19.9*	89±12.9	169.3±15.30*	122±10.7*	87±6.6	150±15.30*
Kidney function mg/dl	Creatinine	0.76±0.11	1.3±0.10	0.8±0.1	1.30±0.17	1.4±0.06	0.767±0.06	1±0.2
	Urea	64±6.4	60±2.78	53.6±4.92	64.95±5.5	60±8	65±5.29	68±3.6

Values = mean± standard deviation *= P < 0.5

4. Conclusion

Treatment and eradication of Brucellosis is difficult due to intracellular bacteria *Brucella* Spp. which are localized in macrophage cell. Due to high rat brucellosis relapse infection in cases treated with traditional antibiotics this encouraged us to seek a new treatment method.

Gelatin nano particles loaded with Doxycycline and streptomycin seem to be a promising strategy. Treatment with the proposed technique resulted in reducing *Brucella* C.F.U significantly after four shoots only in infected treated group.

Although there was a significant reducing number of Brucella viable counts from different organs, the gained results indicated that none of the treatment regimens achieved complete elimination of *Brucella* infection in animals under study. We recommend to retrying gelatin drug delivery system method with more shoots than four with the same antibiotic or other antibacterial agents encapsulated in gelatin nanoparticles carrier.

5. Conflicts of interest

There are no conflicts to declare

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