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Efficiency of silver nanoparticle combined with ciprofloxacin in treatment mastitis induced by *E. coli* O157:H7 isolated from milk of bovine mastitis

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Abstract

In order to determine the efficiency of silver nanoparticles in mastitis treatment induced by *E. coli* O157:H7, 30 animals were infected intramammary with 1×10^{12} cfu/ml of virulence *E. coli* O157:H7 isolated from milk of bovine mastitis and at 48hr post inoculation, the animals were divided randomly into four groups equally, 1st group treated with AgNPs orally and Ciprofloxacin via intramammary 2nd group treated as 1st group but AgNPs were intramammary 3rd group left without treatment, 4th group was inoculated intramammary with 0.05ml of sterile normal saline and served as control negative, five animals from each group were sacrificed at 7 and 14 days post infection. Results showed severe pathological lesions with heavy bacterial isolation in oral treatment animals (4.00 ± 0.07 ; 6.00 ± 0.33 LD) and infected non treatment group (5.00 ± 0.13 ; 6.00 ± 0.24 LD) at 7, 14 days post infection respectively, while animals that intramammary treated with AgNPs expressed mild pathological lesions in mammary gland with few bacterial isolation (1.00 ± 0.05 ; 0.00 ± 0.00 LD) it was concluded that AgNPs expressed synergistic action with Ciprofloxacin against mastitis induced by *E. coli* O157:H7.

Keywords: Silver nanoparticle, AgNPs, Ciprofloxacin, Mastitis, *E. coli* O157:H7, milk, bovine mastitis

1. Introduction

The main problem in dairy cattle is mastitis that causes important losses including high cost of treatment [1, 2]. changes in physical and chemical composition of the milk as a result of pathological changes in the udder, in addition to zoonosis public health important [3,4]

The etiology of mastitis were either infectious a (inflammation and abscess formation) or noninfectious agents, (milk stasis) [5], also mastitis can divided into environmental and contagious microorganisms according to type of infection either transient opportunistic or persistent respectively [6].

there are more 137 pathogenic species were responsible for mastitis [7], and approximately 80% of mastitis in cattle cause by *E. coli*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Staphylococcus aureus* and *Streptococcus dysgalactiae* [8,9], The main environmental pathogen is *Escherichia coli* (*E. coli*) [10]. Which is a communal microorganisms found in the gastrointestinal tract of humans and certain strains of *E. coli* are highly pathogen to human such as *Escherichia coli* O157:H7 (STEC O157) that cause bloody diarrhea hemolytic uremic syndrome (HUS) and hemorrhagic colitis [11]. There were several outbreaks of foodborne disease cause by consumption of contaminated milk particularly with *E. coli* O157 in different countries [11, 12].

There is no efficient vaccine against causative agents of mastitis therefore controlling of these disease was depend on antibiotic treatment [13].

Prolong application of the drugs lead to multi drug resistance pathogen [14]. That associated with high morbidity and mortality rate in addition to high cost of treatment in development countries [15].

Due increase multidrug resistance pathogen, alternative safe and less costly effective bactericidal agents were required to treatment animals and human disease, such as plant extracts (Indirubin) [16], and metal therapy including Silver ions [17, 18], it was found that AgNPs expressed broad spectrum antimicrobial activities [19] and it can killed more 650 varies type of pathogens including viruses, bacteria, parasites, fungi and molds [20].

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In Iraq, contaminated of animal food origin commonly occur due to unhygienic condition in the slaughtered animals and milking processing, therefore these food form a risk for health consumers, few studies were performed on treatment mastitis induced by *E. coli* O157:H7 with AgNPA combine with Ciprofloxacin, therefore the aim of the current study was to determine the efficiency of Silver nanoparticle combine with antibiotic in treatment of mastitis induced by *E. coli* O157:H7 in mice.

2. Materials and methods

The study was conducted at College of Veterinary Medicine/ University of Baghdad, Zoonotic diseases Unit laboratory, Baghdad, Iraq, from 2 February 2017 to 14 March 2017. Preparation of AgNPs and Ciprofloxacin It was mixed 0.05ml of AgNPs with 0.05 ml of ciprofloxacin and the solution left at room temperature for 2 hours for homogenization.

2.1 Bacterial strain

E. coli O157:H7 strain was isolated from Milk of lactating cows suffering from mastitis and PCR identification by Abdullah, (2017), these strain was examined its virulence by I/P inoculation mice with 0.2ml of bacterial suspension and re isolation these pathogen again from internal organs and these process was repeated until death of infected animal

2.2 Experimental design

Forty white female mice were used, 30 of them were inoculated intramammary with 1×10^{12} cfu/ml of virulence *E. coli* O157:H7 isolated from bovine mastitis milk, at 48hr post inoculation all animals were randomly divided into four groups equally as following

1st Group was treated orally with 0.05ml of AgNPs with size

40 nm and intramammary treatment with 0.05ml of Ciprofloxacin daily for 2weeks.

2nd Group was intramammary treatment mixed of 0.05 ml of AgNPs, 40 nm diameters with 0.05ml of Ciprofloxacin daily for 2 weeks.

3rd Group was left without treatment and served as control positive group.

4th Group was intramammary inoculated with 0.1ml of sterile normal saline and served as control negative group, five animals from each group were sacrificed at day 7 and 14 post inoculation, and pieces from mammary glands were taken for bacterial isolation and other pieces with size 1x1x1cm were fixed in 10% formalin for pathological examination according to Luni, [47].

2.3 Statistical Analysis

The Statistical Analysis System- SAS [48], program was used to effect of difference factors in study parameters (Lesion degree-LD). Least significant difference –LSD test was used to significant compare between means in this study.

3. Results

The result showed severe lesion in the infected mammary gland of control positive animal at 7 days post infection (5) and these lesions become very sever at 14 days post infection (6), and the intensity of lesion in control positive animals significantly intense as compared with infected animals after 7 days (1) of intramammary treatment with combination of AgNPs and Ciprofloxacin also after 14 days of treatment (0). While in the infected animal treated orally with combination of AgNPs and Ciprofloxacin showed sever lesion (4) at 7 and 14 days of treatment as showed in table below:

Table 1: Effect of treatment and day in Lesion degree-LD

Course of treatment (days)	Control positive	Rout of treatment with AgNPs and Ciprofloxacin		LSD value
		Intramammary	Orally	
7 days	5.00 ± 0.13	1.00 ± 0.05	4.00 ± 0.07	2.058 *
14 days	6.00 ± 0.24	0.00 ± 0.00	6.00 ± 0.33	2.617 *
LSD value	1.750 NS	1.00 NS	1.62 *	---

LD: Lesion degree

LD 6: Abscess formation and fibrosis

LD 5: Neutrophil infiltration, and abscess formation

LD 4: Abscess formation

LD 1: mononuclear cell infiltration

LD 0: No clear lesion

* ($P < 0.05$), NS: Non-Significant.

3.1 Bacterial isolation

The result revealed severe bacterial isolation from mammary gland of control positive animals and severe to moderate bacterial isolation from mammary glands of infected animal orally treatment with AgNPs and intramammary treatment with 0.05ml of Ciprofloxacin while mild or no bacterial isolation were recorded in mammary glands of animal intramammary mixed treatment with AgNPs and Ciprofloxacin.

3.2 Pathological examination

• At 7days post infection

Histological section in the mammary gland showed inflammatory cells particularly neutrophils infiltration in the interstitial tissue with hyperplasia of epithelial cells of alveoli that complete occluded their lumen (Fig. 1), in other animals, it was recorded abscess surrounded by fibrous connective

tissue that destroyed the alveoli and extended to adipose tissue (Fig. 2) and the abscess replacement of glandular tissue in addition to neutrophils in the lumen of alveoli in addition to vascularization, desquamation and necrosis of epithelial cells of the alveoli (Fig. 3).

• At 14 days post infection

It was found mononuclear cells in milk material in the lumen of mammary duct that expressed hypertrophy with vascularization of their epithelial layer and inflammatory cells and fibrous connective tissue proliferation in the interstitial tissue (galatophoritis) (Fig. 4) and inflammatory cells particularly neutrophils and macrophages reach subcutaneous tissue and muscular layer, in other section.it was seen hyperplasia of epithelial cells of alveoli with severe inflammatory cells infiltration in the interstitial tissue with fibrosis in other animals, the main lesions characterized

vascularization and desquamation of epithelial cells of alveoli with fibrosis of interstitial tissue infiltrated by mononuclear cells and neutrophils (Fig. 5) and granulation tissue infiltrated with mononuclear cells that replacement of alveoli and adipose tissue (chronic mastitis) (Fig. 6, and 7), in other section, it was reported show vascularization of hyperplasia desquamous epithelial layer of alveoli surrounded by marked fibrosis infiltrated by mononuclear cells that extended to adjacent skin region (Fig. 8).

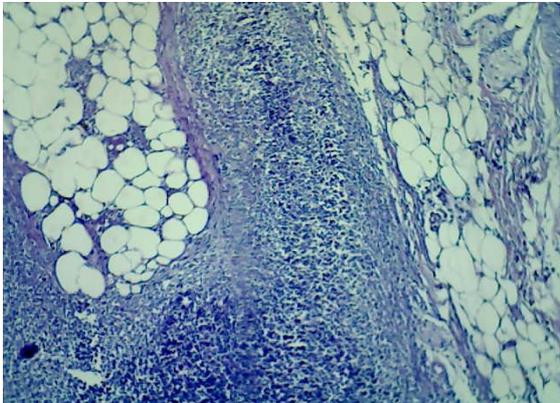


Fig 1: Histological section in the mammary gland of animals at 7 days post infection shows neutrophils infiltration in in the interstitial tissue and in the lumen of the alveoli which expressed hyperplasia of their epithelial cells that closed their lumen (H&E stain 400X).

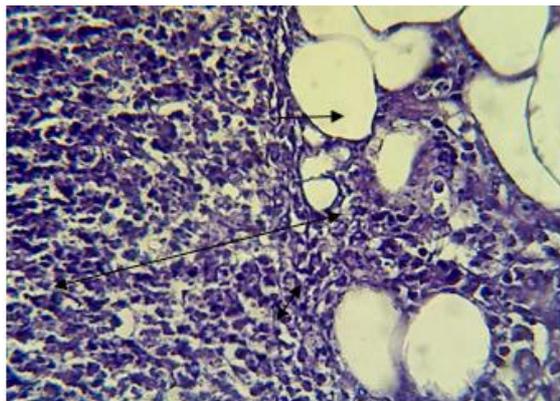


Fig 2: Histological section in the mammary gland of animals at 7 days post infection shows abscess surrounded by fibrous connective tissue that destroyed the alveoli extended to adipose tissue (H&E stain 100X).

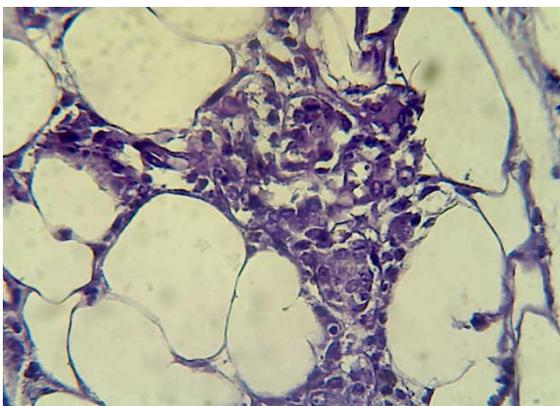


Fig 3: Histological section in the mammary gland of animals at 7 days post infection shows marked neutrophils infiltration in the lumen of the alveoli and in the interstitial tissue in addition to vacuolation, desquamation and necrosis of epithelial cells of the alveoli (H&E stain 400X). At 14 days post infection

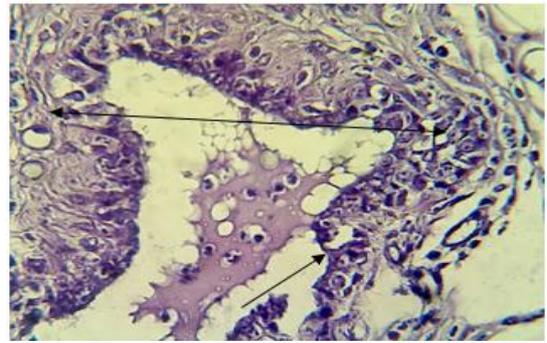


Fig 4: Histological section in the mammary gland of animals at 14 days post infection shows mononuclear cells in milk material in the lumen of mammary duct that expressed hypertrophy with vacuolation of their epithelial layer and inflammatory cells and fibrous connective tissue proliferation in the interstitial tissue (galatophoritis) (H&E stain 400X).

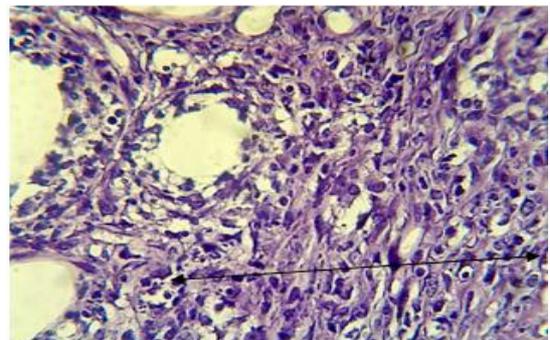


Fig 5: Histological section in the mammary gland of animals at 14 days post infection shows vacuolation and desquamation of epithelial cells of alveoli with fibrosis of interstitial tissue infiltrated by mononuclear cells and neutrophils (H&E stain 400X).

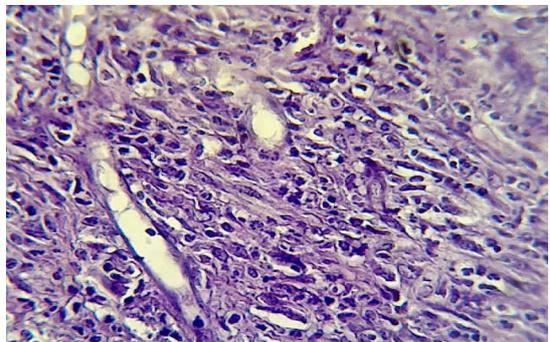


Fig 6: Histological section in the mammary gland of animals at 14 days post infection shows granulation tissue infiltrated with mononuclear cells that replacement of alveoli and adipose tissue (H&E stain 400X).

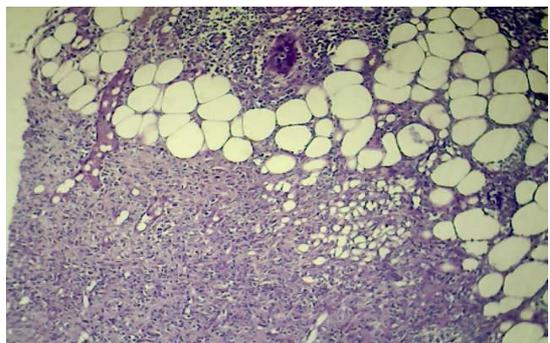


Fig 7: Histological section in the mammary gland of animals at 14 days post infection shows severe fibrosis in the interstitial and adipose tissue that replacement of mammary alveoli infiltrated with mononuclear cells (chronic mastitis) (H&E stain 100X).



Fig 8: Histological section in the mammary gland of animals at 14 days post infection show vacuolation of hyperplasia desquamated epithelial layer of alveoli surrounded by marked fibrosis infiltrated by mononuclear cells that extended to adjacent skin region (H&E stain 100X).

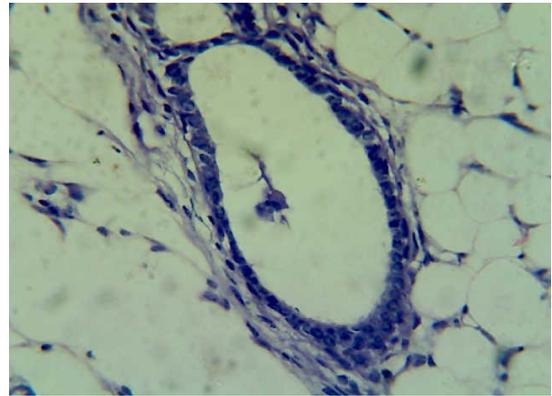


Fig 11: Histological section in mammary gland of animals at 7 days post infection and intramammary treated with mixed of AgNPs and Ciprofloxacin shows no clear lesions in lactiferous duct (H&E stain 400X)

- **Intramammary treatment**
- **At 7 days post infection**

Histological section in mammary gland expressed no clear lesions (Fig9), in other animals, few mononuclear cells infiltration were seen in the interstitial tissue and adipose tissue (Fig:10) as well as no clear lesions in the lactiferous duct (Fig. 11).

- **At 14 days post infection**

No clear lesions were reported in the alveoli (Fig. 12) and lactiferous duct (Fig. 13)

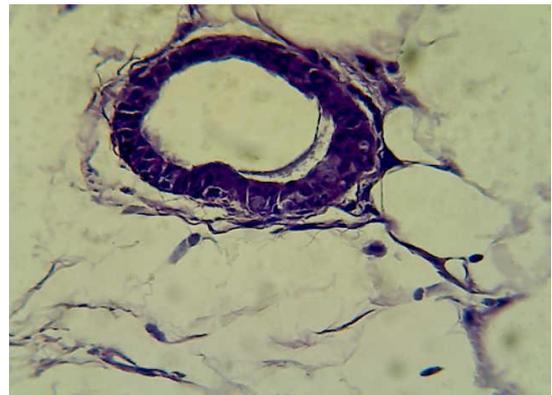


Fig 12: Histological section in the mammary gland of animals at 14 days post infection and intramammary mixed treated with AgNPs and Ciprofloxacin shows no clear lesions (H&E stain 400X).

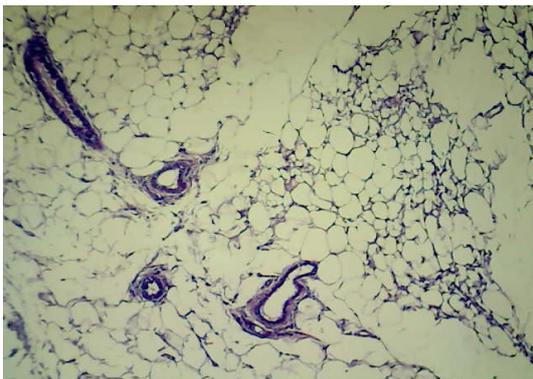


Fig 9: Histological section in the mammary gland of animals at 14 days post infection and mixed treated with AgNPs and Ciprofloxacin shows no clear lesions (H&E stain 100X).

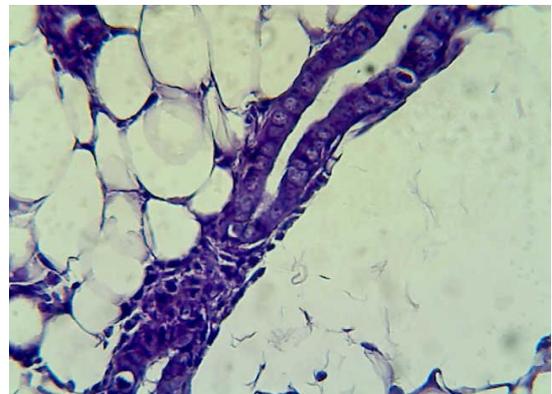


Fig 13: Histological section in the mammary gland of animals at 14 days post infection and intramammary mixed treated with AgNPs and Ciprofloxacin shows no clear lesions in lactiferous duct (H&E stain 400X).

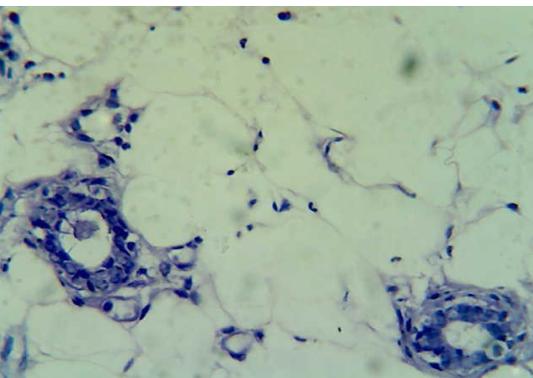


Fig 10: Histological section in mammary gland of animals at 7 days post infection and intramammary treated with mixed of AgNPs and Ciprofloxacin shows few inflammatory cells in interstitial tissue and adipose tissue (H&E stain 400X).

- **Oral treatment**
- **At 7 days post treatment**

The main lesions characterized by abscess formation surrounded by fibrous connective capsule in the interstitial tissue and extended to adipose tissue (Fig. 14, and 15), in addition to neutrophils in the lumen of alveoli that showed vacuolation and desquamation epithelial cells in addition to congested blood vessels in adipose tissue (Fig. 16)

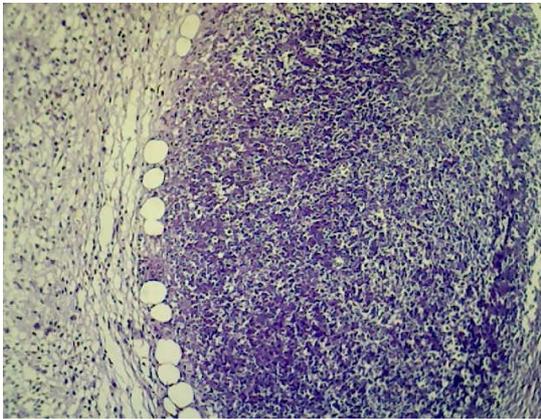


Fig 14: Histological section in the mammary gland of animals at 7 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows abscess surrounded by thick fibrous connective capsule (H&E stain 100X).

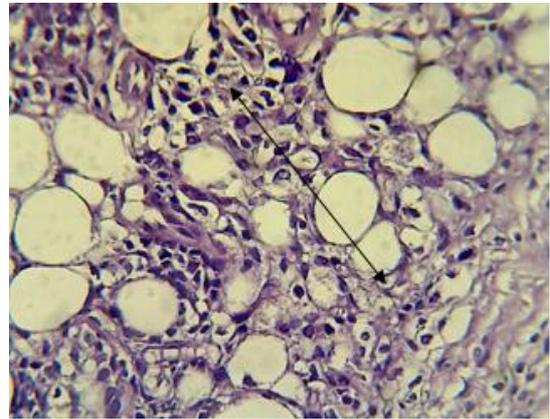


Fig 17: Histological section in the mammary gland of animals at 14 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows vacuolation of hyperplasia of epithelial cells of the alveoli with proliferation of fibrous connective tissue infiltrated with inflammatory cells in the interstitial tissue (H&E stain 400X).

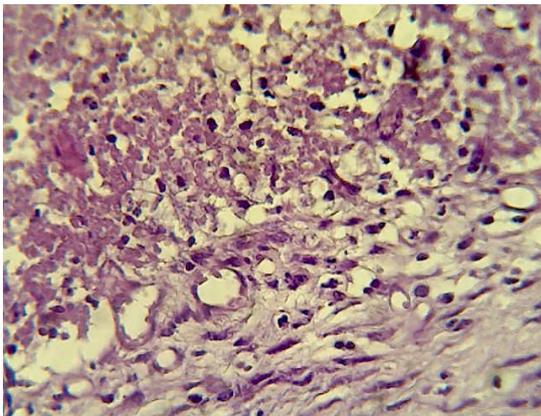


Fig 15: Histological section in the mammary gland of animals at 7 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows abscess surrounded by thick fibrous connective capsule (H&E stain 400X).

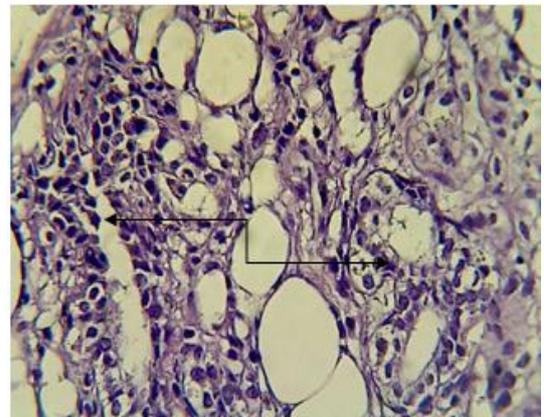


Fig 18: Histological section in the mammary gland of animals at 14 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows vacuolation and desquamation of hyperplasia of epithelial cells of the alveoli with proliferation of fibrous connective tissue infiltrated with inflammatory cells in the interstitial tissue (H&E stain 400X).

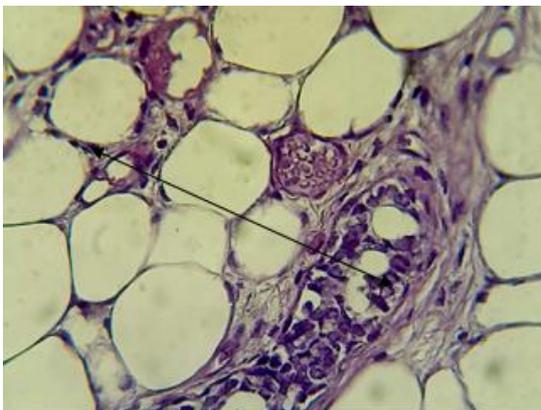


Fig 16: Histological section in the mammary gland of animals at 7 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows vacuolation and desquamation of epithelial cells of alveoli with congested blood vessels (H&E stain 400X).

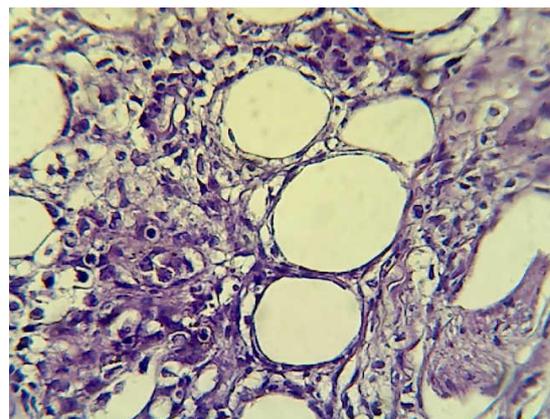


Fig 19: Histological section in the mammary gland of animals at 14 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows inflammatory cells infiltration in fibrosis of interstitial tissue with hyperplasia of epithelial cells of alveoli that lead to occluded its lumen (H&E stain 400X).

• At 14 days post treatment

Histological section in the mammary gland showed vacuolation of hyperplasia of epithelial cells of the alveoli with proliferation of fibrous connective tissue infiltrated with inflammatory cells in the interstitial tissue Fig. 17), in other section occluded of lumen of alveoli occur by hyperplasia of epithelial layer (Fig. 18) it was found that fibrosis replacement of the alveoli. (Fig. 19) in addition to severe fibrosis around dilated lactiferous duct infiltrated by inflammatory cells (Fig. 20).

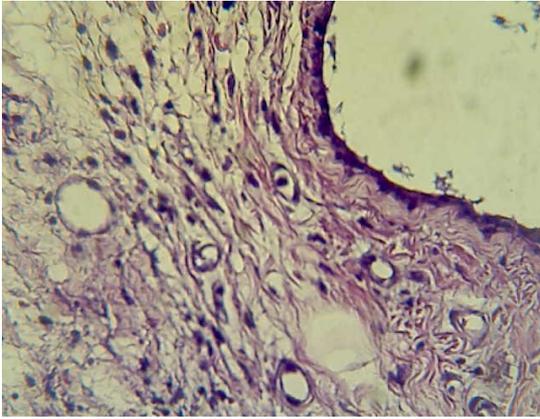


Fig 20: Histological section in the mammary gland of animals at 14 days post infection and oral mixed treated with AgNPs and Ciprofloxacin shows severe fibrosis around dilated lactiferous duct infiltrated by inflammatory cells (H&E stain 400X).

4. Discussion

The current result showed that animals treatment intramammary with combination AgNPs with Ciprofloxacin showed mild or no bacterial isolation, These result may indicated that synergistic activity between AgNPs and Ciprofloxacin which are in agreement with Namasivayam *et al*^[19] who recorded antimicrobial activity of AgNPs alone or combine with Ciprofloxacin against multidrug resistance *E. coli*, *S. aureus* and *P. aeruginosa* also Fayaz *et al.*^[21] found synergistic antimicrobial activity of combination AgNPs with Ciprofloxacin.

Also it was recorded that AgNPs can increase bactericidal activity of certain antibiotic such as amoxicillin, erythromycin, vancomycin, clindamycin and penicillin Graves *et al.*^[22].

The efficacy treatment of mastitis induced by *E. coli* O157:H7 isolated from bovine mastitis milk was similar to those reported by Rajawat and Qureshi^[23] who investigated that the ability of ampicillin to lysis cell wall of gram negative bacteria including *S. typhi*, was increased when it combined with AgNPs that lead to increase concentration of these antibiotic in the site of infection, The present result may supported idea that AgNPs are a good bactericidal agents against multidrug resistance pathogens, these idea was in consistent with Namasivayam *et al.*^[19], Amany *et al.*^[24] who reported that AgNPs are a better bactericidal for most multi drug bacterial resistance such as *E. coli* and *Staph aureus*.

4.1 Pathological examination

The lesions in the mammary glands of infected animals in the present study may indicated that the local isolates strain of *E. coli* O157:H7 is highly virulence that overcome the normal defense mechanism of mammary gland and induced tissue damage, these lesions were similar to those reported by Ravikumar *et al.*^[25] in rabbit experimentally infected by *E. coli* strains O9 and O147 who they found that the main lesions characterized by hyperemia, outer lobular, interlobular edema and PMNs infiltration in the acini and interstitial tissue. In current finding, severe suppurative inflammation with abscess formation at 7 days post infection these result may due to bacterial cellular products that attract the neutrophils to infected site which their lysosomal enzyme can progress the tissue damage in addition to oxidative stress generated by phagocytosis process as well as protease enzyme of immune cells^[26].

The present result showed that local strain of *E. coli* O157:H7 cause acute mastitis these result may due to virulence factor

of such as LPS which is responsible for acute inflammatory reaction, these idea was agreement with Vangroenweghe, *et al.*^[27] who demonstrated that LPS of gram negative bacteria can stimulated production of pro inflammatory cytokines which response for acute mastitis in cattle also Jin-lun *et al.*^[16] reported that LPS of *E. coli* induced thickness in the wall of the alveoli due to neutrophils and macrophages infiltration, also the host response to infection associated with activated phagocytic cells that engulf and clearance the infectious agent^[28, 29].

The present study revealed fibrosis of mammary gland, mononuclear cells infiltration vacuolation and desquamation of epithelial cells of alveoli at day 14 post infection, these result may indicated that chronic mastitis occur at this period as a result neutrophils and damage epithelial cells produced chemo attracted factor to the macrophages that engulfed the bacteria and tissue debris with produced large amount of pro-inflammatory cells that exaggerated the inflammatory reaction also the macrophage produced growth factors that facilitated proliferation of fibrous connective tissue in order to repair the damage tissue^[29,30].

The current result showed that no clear lesions in the mammary glands of infected animals intramammary treatment with AgNPs combined with Ciprofloxacin these result may be indicated that synergistic action between AgNPs and Ciprofloxacin drug, as a result of these particles can facilitated action of these antibiotic, these idea was agreement with Li *et al.*^[31] who demonstrated that hydroxyl and amino groups of amoxicillin can interact easily with AgNPs that facilitated enter these antibiotic to bacterial cells and they suggested that AgNPs can act as antibiotic carrier. Other studies revealed that AgNPS act synergistic with antibiotic drugs^[19, 21].

The present finding demonstrated that severe lesions in the mammary glands of infected animals orally treatment with AgNPs and intramammary treatment with low concentration of Ciprofloxacin at 7 and 14 days post infection, these result may indicated that the antimicrobial activity of AgNPs was route administration dependent, these result may due to differences in concentration of the AgNPs that reached the infected site in the intramammary treatment infected animals, these idea was agreement with Rai *et al.*^[20]; Nateghi and Hajimirzababa^[32] and Raza *et al.*^[33] who showed that the bactericidal efficiency of AgNPs can influenced by their concentration, shape, size and colloidal state, also Ahamed *et al.*^[34]; Johnston *et al.*^[35] demonstrated that the concentration of AgNPs can influence on their antimicrobial activity.

Also the present evidence was agreement with Çiftçi *et al.*^[36]; Paul *et al.*^[37], Çiftçi *et al.*^[36] who recorded that concentration of AgNPs induced permanent damage to bacterial cells ranged between 40–50 µg/mL; also they recorded that this concentration can cause highly oxidative stress.

High concentration of AgNPs associated with the amount of these agent enter the target cell cytoplasm, intracellular accumulation of AgNPs lead to disturbance function of vital bacterial cells^[38, 39], interaction AgNPs with ribosome lead to inhibit protein synthesis^[20] also attachment of AgNPs with DNA cause denaturation DNA and prevent bacterial replication^[40,41].

Also the absent pathological changes in the mammary glands of intramammary treatment infected animals may be indicated that 0.05ml of AgNPs is completely destroyed the bacteria as a result high concentration of AgNPs enter the bacterial cells, The main force that facilitated adhesion between AgNPs and bacterial cell membrane is electrostatic attraction between

positive charge of AgNPs and negative charge of bacterial cells^[42], these interaction lead to shrinkage the bacterial cytoplasm and detachment of its membrane that lead to rupture bacterial cell wall^[43].

Also increase cellular accumulation of AgNPs generated oxidative stress^[44], which considered one important mechanism by which AgNPs kill the bacteria due to oxidative stress cause oxidative DNA damage and prevent bacterial replication^[45].

It concluded that combination of AgNPs with Ciprofloxacin expressed synergistic action against *E. coli* O157:H7

However, the mechanism of antibacterial activity of AgNPs including, attachment of these particles with bacterial surface, intracellular penetration of AgNPs, damage mitochondria, ribosomes and vacuoles, induce oxidative stress by AgNPs and cytotoxicity, changes transduction signal pathways in addition to modulate immune response that inhibited bacterial growth^[46].

5. Conclusion

E. coli O157:H7 strain isolated from bovine mastitis was highly sensitive to Ciprofloxacin (CIP10) (CIP5) also highly susceptible to AgNPs particularly when it combine with antibiotic (Ciprofloxacin) drug, Antimicrobial AgNPs activity was route dependent there for Intramammary inoculation of AgNPs was highly efficiency in treatment mastitis.

6. Acknowledgement

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