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Histopathological changes after treatment of *Mycoplasma bovis* infected Does with Zinc oxide nanoparticles as a new tool.

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Mycoplasma bovis mastitis is highly contagious, results in a severe milk production drop in an affected cow. Mastitis in dairy animals is causing heavy economic losses worldwideby loss of milk production, treatment costs and premature culling of chronically infected animals. Nanotechnology has the potential to enable revolutionarychanges in near future given drugs and vaccines can be more effective in treating and preventing the diseases than current technologies, thus reducing cost.Twenty multiparous rabbit does were used after giving birth. The aim of study is to evaluate efficiency of antibiotic nanoparticle on induced rabbit mastitis infected with *M. bovis*. Animals were divided into four groups' five rabbits each: Gp. 1 was a control negative group. Gp. 2 is the control positive which was inoculated intraperitoneally with freshly prepared 10^6 cfu *M. bovis*. Gp. 3 was inoculated intraperitoneally with freshly prepared 10^6 cfu *M. bovis* for 4 days and then treated with lincospectin antibiotic for 5 days. Gp. 4 was inoculated intraperitoneally by freshly prepared 10^6 cfu *M. bovis* for 4 days and then treated with lincospectin + zinc oxide (ZnO) nanoparticle for 5 days.Animals were sacrificed after 2 weeks of experiment and udder tissue samples were collected. Histopathological findings, Gp.2, rabbits exhibited histopathological changes in different organs in addition to mammary glands causing mastitis. In Gp.3, there were edema, mononuclear cells infiltration and mild fibroblastic proliferation in the interstitial tissue of the mammary gland. Moreover, some acini were almost devoid of milk secretion. Gp.4, there was mild fibroblastic proliferation in the interstitial tissue and little milk secretion in the mammary acini.We concluded that the linco-spectin nanoparticle was effective against *M. bovis*. Furthermore, nanoparticles tagged with antibiotics have been shown to increase the antibiotic interaction, and facilitate binding of antibiotics to bacteria. ZnO-NPs could be formulated in a suitable treatment of mastitis caused by *M. bovis* in dairy cattle.

Keywords: *Mycoplasma bovis*, Zinc Oxide, nanoparticles, mastitis.

Introduction

Mycoplasma bovis is one of the most important pathogenic bovine mycoplasma. It is a wall-less bacterium causing bovine mycoplasmosis, showing clinical manifestations in cattle leads to economic losses to dairy industries [1]. Antibiotic treatments are not efficacious. It mainly colonizes the bovine respiratory mucous membrane [2] and has been associated with genital disorders and abortions [3] bovine pneumonia, reduction of semen fertility [4], arthritis [5], decubiti abscesses [6], keratoconjonctivitis [7], otitis [8], poly arthritis [9] and mastitis [10].

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Lincosamides are broad spectrum antibiotic and Linco-spectin is a combination of lincomycin and spectinomycin. They are indicated in treating serious infections caused by susceptible strains of *Streptococci*, *Pneumococci*, and *Staphylococci* [11]. They are often used as a supportive treatment in preventing chronic respiratory disease associated with *Mycoplasma* and *Coliform* infections in chickens [12].

Several strains of *Mycoplasma* were found to be sensitive to a combination of lincomycin spectinomycin-tylosin [13]. A rabbit model was used in previous studies to detect the mastitogenic potential of a *mycoplasma* viz, *M. capricolumcapripneumoniae* [14], and also of *M. canadense* [15]. The study on *M. canadense* revealed subacute to chronic histopathological reaction in rabbit mammary glands with inflammatory response mainly lymphocytic and macrophagic [15] where as that of *M. capricolum capripneumoniae* recorded a neutrophilic infiltration in acinar lumen and interacinar tissues [14].

M.bovis mastitis is highly contagious and results in a severe milk production drop in an affected cow [16]. Also, causes both sub-clinical and clinical forms of mastitis. *Mycoplasma* in a herd can include: resistant to therapy, involve all four quarters/multiple quarters at the same time, not painful, cows with fever and off feed, rapid decline in milk production, abnormal milk that is often brown to tan with flaky sediment in watery or serous fluid. Some milk samples when allowed to settle may appear to have a sandy, granular appearance [17]. Mastitis in dairy animals is a multietiological in nature and causing heavy economic losses worldwideby loss of milk production, treatment costs and premature culling of chronically infected animals [10].

Nanotechnology use of materials with dimensions on the atomic or molecular scale become increasingly utilized for medical applications and of great interest as an approach to killing or reducing activity of numerous microorganisms [18]. The use of nanotechnology in medicine has great potential, especially in medical microbiology. Nanoparticleshave been beneficial due to their antimicrobial effect with low toxicity against the host and their ability to specific targets[19]. The aim of study is to evaluate the efficiency of antibiotic nanoparticle on induced rabbit mastitis infected with *Mycoplasma bovis*.

Materials and Methods

Animals

Twenty multiparous Newzeland rabbit does were used after giving birth. The rabbits were housed individually in ventilated wire cages at a temperature of 23 ± 3°C and humidity at 50–70 %. Rabbits were fed pellet feed and offered water ad libitum. The experiment was performed according to the guidelines of the Institutional Animal Care and Use Committee of Cairo University, Egypt.

Inoculated Bacteria

Mycoplasma bovis was prepared 10^6 cfu of freshly *M. bovis*provided from Animal Reproduction Research Institute, Agriculture Research Center, Giza, Egypt.

Antibiotic nanoparticles

Zinc oxide nanoparticles were kindly obtained from Dr. Abdel Salam Almuhammady Arab Center for Nanotechnology, Cairo University. Scanning electron microscopy (Hitachi S2150, Krefeld, Germany) wasused to image the size and morphology of the Zinc oxide nanoparticles.

Experimental design

Animals were divided into four groups' five rabbits each:

Gp. 1 was a control negative group not inoculated.

Gp. 2 is the control positive group which was inoculated intraperitoneally (I/P) for 4 days by 1mL of freshly prepared 10^6 cfu M. bovis (Fig. 2).

Gp. 3 was inoculated I/P by 1mL of freshly prepared 10^6 cfu M. bovis for 4 days and then treated with 1 ml of lincospectin antibiotic for 5 days.

Gp. 4 was inoculated I/P by 1mL of freshly prepared 10^6 cfu *M. bovis* for 4 days and then treated with 1 ml of lincospectin + zinc oxide (ZnO) nanoparticles with (8µg/ml) concentration for 5 days.

Animals were sacrificed at the end of the experiment after 21days of inoculation and udder tissue samples were collected.

Histopathology

Tissue samples from themammary gland of rabbit were collected and fixed in 10% neutral formalin buffer. Tissues were then processed by paraffin embedding technique and sectioned by microtome (Leica 2135, Germany) at 4 µ thick. Tissue were examined by light microscope and photographed by digital camera (Olympus XC30, Tokyo, Japan).

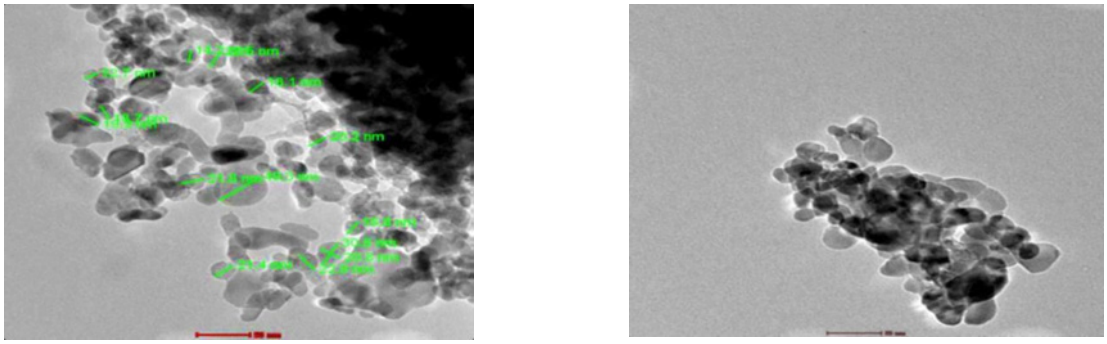

Fig. 1. Scanning electron microscope images of zinc oxide nanoparticles (ZnO-NPs).

Fig.2. Inoculated I/P by freshly prepared 10^6 cfu *M. bovis.*

Results

Histopathological findings

Gp.1, the mammary gland demonstrated normal histological structure with milk secretion in mammary acini in the control group (Fig. 3a).

Gp.2, rabbits inoculated with *M. bovis* exhibited histopathological lesions in different organs in addition to mastitis. The liver of rabbit demonstrated periportal mononuclear leukocytic infiltration and Kupffer cell hyperplasia. In the Lungs, the interstitial tissue and alveolar wall were infiltrated by mononuclear leukocytes together with collapse of some alveoli and dilatation of other forming giant alveoli. In the kidneys, there were proliferation of glomerular cells and glomerular tuft adhesion to Bowman's capsule. In addition, the renal tubular epitheliums were swollen with closure of tubular lumen (Fig. 4a &b).

In Gp.3, inoculated with *M. bovis* and treated with lincospectin antibiotic, there were edema, mononuclear cells infiltration and mild fibroblastic proliferation in the interstitial tissue of the mammary gland. Moreover, some acini were almost devoid of milk secretion and also showed similar lesion to Gp. 2, (Fig. 3b).

Gp.4 infected and treated with antibiotic combination with zinc oxide (ZnO) nanoparticles, there was mild fibroblastic proliferation in the interstitial tissue and little milk secretion in the mammary acini (Fig. 3c & Fig.5, 6a &b).

Discussion

Combining antibiotics with nanoparticles show great promise for treating a wide variety of bacterial and fungal pathogens that are not easily killed by routine antimicrobial agents [20]. Nanoparticles (NPs) have been shown to be a safe and effective alternative therapy

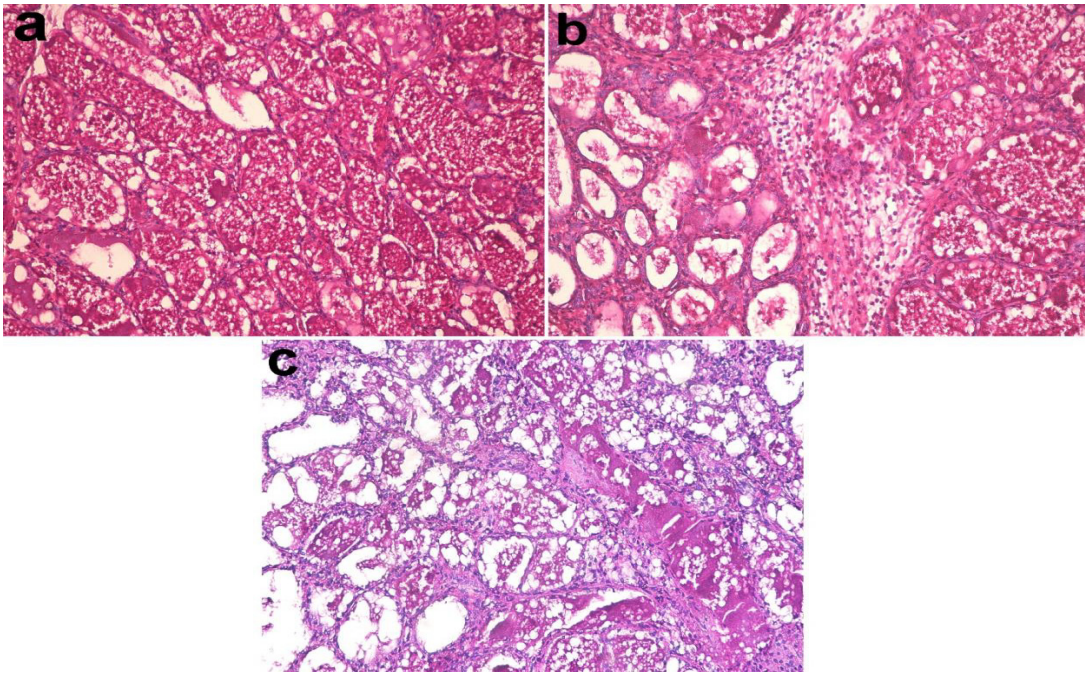

Fig.3. a. Gp.2 rabbit inoculated with M. bovis showing normal structure of mammary acini.
b. Gp.3 inoculated with M. bovis and treated with lincospectin antibiotic showing edema and inflammatory cells infiltration in the interstitial tissue of the mammary gland,
C. Gp.4 inoculated with *M. bovis* and treated with antibiotic combined with Zinc Oxide nanoparticles showing few inflammatory cells infiltration and mild fibroblastic proliferation in the interstitial tissue with little milk secretion in the mammary acini.

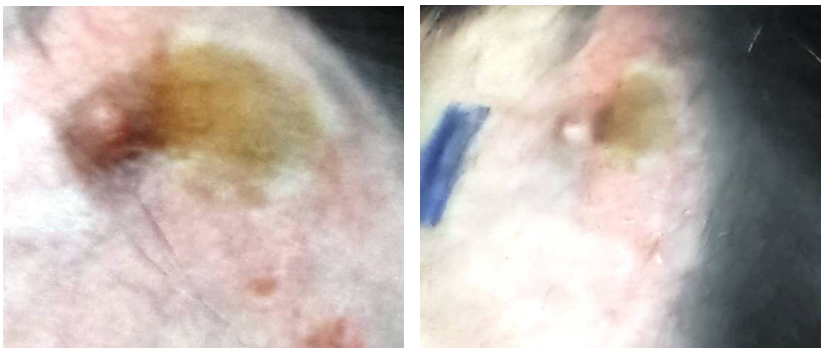

Fig.4. Gp.2, Mastitic cases after infection with *M. bovis.*

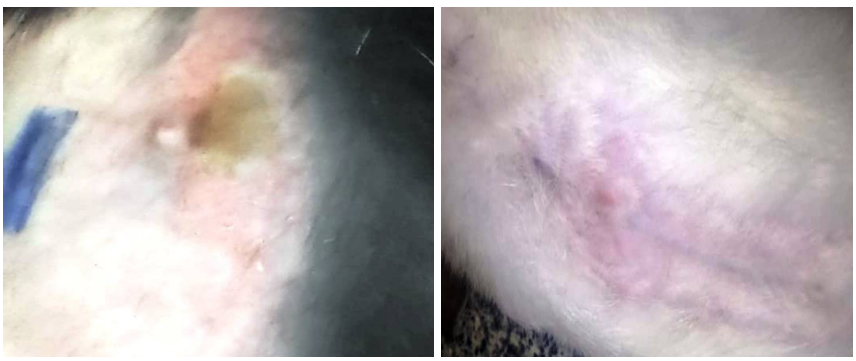

Fig.5. Gp.4, 3 days after treatment with lincospectin + zinc oxide (ZnO) nanoparticles.

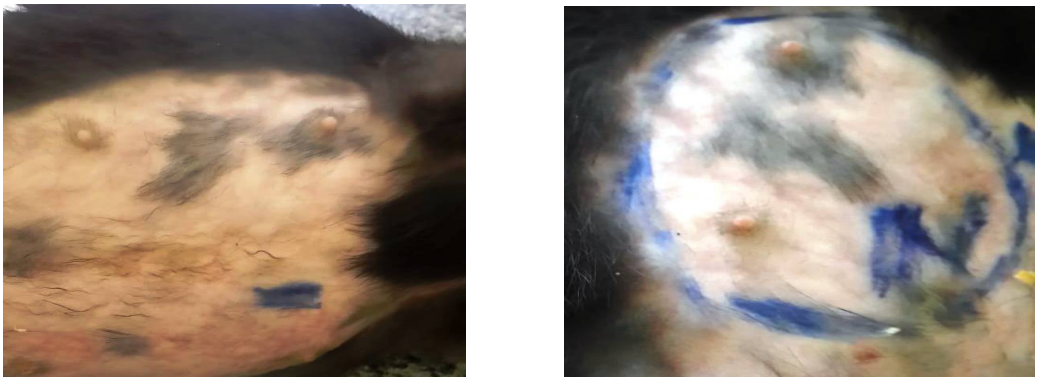

Fig.6. Gp.4, 5 days after treatment with lincospectin + zinc oxide (ZnO) nanoparticles.

against common infectious agents without driving antibiotic resistance in organisms [21]. *Mycoplasma* species havebeen documented as an isolated pathogen from rabbit reproductive tract [22]. *M. bovis* causing chronic mastitis in dairy has becomeincreasingly difficult to treat due to the inability ofcurrent antibiotics to effectively clear infections [23].

Therefore, this study was performed to elucidate the histopathological lesions of *M. bovis* in rabbits in addition to assessing the efficiency of antibiotic nanoparticle. Recent studies have shown that combining nanoparticles with antibiotics not only reduces the toxicity of both agents towards bacterial cells by decreasing the requirement for high dosages but also enhances their bactericidal properties [24]. Combining antibiotics with nanoparticles also restores their ability to destroy bacteria that have acquired resistance to them. The zinc oxide nanoparticles (ZnO-NPs) werebroad spectrum bactericidalhave a wide range of antimicrobial activity against various microorganisms, with low toxicity to human cells [25]. It has ability to improve the immune system andprevent biofilm formation. Furthermore, treatment using zinc was approved by the FDA and nowadays Zn is available as a food additive [26].

The Gp.2 infected with *M. bovis* demonstrated an interstitial mastitis (Fig. 3a& Fig. 4a&b) which resembles the lesions caused by *M. canadense*[15]. It was reported that *M. canadense* causes subacute to chronic histopathological lesions with lymphocytic and macrophagic cells infiltration and moderate fibroblastic proliferation [15].

In Gp. 3 the use of lincospectin was not effective against the bacteria and mastitis was not alleviated (Fig.3b). Generally mycoplasmas are susceptible to antibiotics that affect protein (tetracycline, macrolides, Lincosamides, phenicols) or nucleic acid synthesis (fluoroquinolones) [27]. However antibiotic resistance sometimes develops against these antibiotics by the organism causing a decrease in the effectiveness of certain antimicrobial agents [28].

In Gp.4, infected with M. bovis and treated with antibiotic combinations of zinc oxide nanoparticles, the histopathological lesions were alleviated in which no inflammatory cells were recorded however the fibroblastic proliferation was still present (Fig.3c). (Fig. 5 & 6 a &b) demonstrated enhancing influence of ZnO nanoparticles on the activity of lincospectin against *M. bovis*. ZnO-NPs also were causing destructive oxidative stress to bacterial cells and disrupt the metabolic activity of bacterial cells and therefore inhibit their growth [29].

Therefore it can be concluded that the lincospectin – ZnO nanoparticle was effective against *M. bovis*. Furthermore, nanoparticles tagged with antibiotics have been shown to increase the concentration of antibiotics at the site of bacterium–antibiotic interaction, and to facilitate binding of antibiotics to bacteria.

ZnO-NPs could be formulated in a suitable treatment of mastitis caused by *M. bovi*s in dairy cattle. However, case-controlled and field efficacy studies are needed to evaluate its efficacy and safety.

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التغيرات الهستوباثولوجية في أناث الارانب المصابة بالميكوبلازما بوفس بعد علاجها بجزيئات النانو من أوكسيد الزنك كوسيلة جديدة للعلاج.

أحمد فتحى[١] ، عبد السلام المحمدى [٢] ، منى محمد صبحى[٣] ، هالة فاروق المنياوى[٤] و مروة خطاب[٤]
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يتسبب ميكروب الميكوبلازما اوفس فى ألتهاب الضرع شديد الضراوة ويؤدي إلى انخفاض حاد في إنتاج الحليب في الابقار المصابة. ويسبب خسائر اقتصادية فادحة في جميع أنحاء العالم بسبب فقد إنتاج الألبان وتكاليف العلاج والإعدام المبكر للحيوانات المصابة بهذا المرض المزمن.

وتكنولوجيا النانولديها القدرة على أحداث التغييرات في المستقبل القريب بالنظر إلى الوضع الحالي للبحث والتطوير ويمكن أن تكون الأدوية واللقاحات التي تعتمد على تقنية النانو أكثر فاعلية في علاج الأمراض والقضاء عليها أكثر من التقنيات الحالية ، وبالتالي تقليل التكلفة.

وفى التجربة تم استخدام عشرين أرنبة متعددة الاجنة بعد الوضع مباشرة.

تم تقسيم أناث الارانب إلى أربع مجموعات لكل مجموعة مكونة من خمس أناث .

١. مجموعة الاولى . الضابطة السالبة.

٢. المجموعة الثانية. تم حقنها بميكروب الميكوبلازما بوفس ١٠^٦ بالحقن داخل البطن

٣. المجموعة الثالثة. تم حقنها بميكروب الميكوبلازما بوفس ١٠^٦ بالحقن داخل البطن لمدة ٤ أيام ثم علاجها بمضاد اللينكوسبكتين لمدة ٥ أيام

المجموعة الرابعة . تم حقنها بميكروب الميكوبلازما بوفس ١٠^٦ بالحقن داخل البطن لمدة ٤ أيام ثم علاجها بمضاد اللينكوسبكتين + جزيئات النانو من أكسيد الزنك بتركيز ٨ ميكرون/مللى لمدة ٥ أيام

فى نهاية التجربة تم قتل الارانب وتجميع عينات من الانسجة.

كانت نتيجة الهستوباثولوجى .

فى المجموعة الثانية وجد العديد من التغيرات الباثولوجية فى مختلف الانسجة مع حدوث ألتهاب للضرع

المجموعة الثالثة وجد أوديما و خلايا وحيدة النواة وكذلك خلايا ليمفاوية فى خلايا الضرع والحويصلات خالية من افرازات اللبن.

المجموعة الرابعة كان هناك تكاثر خفيف للأورام الليفية في النسيج الخلوى وإفراز قليل من اللبن في الضرع.

خلصنا إلى أن جسيمات النانو (أوكسيد الزنك) كانت فعالة ضد الميكوبلازما بوفس. علاوة على ذلك ، فقد ثبت أن الجسيمات النانوية المخلوطة بالمضادات الحيوية تزيد من تركيز المضادات الحيوية في مكان التفاعل بين البكتيريا والمضادات الحيوية ، وتسهل في علاج مناسب لالتهاب الضرع الناجم عن الميكوبلازما بوفس فى ابقار الالبان المصابة.