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EXPERIMENTAL PATHOGENICITY OF *MYCOPLASMA BOVIGENITALIUM* ISOLATED FROM BULLS IN LABORATORY RATS AND TREATMENT WITH ANTIBIOTIC NANOPARTICLES

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ABSTRACT

Mycoplasmas are resistance many types of antibiotics, it is very difficult to fight infection resulting in high morbidity. Nanoparticles are a viable alternative to antibiotics and appear to have high potential to solve the problem of bacterial drug resistance. The current study evaluated antimicrobial activity of tilmicosin with carbon nanoparticles on *Mycoplasma bovigenitalium* in vitro and in vivo laboratory rats. Twenty male albino Wistar rats with average body weight 100 g were used, divided into four groups (five rats per group). Group 1 was control negative. Group 2 was infected by intraperitoneal injection of *M.bovigenitalium* at a dose of 10^5 CFU/mL. Group 3 was infected by *M. bovigenitalium* (10^5 CFU/mL) and treated with tilmicosin only (0.5 mg/body weight). Group 4 infected by *M. bovigenitalium* and treated with tilmicosin + Carbon nanoparticle (0.35µg/ml). Tissue samples of testis were collected and fixed in 10 % neutral formalin buffer for histopathology. In Group 1 the testis exhibited normal histological structure. In Group 2 the testis demonstrated massive neutrophilic infiltration in the seminepherous tubules and interstitial tissue and complete necrosis of other tubules. Furthermore, there were areas in seminepherous tubules showed germ cell degeneration and multinucleated giant cell formation. Germ cell necrosis, multinucleated giant cell formation in seminepherous tubules and thickening of interstitial tissue with edema and leukocytes infiltration were also recorded. Bacterial aggregation was observed in the interstitial tissue. In Group 3 there were edema, multinucleated giant cell formation in seminepherous tubules and thickening of interstitial tissue with edema and leukocytes infiltration. Group 4, the seminiferous tubules were lined by spermatogenic cells to sperm formation. Advanced research must be done on antimicrobial nanoparticles will help in control of *Mycoplasma* infection in bovine.

Keywords: *Mycoplasma bovigenitalium, antibiotic, Carbon nanoparticles.*

INTRODUCTION

Mycoplasma microorganisms have been incriminated in various diseases of animals and humans in recent past and it gained importance owing to the inability to diagnose and difficulty to treat (Yatoo *et al*., 2018). *Mycoplasma* is a small prokaryotes lacking cell wall which results in a disease known as mycoplasmosis (Kumar *et al*., 2011). 32 species of Mycoplasma including *M. bovigenitalium* were reported to be of veterinary importance (Auliffe *et al*., 2003) whereas there are around 7 species of *Mycoplasma* reported to cause disease in humans (Embree and Embil 1980).

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Mycoplasma has been associated with reproductive disorders as vulvovaginitis, infertility, endometritis and dystocia (Ghanem *et al.* 2013). The highly contagious nature of *Mycoplasma* spp., their poor responsiveness to treatment and culling for affected stock (Hermeyer *et al*., 2012). *Mycoplasma bovigenitalium* is common in semen, prepuce and vagina of cattle (Parsonson *et al.*, 1974). *M. bovigenitalium* was found to be incriminated in reproductive disorders in cattle but there was no clinical or diagnostic evidence (Nicholas and others 2008).

Mycoplasma bovigenitalium has been isolated from infertile cattle, and seroreactions to *M. bovigenitalium* antigen was more common among infertile cattle (Catania *et al*., 2014). *M. bovigenitalium* has been isolated from cattle associated with reduced fertility, endometritis and granular vulvovaginitis, also from semen samples and from the respiratory tract (Nicholas and others 2008). A previous study

reported that, 9.29% of cows were positive for *M. bovigenitalium* (Macêdo *et al*., 2018). However, the pathogenicity of the most commonly isolated *Mycoplasma bovigenitalium*, from bulls is still vague.

Treatment of *Mycoplasma* is difficult due to lack a cell wall and resistant to some commonly used antibiotics (Marouf *et al.*, 2011). Tilmicosin which is a semisynthetic 16-member macrolide antibiotic is widely used in veterinary medicine (Zhang *et al.*, 2016). Tilmicosin has an antimicrobial activity by inhibiting the protein synthesis of susceptible bacteria through binding with the 50S subunits in the ribosome to block transpeptidation and/or mRNA displacement (Kang *et al*., 2015). Tilmicosin has a broad efficacy spectrum, particularly against *Mycoplasma* (Ziv *et al*., 2010). *M. bovigenitalium* were found to be sensitive to pirlimycin, danofloxacin, enrofloxacin, oxytetracycline, tilmicosin and tylosin, but not to kanamycin (Kawai *et al*., 2014). Therefore the rapid and accurate diagnosis is very important for control and prevention of disease outbreaks (Parker *et al.*, 2018).

Prevalence of drug-resistant bacteria decreases effectiveness of treatments. New improvements in this problem based on metallic nanoparticles represent an effective solution for overcoming bacterial resistance (Allahverdiyev *et al*., 2011). The antibacterial mechanisms of nanoparticles (NPs) are oxidative induction, metal ion release, and non-oxidative (Wu *et al*., 2016). The multiple simultaneous mechanisms of action against microbes would require multiple simultaneous gene mutations in the bacterial cell for antibacterial resistance to develop; therefore, it is difficult for bacterial cells to become resistant to NPs (Wang *et al.*, 2017).

Carbon-based nanoparticles used in biomedical applications in drug and gene delivery. The application as drug delivery is very common in carbon nanoparticles, especially the grapheme nanoparticles. The structure of six-atom rings can be considered as a planar aromatic macromolecule loading capability to a variety of drugs (Yun and Huang, 2016).

Mycoplasma microorganisms are of major concern nowadays due to the emerging antibiotic resistance of *Mycoplasma* which would result in outbreaks and help in spreading the infection (Yatoo *et al*., 2019). Therefore, the aim of this study was to evaluate the antimicrobial activity of tilmicosin and tilmicosin nanoparticles on *Mycoplasma bovigenitalium* in vivo using laboratory rats.

MATERIALS AND METHODS

Animals:
Twenty male albino Wistar rats with average body weight 100 g were used. The animals were housed in plastic cages at room temperature 25- 27C^{O}, and relative humidity 50– 60%. Rats had free access to water and maintenance ration. This study was performed in accordance with the Institutional Animal Use and Care Committee (IACUC) guidelines, Cairo University.

Bacterial strain:
Mycoplasma bovigenitalium was obtained from Animal Reproduction Research Institute, Agriculture Research Center in Giza Egypt.

Antibiotic nanoparticle preparation:
Antibiotic tilmicosin with carbon nanoparticles were kindly obtained from Dr. Abdel Salam Almuhammady Arab Center for Nanotechnology, Cairo University. Scanning electron microscopy (Hitachi S2150, Krefeld, Germany) was used to image the size and morphology of the Carbon nanoparticles.

Experimental design:
Twenty rats were divided into four groups (five rats per group).

Group 1 was control negative.

Group 2 was infected by intraperitoneal injection of *M.bovigenitalium* at a dose of 10^5 CFU/mL.

Group 3 was infected by *M. bovigenitalium* (10^5 CFU/mL) and treated with tilmicosin only (0.5 mg/body weight).

Group 4 infected by *M. bovigenitalium* and treated with tilmicosin + Carbon nanoparticle (**0.35µg/ml**).

Histopathology:
Tissue samples of testis were collected and fixed in 10 % neutral formalin buffer. After fixation, tissues were processed by paraffin embedding technique and sectioned by microtome (Leica 2135, Germany) at 3 µm thick sections. Tissue sections were then stained by hematoxylin and eosin stain and examined by light microscope (Olympus XC30, Tokyo, Japan). Lesions were photographed by digital Camera (Olympus XC30, Tokyo, Japan).

RESULTS

Macroscopic findings:

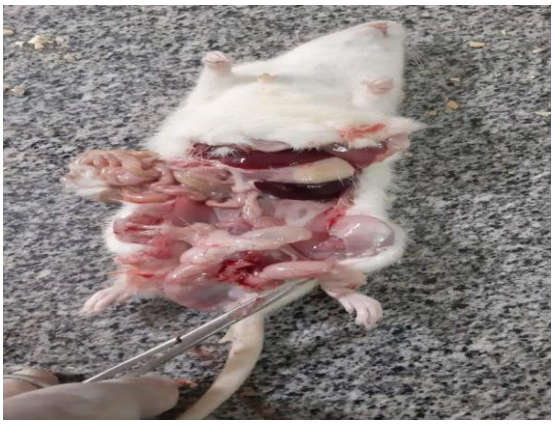

Photo 1: Group 1 as control negative with normal testis.

Photo 2: Group 2 infected I/P with *M.bovigenitalium* showed congested left testis and enlargement of right testis.

Photo 3: Group 3 infected by *M. bovigenitalium* and treated with tilmicosin only showed mild congestion in the left testis.

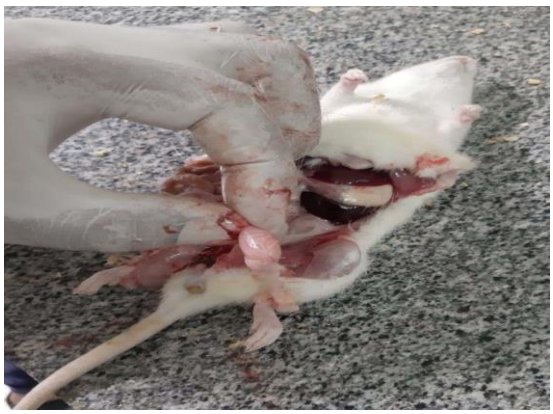

Photo 4: Group 4 infected by *M. bovigenitalium*, treated with tilmicosin and Carbon nanoparticle.

Histopathological findings:

In the **Group 1 (**control group**)**, the testis exhibited normal histological structure in which the seminiferous tubules are lined by spermatogenic cells up to sperm formation (Fig. 1).

In **Group 2** (infected group), the testis demonstrated massive neutrophilic infiltration in the seminepherous tubules and interstitial tissue and complete necrosis of other tubules (Fig. 2). Furthermore, there were areas in which the seminepherous tubules showed germ cell degeneration and multinucleated giant cell formation (Fig. 3). Germ cell necrosis, multinucleated giant cell formation in seminepherous tubules and thickening of interstitial tissue with edema and leukocytes infiltration were also recorded. Bacterial aggregation was observed in the interstitial tissue (Fig. 4).

In Group 3 inoculated with *M. bovigenitalium* and treated with tilmicosin only, there were edema, multinucleated giant cell formation in seminepherous tubules and thickening of interstitial tissue with edema and leukocytes infiltration were demonstrated (Fig. 5).

Group 4 infected by *M. bovigenitalium* and treated with tilmicosin + Carbon
Nanoparticles the seminepherous tubules are lined by spermatogenic cells to sperm formation (Fig. 6).

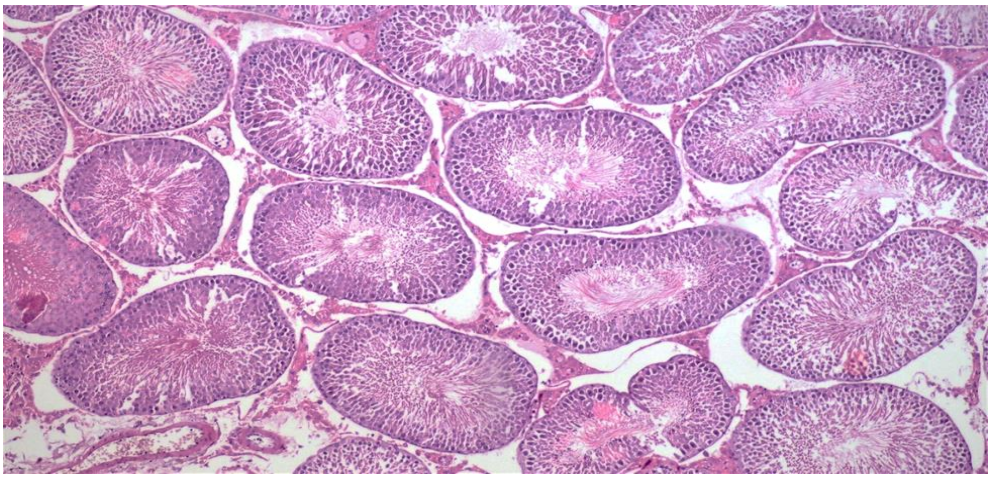

Fig. 1: Testis of rat in **Group 1** (control group) showing normal histological structure in which the seminepherous tubules lined by spermatogenic cells up to sperm formation (H and E stain X 100).

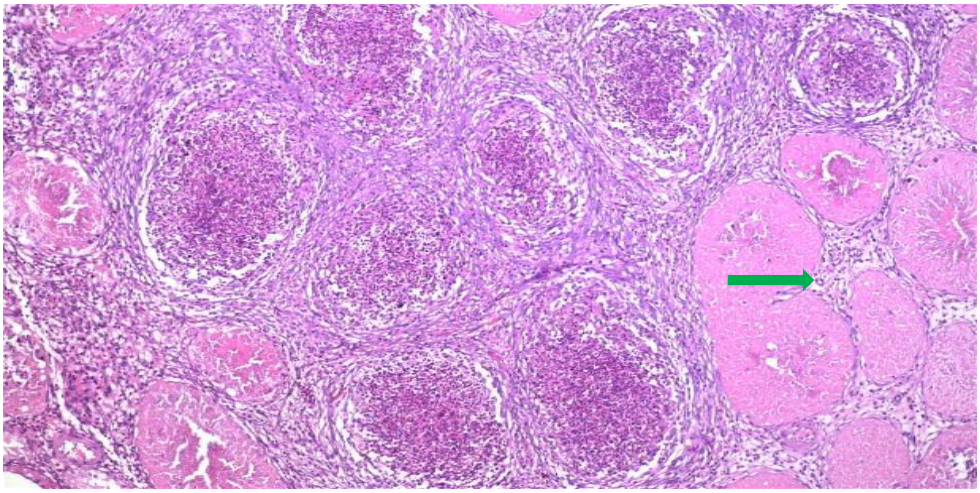

Figure 2: Testis of rat in **Group 2** (infected group) showing massive neutrophilic infiltration in the seminepherous tubules and interstitial tissue (arrow head) and complete necrosis of other tubules (arrow) (H and E stain X 100).

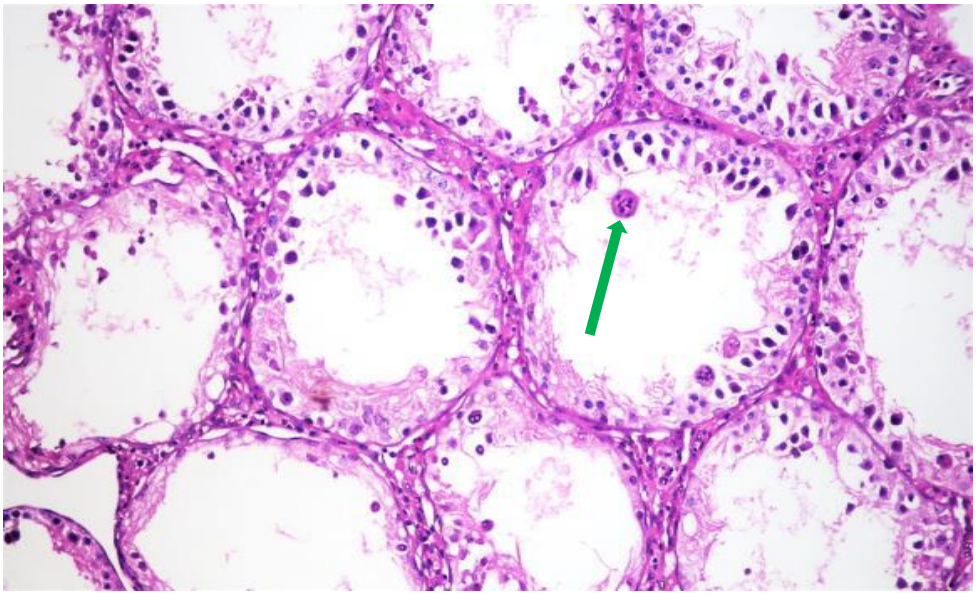

Figure 3: Testis of rat in **Group 2** showing germ cell degeneration and multinucleated giant cell formation (arrow) in seminepherous tubules (H and E stain X 250).

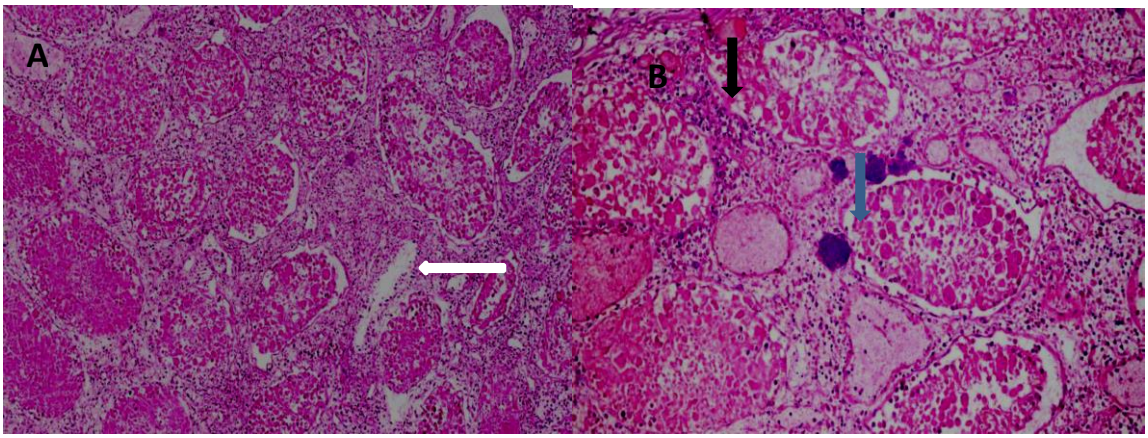

Figure 4 (A & B): Testis of rat in **Group 2** showing germ cell necrosis, multinucleated giant cell formation (black arrow) in seminepherous tubules and bacterial aggregation in the interstitial tissue (white arrow) (H and E stain X 100).

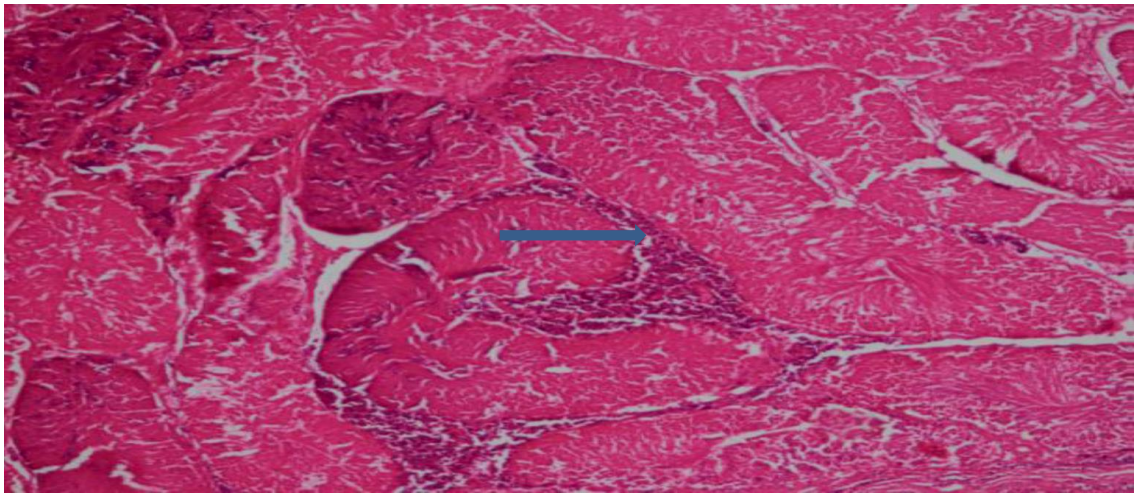

Figure (5): Group 3 testis of rat showing few leukocytic cells in the seminepherous tubules (H and E stain X 100).

Figure (6): Group 4 testis of rat showing normal histological structure (H and E stain X 250).

DISCUSSION

Mycoplasmas can cause serious disease in cattle herds resulting in significant negative economic and welfare impacts (Parker *et al.*, 2018). *Mycoplasma bovigenitalium* has been associated with infertility, abortion, endometritis, seminal vesiculitis, and impaired spermatozoa motility in cattle (Ruhnke, 1994). It is common in semen, prepuce and vagina of cattle (Parsonson *et al*., 1974). Blom and Ernø (1967) isolated *M. bovigenitalium*, from a case of bovine seminal vesiculitis. *Mycoplasmas* are difficult to isolate from tissues highly contaminated with other bacteria (Thiede *et al*., 2002). Mycoplasmas have ability to modulate host immune responsiveness enabling them to suppress or evade host defense mechanisms and establish chronic, persistent infection (Razin *et al*., 1998).

NPs can combat bacterial and microbial resistance also can act as a "medium and carrier" of antibiotics. NP carriers can help to target antibiotics to an infection site, minimize side effects and blood drug level maintained in large range that can exceed the maximal tolerated dose (Wu *et al*., 2017). Carbon-based nanoparticles graphene, has a large surface area and available π electrons, which make a smart nanomaterial for a wide range of biomedical applications, including drug delivery, biomolecules sensing, cancer therapy and so on (Chen *et al.*, 2018).

In Group 2 (infected group), the testis demonstrated massive neutrophilic infiltration in the seminepherous tubules and interstitial tissue and complete necrosis of other tubules (Fig. 2 & Photo, 2). Furthermore, there were areas in which the seminepherous tubules showed germ cell degeneration and multinucleated giant cell formation (Fig. 3). Germ cell necrosis, multinucleated giant cell formation in seminepherous tubules and thickening of interstitial tissue with edema and leukocytes infiltration were also recorded. Bacterial aggregation was observed in the interstitial tissue (Fig. 4).

In Group 3 inoculated with *M. bovigenitalium* and treated with tilmicosin only, there were edema, multinucleated giant cell formation in seminepherous tubules and thickening of interstitial tissue with edema and leukocytes infiltration were demonstrated (Fig. 5 and photo, 3).

Group 4 infected by *M. bovigenitalium* and treated with tilmicosin + Carbon Nanoparticles the seminiferous tubules are lined by spermatogenic cells to sperm formation (Fig. 6 and photo, 4). Carbon, CNTs have attracted various drug molecules into the living cells because their natural morphology facilitates non-invasive penetration across the biological membranes (Liu *et al*., 2013). Non-covalent interaction facilitates the controlled release of the drug in the acidic condition of lesion sites (Panczyk *et al*., 2016).

Advanced research must be done on antimicrobial nanoparticles will help in control of *Mycoplasma* infection in bovine.

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العدوى التجريبية للجرزان باستخدام الميكوبلازما بوفيجنتاليم المعزولة من الطلائق وعلاجها باستخدام مضاد حيوى بجزيئات النانو

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الميكوبلازما مقاومة لأنواع كثيرة من المضادات الحيوية ، ومن الصعب مكافحة العدوى والتي تؤدي إلى ارتفاع معدلات الإصابة بالميكوبلازما. وتعد الجسيمات النانوية بديلاً قابلاً للتطبيق للمضادات الحيوية لان لديها قدرة عالية على حل مشكلة مقاومة البكتيريا للمضادات الحيوية. وهذه الدراسة لتقييم دورالتيلميكوسين كمضاد حيوى للميكوبلازما بوفيجنتاليم مع الجسيمات النانوية الكربونية لاحداث العدوى التجريبية٠ للجرزان الحية بالمعمل ودراسة التأثير الداخلى والخارجى عليها. تم استخدام عدد عشرون ذكراً من فصيلة الوستار البيضاء متوسط وزن الجسم ١٠٠ جرام ، مقسمة إلى أربع مجموعات (خمسة فئران لكل مجموعة). المجموعة الاولى: المجموعة الضابطة السالبة. المجموعة الثانية تم أصابتها بعدوى الميكوبلازما بوفيجنتاليم عن طريق الحقن داخل الغشاء البريتونى بجرعة ١٠^٩ / مل من الميكروب. المجموعة الثالثة تم أصابتها بعدوى الميكوبلازما بوفيجنتاليم أيضاعن طريق الحقن داخل الغشاء البريتونى بجرعة ١٠^٩ / مل وعولجت التيلميكوسين فقط (٠,٥ مجم / وزن الجسم). المجموعة الرابعة تم أصابتها بعدوى الميكوبلازما بوفيجنتاليم أيضاعن طريق الحقن داخل الغشاء البريتونى بجرعة ١٠^٩ / مل وعولجت بالتيلميكوسين + جسيمات الكربون النانوية (٠,٣٥ ميكروجرام / مل). فى نهاية التجربة تم قتل الجرزان وجمع عينات من أنسجة الخصية ووضعها في ١٠ ٪ محلول الفورمالين. بعد التثبيت ، تمت تقطيع الأنسجة عند سمك ٣ ميكرون ثم صبغها بصبغة الهيماتوكسيلين والايوسين وفحصها تحت الميكروسكوب الضوئي. أظهرت نتائج المجموعة الاولى أن الخصية طبيعية الانسجة. في المجموعة الثانية ، أظهرت الخصية تجمع عدد ضخمً من الخلايا المناعية في والأنسجة وتنقرز تام فى الأنابيب. علاوة على ذلك وجود مناطق في الأنابيب شبه المصلية أظهرت وجود الخلايا الجرثومية محاطة بخلايا مناعية عملاقة متعددة النوى. مع وجود تضخم فنسيج الخصية وانتشاركريات دم بيضاء. وقد لوحظ التجميع البكتيري في النسيج الخلوي. في المجموعة الثالثة كان هناك أوديما وأظهرت الخلايا المناعية العملاقة متعددة النوى في الأنابيب شبه المميتة مع تضخم فى جدار الانابيب للخصية وتجمع لكريات الدم البيضاء. المجموعة الرابعة وجد بهاالعديد من الخلايا المنوية مصطفة فى انابيب الخصية لتشكيل الحيوانات المنوية. يوصى باستخدام جسيمات النانو المضادة للميكروبات فى الابحاث المتقدمة للسيطرة على عدوى الميكوبلازما في الماشية.