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Contents

Evaluation of antifungal activity of nanobiosilver particles to treat Candida albicans releated urogenital infections in female rat model	1-5
Bugrahan Bekir Yagci, İbrahim Mert Polat, İlknur Pir Yagci, Elif Bulut, Mustafa Turk	
Black Seed (Nigella Sativa) and Immunomodulatory Effect	6-9
Serife Tutuncu	
Investigation of The Effects of Some Intrafollicular Growth Factors (GDF-9, GATA-4, GATA-6, IGF-I, IGF-II) on Etiopathogenesis of	10-15
Cystic Follicular Ovarian Degenerations in Cows	
İbrahim Mert POLAT, Mehmet Rıfat Vural	
Investigation of Systemic Toxic Effects of Nanobiosilver Use in Rodent Models	16-20
Yasemin Ozdemir, Husamettin Ekici, Bugrahan Bekir Yagci	

The Effects of Lactation and Body Condition Score Changes on Embryonic Death Rates in KWPN Mares

Serhan Durmaz, İbrahim Mert Polat, Ömer Korkmaz, İlknur Pir Yagci, Taha Burak Elifoglu, Eser Akal, Muzaffer Celebi



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Content

1)	Articles Evaluation of antifungal activity of nanobiosilver particles to treat Candida albicans releated	Page
	urogenital infections in female rat model Bugrahan Bekir Yagci, İbrahim Mert Polat, İlknur Pir Yagci, Elif Bulut, Mustafa Turk	1
2)	Black Seed (Nigella Sativa) and Immunomodulatory Effect Serife Tutuncu	6
3)	Investigation of The Effects of Some Intrafollicular Growth Factors (GDF-9, GATA-4, GATA-6, IGF-I, IGF-II) on Etiopathogenesis of Cystic Follicular Ovarian Degenerations in Cows İbrahim Mert POLAT, Mehmet Rıfat Vural.	10
4)	Investigation of Systemic Toxic Effects of Nanobiosilver Use in Rodent Models Yasemin Ozdemir, Husamettin Ekici, Bugrahan Bekir Yagci	16
5)	The Effects of Lactation and Body Condition Score Changes on Embryonic Death Rates in KWPN Mares	
	Serhan Durmaz, İbrahim Mert Polat, Ömer Korkmaz, İlknur Pir Yagci, Taha Burak Elifoglu, Eser Akal, Muzaffer Celebi	21

Evaluation of antifungal activity of nanobiosilver particles to treat Candida albicans releated urogenital infections in female rat model

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Abstract

Candida species are the most common encountered agent of fungal infections. Catheter related infections affect over one million patients in Europe and US annually. Candida species infections are responsible for over 63 % of fungal infections in veterinary practice. The aim of this study was to investigate the therapeutic efficacy of nano biosilver particles in urinary catheter related cystitis and vulvo vaginitis of *Candida albicans* (*C. albicans*), which is common for human and veterinary medicine is very hard to treat. Thirty healthy adult female Wistar rats were used in the study. The rats were divided into three groups; experimental group with *C. albicans* inoculation and treatment (n = 10) and positive control group *C. albicans* inoculations without treatment (n=10) and negative control group with urinary catheter placed without any pathogen inoculation (n=10). Nanobiosilver was administrated after the establishment of infection. Vulvovaginal and urinary bladder tissues collected at necropsy were process for Gomori staining histopathology and electron microscopy. Rats received inoculation exhibited significant outcomes associated with fungal infections compare to negative control rats. Results of control group were between physiological limits. *C. albicans* was detected in urine samples at 72nd hours after inoculation in experimental group but not observed at 7th day in animals treated with nanobiosilver. In conclusion, due to its safety, efficacy and lack of systemic effects, nanobiosilver could be an excellent alternative for the initial treatment of catheter related candidiasis in veterinary medicine.

Keywords: Nanobiosilver, Candida albicans, catheter related infection, treatment.

INTRODUCTION

Candida species are the most common among fungal infections (Achkar and Fries, 2010). Candida albicans is the most common isolated infectious agent among Candida species regarding diseases (Wang and Fries, 2011). Dimorphic structured agent can colonize in gastrointestinal system and reproductive path (Sobel 2006). C. albicans can be determined as mucocutaneus form which is not life threatening to life-threatening invasive forms with respect to patient's immunologic state or comorbid diseases (Achkar and Fries, 2010). Mucocutaneus Candidiasis has two groups; urogenital and non-urogenital forms. Nonurogenital Candidiasis usually effects oropharyngeal region in immunosuppressive patients where urogenital Candidiasis is responsible for vulvovaginitis in women and balanitis and balanoposthitis in men as well as in dogs and cats (Sobel et al., 1998). In veterinary practice, Candidiasis causes urinary tract infections, peritonitis, cutaneous and mucocutaneous infections, gastrointestinal overgrowth, ulcerative glossitis, keratitis (Seyedmousavi et al., 2018). Urinary tract infections are widely seen in hospitalized patients. Catheter-related urinary tract infections are seen over one million patients in Europe and US. Candida albicans is one of the most frequent among these infections (Tambyah et al. 2002). Catheters are used in woman of inpatients to provide urine flow and output (Saint et al., 2000). Catheters are suitable for microorganism and biofilm formation. penetrations Numerous microorganisms cause urinary tract infections via catheters. Candida eradication will be very hard if it manages to biofilm formation (Mah et al., 2003).

Candida identification in urine may cause different clinical manifestations (Kauffman et al., 2011). Firstly, agent penetrates to urinary tract via catheter and forms biofilm. Most patients are asymptomatic at this stage. *C.albicans* is excreted with urine in these patients which is called candiduria. Afterwards, it may cause a bladder infection, reach the kidneys with ascendant path and cause pyelonephritis. At this stage clinical symptoms are visible and needs treatment. The worst manifestations is the agent's distributing to whole body using hematogenous paths which is life-threatening. As an alternative, vaginal *Candidiasis* can be seen due to candiduria (Nobile et al., 2008).

Candida albicans was isolated in 89% Australian and US, % 88 Austrian, 84% Italian and 44% Turkish vulvovaginal Candidiasis cases (Achkar and Fries, 2010). Most common clinical symptoms are pruritis in vulva and dysuria. Vulvo-vaginal erythema, edema, fissures and dens vaginal discharge are common findings in physical examination (Eckert et al., 1998). Similarly, balanitis cases caused by candidiasis are not specific. Generally local pruritis and dermatitis are seen. Fungal infections of urinary system usually do not cause systemic clinical symptoms. Fever, dysuria and leukocyturia and similar symptoms can be seen but not in all patients with candiduria. Candiduria is usually seen in long time hospitalized patients with catheters (Lisboa et al., 2009).

There are many research about biofilm formation and medical equipment related infections like urine catheters, vascular stents, cerebrovascular shunts, joint implants. Catheter-related urinary tract infections are

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the most common among them and seen as 70% (Kojic and Darouiche, 2004; Dudeck et al., 2011; Siddiq and Darouiche, 2012). Candida biofilm formations are investigated repeatedly in vitro biofilm infection models in medical equipment. The biggest issue here is not achieving a response with antifungal treatment after biofilm formation (Kojic and Darouiche, 2004; Siddiq and Darouiche, 2012).

Using rat models have advantages for usage and give useful information to determine pathogenesis of urinary tract diseases caused by candida species, pharmacokinetics of antifungal drugs and immune response in both human and domestic animals (Dudeck et al., 2011). Besides, plenty of candida models affect kidneys via hematogenous paths due to intravenous candida inoculation. Meaning that this happens with ascendant way (Naglik et al., 2008). Whereas catheter-related candida infections happens with descendant way. Descendent developing urogenital candidiasis is rarely spreadable hematogenous (Wang and Fries, 2011).

There are many treatment options regarding to localization of the infection, form and severity. Most of the antifungals have short and long term side effects. Biosilver particles under 2-100 nm size act as bactericide and fungicide due to increasing area with bacteria and fungus which is much larger than itself. This product can gain antimicrobial feature with the conductivity specialty of silver. Once biosilver particles are over any substance surface, very strong antibacterial effect is provided. Silver ions do not harm any cells because of cell the membrane in body. Consequently, biosilver is considered to be harmless for human, animals, plants, in other words multi cell organisms. Biosilver structure do not change during the process as it does not go into any chemical reaction and works continuously, no need to add more silver into the system. It was reported that it has killed 650 known pathogens in a very short time whether they were mutational or not. Silver (Ag) compounds have a wide spectrum of antimicrobial activity against bacteria, virus and fungus which is called "oligo dynamic activity" (Chen et al., 2006). It was reported that antifungal activity of nano particles against yeast and fungostatic reaction were due to membrane structure effect with a mechanism of electrostatic interaction of oxide particles on fungus cell (Raju and Rajappa, 2011).

In this study, it was aimed to investigate the effects of biosilver compound which does not have a systemic effect in hard to treat urine catheter-related cystitis and vulvovaginitis infections of *C. albicans* that is widely seen in human (especially women) and domestic animals by using rat model.

MATERIALS AND METHODS

Animals

Modeling of urogenital *C. albicans* infection and sample collection was performed at Kırıkkale University Hüseyin Aytemiz Experimental Research and Application Center, Turkey. Thirty Wistar adult female rats with weighing of 300–350 gr. were used in the study. During the study, rats were separated in cages, fed with commercial pellet food and given *ad libitum* water. Animals showing any disease symptoms (nasal discharge, mucosal paleness, hyperemia, apathy etc.) were excluded from the study. The rats were divided into three groups; experimental group with *C.albicans* inoculation and treatment (n=10) and positive control group *C.albicans* inoculations without treatment (n=10) and negative control group with solely urinary catheter placed without any pathogen inoculation (n=10).

Preparation of Urinary Catheter

Single dose of 250 mg/kg cortisone acetate was applied subcutaneously to the rats at the day of urinary catheter placement. Gentamicin 80 mg/kg was also applied subcutaneously twice a day to prevent systemic bacterial infections. In addition, 0.9 mg/ml Penicilin G was added to drinking water. Animals were examined in terms of stress every six hours during the time urinary catheter was placed. Catheter region was controlled in terms of inflammatory and purulent changes.

Insertion of urinary catheter

General anesthesia was given with Xylazine (5 mg/kg) and Ketamine (45 mg/kg) before the administration. Necessary asepsis and antisepsis were performed with surgical scrub from midline to tail. A silicone catheter (Instech Solomon, 3.5 Fr, female luer, round tip, gas sterilized) was placed in urethra and stabilized with 4/0 surgical prolene suture. After urinary catheterization, Elizabeth collar was put on to the rate

Inoculation of Candida albicans

After the insertion of urinary catheter, *Candida albicans* broth consisting 10⁷/mL cells were given via catheter inside the bladder. Furthermore, same doses of inoculum were administered cranial vagina. After inoculation, rats were tracked for monitored for sign of distress for two hours and then placed back to their cages.

Biosilver synthesis

100 mg silver nitrate (Merk) was dissolved in pure water and diluted to be 100 ppm. 10 ppm pure water solution of ascorbic acid was prepared. 6 g/L of root and body *Cotinus coggygria* plant was boiled for 15 min. Liquid part was vaporized in rotary evaporator and extract was obtained. Afterwards, 100 ml of 100 ppm silver solution, 10 mg cotinus plant extract, 100 ml ascorbic acid 0,1 M solution, 13,4 mg NaHPO4, 180 ml 0,15 molar NaCl solution were mixed in a beaker. The pH of solution was set to 7,8 using NaOH. It was mixed in magnetic mixer in 38.6 °C for 28 hours using magnetic fish. One ml (3,5 μgr/mL) of biosilver was administrated to bladder via catheter.

Fungal Culture and Urine Analysis

After C. albicans inoculation, urine samples were collected via urinary catheter at 72nd hour and 7th day after all study groups and analyzed with using commercial urine dipsticks (Mission® Urinalysis Reagent Strips, ACON Laboratories, Inc. San Diego, USA). Microbiologic analyses were performed in euthanized rats vagina, urinary tissues and urine catheters to obtain C. albicans microorganism load. Urine sample, collected in sterile conditions and sent to lab the same day was inoculated in sabauraud dextrose agar (SDA) and blood agar and incubated for three days in 37 ⁰C. At the end of three days, colonies appeared in SDA and blood agar. Colonies were gram stained. At the same time, a small inoculum from an isolated colony is suspended in 0,5 mL of sheep serum and is incubated at 37 °C for two hours and investigated in terms of germ tube formation as well.

Tissue Collection

Rats were sacrificed in 4th day after inoculation and rats of experimental group in 7th day of nanobiosilver treatment to determine whether infection modeling were formed and examined the histological changes in tissues. For this reason, rats were euthanized under general anesthesia. Urogenital tissues were collected and transported to the

laboratory immediately for histopathological examination.

Electron Microscope Investigation

Catheters were left for drying at room temperature after removal procedure. They were cut longitudinally under dissection microscope by microtome knife in order to scan its internal surface. Afterwards, catheters had been coated with gold by gold coating machine. The images of internal surfaces were taken by scanning electron microscope for displaying the biofilm formation of *C. albicans* (Jeol SEM).

Histopathologic Examinations

Bladder and urethra was examined histopathologically to determine host response for urinary catheter-related *C. albicans* infection. Taken tissues after euthanasia were fixed in 10% formalin solution and paraffin blocks were prepared for examination. They were stained with methenamine silver and histopathologic changes were determined and *C. albicans* was visualized.

Ethics committee approval

Th study was approved by Kırıkkale University Local Ethics Committee for Animal Experiments with approval number 16/21 on 25th February 2016.

RESULTS

After inoculation, rats were tracked for stress for two hours and taken to their cages. None of any critical stress symptoms was observed. Results of urine analyzes of rats are given in Table 1. Urine and vaginal samples of the experimental and positive control group the colonies of C. albicans were appeared as white to cream, shiny, highconvex and S type. Gram blue stain was used on fixed urine and vaginal smears from the colonies. Also, a small inoculum from an isolated colony were suspended in 0.5 mL of sheep serum and were incubated at 37°C for two hours. Small tubes were seen projecting from some of the yeast cells which is characteristic of C. albicans. No growth was detected in experimental and control group samples taken seven days after biosilver administration. Although colony formation was specific to C. albicans and yeast cells were seen in gram stained samples, small amount of inoculum was taken from colonies.

Table 1. Urine analyzes result. I: 72^{nd} hour after *C. albicans* inoculation, II: 7^{th} day after biosilver administration, III: 72^{nd} hour after catheter application, IV: 7^{th} day after catheter application.

Parameter		Group =20)	Control Group (n=10)	
	I	II	Ш	IV
Leucocyte	+/+++	-	1	-/+
Protein	+/++	-	-	-/+
pН	6,0/7,5	6,0/7,0	6,0/7,0	6,0/7,5
Erythrocyte	-/+	-	-	-
Density	1025/1030	1015/1025	1015/1025	1015/1030
Ketone	-	-	-	-
Glucose	-	-	-	-
Bilirubin	-	-	-	-
Urobilinogen	-	-	-	-
Nitrite	-	-	-	-

DISCUSSION

Raju and Rajappa (2011), specified gomori methenamine silver as the most suitable method to determine *C. albicans* in tissues. Gomori methenamin silver, oxidases fungus cell wall and aldehyde groups come off and these aldehyde groups gets into reaction with silver nitrate and the agent is visible (Nassar et al., 2006). In this study, euthanasia was performed under general anesthesia to the animals on 4th day after urogenital tract infection of *C. albicans* and seven days after nanobiosilver treatment. Gomori methenamin silver was used to detect *C. albicans* in urogenital tissues. Especially in the 4th day samples of *C. albicans* formations as dark black brown buds were detected (Figure 1A).

In the study, tissue samples taken 7th day after nanobiosilver administration were gomori methenamine silver stained and no C. albicans like structures were detected in microscopic examination of histopathologic samples and they seem like tissues of control group (Figure 1 B and C). At the end of the study, urethral catheters placed in control group and catheters of study group taken out at seventh day after nanobiosilver administration were examined by electron microscobe. A film layer was detected in internal side of urethral catheters of control group showing microbial growth. There was no such layer formation in the catheters of nanobiosilver administrated study group animals. Extracellular mucopolysaccharide biofilm formation on the surface of long term used urinary catheters are the major cause of candiduria or bacteriuria in human as well as in domestic pets. Tomm-harsfall proteins, magnesium and calcium ions that are in the urine structure get into that material formation. After placing catheter, there is a rapid biofilm formation that makes a large and rough path on catheter's external and internal surface that enables microorganisms to hold and grow easily (Nicolle, 2014; Rudramurthy et al., 2016). In this study, biofilm formations on the internal surfaces of control group catheters were detected by electron microscope examination (Figure 1D). On the other hand, no biofilm formation was detected in biosilver administrated study group catheters' internal surface (Figure 1E).

Catheters provide a substrate for adherence of microorganisms and proliferation of biofilms. When growing as a biofilm, Candida spp. is difficult to eradicate due to inherent drug resistance and immune tolerance. Resistance due to most antifungal and antibacterial agent usage is a major issue today. Improper use of antifungal drugs increases the number of resistant microorganisms (Chen et al., 2006; Shi et al., 2010). Moron et al. (2005) states that this will be threatening in long term as antimicrobials are very few for different species. Nabawy et al. (2014) states that researches concerning antimicrobial metallic particles that are against resistant strains of large surfaced and volume areas are promising. Keuk-Jun et al (2008), made clinical isolations in ATCC cell culture and showed that silver particles are effective against T. mentagrophytes and Candida albicans, they also stated that particles act by effecting mycelium and further investigations should be conducted in their study concerning antifungal effect of biosilver particles. Bubenik et al. (2007) reported that daily increase of urinary tract infections in catheterized dogs were 27%. Duration of the catheterization leads to resulted in more complicated infections and cost. According to results of this study, contribute with previous study (Bubenik et al., 2007), it was shown that biosilver complex could be effective and alternative in C. albicans treatment. Lee et al. (2010)

stated that antifungal inhibitory concentration of nanobiosilver is about 20-30 times less than fluconazole. This gave us the idea that nanobiosilver is much safer than the antifungal drugs as it is used as very little doses. Nanobiosilver particulates have significant antifungal activity due to skin penetration while having lower systemic toxicity than other antifungal agents (Lee et al., 2010).

During recent years nanobiosilver particulates have attracted attention due to its unique physical and chemical

properties (Stoimenov et al., 2002; Tak et al., 2015). For this reason, we investigated effectiveness of nanobiosilver complex and positive results were found especially for treatment of *C. albicans* infections.

We concluded that nanobiosilver is an effective chemical agent in treatment of *C. albicans* infections. Nanobiosilver can be an alternative for catheter-related candidiasis in woman and domestic pet species devoting to its safety, effectiveness and not having systemic effects.

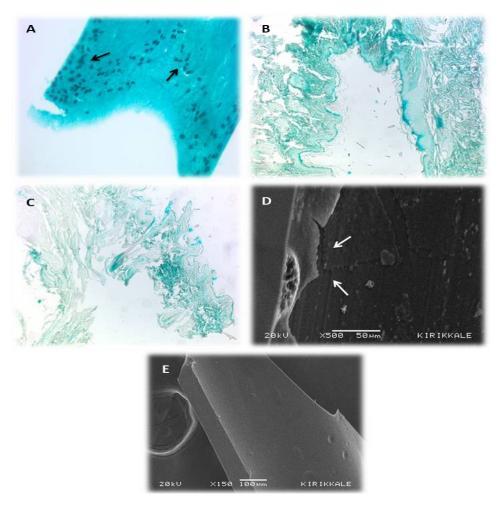


Figure 1. Electron Microscope and Histopathologic results

A. Study group; *C. albicans* infection (gomori methenamin silver stain) (Arrow: *C.albicans*), B. Study group; urethra after administration normal appearance gomori methenamin silver stain), C. Control group; uretra (gomori methenamin silver stain), D. Internal surface of urethral catheter from control group (Arrow: biofilm layer), (Scanning electron microscope), E. Internal surface of urethral catheter from study group after biosilver administration (Scanning electron microscope).

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Black Seed (Nigella Sativa) and Immunomodulatory Effect

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Abstract

Nigella (Nigella sativa) plants and oil for centuries in Africa, Asia and the Middle East today is by the United States and millions of people in Europe "to promote health" was a plant used and has been a material referenced in terms of health. As a result of scientific research, it was suggested that the seeds of vitamins, active substances and essential fatty acids should be consumed by scientists due to acid. Nigella sativa has significantly increased the total lymphocyte amount and has been supported by studies that have an immunomodulatory effect on both humans and animals. Healthy life support issues to the forefront of popular Cumin seed, which is an important component of thymoquinone (TQ) has been preferred due to potential medicinal properties as a source of healing. In the scientific researches, seedlings of black seed, vitamins, active substances and essential fatty acids may be useful to be consumed due to the products.

Keywords: Nigella sativa, immunomodulatory, thymoquinone

INTRODUCTION

Medicines derived from plants and plant extracts have traditionally been used for many years in the treatment of diseases. In the field of science, the mechanisms and active components of plants are tried to be understood in recent years (Abuharfeil et al., 2001). In many studies, herbal medicines have been shown to have many beneficial effects including antioxidant, anti-inflammatory, anti-carcinogen, antibacterial and immunomodulatory (Abuharfeil et al., 2001; Al-Ali et al., 2008; Al-Asoom et al., 2014). There are many plants used for traditional treatment purposes; one of them is *Nigella Sativa* (Nigella) plant, which belongs to the family Ranunculuceae (Al-Asoom et al., 2014; Al-Gaby, 1998; Al-Ghamdi, 2001).

Today, health problems due to technological advances have led researchers to seek different solutions. The most important tendency in this field is alternative medicine and traditional methods with products of natural origin. These products have become very popular around the world due to the benefits they provide to human health. Recently, the daily use of plant-based food products has increased, which does not carry a health risk and has no excessive side effects. In addition, these products are used as therapeutic and supportive food. Since ancient times, various plants have been used to treat different ailments. Similarly, today, plants are treated and the related industry is growing rapidly from year to year (Al-Kushi, 2013). In developing countries, the majority of the population uses herbal medicines to treat different basic medical problems (Alshatwi, 2013). One of the underlying causes of this; according to chemical drugs, herbal treatments are more effective, safer, less toxic, easily usable and affordable (Baytop, 1984). For this reason, serious research is carried out on the therapeutic potentials and medical uses of plants in medicine.

It is known that Nigella sativa seeds are used to support

healthy living, active and healthy aging, improve quality of life, and most importantly to help prevent diseases, i.e. preventive medicine. Thymoquinone, an important component of Nigella sativa seed oil, which is at the forefront of supporting healthy life, is preferred due to its potential medical properties. As a result of the researches, the active ingredient found in the plant seed is one of the products that may be beneficial to be consumed due to vitamins and fatty acids. Thanks to the clinical findings, the scientifically proven of pharmaceutical activities of the Nigella sativa seed have also been a preferred plant for treatment purposes. One of the important features of Nigella is the immunomodulatory effect of the substances in its composition (thymoquinone). (Al-Asoom et al., 2014; Boskabady et al., 2011; Cheikh-Rouhou et al., 2007; Çelik et al., 2014; Elkadi and Kandil, 1987). Many years ago, studies have started to be done that black seed can increase response in humans. Recently, immunomodulatory effect and protein structure of all extracts of black seed has been analyzed in vitro (Ghosheh et al., 1999). As a result of the examinations, the black seed oil increases T cell proliferation and therefore triggers cellular immunity; on the other hand, it has been reported that it suppresses B cells, i.e. humoral immunity (Al-Asoom et al., 2014). These findings, obtained through in vitro studies, were also supported as in vivo (Abuharfeil et al., 2001; Gholamnezhad et al., 2014; Girgin et al., 2008). Based on recent in vitro and in vivo data, it is possible to say that black seed can increase cellular immunity while humoral immunity. suppressing However. experimental studies are needed to confirm this hypothesis. Thanks to the studies to be carried out in this way, the immunomodulating effects of black seed can be measured based on the natural immune reaction mediators in diseases.

Chemical Structure of Thymoquinone

Nigella sativa, commonly bred in middle eastern and West Asian countries and popularly known as Nigella, used for bronchial asthma, cough, headache, toothache, nasal congestion, infections, obesity, back pain, is used in the treatment of hypertension, diarrhea, digestive system problems and numerous types of cancer (Güzelsoy er al., 2018). In addition, Black Seed is used as a food additive and spice (Abuharfeil et al., 2001; Grover and Yadav,2004; Guyton and Hall, Haq et al., 1995; Haq et al., 1999; 2001; Hawsawi et al., 2001).

The part of the Nigella sativa species of the Ranunculacea (Buttercups) family, which is used as food, is the seed. Nigella seeds; It consists of 36-38% fats, proteins, alkaloids, saponins and 0.4 -2.5% fatty acids. The majority of fats are unsaturated fatty acids. Although it contains many components in its structure, the main factor is thymoquinone (27.8% 57%) (Celik et al., 2014). Black seed seeds are especially rich in oleic acid, linolenic acid, linoleic acid, arachidonic acid, palmitoleic acid and stearic acid, which are fatty acids (Hosseinzadeh and Parvardeh, 2004). The biologically active compounds of black cumin include thymoquinone, thymohydroquinone, ditimokinone (Kanter, 2009; Kaya et al., 2003). The nitroseutic substance in the seeds is considered to be thyroxinone (Figure 1). Seeds also contain carotene and potassium, phosphorus, calcium and iron, which are converted into vitamin A in the liver (Kenawy et al., 2014). It has been shown in studies that cytotoxic effect against various cancer cells (Mills and Bone, 2010) increases cellular activation and tumor-specific antibodies production. Salemi and Hossainb (2000) have shown that seed extract and seed oil have an antiviral and antimicrobial effect in their work (Mohammed et al., 2010).

Thymoquinone (TQ)

Figure 1. Molecular structure of thymoquinone

In many studies, it has been tried to show certain neuropharmacological effects of the active substance and it has been revealed that thymoquinone can be used as an anticonvulsant in mild epilepsy (Çelik et al., 2014; Haq et al., 1999). It is also reported to have a healing effect in neuropathic pain (Haq et al., 1999).

Today, gastrointestinal system problems are quite common and play a role in the pathogenesis of most diseases. There are various causes of stomach problems such as stress, smoking, malnutrition, infections and nonsteroidal anti-inflammatory drugs. Studies have shown that black seed and its active ingredient, thymoquinone, play

an important role in preventing gastric mucosa damage by decreasing the increased stomach acidity. It is reported that this protective effect may be achieved by increasing the arachidonic acid bioavailability of the gastric mucosa (Al-Ghamdi, 2001).

Physicochemical Properties of Nigella Sativa Seed

When the physical properties of black seed oils were examined, there was no significant difference between the oils obtained by the cold press method and the physical properties of the oils obtained by solvent extraction. In the studies conducted, it has been stated that black seed oil has high UV absorption due to its color composition and can provide sun protection effect with this feature. Regarding oxidative stability, studies carried out with rancimat device showed up to 55 hours of durability. It can be stored for long periods of time due to its high amount of natural antioxidants and low amounts of unsaturated fatty acids. Thanks to this feature, it prevents many vegetable oils. (Ojha et al., 2015).

Considerations in the Hand of Nigella Sativa Seeds

It has been reported that the environmental conditions, climate, light, sea height, distance from the equator, soil condition, soil reaction, condition of water and minerals that are effective in the development of plants such as black seed. At the same time, factors such as the age of the plant, physiological development, harvest time, processing and drying operations have a positive or negative effect on the normal development of the plant and the synthesis of valuable substances, the quantity and quality of the essential oil obtained. Therefore, the number of essential oils such as nigellone and thymoquinone, which are active ingredients in the composition of black seed grown in different geographical regions, may also vary. In studies on the chemical composition of black seed seeds grown in different regions of our country, it is reported that the amount of essential oil varies between 0.09% and 0.36% (Alshatwi, 2013).

Effect of Nigella Sativa on Immune System

In the study on humans, Nigella sativa seed oil has a CD4 (auxiliary lymphocyte) / CD8 (suppressive lymphocyte) rate of 55%; natural killer (NK) cells were found to increase by 30%. When nigella sativa seed's juicy extract was taken for a week, the amount of natural killer cells and cytotoxicity against YAC-1 tumor cells increased nearly twice (Osman et al., 2012). Black seed oil directly enhances immune resistance in the elderly. It has been reported that it prevents a decrease in the number of hemoglobin and total leukocytes caused by "cisplatin" in mice. The total number of leukocytes was increased by 3.2% in mice given black seed. Black seed has the effect of enhancing the effect of T cells in the immune system (Paarakh, 2010).

It is determined that black seed activates T lymphocytes for the IL-3 release while it has no activating effect in the IL-2 release and its proteins significantly increase the amount of total lymphocytes, and in this respect, it has an immune-modulating effect in both humans and animals (Randhawa and Alghamdi, 2011; Salama, 2010). In studies conducted, it was observed that black seed extract increased T cell population, CD3, CD4 and CD8 surface antigens and the number of immune system cells. As a reason for this increase, it was found that thymoquinone stimulates

hematopoiesis and thus the immune system-related cells are affected and the increase is shaped (Paul, 2003; Rahmani and Aly, 2015). In allogenic cell cultures and in vivo studies using black seed proteins, it causes large amounts of IL-1 β and TNF- α expression. When activating the T lymphocytes of the black seed in the direction of IL-3 secretion, IL-2 has no activating effect towards release and their protein significantly increases the total amount of lymphocytes, in this respect, both in humans and in animals modulator (Randhawa and Alghamdi, 2011; Salama, 2010).

In another study on mice reported that black seed prevented decrease of hemoglobin amount and total leukocytes caused by Cisplatin (Mills and Bone, 2010). In this study, black seed seeds increased CD3 + T cells, CD4 + helper T cells, CD8 + suppressor to cytotoxic T cells and their ratio to each other (CD4 + / CD8 +) and increased the total leukocyte count.

A number of studies have been carried out to show the effects of Nigella and thymoquinone on the immune system. In previous studies, thymoquinone has been shown to have important effects on the immune system. This effect is defined by the active substance as suppressor or activation on T cells, B cells, and cytokines involved in the immune system (Salem, 2005).

Based on recent in vitro and in vivo data, it is likely to say that black seed can increase cellular immunity while suppressing humoral immunity. However, more studies are needed to confirm this hypothesis. Thus, the immunomodulatory effects of black seed can be measured based on the natural immune reaction mediators in diseases. In the literature studies, different application methods (orogastric, intraperitoneal, subcutaneous, inhalation etc.) of the active substance were and their effects were observed in order to investigate the immunomodulatory effects of black seed (Salemai and Hossainb, 2000; Swamy and Tan, 2000; Woo et al., 2000; Zohary et al., 2012).

CONCLUSION

In most of the studies on thymoquinone obtained from black seeds and seeds; It is reported that black seed has many beneficial pharmacological activities and can show toxic effects only in very high doses. It is reported that thymoquinone maybe contact allergen. Since the reliability of oral and dermal exposure is not sufficient, it is not recommended to use it during pregnancy and breastfeeding. It has been shown by studies that black seed significantly increases the total amount of lymphocytes; in this respect, it has an immunomodulatory effect in both humans and animals. However, more studies are needed to better understand its effects on metabolic pathways. In addition to use the black seed seeds and its effective components as medicines determined they are determined and standardized to have more advanced research methods, including clinical and toxicological studies to pass; quality, effectiveness and safety should be evaluated. It has been reported that the daily use of black seed seeds 30 mg / kg in humans can activate the immune system, but more studies are needed on this subject.

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Investigation of The Effects of Some Intrafollicular Growth Factors (GDF-9, GATA-4, GATA-6, IGF-I and IGF-II on Etiopathogenesis of Cystic Follicular Ovarian Degenerations in Cows

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Abstract

The aim of the presented study was to investigate the efficacy of intrafollicular GDF-9, GATA-4, GATA-6, IGF-I and IGF-II levels on etiopathogenesis of follicular cystic degenerations in dairy cows in accordance with some puerperal physiology parameters. After calving, all the cows were screened for the preovulatory and cystic follicles via ultrasonography with 5 MHz transrectal linear probe for the evaluation of ovarian activity at the days of postpartum 14, 24, 34, 44 and 55. Preovulatory and cystic follicular fluids were collected by aspiration via transvaginal ovum pick up method. Furthermore, uterine involution and vaginal discharges were evaluated by clinical examinations. Intrafollicular expression of GDF-9, GATA-4, GATA-6 and IGF-II were estimated by Western-blot assay and IGF-I levels were determined by ELISA. In cyst group, densitometric evaluation revealed that the expression of intrafollicular GDF-9, GATA-4 and GATA-6 and IGF-I levels were significantly lower than the control group (p<0.01). According to ovarian examinations, in cyst group, it was observed that at the day of postpartum 14 and 24, the difference in follicular diameters were significant between groups (p<0.01) and the follicles moved directly to cystic structure at the days of postpartum 24 or 34, is originated from same follicles at the day of postpartum 14. Involution process was observed to be slightly lower in the cyst group, but, it was determined that there was no difference in uterine involutions among the study groups (p>0.05). In follicular cyst group, especially at the days of postpartum 24 and 34, the observation of severe mucotic vaginal discharges was of the preovulatory follicle group and also it has been suggested that these proteins may play an active role in the etiopathogenesis of ovarian cysts and these findings may be associated with physiological parameters as well.

Keywords: Cow, postpartum, follicular cyst, follicular fluid, transforming growth factor family, IGF-1, ELISA, western blot.

INTRODUCTION

Follicular cysts of the ovary are anovulatory structures, which are identified for many mammalian species as well as cows. The incidence of ovarian follicular cysts in dairy cattle showed a range from 5.6% to 18.8% (Lopez-Diaz et al. 2002). The time which deflection lapse between diagnosis, treatment and insemination of the cows with follicular cyst can take up to 50-70 days. This situation causes significant economic losses in terms of dairy cattle industry (Vanholder et al. 2005). The effect of disruptions in some possible mechanisms in the process of formation of anovulatory follicles and permanent ovarian cysts in cows are aimed to be explained. These are categorized under three main themes such that preovulatory luteinizing hormone (LH) response from the pituitary to the estradiol in the new preovulatory follicles developing in the postpartum period is not observable, LH release problems occur due to the inadequacy of gonadotropin release from the hypothalamus despite the presence of estradiol, and sometimes anovulation problems arise from the lack of ovarian response despite the presence of gonadal response (Rizzo et al. 2010; Paredes et al. 2011).

During folliculogenesis, gonadotropins and metabolic hormones interact with local gonadal somatic cells at the receptor level, and thus externally they control the stimulation and inhibition of autocrine and paracrine signals. However, such a control mechanism is ensured by some intraovarian factors. For example, the ovarian

somatic cells and some factors secreted from the oocyte in this interaction directly affect the function of the hypothalamic-pituitary-gonadal axis although to which size the follicle will grow, and at which stage it will be ovulated is closely related to the amount of intra-follicular estradiol. This situation highlights the effects of intraovarian regulation mechanisms on folliculogenesis and ovulation process (Scaramuzzi et al. 2011). Follicular development as a fertility parameter along with pubertas is a phenomenon during which oocyte gains the ability to ovulate. One of the evidences and most important indicators for this is the rapid increase in the production and activities of transforming growth factor-beta family proteins (TGF-β) secreted from the oocyte and other functional somatic cells of the ovary. These proteins play an important role in the intraovarian regulation mechanisms. Some of these duties are; granulosa and theca cell proliferation, follicle selection, follicle development and structural strength, steroidogenesis, oocyte maturation, ovulation and luteinization (Hunter et al. 2004; Webb et al. 2007; Webb and Campbell, 2007). Growth and Differentiaon Factor-9 (GDF-9), which plays a role in regulating the phenotype of the oocyte, is synthesized from the oocyte and surrounding cumulus cells. The process of regulation of this phenotype involves supporting of a healthy formation of the oocyte and its gradual maturation. Thanks to the Cumulus cells and the follicular cells which face the lumen, such a

gradual maturation is regulated (Juengel & McNatty, 2005). GATA 4 and 6 play a key role in directing endocrine, paracrine and autocrine signals in the ovary (Tremblay & Viger, 2003). It is highly probable that the follicle which is under the extreme effect of FSH (folliclestimulation hormone) or highly susceptible to FSH stimuli due to differentiation properties of FSH characterized for anti-apoptotic, proliferative, aromatase enzyme production and the formation of LH receptors in the granulosa cells of the follicle would be the dominant follicle (Son et al. 2011). Follicle stimulating hormone and Insulin Like Growth Factor-I (IGF-I) support each other's effects on proliferation, and reproduction of steroids, activin and inhibin in bovine granulosa cells (Fortune et al. 2004; Beg and Ginther, 2006). IGF actions increase as the follicles grow. As the concentration of IGF-I in circulation increases, follicular growth also increases (Nicholas et al. 2002). In cows and sheep, IGFs are secreted from somatic cells of the follicle under the influence of insulin as well as oocyte-induced GDF-9 and Bone Morphogenetic Protein-6 (BMP-6) and BMP-15, together with insulin (Webb et al. 2007, Scherzer et al. 2009). While plasma IGF-I level is of critical importance for follicular development, it is also directly related to energy status and liver health (Butler, 2000; Zulu et al. 2002).

In the presented study, it is aimed to compare the expression levels of intra-follicular GDF-9, GATA-4, GATA-6, IGF-I and IGF-II, which have important roles in the intraovarian regulation mechanisms in the physiological process in dairy cows with high metabolic activities and are thought to have an effect on the etiopathogenesis of cystic ovarian degenerations, in cystic and healthy dominant follicles, within the framework of postpartum involution, ovarian activity and puerperal physiology.

MATERIALS AND METHODS

Animals and Grouping

The study was conducted in a commercial farm engaged in intensive dairy cattle breeding. Lactating Holstein cows in the postpartum period, which have given at least 1 and at most 4 births, were used as animal material. Cows in the dairy were housed in separate sections according to their yield characteristics and age. In the study, ovarian activities were monitored by means of regular gynecological examinations at ten-day intervals from the 14th day of postpartum, performed for every cow which has given birth to a calf. The diagnosis of cystic follicles was made according to Day's (1991). Accordingly, in the postpartum period, the cows with a persistent follicular structure of 2.5 cm and more in diameter in their ovaries as identified in the examinations performed at ten-day intervals, have formed the cyst group (n=10), whereas the cows with a physiological preovulatory follicular structure of 1.5-2.0 cm in diameter in their ovaries and indicating the clinical findings of oestrus have formed the control group (n=10).

Gynecological Examinations

The postpartum period, was monitored through the rectal palpation and ultrasonographical (ALOKA® PS2) examinations of the ovaries, uterine horns, uterine body and cervix uteri starting from the 14th day. Measurements of uterine horns diameters were made according to Kamimura et al. (1993). During the examinations, 8 mm and larger follicles in both ovaries of the cows were recorded.

Follicular Fluid Aspiration

Follicular fluid aspiration was performed transvaginally using the Ovum Pick Up (OPU) method (Seneda et al. 2003). For the purposes of follicle aspiration, 6 cm (length) 18 G (1.250 mm outer diameter) cannulas. Follicular fluids were transferred into pure serum tubes (BD Vacuteiner®-365815) and centrifuged at 1300 g for 13 minutes and storedat-20°Cuntilanalysis.

Sulfate-Poly Sodium Dodecyl Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Methods Proteins were separated in 10% SDS-PAGE. The same volume of follicular fluid was applied to each well. After electrophoresis, proteins were transferred to nitrocellulose membranes (Nitrocellulose Pure Transfer Membrane, Santa Cruz Biotech) and blocked with 3% bovine albumin in TBST. Goat polyclonal IgG anti-mouse GATA-4, goat polyclonal IgG anti-human GATA-6, GDF-9 and IGF-II were used as a primary antibody and bovine anti-goat IgG-HRP (Santa Cruz Biotech) was used as a secondary antibody. Proteins were detected by Luminol Reagent (Santa Cruz Biotech). Protein bands were scanned using a Bio-Rad GS-800 densitometer and signal intensity was determined with Quantity One Software (Bio-Rad) to compare expression levels among groups.

IGF-I (Insulin-like Growth Factor I) analysis of Follicle Fluid

IGF-I levels in follicular fluids were measured using the ELISA (Enzyme-linked immunosorbent assay) technique using a commercial kit (Cusabio®, CSB-E08893b). The absorbance values of the samples were measured at 450 nm with the ELISA MAT-2000® microplate reader. Absorbance values were calculated in nanogram/milliliter using Ridawin® package program.

Statistical Analysis

The SPSS was used for analysis of data (v14.1; Chicago, IL). Firstly, data were checked for equality of variance trough Levene's test. The Shapiro-Wilk test was performed for normality of original and logarithmic values. Student's t-test was used when data were normally distributed, and the Mann-Whitney U test was used when data were not normally distributed. Descriptive statistics for each variable were calculated and presented as the "mean \pm SEM." Probability values of P<0.05 were considered as statistically significant.

RESULTS

Ultrasonographical Findings

Although there was no statistically significant difference in terms of both uterine horns and cervix involution between cyst and control groups, it is determined that uterine measurement values were higher in the cyst group on 54th day of postpartum period compared to the control group (p>0.05). It was observed that the course of the uterine involution process was similar in both groups. However, it was determined that the rate of involution occurred in a more horizontal curve in the cyst group (Table 1). In the ovarian examinations of the cyst and the control group, which were performed by means of ultrasonography from the 14th day until 54th day of postpartum period; luteal structures were identified in the examinations of generally aspirated follicular structures, which conducted ten days later. It was observed in the examinations of these cases, which were conducted 20 days after aspiration, that corpus

luteum disappeared and a new follicular structure bigger than 8 mm was formed. While this observation revealed a statistical difference in terms of follicular numbers compared to the control group on 44^{th} day of postpartum period during which the first cycles were concentrated after aspiration in the cystic group (p<0.01), it was found that the said difference disappeared in the examinations

conducted on the 54th day of postpartum period when normal cycles started to occur (Table 2). In the cyst group, the follicle diameters which would transform into a cystic structure were found to be significantly larger compared to the control group as of 14th day of postpartum period (p<0.01) (Fig1).

Table 1. Ultrasonographical findings of cervix and uterine horns during post calving periods in cystic and control cows. $(\bar{x}\pm S\ \bar{x})$: mean \pm standard error.

Examination days (post calving days)	Cervix (cm) (\overline{x}\pm S)		Uterine horns (cm) $(\overline{x}\pm S\ \overline{x})$	
	Cystic Group (n=10)	Control Group (n=10)	Cystic Group (n=10)	Control Group (n=10)
14 th	4,08±0,36a	4,04±0,19 ^a	6,90±0,67ª	6,33±0,50 ^a
24 th	3,84±0,39a	3,62±0,33ª	5,80±0,62 ^b	6,46±0,52 ^b
34 th	4,14±0,33ª	3,90±0,21ª	5,60±0,48°	4,65±0,17°
44 th	4,42±0,41 ^b	3,37±0,21ª	5,30±0,43 ^d	4,65±0,33 ^d
54 th	4,36±0,41 ^b	3,70±0,14a	5,10±0,44e	4,80±0,28°

^{a,b,c,d,e}: Means with different superscripts in a column are significantly different (P<0.05).

Table 2. Mean numbers of follicles in cystic and control cows during post calving period. $(\bar{x}\pm S\bar{x})$: mean \pm standard error

Examination days (post calving days)	Cystic Group(cm) (n=10) (x±S x̄)		Control Group(cm) $(n=10) (\overline{x}\pm S \overline{x})$	
	Right Ovary	Left Ovary	Right Ovary	Left Ovary
14 th 2,14±0,26 ^a		2,57±0,37ª	1,85±0,34ª	2,75±0,31a
24 th	2,30±0,30ª	2,30±0,21ª	1,70±0,21ª	2,40±0,27ª
34 th	2,40±0,34ª	2,30±0,21ª	1,70±0,30°	2,60±0,27ª
44 th	1,70±0,26ª	1,90±0,23ª	2,00±0,26 ^b	2,80±0,25 ^b
54 th	1,80±0,25ª	2,20±0,20ª	2,20±0,29ª	2,30±0,30 ^a

^{a,b}: Means with different superscripts in a line are significantly different (P<0.05).

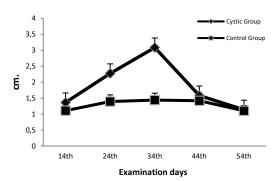


Figure 1. Follicular diameters in cystic and control groups.

Intra-follicular IGF-I and IGF-II Findings in Cyst and

In the cyst and control groups, intra-follicular IGF-I levels were calculated to be 20.9±1.01 ng/ml in the cyst group

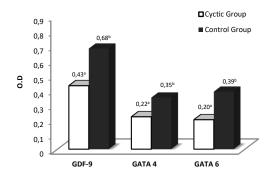


Fig 2. Expression levels of proteins in follicular fluids of bovine

cystic follicles . a,b : Means with different superscripts are significantly different (P<0.01). O.D: optical density.

and 27.7±1.55 ng/ml in the control group. Average intrafollicular IGF-I levels were determined to be statistically significant between the groups (p<0.01). As for IGF-II, it could not be determined by Western Blot Method.

Western Blot Findings in GDF-9, GATA-4 and GATA-6 Proteins in Follicle Fluid and Densitometric Evaluation of Bands

It was determined that the densitometric values of GDF-9, GATA - 4 and GATA - 6 protein bands obtained from follicular fluids of cyst group were lower compared to the control group (p <0.01). Densitometric measurement values of intra-follicular growth factors such as GDF-9, GATA-4 and GATA-6 are shown in Figure 2.

DISCUSSION

In the examinations made for cyst group on the 24th, 34th, 44th and 54th days of postpartum period, follicular cysts with a follicular diameter of 2.5 cm and above showed a steady growth curve in the following examinations, while the follicles below 2.5 cm showed a faster growth curve. In this group, the recurrence was determined for two out of ten subjects which underwent aspiration. Corpus luteums occurred in eight cases which did not exhibited recurrence after aspiration. It was determined that the life span of the corpus luteum was within a normal cycle for two cases which could be monitored during the study period and that it was directed to a healthy cycle in the 20-day period following aspiration. Completion of the postpartum period within physiological limits is the only way to achieve economic goals in dairy cattle breeding. Controlling the factors which affects this process ensures that fertility parameters remain within acceptable limits (LeBlanc et al. 2002; Ingvartsen and Moyes, 2012). lipopolysaccharides produced particularly by gram negative bacteria in the presence of contamination and infection of the uterus in the process of involution negatively affect ovarian and pituitary functions (Sheldon et al. 2002).

Although postpartum uterine dimensions vary according to the breed and genetic characteristics of the cow, it is generally identified in ultrasonographic measurements of the uterus to be approximately 15 cm, 9-1 cm, 7-8 cm, and 5-6 cm on the 2nd, 10th, 30th and 60th days of postpartum period respectively (Arthur et al. 1982). Olson et al. (1986) reported these measurements to be 3-4 cm and 2-3 cm on the 30th and 40th day of postpartum period respectively. In the presented study, uterine horn diameter was reduced from 6.9±0.67 cm to 5.1±0.43 cm measured for cyst group respectively on 14th and 54th day of postpartum period; whereas it was reduced from 6.33±0.5 cm to 4.8±0.28 cm measured for control group respectively on 14th and 54th day of postpartum period; As for cervical uteri diameters; it was determined to be 4.08±0.36 cm and 4.36 ± 0.41 cm measured for cyst group respectively on 14th and 54th day of postpartum period; whereas it was determined to be 4.04±0.19 cm and 3.70±0.14 cm measured for control group respectively on 14th and 54^{th} day of postpartum period. These measurements for both study groups are similar to the measurement findings by Zhang et al. (2010), Olson et al. (1986) and Senosy et al. (2009). The cervix measurements, which started to decrease in parallel with the control group on the 14th day of postpartum period, exhibited a different increase than the control group on the 24th day of postpartum period. The slow involution process in the cyst group and the increase in cervix diameters as of the 24th day of postpartum period, cysts began to form structurally; the edema which exhibits increase in the uterus tissue especially under the reflection of intra-follicular steroids after the 24th day of postpartum period when cysts start to form structurally is considered that it could change the measurement values and involution

curve in cases with tendency towards the cyst.

The follicular activity in the ovaries in the postpartum period and selection of dominant follicle depend on the frequency of gonadotropic hormone release (Crowe 2008), and the selection of the dominant follicle occurs as of the 10th day after birth. The first ovulation after delivery is formed in the second week of postpartum period (McDougall et al. 1995). However, the first cycles in the postpartum period are short whereas the life span of the corpus luteum after possible ovulation may be even shorter (Wathes et al. 2003). Thiengtham et al. (2008) reported that the first dominant follicle selection and early ovulation occurred between 12th to 15th days of the postpartum period, while Sakaguchi (2011) reported that they observed initial ovulation on the 18th day of the postpartum period, but the first cycles were short. Lee and Kim (2006) stated that how many times a cow gives birth to a calf and its milk yield play an important role in the formation of cystic follicular structures; accordingly, they proposed that the incidence of cyst also increases as the number of births given increases. In this study, the fact that the ovaries were active for the majority of the subjects on the 14 th day of postpartum period and the follicle diameters were generally larger than 1.5 cm in the cyst group was in parallel with the findings of Sakaguchi (2011) and Thiengtham et al. (2008). However, it was determined to differ from the control group due to the fact that initial ovulations are not observed in the early period. For the cyst group in the presented study, the mean diameter of the largest follicles was measured to 1.85±0.28 in the examinations made on the 14th day of the postpartum period and it was significantly different from the healthy group (p <0.01).

Insulin and IGF-I receptors are specifically available in the cow ovary. Insulin plays an indirect role in follicle development by acting on IGF-I receptors (Spicer and Echternkamp, 1995). Insulin-like growth factors influence the release of FSH and LH and thus support the steroidogenesis process in follicular cells, the increase in the number and function of LH receptors. However, IGF (insulin-like growth factor) system plays an important role in follicle selection and ovulation by stimulating the aromatase system together with carrier proteins (IGFBP = Insulin-like growth factor-binding protein) (Zulu et al. 2002b). The IGF-I system plays a central role in the regulation of TGF-β bioactivity during the growth and development of the follicles, thanks to specific extracellular proteases. While IGF-I plays an important role in the selection of dominant follicles, intra-follicular IGF-I levels show a positive correlation with the follicle diameter in physiologically healthy follicles. Intra-follicular IGF-I levels in cystic structures and physiological dominant follicles differ in cows (Perks et al. 1999). Eden et al. (1988) reported that intra-follicular IGF-I levels in women with polycystic ovary syndrome are the same as healthy individuals and that there is no intra-follicular difference, while Ortega et al. (2008) stated that the opposite is valid for the cows. Bovine granulosa cells can synthesize IGF-I carrier proteins (Santiago et al. 2005). Possible insufficiency of the expression of carrier proteins in granulosa cells in follicles with tendency towards cystic structure and irregularity in the IGF system suggests that the cystic structures may be an indicator of the decrease in intra-follicular IGF levels in the postpartum period. In this study, the lack of ovulation in cystic subjects on the 14th day of postpartum period can be associated with the increase in the number LH receptors due to a probable decrease in IGF-I level in blood and lack of response to LH, and low intra-follicular IGF-I levels in cystic cases,

LH surges and preservation of the presence of oocyte with a view to inhibiting premature differentiation of granulosa cells as a result of increase in intra-follicular estradiol upon second release of FSH.

The functions of GDF-9, a member of the TGF- β parent family, ensure that proteins and binding proteins bind to receptors during follicle development. These receptors are frequently available in oocyte, granulosa cell and theca cells. However, these receptors interact with different proteins at different stages of development of the follicles, and their distribution differs depending on the species (Dijke et al. 2000). GDF-9, which plays a role in regulation of the quality and function of the oocyte, is produced by oocyte cumulus cells. GDF-9 takes a part in promoting oocyte health and its gradual maturation (Elvin et al. 2000). Juengel and McNatty (2005) reported that the gradual maturation was regulated by means of the lumenfacing cells of the cumulus cells and follicles, and the factors released from oocyte were restricted and the concentration level of oocyte-derived factors was controlled during this process. Regarding cows and sheep,GDF-9, BMP-6 and 15 are expressed by the oocyte in the early stages of the primordial follicle (McGrath et al. 1995; Jaatinan et al. 1999; Bodensteiner et al. 1999; McNatty et al. 2001). It is reported that GDF-9 deficiencies cause follicular development disorders in women and GDF-9 expression decreases in women with polycystic ovary syndrome (Zhao et al. 2010). In this study, it is anticipated that the results obtained for follicular cysts and after treatment of follicular fluids extracted from preovulatory healthy follicles with GDF-9 antibody are similar to the findings of Juengel and McNatty (2005), and the oocyte could be degenerated and aged in cystic structures, and accordingly, the required signals for establishment of relation between oocyte and somatic cells and protein expressions could not be made satisfactorily and remain persistent in the ovaries with no tendency for ovulation. In the study, it is known that inhibin alpha expression is high in follicular fluid analyzes and immunohistochemical studies on cystic and granulosa cell tumors, although the level of inhibin alpha expression is not checked (Matzuk et al. 1992). As is known, inhibin alpha uses common receptors with members of the TGFbeta family regarding its intra-follicular actions (Stenvers and Findlay, 2009), and it is suggested in this study for the cyst group that the preferable binding of the probably high inhibin alpha to these receptors might have indirectly blocked the actions of GDF-9.

GATA-4 and 6 are released from granulosa cells and theca cells in the ovary (Heikinheimo et al. 1997; Laitinen et al. 2000). In the presented study, the fact that the expression levels of GATA-4 and GATA-6 proteins in follicular cysts were found to be significantly lower densitometrically compared to the control group (p<0.01) can be associated with the inadequacy of gonadotropin receptors in the cystic follicles. Additionally, the fact that there may be no cellular differentiation at the ovarian level (receptor up regulation) despite the tonic and pulsatile gonadotropin releases in the cyst group can be considered as problems in response to gonadotropins and consequently anovulation of the follicles.

It can be concluded that GATA-4 and GATA-6 expressions and activations are suppressed as a result of gonadotropin receptor deficiency in the presence of possibly low IGF-I levels in terms of intra-follicular molecular regulation mechanisms as well as systemic metabolic changes such as bacterial endotoxins, insulin resistance in the formation of cystic follicular structures in

the early postpartum period. Furthermore, it is suggested that the follicles with tendency towards cysts in the early postpartum period are shaped from the first follicular wave which develops as of 14th day of the postpartum period, and they are intensified in clinical presentation between 24th and 34th days of the postpartum period.

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Investigation of Systemic Toxic Effects of Nanobiosilver Use in Rodent Models

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This study aims to investigate the toxicity of biosilver particles on the rodent model. The study used 10 Guinea pigs for the sensitization test (Guinea pig maximization test) and 80 8-to-12-week-old BALB-C mice for systemic toxicity researches. The mice used in the study were divided into 6 groups: acute, subacute, subchronic experimental groups and the control groups thereof. The experimental animals were given NanoBioSilver intraperitoneal doses of 50 ml/kg, including a single dose for the acute systemic toxicity test and 7 repetitive doses for NanoBioSilver, subacute and subchronic toxicity tests. At the end of the study, liver tissues were sampled from animals, which were then examined histopathologically. Throughout the study, no significant changes were observed in clinical findings of the groups. Also, no significant changes were found in vital tissues of the study groups. In conclusion, biosilver particles were found to have no acute, subacute and subchronic toxic effects on the rodent model.

Keywords: Acute, biosilver, subacute, subchronic, toxicity.

INTRODUCTION

The concept of technology, which is as old as the history of humanity itself, continues to deeply affect the societies through its gradual development. The technology has been developing very rapidly especially in recent times, which makes it difficult to follow those developments on an individual and social basis. Against the backdrop of these developments, Nanoscience and Nanotechnology (N&N) are now regarded as an important technological field that will mark the 21st century (Özer, 2008).

Nanoscience is a discipline which examines the unique behaviors and properties of the materials ranging from 1 to 100 nanometers (one billionth of a meter) in size (Sarsar et

Nanomaterials are designed with a view to make use of the unique properties of the nanoscale. Typically, nanoparticles have a greater chemical reaction, biological activation and catalytic property compared to particles with the same chemical structure but a larger surface area. Yet, even though it is desired to make use of those new properties, changing the size of the materials on this scale may bring about toxicological risks (Garnett and Kallinteri, 2006; Limbach et al. 2007; Nel et al. 2006; Bergin and Witzmann, 2013).

Nanotechnology has achieved tremendous progress in determining the potential negative effects of the biological effects of nanomaterials. Though, an aspect which has not yet been satisfactorily explored so far is physicochemical properties of nanomaterials are changed by physiological environment. This raises new confusion about the solid phase nanomaterials (Braydich-Stolle et al. 2014).

The viability tests, morphological observation and oxidative stress-generating capacity provide indications about the toxicity-generating mechanism of nanomaterials (Schrand et al. 2012). This study aims to investigate the systemic toxicity of biosilver particles on the rodent model.

MATERIALS AND METHODS

Experimental Animals and Grouping

Ethical approval for this study was obtained from the Local Ethics Committee of Kırıkkale University (Decision No: 2018-52). In systemic toxicity tests, 80 8-to-12-week-old mice were used. The experimental animals were grouped as follows: 5 female & 5 male mice in acute toxicity test group, 10 female & 10 male mice in each of the subacute and subchronic toxicity test groups. The mice used in the experiment were selected randomly, marked individually and encaged 5 days before the application.

As experimental animal, the sensitization experiments, however, employed five male and five male albino guinea pigs weighing 300 g to 500 g and dermatologically free of any disease.

The control groups were composed of BALB-C mice having the same characteristics and number with those in biosilver groups. Those groups were administered the same dose of 0.9% sodium chloride (NaCl) instead of biosilver. Also, the control group of the sensitization test was composed of healthy young Guinea pigs, including three males and two females.

Biosilver Synthesis and Extraction

A total of 100 mg silver nitrate (Merk) was dissolved in pure water and diluted to 100 ppm. A 10-ppm solution of ascorbic acid & pure water was prepared. The root and the stem of the Cotinus Coggygria plant were boiled in 6g/L for 6 minutes. The liquid part was evaporated in the rotary evaporator and thus extracted. Then, 100 ml of 100 ppm silver solution, 10 mg of Cotinus plant extract, 100 ml of ascorbic acid, 13.4 mg of 0.1M solution NaHPO4 and 180 ml of 0.15 molar NaCl- solution were mixed in a beaker. And then, the pH of the solution was adjusted to 7.8 using NaOH. The resulting solution was mixed in magnetic stirrer at 38.6 °C for 28 hours using a magnetic stirring bar.

As specified in the standard test protocols TS EN ISO 10993-11 & 10993-12, since biosilver cannot be applied directly to experimental animals, the biosilver was kept in 37 °C for 72 hours before the extraction procedure was performed. According to the section 10.3.1 of the said protocol, the extraction was prepared at a rate of 3 cm²/ml due to the structure of the biosilver used.

Application Method of Biosilver and Determination of Dose Level

The application method and dosage of the biosilver to be applied to the experimental animals were determined based on the standard test protocol TS EN ISO10993-11 (Table 1.). In calculating the right dose (ml/kg body weight) according to this protocol, the animal species, body weight/surface area and the physical & chemical and biological properties of the test sample were considered. Taking into account those factors, biosilver was injected to the mice intraperitoneally at a dose of 50 ml/kg, being once in acute toxicity tests and for 7 days in subacute and subchronic tests.

Sensitization Test (Guinea pig maximization experiment) Preparation of the Experimental Group

The experimental group included only healthy guinea pigs with firm skins. Before the testing procedure, the application sites of the animals were shaved, and 0.1 ml was injected per application site for intradermal injection.

Intradermal induction phase

The shaved region between the two scapulae was divided into sites A, B and C, and each animal received an intradermal injection of Freund's complete adjuvant (sc-24018, Santa Cruz) with physiological saline, biosilver (undiluted extract) and biosilver at 50:50 (volume ratio), Freund's complete adjuvant with physiological saline (50%) emulsion, being 0.1 ml each, respectively.

Local induction phase

Seven days after the intradermal induction phase was achieved, a local application was performed to each animal with test samples impregnated in a gas cloth of approximately 8 cm². The skin was pretreated with 10% sodium dodecyl sulfate (Merck, 151-21-3) to avoid irritation 48 hours before local application. The local application was terminated after 48 hours.

Challenge phase

At 14 days after completion of the topical induction phase, challenge all test and control animals with the test sample. Administer the test sample and a vehicle control by topical application to sites that were not treated during the induction stage, such as the upper flank of each animal, using appropriate patches or chambers soaked in the test sample at the concentration selected in for site C. Dilutions of this concentration may also be applied to other untreated sites in a similar manner. Secure with an occlusive dressing. Remove the dressings and patches after 24 h.

Table 1 Application method and	I docage of the biocilver to I	be applied to experimental animals
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Species	Subcutaneous ml/kg	Intramuscular ml/kg	Intraperitoneal ml/kg	By probe By feeding ml/kg	Intravenous ml/kg
Rat	20	1	20	50	40
Mouse	50	2	50	50	50
Rabbit	10	1	20	20	10
Dog	2	1	20	20	10
Monkey	5	1	20	15	10

Observation of Skin Reactions

Within 24 to 48 hours after dressings were removed, the appearance of the competitive skin regions of both control animals and test animals and the skin reactions, under good lighting, were assessed according to the Magnusson and Kligman scale (Table 2.).

Table 2. Magnusson and Kligman Scale (TS EN ISO 10993-10, 2014).

Patch test reaction	Grade scale
No visible changes	0
Discrete or patched erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

RESULTS

Toxicity Test Results

As a result of systemic acute, subacute and subchronic toxicity tests of the NanoBioSilver, the testing periods required for all three stages were followed up in accordance with the clinical observation criteria in the table specified in TS EN ISO10993-10. Accordingly, the results were systematically evaluated in experimental animals which received NanoBioSilver throughout the testing period.

In terms of the respiratory system, no such findings as abdominal breathing, difficulty breathing, breathing rhythm, breathing stopped or cyanosis were found.

During the experimental period, no such findings as loss of righting, partial or complete disappearance of sensory ability, loss of voluntary motor movements and sensitivity, a pathological condition characterized by hypertonia that makes it impossible to move, imbalance, fatigue, flailing, sweating, etc. were found in the motor activities of the animals.

Also, there were no conjunctivitis, exophthalmus, increased or decreased lacrimation, inflammation of the iris, opacity of the eye, myiasis or mydriasis in experimental animals whose ocular signs were followed. In the wake of the cardiovascular follow-ups performed following applications in the experimental animals that were administered biosilver at the specified test period and doses, there occurred no impaired heart rhythm (arrhythmia or bradycardia), increased heart rhythm (tachycardia) and vasodilatation or vasoconstriction in the vein walls.

Following the administration of biosilver to the intraperitoneal space, no increased or decreased salivation, soft stool, diarrhea, vomiting or diuresis were found in the digestive system.

Live Weight Results

All along the testing period, the weights of experimental animals were followed up, and the weight change rates, liver weights and liver index values are presented below (Figure 1-4). The food and water consumption were found normal in all groups. No out-of-limit weight variation was observed in any mouse between the start and end time of the testing procedure. The liver weights of the experimental animals were also found to fall within normal limits (4 to 6%).

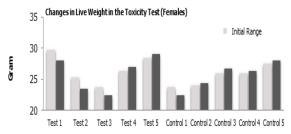


Figure 1. Changes in Live Weight in the Toxicity Test (Females)

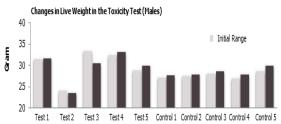


Figure 2. Changes in Live Weight in the Toxicity Test (Males)



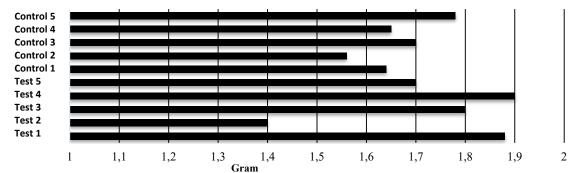


Figure 4. Liver Weights in the Toxicity Test (Males)

Necropsy and Histopathological Results

As a result of the tests performed in terms of acute (7 days), subacute (28 days) and subchronic (90 days) toxicities to determine the systemic toxicity of biosilver, all of the experimental animals were examined postmortem histopathologically.

Of the experimental animals with no pathological condition found in the external examination; the adrenal glands, lymph, all gross lesions, aorta, heart, bone marrow (femur, rib or sternum), brain (representative sites including cerebrum, cerebellum and pons), cecum, colon, duodenum, ileum, jejunum esophagus, eyes, gall bladder, kidneys, liver, lungs and bronchi as well as mammary glands in females and epididymis in males were examined at necropsy and histopathologically, which revealed no findings.

Sensitization Test (Guinea pig maximization experiment) Results

A Sensitization Test (Guinea pig maximization test) was performed using the tested nanosilver, and the test was ended at the end of the 27-day observation period. At the end of the 27-day observation period, the nanosilver tested was evaluated as "0.35 (no visible change)" according to the grade scale presented in the procedure TS EN ISO 10993-10 "Table 2 Magnusson and Kligman Scale". Table 3 presents the values observed in male and female animals according to the Magnusson and Kligman scale throughout the study.

Test An	imals	24 th hour after the dressing was removed	48 th hour after the dressing was removed	Individual Average	Average	Result
	1	1	0	0.5		
Test Male	2	1	0	0.5	0.4	
Maic	3	0	0	0	0.4	
	4	1	0	0.5		0.35
	5	0	1	0.5		
	1	0	0	0		
Test Female	2	0	0	0	0.3	
remaie	3	1	0	0.5	0.5	
	4	0	1	0.5		
	5	0	1	0.5		
	1	1	0	0.5		
Control	2	0	0	0	0.3	0.3
Control	3	1	0	0.5	0.3	0.3
	4	0	0	0		
	5	1	0	0.5		

Table 3. Values observed according to Magnusson and Kligman Scale.

DISCUSSION

Despite the fact that silver has been known for so many years as a powerful antibacterial agent with antifungal effects, it has revived with its biocidal effects in the form of suspension and nanoparticles in the recent years (Navarro et al. 2008).

In their study, Drake and Hazelwood, (2005) reports that toxicity occurs in humans when exposed to very high doses of a form of silver, which is usually biologically available, that since metallic silver products generate very poorly solute or soluble silver ions, exposure to them will not pose any risk to human health, however, when exposed to extremely high doses of silver nitrate, this may lead to a decrease in blood pressure, diarrhea, irritation in the stomach and a decrease in respiratory rate, and that in low doses, but in case of long-term exposure, some of the chronic symptoms would include degeneration in the liver and kidney and ulceration in the stomach. This study, however, revealed that no toxic effects were observed when the mice were intraperitoneally exposed to nanosilver at a dose of 50 ml/kg in acute, subacute and subchronic test procedures.

In their experimental study in which silver nitrate was given to rodents intravenously or by drinking water, Berry et al. (1995) confirmed low nephrotoxicity of silver in the urinary tract, and observed silver deposits in glomerular basement membranes, arteriolar endothelium and elastic laminae with no obvious structural harm. In contrast, no pathological findings were found in the kidneys at the end of the study.

Jiang et al. (2017) reported that nanomaterials such as nanostructured surfaces, nanoparticles and nanocomposites represent new suitable sources for future therapeutics designed for cardiovascular diseases and that nanomaterials can effectively increase the desired cellular responses within the cardiovascular system, leading to increased potential for clinical use, with their unique physiochemical properties and special properties such as surface energy and surface topographies. In parallel with the results presented by Jiang et al. (2017), the present study also revealed that the silver product administered intraperitoneally to the mice did not cause any cardiovascular pathological disorders in animals.

The healing process of skin wounds includes proliferation and reshaping of tissue. It stimulates inflammation of injury and ensures release of proinflammatory cytokines. The proliferation leads to formation of granulation tissue and angiogenesis, which are supported by macrophages. During the re-formation of the tissue, damaged tissue is removed, and the extracellular matrix is rearranged; this last process is controlled by various matrix metalloproteinases (MMPs) and tissue inhibitors. The nanosilver treatment has been found to be beneficial in wound healing since the fact that the healing process is accelerated with short-term inflammation. A thermal harm animal model using male BALB/C mice, bandages coated with nanosilver (14 nm) reduced inflammation and stopped bacterial growth and caused faster healing with reduced scar compared to the control mice (Cameron et al. 2018). The results from sensitization tests performed in this study are in line with this information and prove that the nanosilver product does not cause sensitization on the skin.

However, information on toxic effects of nanosilver in respiratory system of rodents is still too limited. There are evidences that subchronic inhalation of AgNPs causes dose-dependent, mild pulmonary inflammation and changes in pulmonary function, and inhaled AgNPs can enter the systemic circulation to be distributed to extrapulmonary organs such as the liver and brain. On the other hand, minimal or no toxicity has been reported in studies using low inhalation doses (Seiffert et al. 2016). This study, however, shows that no clinical changes were found in respiratory functions of the experimental animals as a result of acute, subacute and subchronic intraperitoneal tests performed.

In the digestive system, NP stability, dissolution and release of potentially toxic ions partly depend on liquid pH, composition and exposure time. The intestinal mucus layer is a complex network containing highly branched glycoproteins, lipids, cellular and serum macromolecules, and constitutes the first barrier for NPs swallowed. The electrical charge of the surface plays a highly important role; the neutral or positive surface charge prevents the mucosa from sticking, supports penetration and prevents the passage

of negative hydrophilic and lipophilic compounds. In their study performed in vitro on the human digestive study performed in vitro on the human digestive system, Walczak et al. (2012) conducted a study showing that 60 nm silver nanoparticles reduce the interaction and aggregation of chlorine ions after digestion of silver ions.

CONCLUSION

To correctly determine the right doses for the intake of nanosilver ions in mammalian digestive systems in in-vivo studies, in-vivo and in-vitro tests should be performed more frequently.

Nine out of ten studies conducted using oral exposure to AgNP in rodents has evaluated the tissue distribution and/or toxicity in healthy animals and focused on modulating effects on inflammation in a colitis model.

The evidences obtained from those studies generally reveal that AGNP is unlikely to have negative effects on host tissues caused by its acute or subchronic oral administration. Moreover, evidences often suggest that the orally-taken AgNP has a low level of tissue distribution and bioavailability.

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The Effects of Lactation and Body Condition Score Changes on Embryonic Death Rates in KWPN Mares

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Embryonic deaths (ED) are one of the most important economic losses in breeding mares. Energy deficit and hormonal changes in the lactating mare is expected to increase the incidence of embryonic deaths. In this study, it was aimed to investigate the liability of ED from lactation and body condition score changes during breeding season in KWPN (Koninklijk Warmbloed Paard Nederland) mares, on which there are a limited number of studies about the reproductive properties in this breed. At the onset of reproductive activity inception in lactation, average body condition score (BCS) was 7.44 ± 0.24 , until the second estrous cycle postpartum it was decreased (6.89 \pm 0.20), and by the end of the breeding season upward tendency (6.96 ± 0.18) was observed. In the non-lactating mares the average BCS was 6.33 ± 0.21 , continuously rising until the end of the breeding season (7.17 ± 0.31). The rate of embryonic deaths were 25.58% in lactating mares (11/43). In the non-lactating mares, total of 16 pregnancies were recorded in any of the embryonic mortality was observed. As a result, the decrease in embryonic death occurred in body weight and BCS, depending on the energy loss in lactating KWPN mares was concluded to be remarkable factors with increasing incidence. Therefore the reproductive status of the mares in the generative approach has been demonstrated that a strategy should be kept in mind strongly.

Keywords: Mare, embryonic death, lactation, fertility.

INTRODUCTION

Embryonic deaths (ED), which result from certain factors related to the first 40 days of pregnancy, are one of the most important causes of economic loss in the equine industry since they require re-insemination or reduce the foal production in mares (Pycock, 2001). The rate of ED's are ranged from 2.5 to 25 % in mares, (Samper et al., 2007), and they predominantly occurred before the 35th day of the pregnancy (Villahoz et al., 1985). The factors that lead to ED are classified into three groups as intrinsic (luteal insufficiency, age, endometrial diseases, lactation and insemination time), extrinsic (stress, nutrition, season, transrectal palpation and factors related to the stallion) and embryonic (chromosomal anomalies) (Samper et al., 2007; Yang & Cho, 2007). It is reported that the decrease in the progesterone level (Allen, 2001), age progression (Hemberg et al. 2004; Morel et al., 2005), and endometrial cysts (Stanton et al., 2004; Samper et al., 2007) increase the rates of ED in lactation period (Morris and Allen, 2002; Heidler et al., 2004; Newcombe and Wilson, 2005; Dirk, 2008). Van Niekerk and van Niekerk (1998) pointed out to low progesterone levels as the possible cause of EDs that occur in lactating mares in early pregnancy period. Researchers attributed this to the problems that arise in corpora lutea development or continuity as a result of low circulatory levels in lactating mares. Dirk (2008) maintains that the energy gap in the lactation period and hormonal and metabolic changes coexisting with lactation could raise the incidence of ED. However, there are a limited number of studies conducted in this subject.

This study aims to present some effects of lactation period and body condition score (BCS) changes on ED in KWPN (Koninklijk Warmbloed Paard Nederland) mares,to

identify the differences between lactating and non-lactating mares and to provide a new insight putting emphasis on the reproductive status into the reproduction methods in equine industry.

MATERIALS AND METHODS

Animals and Study Groups

The present study was conducted in the Southern Marmara region of Turkey. This study was carried out during four breeding seasons on 20 KWPN mares which did not have any contagious diseases between the ages of 6-10, with mean body weight of 642.40±25.48 kg and with BCS ranging between 6 to 8 in Turkish Military Veterinary School. All mares were housed in the same building under identical environmental and nutritional conditions.

In this study, the mares were divided into two groups according to their reproductive status. Group I (n=20) consisted of lactating mares, which conceived one year ago and went through a normal pregnancy period (normal delivery, no postpartum gynaecological and metabolic disorders). Group II (n=14) was formed based on the data collected about non-lactating mares, which were either not bred or did not become pregnant previous year. No artificial lightening was performed and no hormonal application was conducted in order to activate the ovarian activity during the study.

Study design and clinical examinations

Electronic scale was used to determine body weight changes in mares. Body condition scores were determined as of February, the onset of breeding seasons (15th February), until the 60th day of the pregnancy in mares

whichconceived and until the end of breeding season (15th July) in those which did not conceived at every 15 days in line with the method by Henneke et al. (1984) through visual evaluation and grading the fatty tissue of the horse that can be palpated from 1 (excessively thin) to 9 (excessively fatty) subjectively by two different researchers.

Rectal palpation and B-Mode real-time, 5 MHz linear probe transrectal ultrasonography (AGROSCAN AL, FRANCE) and teasing method (Gorecka et al., 2005) were performed daily in lactating mares following foal heat and in non-lactating mares starting from the seven days after the preceding ovulation until the next ovulation for oestrous cycle follow-up. In the study, bred by natural cover was performed every two days upon detection of ≥ 35 mm follicles in the ovaries of the mares until ovulation occurs. In all bred by natural cover procedures, two fertile KWPN stallions which were regularly tested for andrological condition (sperm density was at least 150x106, and motility was at least 70%) were used. The pregnancy examinations were performed with transrectal ultrasonography for the first time 14 days after ovulation in oestrous cycles. The mares which were found to be pregnant were examined again on days 24-27, 33-35, and 60 with the aim of monitoring pregnancy status, embryonic development, twin pregnancy, and embryonic death.

In the ultrasonography examination, pregnancies in which malformed embryonic pouch, echogenic illumination of liquid pouch, prolonged movement of the embryonic pouch and abnormal endometrial fluid collection by days were visualized and in which no heartbeat was heard after 30th day of the pregnancy were accepted as ED (Carnevale et al., 2000).

Ethics committee approval

The study was approved by Ondokuz Mayıs University Local Ethics Committee for Animal Experiments with approval number HADYEK/32.

Statistical Evaluation

The significance of the difference between the study groups was analysed with SPSS package programme (version 16.0) using basic statistical methods and appropriate test statistics (ANOVA, ChiSquare, Student T-Test, Wilcoxon Test, Mann Whitney U Test). The data were given as mean and standard error.

RESULTS

In Group I, it was identified that mean body weight (690.00±5.72 kg) was higher than Group (605.17±12.90kg) (P<0.001). In the mares which foaled, post-partum mean body weight loss was found as Following delivery, no statistical 87.38±1.26 kg. difference was detected between Groups I (649.78±5.56 kg) and Group II (631.33±9.85 kg) mean body weights. Although it was seen that the rate was approximate to mean body weights of the groups at the end of the breeding season (662.83±4.83; 676.75±13.11 kg), the measurements indicated body weight loss in some lactating mares, no body weight loss was found in non-lactating mares in any measurement. In the study, it was observed in lactating mares that at the beginning of breeding activity follow-up, BCS (7.44±0.24) decreased until second post-partum oestrous cycle (6.89±0.20) and increased again until the end of the breeding season (6.96±0.18). In non-lactating mares, it was observed that at the beginning of breeding activity mean BCS was (6.33±0.21) continuously increased until the end of breeding season (7.17±0,31) (Table 1).

Table 1. Body Condition Score profiles and reproductive outcomes of lactating (Group 1) and non-lactating (Group2) mares in various breeding seasons. LM: Lactating mares, NLM: Non-lactating mares.

lactating mares.			1
Results		Group I (LM)	Group II (NLM)
	Starting of Breeding Season	7.44±0.24	6.33±0.21
Body Condition Score profiles	2 nd Estrus Cycle	6.89±0.20	6.83±0.16
	End of breeding season	6.96±0.18	7.17±0.31
	Positive	50 (74.63 %)	21 (75.00 %)
Pregancy rates	Negative	17 (25.37 %)	7 (25.00 %)
	Number of overall mating cycles	67	28
Embryonic death rates $(P < 0.05)$		11 (25.58 %)	N/A
Localization of embryonic death	Same uterine horn	6 of 19	N/A
(P>0.05)	Opposite uterine horn	5 of 24	N/A
Distribution of embryonic deaths according to	Stallion A	7 of 36	
stallions used for mating	Stallion B	4 of 23	

In the study, it was found that the pregnancy rates were similar in Group I and II (74.63% and 75%). From these pregnancy rates that were recorded throughout four seasons, 2 abortions and 5 twin pregnancies seen in Group I and 1 abortion and 4 twin pregnancies seen in Group II were not included in the mean score in order for the data on ED not to change the normal distribution. In the study, while 25.58 % (11/43) ED was observed in the cycles following foal heat in Group I, no ED was observed in any of the 16 pregnancies in Group II. It was observed that of a total of 11 ED, 2 of them (18%) occurred on days 14-23, 4 of them (36%) occurred on days 24-27, 5 of them (46%) occurred on day 28-35

The ED was seen with a higher rate in case that a pregnancy occurs in the uterine horn where the preceding pregnancy developed a year ago in the lactating KWPN mares. In the study, from the factors that could influence the ED in lactating KWPN mares, the age of the mare, the stallion used in insemination, the months of breeding seasons and breeding season were found statistically insignificant.

DISCUSSION

It is reported that the rate of ED increase as the bodyweight decreases in the early periods of the pregnancy in mares (Ashworth, 1994; Newcombe, 2000). A continuous body weight loss was reported in the first two weeks of the lactation period and then increase after pregnancy and in mares (Heidler et al., 2004, Deichsel and Aurich, 2005). In the study, it was seen that the mean body weight in the lactating group was 14% higher compared with the non-lactating mares (P<0.001). It was reported that this difference resulted from the weight of mares and the amniotic fluid, which are the physiological outcomes of the

pregnancy (Brinsko et al., 2010). In the study, no statistical difference was detected between the mean body weight at the beginning and end of the breeding season in both groups while post-partum mean body weight loss was found as 87.38±1.26 kg. It was seen that these findings are similar to the findings by the researchers who state that lactation does not result in a significant body weight loss in the mares (Doreau et al 1988; Heidler et al., 2004). In the study by Newcombe and Wilson (2005) who reported the contrary, it was found that the ED rate in parallel with body weight loss in the lactating mares was higher (31.4% vs 15.4%) and this was attributed to the body weight changes that occurred. It was reported that this difference in the body weight gain in the lactating mares might be resulting from the energy gap due to lactation (Doreau et al., 1990).

In this study, it was concluded that the body weight loss detected in some lactating mares in line with the findings by Heidler et al. (2004) despite the diet prepared taking the reproductive status into the consideration and the lower rates of body weight increase in the lactating mares at the early pregnancy period, consistent with the findings by Newcombe and Wilson (2005), are the potential factors that could trigger ED.

Number of studies reported that the ED are increase rates in the mares during lactation (England, 1996; Van Niekerk and van Niekerk. 1998: Morris & Allen. 2002: Heidler et al., 2004; Newcombe and Wilson, 2005; Dirk, 2008), some studies reported the contrary are available as well (Hemberg et al., 2004 and Yang & Cho, 2007). In this study, while ED was seen 11 out of 43 pregnancy (25.58%) which conceived following the cycles after foal heat, no ED was seen in any of 16 non-lactating mares. This finding is in parallel with the studies revealing that ED rates are affected from lactation. A previous study (England, 1996) suggested that the ED rate in the mares in which the preceding pregnancy developed in the same horn a year ago is two folds of the pregnancies developed in a different uterine horns. Also, England (1996) attributes the high rate of ED in lactating mares to the adverse effect of a new pregnancy in the uterine horns where another pregnancy developed a year ago on the embryonic development. Newcombe (2000) also obtained similar findings. In this study, consistent with England (1996) and Newcombe (2000), it was observed that the rate of ED in the pregnancies developed in same uterine horn with the preceding pregnancy is 1.5 folds of the pregnancies that developed in a different uterus horn in lactating KWPN

Newcombe and Wilson (2005) associated the high level of ED in lactating mares with the negative impact of energy gap that arose out of lactation on body weight. Similarly, in this study, consistent with findings by Newcombe and Wilson (2005), while no body weight loss was observed in non-lactating KWPN mares, body weight was decreased in some lactating KWPN mares by the measurements performed during the breeding seasons. Additionally in this study, It was found that the rate of body weight increase between 0-60 days of pregnancy was lower in lactating KWPN mares compared to non-lactating mares.

Also, another significant finding in our study was ED occurred in 2 out of 4 mares (50%) which were bred and became pregnant at the end of lactational anoestrus period. This finding is in parallel with the study by Nagy et al.

(1998) who reported that ED rates are high in the mares which were bred and became pregnant in the cycles in which luteal activity lasted longer than normal. Although the findings of the studies by Hemberg et al. (2004) and Yang & Cho (2007) suggested that ED rates are lower in lactating mares compared with the non-lactating mares and revealed a large majority of the non-lactating mares experienced chronic infertility problems. Similarly, the study by Yang & Cho (2007) associated the higher rate of ED in non-lactating mares than the lactating mares with this reason. Besides, the studies which investigated the effect of lactation on the ED presented that embryonic deaths were not related to the age of the mares and any specific month of breeding season (Morris and Allen, 2002; Hemberg et al., 2004; Yang and Cho, 2007). Consistent with these findings, no statistically significant correlation was found between the age of mares, stallions used for mating, breeding season months and years in this study.

Although prostaglandin secretion varied according to the oestrous cycle stages in mares, the sensitivity of endometrial response to oxytocin and prostaglandin secretion capacity can be estimated. Oxytocin receptor concentration of mare endometrium reaches 25-30 % maximally during luteolysis (Starbuck et al., 1998; Sharp et al. 1997). Additionally, when bonded to the membrane receptors in combination with G proteins, oxytocin activates the prostaglandin synthesis (Gimpl and Fahrenholz, 2001). In this study, it can be concluded that uterus status, which is required for implantation due to impact of pulsatile oxytocin secretion of in lactating mares, is not sufficient under the negative influence of the prostaglandins secreted.

Consequently, it has been concluded that the decrease in the body weight and BCS in lactating KWPN and lower rates of body weight gains in the early period of pregnancy as a result of energy loss, conception at the end of lactational anoestrus and development of pregnancy in the same uterus horn where the preceding pregnancy developed previous year are the factors that contribute to the incidence of ED rates in lactating mares. Therefore, reproductive status should definitely be taken into consideration in the reproduction strategy approaches in KWPN mares

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Content

	Articles	Pages
1)	Evaluation of antifungal activity of nanobiosilver particles to treat Candida albicans releated urogenital infections in female rat model Bugrahan Bekir Yagci, İbrahim Mert Polat, İlknur Pir Yagci, Elif Bulut, Mustafa Turk	1
2)	Black Seed (Nigella Sativa) and Immunomodulatory Effect Serife Tutuncu	6
3)	Investigation of The Effects of Some Intrafollicular Growth Factors (GDF-9, GATA-4, GATA-6, IGF-I, IGF-II) on Etiopathogenesis of Cystic Follicular Ovarian Degenerations in Cows İbrahim Mert POLAT, Mehmet Rıfat Vural.	10
4)	Investigation of Systemic Toxic Effects of Nanobiosilver Use in Rodent Models Yasemin Ozdemir, Husamettin Ekici, Bugrahan Bekir Yagci	16
5)	The Effects of Lactation and Body Condition Score Changes on Embryonic Death Rates in KWPN Mares	
	Serhan Durmaz, İbrahim Mert Polat, Ömer Korkmaz, İlknur Pir Yagci, Taha Burak Elifoglu, Eser Akal, Muzaffer Celebi	21