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The Impact of Cytopathogenic and Non-Cytopathogenic Biotypes of Bovine Viral Diarrhea Virus on Total Antioxidant Capacity of Bovine Oocytes *In-Vitro*

Amirmahdi Roshanzamir[1], Massoud Talebkhan Garoussi[1*], Jalil Mehrzad[2]

[1] Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.

[2] Section of Immunology, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Abstract

Background :Bovine Viral Diarrhea Virus (BVDV) is the most significant pathogen in dairy cattle herds.

Objectives: The objective of this research was to examine the impact of cytopathic (CP) and non-cytopathic (NCP) biotypes of BVDV on the total antioxidant capacity of bovine oocytes *in vitro*.

Materials & Methods: Oocytes were obtained from slaughtered bovine ovaries, washed, and matured in maturation medium. The oocytes were divided into five distinct groups, each consisting of at least 60 oocytes. The control group was not exposed to any BVDV biotypes. Oocytes were challenged with cytopathic (CP) and non-cytopathic (NCP) BVDV at two distinct concentrations of 10^4 and 10^5 tissue culture infectious doses (TCID) 50/mL. To determine the antioxidant capacity of oocytes, a Total Antioxidant Capacity Assay was conducted after two hours of incubation. Graph Pad Prism 8.4.3 was utilized to analyze the data. A one-way ANOVA was performed, accompanied by post-hoc Tukey's HSD test.

Results: Results indicated that only the CP biotype of BVDV significantly decreased the total antioxidant capacity of infected oocytes when compared to the control group whereas the NCP biotype did not significantly alter the total antioxidant of infected groups.

Conclusion: Our study showed that only CP BVDV in 10^4 and 10^5 $(TCID)_{50}$/mL doses had an effect on the infected oocytes and decreased the oocyte's total antioxidant capacity significantly while the NCP biotype did not significantly alter the infected oocyte's total antioxidant capacity.

Keywords: Bovine viral diarrhea virus, Cytopathic, Non-cytopathic, Oocyte,Total Antioxidant Capacity Assay.

Introduction

Bovine viral diarrhea virus (BVDV) is a highly consequential pathogen in the dairy cattle industry, accountable for a diverse array of clinical manifestations spanning from subclinical, moderate infections to severe, acute disease (Abdelsalam *et al.*, 2020). BVDV belongs to the Pestivirus genus of the Flaviviridae family, which is characterized by its single-stranded RNA genome, enveloped structure, and the presence of two biotypes: non-cytopathogenic (NCP) and cytopathogenic (CP) depending on their effect on cultured cells. The NCP biotype is prevalent and often leads to transient infections, while the CP biotype is associated with fatal mucosal disease (Dabiri *et al*., 2021, Talebkhan Garoussi et al., 2011). Transmission occurs through direct contact with infected animals or fomites, with persistently infected (PI) calves playing a critical role as viral reservoirs. The economic effect of BVDV is profound, stemming from reduced reproductive performance, increased mortality and immunosuppression which exacerbates secondary infections (Veysi and Hajibemani 2020). Control measures include vaccination, biosecurity, and the identification and culling of PI animals. Despite these efforts, BVDV remains a pervasive challenge in terms of its extensive host range and genetic diversity (Meyer *et al*., 2021).

The effect of BVDV on the reproductive tract and also mammary glands of cattle is a critical concern in veterinary medicine and animal husbandry. BVDV is known to cause a range of reproductive disorders, including infertility, abortions, and congenital abnormalities. The virus

can cross the placental barrier, leading to fetal infection, which can result in the birth of PI calves (Oguejiofor *et al*., 2019). These PI calves often appear normal at birth but can shed the virus throughout their lives, serving as a source of infection for the rest of the herd (Garoussi *et al*., 2019). The effect of virus on the reproductive tract can manifest in the form of ovarian dysfunction, decreased sperm quality, and disruption of the estrous cycle. Moreover, BVDV was associated with the development of uterine diseases, such as endometritis, which further impairs reproductive performance (Oguejiofor *et al*., 2019). The economic implications of these reproductive challenges are substantial, as they can lead to decreased calving rates, increased culling of affected animals, and overall reduced productivity of the herd.

BVDV has a significant effect on the quality of bovine oocytes, which is crucial for the reproductive success of cattle. Studies showed that BVDV infection can lead to a decrease in the developmental competence of oocytes, affecting their ability to be fertilized and develop into viable embryos (Pinto *et al*., 2017). The virus can cause direct cytopathogenic effects on the oocytes or indirectly affect them by altering the ovarian follicular environment. This can result in a reduced number of oocytes retrieved during ovum pick-up procedures and a lower proportion of oocytes reaching maturity (Kagawa *et al*., 2022). Moreover, the presence of BVDV in the follicular fluid was associated with alterations in the levels of critical growth factors and cytokines that are essential for oocyte maturation (Wang and Pang 2024).

Oxidative stress occurs when the cellular antioxidant defenses fail to counterbalance the production of Reactive Oxygen Species (ROS), which has the potential to cause injury to the cell. Overproduction of ROS, which are chemically reactive molecules involved in cell signaling

and homeostasis, can be detrimental to DNA, lipids, proteins, and cell structures (Dayal *et al*., 2014). Total Antioxidant Capacity (TAC) is an indicator of the cell's capability to counteract ROS, as antioxidants inhibit the progression of oxidative damage (Asadi *et al*., 2017). Viral infections, including those caused by BVDV, can disrupt this delicate balance, leading to increased oxidative stress. This can result in the overproduction of ROS. If there were no adequately counteracted by antioxidants, the cellular damage can be induced and affect various physiological processes (Rajput *et al*., 2014). It is imperative to comprehend the intricate relationship among oxidative stress, TAC, and viral infections in order to devise efficacious therapeutic approaches for the management of viral diseases. BVDV is known to induce oxidative stress in various cell types, and, probably, bovine oocytes are similarly affected (Al-Kubati *et al*., 2021). An imbalance between the generation of ROS and the antioxidant defenses of bovine oocytes has the potential to impair normal cellular activities, including those essential for successful fertilization and early embryonic development (Rajput *et al*., 2014). Understanding how BVDV affects oocytes' levels of oxidative stress is crucial because of their critical function in reproduction.

The purpose of this work is to examine how BVDV affects the overall antioxidant capacity of infected bovine oocytes.

Material and Methods

Bovine ovaries were acquired from a commercial slaughterhouse in the suburban areas of Tehran and Alborz provinces immediately after slaughter and were submerged in phosphate-buffered saline (PBS). Thereafter ovaries were transferred to the laboratory in one hour, and subjected to

two more washes in fresh PBS containing streptomycin at a concentration of 100 mg/mL, penicillin at a concentration of 100 IU/mL, and fungizone at a concentration of 25 µg/mL. Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles measuring 3-6 mm in diameter using an 18-gauge needle connected to a 10 mL syringe. Before starting any experimental procedures, follicular fluid extracted from each batch of ovaries was regularly tested using the Garoussi and Mehrzads' methodology for the presence of BVDV antigen using Polymerase Chain Reaction (PCR) (Garoussi and Mehrzad 2011).

Washing Oocytes

Before the in vitro maturation of oocytes, they were subjected to a washing procedure based on the Stringfellow method (Stringfellow *et al*., 1997). This method involved ten cycles of washing. Initially, the oocytes were grouped into sets of ten and placed within one-milliliter droplets of a washing medium. Subsequently, they underwent a single wash in an antiseptic solution for 90 seconds. Per the protocol, the washing medium contains 100 mL of PBS, 4 g of Bovine Serum Albumin (BSA), 10000 IU of Penicillin G Potassium, and 10 mg of Streptomycin sulfate. Also, the antiseptic solution contains 100 mL of Hank's Balanced Salt Solution (HBSS), 0.4 mL Trypsin-EDTA (0.25%), Phenol red, 10000 IU Penicillin G Potassium, and 10 mg Streptomycin sulfate.

In Vitro Maturation (IVM)

Using the established procedures outlined by Garoussi and Mehrzad (Garoussi and Mehrzad 2011), bovine oocytes were matured *in vitro*. Oocytes were immersed in a maturation media and

repeatedly washed, surrounded by layers of dense follicular cells. Tissue culture medium-199 (TCM-199) supplemented with Earle's salts, 10% BVDV-free fetal bovine serum (FBS), 1 µg/mL estradiol, 60 µg/mL Folltropin, 2 IU/mL HCG, 50 ng/mL EGF, 0.4 mM glutamine, 0.2 mM sodium pyruvate, and 50 µg/mL gentamicin made up the maturation medium. Four-well culture containers containing 700 µL of the maturation medium were used to culture the oocytes in a controlled incubator. The incubation conditions consisted of 38.5°C temperature, 5% carbon dioxide (CO2) concentration, and 100% humidity for 24 hours. After maturation, the cumulus cells were removed from the oocytes by vortexing them for 90 seconds in 2 mL of minimal essential medium (MEM) containing 2% BVDV-free Fetal Calf Serum (FCS).

Virus Culture and Preparation

The Madin-Darby bovine kidney (MDBK) cell line was used for the Bovine Viral Diarrhea Virus (BVDV) laboratory culture. The CP and NCP biotypes of the BVDV were obtained from the Department of Virology, Faculty of Veterinary Medicine, University of Tehran, and the Virology Department of Razi Vaccine and Serum Research Institute, respectively. Initially, MDBK cells were cultured in Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum. Adherent cells were subcultured after reaching 70-80% confluence. The virus was propagated in MDBK cells and frozen at -70°C. After eight passages, the presence of the virus was confirmed using reverse transcription-polymerase chain reaction (RT-PCR). The virus was then frozen and titrated using the Reed-Muench formula (Reed and Muench 1938).

Experimental Groups

The experimental groups in this study were established based on CP and NCP biotypes of the BVD virus, with two different viral doses (10^5 and 10^4) expressed as 50% tissue culture infectious dose ($TCID_{50}$/mL), as described by Garoussi and Mehrzad (Garoussi and Mehrzad 2011).

The oocytes were divided into five distinct groups, each consisting of at least 60 oocytes, and replicated three times. The control group was not exposed to any BVDV biotypes. The second and third groups were infected with 10^4 and 10^5 $TCID_{50}$/mL of NCP BVDV, respectively. The fourth and fifth groups were infected with 10^4 and 10^5 $TCID_{50}$/mL of CP BVDV, respectively. The duration of infection in each group was set for 2 hours.

Total Antioxidant Capacity Assay (TAC)

To assess the samples' overall antioxidant capacity with and without extract, the TAC test was used. TAC test was performed using the Kiazist™ Total Antioxidant Capacity Assay kit. This experiment's concept and methodology include the reduction of Fe+2 ions. The process produced a dye when the chromogen 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was used as an appropriate substrate for the peroxidase enzyme. The absorbance of light at 593 nm was measured, and the Optical Density (OD) was recorded. A higher antioxidant power in the sample indicates the increased involvement of the chromogen in the reactions, resulting in higher OD values. An optimization process was performed to quantify the antioxidant levels in the samples, and a standardized method was employed for reporting the results. Utilizing a standard Working solution, light absorption was measured. In order to conduct the assay, five microliters of the sample were added to a 96-well plate, while five microliters of PBS were added to the wells

serving as the control group. Afterward, 250 µl of the working solution was added to each well. The plate was incubated for approximately 5 minutes at ambient temperature. The concentration of Fe^{+2}, representing the antioxidant capacity and the presence or absence of antioxidants in the samples, was determined and reported as µM Fe^{+2} /L.

Statistical Analysis

The results were analyzed using Graph Pad Prism version 8.4.3. One-way ANOVA with post-hoc Tukey's HSD test was used to determine the statistical significance between groups. A P value of ≤ 0.05 was considered significant. The results were presented as means ± SD.

Results

Total Antioxidant Capacity Assay (TAC)

Table 1 displays the effect of several biotypes of BVDV on oocytes. The control group's Total Antioxidant Capacity Assay (TAC) was 659.3±50.44. TAC levels were similar in the control group and 10^4 $TCID_{50}$/mL, and 10^5 $TCID_{50}$/mL non-cytopathic (NCP) groups, with no significant differences among them ($P>0.05$). Nevertheless, a significant decrease in TAC was seen between 10^4 $TCID_{50}$/mL, and 10^5 $TCID_{50}$/mL CP groups compared to the control group ($P\leq0.05$) Table 1).

Table 1. The effect of CP and NCP BVDV on the total antioxidant capacity of bovine oocytes

Control	10^4 NCP	10^5 NCP	10^4 CP	10^5 CP

659.3±50.44[a]	582.3±48.25[a]	640.7±47.54[a]	488.1±40.25[b]	447.6±20.79[b]

[a,b] symbols on line with different alphabetic letters are significantly different.

Discussion

The current study investigated how CP and NCP BVDV biotypes affected the overall antioxidant capacity of bovine oocytes in vitro. We measured the TAC of bovine oocytes following BVDV infection to assess their overall antioxidant response, focusing on endogenous mechanisms rather than the effects of exogenous antioxidants. TAC was chosen because it is an excellent representative of the capacity to combat oxidative stress, and it provides a comprehensive measure of both enzymatic and non-enzymatic antioxidants within the cells and here oocytes, reflecting their natural defense mechanisms against oxidative stress (Ghorbanian *et al*., . 2017) (Lira Ferrari and Bucalen Ferrari., 2011). While specific markers like Malondialdehyde (MDA) could offer additional insights into oxidative damage, we focused on TAC as it effectively gauges the oocytes' capacity to counteract oxidative stress. This research showed that although the NCP biotype did not have a comparable impact, varying amounts of CP BVDV might influence the overall antioxidant capacity of bovine oocytes. In brief, both doses of the CP biotype decreased the total antioxidant capacity of the infected oocytes significantly (Table 1).

The complex interaction between the body's antioxidant defense systems and the production of ROS gives birth to the complex phenomena known as oxidative stress (Bohm *et al*., 2023). ROS are chemicals that are produced naturally during cellular metabolism, such as mitochondrial respiration, enzymatic reactions, and inflammation. While ROS serve as important signaling

molecules and contribute to normal cellular functions, their excessive production or impaired elimination can lead to oxidative stress (Szarka *et al.*, 2022).

ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, have the potential to damage cellular components in terms of their reactivity (Katakwar *et al.*, 2016). Nucleic acids, proteins, and lipids are especially susceptible to oxidative damage. Amino acid residues within proteins are susceptible to oxidation, which can result in structural and functional changes. The oxidation of lipids can lead to the production of reactive products, including lipid peroxides, which have the ability to disrupt the integrity and function of cell membranes (Gaschler and Stockwell 2017). Oxidative damage to DNA can result in mutations and the alteration of nucleotide bases and DNA strand breaks, both of which are detrimental to genetic integrity (Rahimian *et al.*, 2020). As an elaborate antioxidant defense mechanism, cells have developed to counteract the harmful effects of ROS. There are both enzymatic and non-enzymatic components in this system (Khazaei and Aghaz., 2017). Enzymatic antioxidants, including catalase, superoxide dismutase, and glutathione peroxidase, collaborate in order to catalyze the conversion of ROS into less deleterious forms. Vitamins C and E, glutathione, and a variety of phytochemicals are non-enzymatic antioxidants that function as scavengers of ROS by donating electrons to stabilize and neutralize free radicals (Gupta *et al.*, 2021). However, under conditions of increased ROS production, such as during exposure to the environmental toxins, chronic inflammation, or viral infections, the antioxidant defense system can become overwhelmed. This results in oxidative stress due to an imbalance between ROS production and antioxidant capacity (Alfadda and Sallam., 2012). A cascade of detrimental events, including the dysregulation of

cellular processes and the activation of stress signaling pathways, can be initiated by oxidative stress.

The current investigation demonstrated that CP BVDV had a substantial impact on the overall antioxidant capacity of bovine oocytes. Recent research provided valuable insights into the effect of BVDV infection on oxidative stress and antioxidant capacity in animal cells. A study done by Schweizer found that CP BVDV induces apoptosis in cultured cells through oxidative stress, caspase activation, and DNA fragmentation. This process is characterized by an increase in ROS levels early in apoptosis, preceding caspase activation and DNA fragmentation. Certain antioxidants, such as butylated hydroxyanisole and ebselen, were effective in protecting cells from apoptosis induced by the CP biotype of BVDV. On the other hand, antioxidants like N-acetylcysteine, pyrrolidine dithiocarbamate, lipoic acid, dihydrolipoic acid, and tiron were found to be ineffective in preventing apoptosis. These findings suggest that oxidative stress plays a crucial role in the apoptotic cell death induced by the CP biotype of BVDV (Schweizer and Peterhans., 1999). A study done by Villalba et al., . presents responses to CP BVDV infection in MDBK cells. Notably, the study observed a downregulation of genes involved in reactive oxygen species metabolism suggesting increased oxidative stress (Villalba et al., 2016). It was demonstrated that BVDV infection interferes with cellular metabolic processes, increasing the generation of ROS, hence causing oxidative stress, which aligns with the current study (Liu *et al*., 2018). Furthermore, the excessive production of ROS triggered by BVDV infection can initiate intracellular signaling pathways that mediate various cellular responses, including inflammation, and programmed cell death (Zhou *et al.,* 2017).

Moreover, BVDV infection in cattle has been linked to several clinical symptoms, such as reproductive problems, respiratory diseases, and immunosuppression. The observed consequences may be associated with the oxidative stress caused by BVDV since oxidative stress has been involved in these symptoms (Pinior and Köfer., 2016). Recent research provided new insights into the involvement of BVDV in initiating mitophagy, a specific kind of autophagy that targets the breakdown of impaired or harmed mitochondria. Since mitochondria are essential for controlling ROS generation and antioxidant defense mechanisms, the activation of mitophagy by BVDV infection may affect the cellular response to oxidative stress (Li *et al*., 2024). Oxidative stress was recognized as a critical factor in reproductive biology. In the context of oocyte quality and embryo development, oxidative stress can have profound consequences (Jiang *et al*., 2021). Oocytes, being highly metabolically active cells, are particularly vulnerable to oxidative damage in terms of their limited antioxidant capacity. Consequently, any disruption in the delicate balance between ROS production and antioxidant defense mechanisms can significantly impact oocyte function, and subsequent embryo development (Barrozo *et al*., 2021).

Antioxidants, such as superoxide dismutase, catalase, glutathione, and vitamin E, play crucial roles in neutralizing ROS and protecting against oxidative damage (Dontha 2016). However, BVDV infection disrupts the antioxidant defense system, leading to a diminished ability of oocytes to scavenge ROS effectively. This compromised antioxidant capacity further exacerbates oxidative stress within oocytes, amplifying the detrimental effects on oocyte quality and reproductive success (Altamiranda *et al*., 2013).

Conclusion

To conclude, only the CP biotype of BVDV can alter the total antioxidant capacity of the infected oocytes significantly, but the NCP biotype did not have such an effect. The current study demonstrated that the BVDV's CP biotype reduced the overall antioxidant capacity of infected oocytes as compared to the control group. However, further basic research is needed to determine the actual processes behind the loss of total antioxidant capacity in BVDV-infected oocytes.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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تأثیر بایوتایپ های سایتوپاتیک و غیر سایتوپاتیک ویروس اسهال ویروسی گاوان بر ظرفیت آنتی اکسیدانی تام تخمک های گاو شیری در شرایط آزمایشگاهی

امیرمهدی روشن ضمیر[1]، مسعود طالب خان گروسی[1*]، جلیل مهرزاد[2]

[1]گروه مامایی و بیماری های تولید مثل، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران

[2]بخش ایمونولوژی، گروه ایمونولوژی و میکروبیولوژی، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران

چکیده

پیشینه : ویروس اسهال ویروسی گاوی (BVDV) مهم ترین پاتوژن در گله گاوهای شیری است.

اهداف: هدف از این تحقیق بررسی تاثیر بیوتیپ های سیتوپاتیک (CP) و غیر سیتوپاتیک (NCP) ویروس BVD بر ظرفیت آنتی اکسیدانی تام تخمک های گاو در شرایط آزمایشگاهی بود.

مواد و روش کار: اووسایت ها از تخمدان گاو های کشتار شده اخذ گردیده و سپس شستشو داده شده و در محیط کشت بلوغ بالغ گشتند. تخمک ها به پنج گروه مجزا تقسیم شدند که هر گروه شامل حداقل 60 تخمک بود. گروه کنترل با هیچ بایوتایپ ویروس BVD آلوده نگردید. تخمک ها در گروه آزمایش با دو بایوتایپ CP و NCP در دو غلظت عفونی کشت بافت (TCID) 50/ml

مختلف (10^4 و 10^5) آلوده شدند. برای تعیین ظرفیت آنتی اکسیدانی تخمک ها، سنجش ظرفیت آنتی اکسیدانی کل پس از دو ساعت انکوباسیون انجام شد. برای تجزیه و تحلیل داده ها از Graph Pad Prism 8.4.3 استفاده شد. یک آنالیز واریانس یک طرفه همراه با تست HSD توکی انجام شد.

نتایج: نتایج نشان داد که تنها بایوتایپ CP از BVDV به طور قابل توجهی ظرفیت آنتی اکسیدانی تام تخمک های آلوده را در مقایسه با گروه کنترل کاهش داد در حالی که بایوتایپ NCP چنین تاثیری را نداشت.

نتیجه گیری نهایی: مطالعه ما نشان داد که تنها CP BVDV در دوزهای 10^4 و 10^5 $(TCID)_{50}/mL$ بر روی تخمک های آلوده تأثیر داشت و ظرفیت آنتی اکسیدانی کل تخمک را به طور قابل توجهی کاهش داد در حالی که بایوتایپ NCP چنین تاثیری را بر روی تخمک ها نداشت

کلمات کلیدی: اسهال ویروسی گاوان، ظرفیت آنتی اکسیدانی تام، تخمک، سایتوپاتیک، غیر سایتوپاتیک.