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In vitro 3D Spheroid Wound Modeling: An Alternative to Experimental Animal Studies

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ABSTRACT

Laboratory animals have frequently been used in scientific and preclinical pharmaceutical drug safety and efficacy research. Although the introduction of new in silico assays and computer modeling for drug discovery has shown promise in reducing laboratory animal trials, there is still a need to develop *in vitro* alternatives to in vivo animal models. The *in vitro* spheroid wound model is one of the best options for developing alternative techniques to animal research as it is the most widely used laboratory animal model. The aim of the study is to using 3D *in vitro* wound modeling as an alternative to in vivo wound healing assays. In the study, a three-dimensional cell culture (organoid culture) with cell/cell and cell/matrix junctions was generated using the most common Fibroblast and HaCaT cell lines hanging drop technique to replicate the healing stages in the injured skin area. After spheroid epidermal structures were formed, inhibitors and activators were added to the culture medium and their effects on the wound line and 3D cells produced were determined. It was noted that the number of spheroid structures increased significantly and cell-cell interactions became visible in the additional activator groups compared to the control groups. When the inhibitor-treated group was compared with the control groups, it was observed that the formed structures completely disappeared or decreased in amount and cell-cell interactions could not be established**.** In conclusion, this study offers an alternative to using laboratory animals to evaluate potential medicines and/or extracts in wound healing experiments.

INTRODUCTION

The wound is a phenomenon that will never lose its importance as long as life exists, and the efforts to understand healing and regeneration mechanisms have been grown out of a long-held fascination and remain current. The wound is the breakdown of living tissue's anatomical and functional integrity (Robson et al., 2001). Wound formation can be superficial or deep, depending on the degree of the force applied to the tissue (Ekizoğlu and Arican, 2009). Continuous therapy research and innovative applications for wound and wound healing have been conducted utilizing various materials, methodologies, and animal models. In vivo and in vitro modeling are the most preferred ways for wound creation and healing modeling. In vivo modeling can be defined as the designation of the experimental mechanism for the processing or development process of some physiological or pathological phenomena using biological similarities between animals and humans (Kaya and Çevik, 2011). The animals used in animal experiments are generally mice, rats, guinea pigs, rabbits, cats, dogs, chickens, sheep, and pigs. The most preferred of these are mice, rats, and guinea pigs. In vitro is a term that means in the laboratory or artificial conditions, and in vitro modeling is created outside of the living organism in a controlled environment, usually using Petri dishes, flasks, or test tubes. In vitro wound modeling involves cell and tissue cultures as with 3D matrices and is often used to assay intercellular interactions and intracellular signal transduction (Jing et al., 2014). Fibroblasts, endothelium and keratinocytes are the most successful cells in wound healing, making them the most widely used cell types in in vitro wound modeling. In these models, the cells are employed separately or in combination (Inan and Duman, 2020). The

wound healing phenomena in experimental animals, which is frequently utilized in vivo modeling for wound formation and repair, differs from that in humans. Contrarily, human wound healing is typified by the production of granulation tissue, whereas experimental animals rarely experience this type of healing; instead, they have contraction. Pigs are the most closely related experimental animal model to human physiology, although they are not usually chosen because of the challenges associated with study design, care, and application. Since the healing process after wound formation is a cellular phenomenon, the proliferation of cells active in this process, cell-cell interactions, intercellular signal pathways, cell-matrix interactions, and the activities of cells in the region are evaluated. In vitro wound healing modeling allows the observation of cellular activities and gives a great advantage to see which cells play an active role directly in the region. In vitro experiment designs prevent ethical concerns in vivo studies and animal use is reduced by acting in the light of human experimental technique principles (4R Principles).

In this study, it is aimed to construct a 3D skin matrice and to make it a valid in vitro healing model by testing the effects of well-known healing inhibitor/activator compounds. It is envisaged that this model can be conveniently used in further wound experiments, without the need for in-vivo experiments.

MATERIALS AND METHODS

Fibroblast and keratinocyte cell lines were chosen to create 3D spheroid forms because they represent wound healing and complete skin regeneration stages, respectively. Cell lines L929 (NCTC clone 929) and Hacat (CLS; 300493) were obtained from the cell culture archive of The Center for Application and Research of Scientific and Technological Researches (KÜBTUAM) at Kırıkkale University. All cell culture work was performed in the Class IIB biosafety cabinet in accordance with biosafety regulations (Fig. 1). For the cultivation of the cells; DMEM (Dulbecco's Modified Eagle's Medium, 4.5 g/L Glucose, w/ Sodium Pyruvate, w/out L- Glutamine), 10% polyline-streptomycin, 1% penicillin-streptomycin were used. Briefly, 5ml culture medium was added, and following the necessary warming to 37ºC in an incubator, Thawed cells were washed in PBS and following centrifugation, the cell pellet was resuspended as being their concentrations of 10^6/ml each cell culture suspension. Each fibroblast and skin epithelia cell was cultured indivudually at 37°C 5% CO_2 and they were trypsinized when their confluence reached 80%. Matrigel (Corning Matrigel matrix) and platelet-rich plasma were used to create 3D cytoskeletons and replicate the intercellular matrix of the skin. PRP was prepared as follows; 10 ml total blood was collected by venipuncture and the frozen cells were transferred to the PRP kit tube (Rich Cell Prp Kit 15cc; PRP15/Lot:316/2102-01) and centrifuged at 570g -7 minutes according to the kit instructions. The remaining platelet-rich plasma was collected into a separate falcon tube using a syringe with a 21G needle. Then 2 ml solution of 0.06 mM CaCl2 2H2O and 2% penicillin/streptomycin were added. The gel was kept at room temperature for two hours for gel formation. Trentilin Ampoule (active ingredient: Pentoxyfilin 100mg/5ml) was used by suspension at a rate of 10µg/ml to increase cellular activity during wound healing. As an inhibitor, 6% hydrogen peroxide was used at a rate of 0.4µM/L. In the study, spheroids were prepared using both matrigel and PRP and were allocated into three groups each as follows; control group, activator group, and inhibitor group.

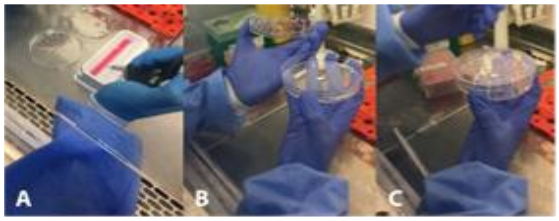

Figure 1. Stages of creating spheroids with the hanging drop technique**.** A) Transfer from cell suspension to petri dish for hanging drop technique. B) Inverting the petri dish in which the cell suspension was transferred. C) View of the petri dish after the formation of hanging drops

PRP–Fibroblast Group

Previously frozen fibroblast(L929) cells were thawed and transferred into a T-75 flask and the study was initiated. The culture medium was changed and the transplanted cells were passaged until they covered 80% of the flask surface. Fibroblast cells reaching 80% growth were trypsinized and separated from the flask surface. 15ml was taken into a flask and centrifuged. The medium formed at the upper end was poured off and the cell pellet at the bottom was diluted with 1 ml medium. To determine the cell viability, cells were stained with trypan blue and live cells were counted. The result was found to be 4.5x106. Fibroblast cells (L929) were suspended 100µl in 24-well plates at 40,000 cells per well, 100µl was spilled into the bottom of the first six wells and the plate was placed in an oven (4% CO2 at 37°C for 2 hours). After removing the plate from the oven, 100µL of PRP in gel form was added to the fibroblasts in the first two wells of the plate and 100µL of PRP with 10µg/ml Trentilin was added to the fibroblasts in the next 2 wells and the surface was covered. The third two wells were closed by adding 100µL of PRP to which 0.4µM/L hydrogen peroxide was added and after this process, the study was terminated by adding another 100µL of fibroblast cell suspension to all wells. After the procedure, fibroblasts were examined under a microscope and photographed and recorded at 0, 24, 48, and 72 hours. The spheroid diameters were measured with the 'Image J' program and recorded and graphed in Microsoft Excel. After the procedure, fibroblasts were examined under a microscope and photographed and recorded at 0, 24, 48, and 72 hours (Fig. 2). The resulting spheroid diameters were measured with the 'Image J' program and recorded and graphed in Microsoft Excel (Fig. 3).

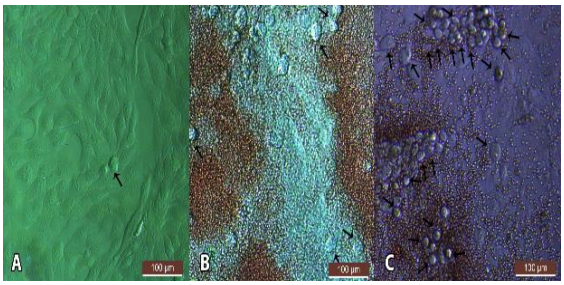

Figure 2. PRP – Fibroblast (L929) Group Spheroid formation Inverted Microscope Image (100 µm) Indicator group B) Control group C) Activator group

Figure 3. Images of measurement in 'Image J'

Matrigel - Fibroblast Group

Previously frozen fibroblast(L929) cells were thawed and transferred to a T-75 flask and the study was started. The culture medium was changed and passaged until the transplanted cells covered 80% of the flask surface. Fibroblast cells reaching 80% growth were trypsinized and separated from the flask surface. After staining with trypan blue, viability determinations, and cell counts were performed. The result was found to be 3.9 x 10^6. After cell counting, the cell suspension was diluted to 1.25 x 105 in 20 µL with the addition of a culture medium. Using the resulting cell suspension, 20 µL droplets were added to the lid of a 10 cm diameter sterile petri dish to form spheroids by the hanging droplet method. 5ml PBS was added to the petri dish bottom to maintain ambient humidity during the propagation of the cell clusters. The lid was quickly inverted and closed without deteriorating the drop structure and left to reproduce for 72 hours in a study with 4% CO_2 at 37°C. For this, the matrigel matrix at -20 ºC was replaced to +4°C the night before. The pipette tips to be used before the procedure were cooled sterile at -20 ºC for 10 minutes and all procedures with the matrigel matrix were performed on an ice battery. The wells to be used on the plate with 24 wells were marked and placed on the base with a 100 µL matrix pipette, and the plate 37 ºC was removed and waited for 30min. Thus, the matrix was gelled, which is found in liquid form in a cold environment. At the end of 30 minutes, the spheroids in the Petri dishes we had prepared before were taken with a 21G needle tip and placed in the middle of the gelled matrigels, taking care not to form bubbles. The second spheroid was added just above the previous spheroid following the same steps as before. Added to the control wells; 500µL of Trentilin Ampoule (Trentilin Ampoule: Pentoxyfilin 100mg/5ml) suspended with 10µg/ml standard culture medium. Added to the migration activation group 500µL of %6 H2O2 suspended at 0.4µM/L was added to the migration inhibition group. The areas of the spheroid structures formed were photographed during the 0, 24, 48, and 72 hours (Fig. 4). The photographs taken were measured and graphed with the 'Image J' program (Fig. 3).

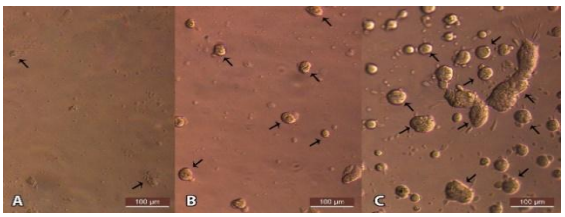

Figure 4. Matrigel–Fibroblast (L929) Control Group Spheroid formation Inverted Microscope Image (100 µm) A) Indicator group B) Control group C) Activator group

PRP–Fibroblast–HaCaT Group

Previously frozen HaCaT and fibroblast (L929) cells were dissolved in a hot water bath and cultured in T-75 flasks. Cells that reached 80% proliferation were trypsinized, and cell counts were 3.6 x 10^6 for HaCaT; and 4.9x10^6 for fibroblast (L929). L929 fibroblast cells and HaCaT cells were previously passaged and were dissolved and transferred into T-75 flasks and the process started. The stock cells at -80ºC were removed and dissolved in 37ºC water bath. The previous cell transfer procedure was followed, and nd suspended when they reached 80% proliferation; the viability of the cells was measured. Trypan blue was used for vitality measurement and cell count. 10µl of HaCaT cell suspension and 10µl of trypan blue were taken into an Eppendorf tube. 10µl were taken from the new suspension and added to the 'A' compartment of the cell, counting slide and counting with a hemocytometer (3.6 x 10^6). At the same procedure was performed with the L929 fibroblast cell line (4.9 x 10^6). For 24 well plates, new cell suspensions were prepared with 40,000 cells per well, and 100µL of the fibroblast suspension was added to the desired wells in the plate, and the plate was incubated at 37 ºC for 2 hours. The cell surfaces were coated by adding 100 µL each of the previously used PRP Activator- inhibitor and control group. 100µL of HaCaT cell suspension was added. The diameters of the spheroids were evaluated by photographing the hours 0, 24, 48, and 72. Measurements were made with the 'Image J' program, graphed the results. The photographing and examination of the spheroid structures were carried out with an inverted microscope (Marka: Leica Model: DMI 4000 B) (Fig. 5). Spheroid density measurement and graphing in a microscopic field were performed with the " Image J " program (Fig. 3). The areas and environments of the spheroids were measured as quantitative evaluation criteria. The measurements were recorded in Microsoft Excel and compared (Fig. 6 and Fig. 7).

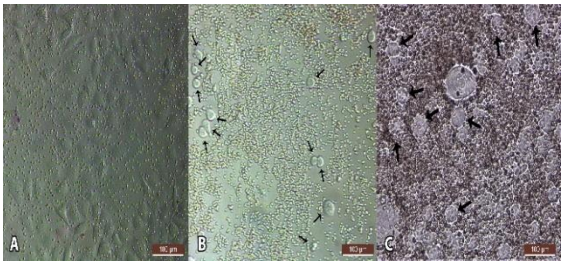

Figure 5. Fibroblast (L929)–PRP-HaCaT Control Group Spheroid formation Inverted Microscope Image. A) Indicator group B) Control group C) Activator group

GROUPS/AREA	24.hour	48.hour	72.hour
CONTROL GROUP PRP-FIBROBLAST	4492	7000	5532
CONTROL GROUP FIBROBLAST-MATRIGEL	12.699	21.118	18.076
CONTROL GROUP FIBROBLAST-PRP-HACAT	25.984	10384	24.864
ACTIVATOR PRP-FIBROBLAST	6956	23925	6643
ACTIVATOR FIBROBLAST- MATRIGEL	17.952	56.016	47.632
ACTIVATOR FIBROBLAST-PRP-HACAT	14.496	9.676	5.268
INDICATOR PRP-FIBROBLAST	4779	3968	0
INDICATOR FIBROBLAST- MATRIGEL	0	0	0
INDICATOR FIBROBLAST-PRP-HACAT	4.780	3.904	0

Figure 6. Area measurements were calculated and tabulated in micrometersquare with the 'Image j' program

GROUPS/ ENVIRONMENTAL MEASURE	24th hour	48th hour	72th hour
CONTROL GROUP PRP-FİBROBLAST	238.761	298.451	263.894
CONTROL GROUP FİBROBLAST-MATRİGEL	436.123	539.886	489.230
CONTROL GROUP FİBROBLAST-PRP-HACAT	571.770	361.283	559.203
ACTIVATOR PRP-FİBROBLAST	296.881	549.779	289.027
ACTIVATOR FİBROBLAST-MATRİGEL	543.035	930.224	858.649
ACTIVATOR FİBROBLAST-PRP-HACAT	427.257	348.717	259.181

Figure 7. Environmental measurements were calculated and tabulated in micrometers with the 'Image j' program

RESULTS

Microscopic examination showed that cell-cell complex structures formed spheroids in all three methods. It was also revealed whether the formed spheroid structures increased or not by using activators and inhibitors. Activator and inhibitor substances were selected from substances that promote and inhibit healing in the wound healing process. Spheroid structures (spherules) formed by the proliferation of cells and their interaction with each other were significantly increased and enlarged in the activator groups using the preparation that clinically activates wound healing. In the inhibitor groups, spheroid formation was seen in some groups, but in small numbers and in small shapes, while in some groups it was seen in very small structures or not seen because it disrupted the structure that would form a skeleton for the cells used and caused death in the cells. Since our aim was not to measure the drug effect, no further evaluation was made on this issue.

As seen in the microscopic examination, it was seen that activator and inhibitor substances that promote wound healing can increase or decrease the formation of cell-cell complex structures in 3D cell culture and increase or decrease migration to the wound site. The area and perimeter measurements of the spheroid structures formed in our study were made with 'Image J'.

The results were compared graphically. In this comparison, the results showed that spheroid formation occurred in the groups in which the gel form of PRP was used as a cell support network, but spheroid structures were not as large as in the groups in which matrigel was used as a cell support network. Although smaller spheroids were formed in the groups using PRP compared to the groups using matrigel, it has an advantage over matrigel, which is that spheroid structures are formed and prominent in the inhibitor groups in the groups using PRP.

In our study, fibroblast and keratinocyte cells, which are most effective in wound healing, were used and the spheroid structures formed by them were examined. The size and multiplicity of the spheroid structures formed give us information about the speed and formation of the migration phenomenon. PRP and Matrigel used in the study form a skeleton for the cells to form a complex structure. Matrigel fills the cavity of the colloagin and PRP contains platelets that accelerate cell migration to the wound area. The activator and inhibitor substances used are clinically used materials.

When the control groups were examined, it was observed that spheroid formations were present but not as large and prominent as in the matrigel control group. Another difference between the control groups was that the spheroid structures formed in the study with HaCat were more prominent and larger than the fibroblasts in the study with fibroblasts only. It was observed that the spheroids formed in the activator groups were small but numerous at the 72nd hour, while at the 24th and 48th hours, the formations had just started and larger structures were also observed. In the inhibitor group studies, no results could not be reached because the inhibitor we used in the matrigel group disrupted the matrigel form, but in studies with fibroblast and keratinocyte cells, spheroid formations were observed at 24 and 48 hours, but the spheroid structures formed were small structures. At 72 hours, no spheroid structures were observed and the structures formed were observed to be disrupted.

DISCUSSION AND CONCLUSION

Wound formation, the healing mechanism, and the quest for healing treatment approaches are among the most frequently investigated and experienced issues worldwide (Daunton et al., 2012). Wound modeling uses a variety of models, including in vivo, in silico, ex vivo, and in vitro (Ud-Din and Bayat, 2017).

In vivo modeling is the method of preference for studying complicated events in wound healing. Although in vivo investigations are favored, the skin structures of experimental animals do not demonstrate physiology in wound formation and healing in people because they are not similar to those in humans, and the cases that arise during the healing process differ from humans (Greenhalgh, 2005).

The group of rodents that are often studied are creatures because they are easy to produce and durable, partly because of the ease of modeling. The fact that wound healing in rodents is by contraction, which does not develop granulation tissue as in humans, as well as the different placement of skin add-on glands and layers, has a negative impact on the study's results (Dorsett-Martin, 2004) Its skin composition is the closest to that of a living pig. The dermis and epidermis are thick in humans and pigs, but thin in rodents. In rodents, wound healing is accomplished by contraction; in humans and pigs, it is accomplished by granulation and epithelialization. Although the architecture of human and pig hair follicles are similar, one feature that sets rodents apart from humans is the abundance of hair follicles. While apocrine sweat glands are confined to the udder in rodents, they are widely distributed throughout the human perineal and axillae (Dorsett-Martin, 2004; Sulivan et al., 2001; Wong et al., 2011). When Google Scholar searches based on the last 5 years, it is seen that there are 18,800 results under the name of 'wound healing mouse model', 19,200 results in the 'wound healing rat model' search, 18,200 results in the 'wound healing rabbit model' search and 17,700 results in the wound healing pig model search. The use of animals in studies brings ethical problems and causes undesirable situations to develop during studies. In the interventions performed during the study, the development of sepsis in animals, shock development during the intervention, or

interventions do not have the desired effects on the animal, and the intervention is completely tolerated by the animal (Kaya and Çevik, 2011). Situations such as the care and feeding of animals, the replacement of dead animals, and the repetition of work require extra time and extra-economic burden (Yeğen and Gören, 2005). Sauer and his friends, in their study, argued that animal experiments should be applied after all alternative methods have been tried. In experimental animal modeling, the inability to prevent the animal from being affected by environmental conditions, the fact that the drug applied in drug applications is metabolized first, and the full effect cannot be clearly demonstrated, the animals' reactions to the applications are restrictive factors in studies. In light of all this, the use and development of cell cultures reflect all the characteristics of human tissues, and cells have started to come to the fore in recent years. Considering the ethical concerns in animal studies and the Principles of Human Experimental Technique (4R Principle), the move of the experiments to cell culture eliminates these concerns. Cell culture studies dating back a long time are used in 2D to study the activities, structures, and development of cells in living tissue (Duval et al., 2017). Cell culture studies used in many different fields such as toxicological, pharmacological, microbiological, and oncological examinations and paving the way for important developments in living life are also used in wound modeling and the study of cellular activities developing in wound healing (Huh et al., 2011; Duval et al., 2017). Until recent years, these studies have been modeled in 2D cell culture, first causing mechanical damage to the cells covered in the base to characterize wound formation, and then looking at the migration of cells to this region, the cellular effects of drugs used or developed, whether they are toxic, and cellular responses to mechanical injuries (Liang et al., 2007; Jonkman et al., 2014). Although measurable results were obtained through these studies, it was concluded that 2D cell cultures are alternatives in terms of easy repeatability to animal experiments, control of environmental conditions, and economics, but are incomplete in reflecting the complex event developing in the in vivo environment (Mazzoleni et al., 2009). The fact that cell reproduction is limited to the media in the culture dish and the base in the culture container and the limited cell and cell ECM interaction is one of the limitations of 2D cell cultures in reflecting the organoid structure in life (Bonnier et al., 2015; Choi et al., 2010). As a result of the elimination of these limitations and the increase of studies on in vivo organoid structure and reactions, and as a result of developing research techniques, 3D cell cultures were started to be made (Duval et al., 2017). As a result of cell-cell and cell-ECM interactions in spheroid structures formed in 3D cell cultures, it has been observed that many cellular behaviors such as proliferation, polarization gene, and protein expressions provide traceability as in vivo conditions (Steinwechs et al., 2016; Marby et al., 2016). The basis of 3D cell culture is the secretion of structural proteins and molecules in vivo conditions of spheroids formed in an extracellular matrix-like structure (liquid, solid) (Weaver et al., 1997; Lin et al., 2008). In many studies using 3D cell cultures, 3D cell cultures were closer to in vivo cases than 2D cell cultures, and the results were close to the results of experimental animal studies (Polat, 2020). In light of these studies, this study was carried out to investigate whether 3D wound modeling can be performed and how the cells that play an active role in wound healing will react to the factors used in wound healing that accelerate healing. The culture of the cells most involved in wound healing in 3D in three different cell collections created using two different scaffoldings and the effect of substances that accelerate and inhibit wound healing were examined. The multiplicity and size of spheroid structures formed in the evaluation of the study groups were evaluated. Since the migration and proliferation of fibroblasts in wound healing were related to activation, the evaluation was accurately proportioned with the number and size of spheroids. The fact that while the number of spheroids was evaluated qualitatively, the area and environment measurements of the spheroid structures were evaluated quantitatively with the 'Image J' program (Fig. 8-13). The more spheroid structures formed or larger, the more fibroblast activity they had and were evaluated as migration. The PRP part of the blood used in this study increased the activation of fibroblast cells and acted as a skeleton for cell connections (Fig. 10). PRP has been shown in studies with positive effects on wound healing (Hudgens et al., 2016). Spheroid formation was observed in this 3D cell culture study, and fibroblast migration increased with the activator (Fig.11and Fig. 13). PRP- HaCaT- It was observed that the size and number of spheroid structures formed in the fibroblast group were more intense than in the study in the fibroblast –PRP group. It was thought that the source of this may be the formation of cross-bonds between the HaCat and fibroblast cells that allow scar formation (Ghahary and Ghaffari, 2007). The group where fibroblast migration is best evaluated and seen is the working group with matrigel (Fig. 12 and Fig. 13). Spheroid formation was seen in other groups, but it was not as pronounced in size and number as in the matrix. When scratch modeling was required to be performed for wound work and to see the migration on this line, the cells were collected at the edges due to the gel-shaped structures, and this study could not be done due to the deterioration of the gel form. The environment and areas of the spheroid structures formed in the study were calculated, and it was observed that the matrigel group also stood out compared to these (Fig. 6 and Fig. 7). In the case of migration in the wound, it was considered acceptable as a model used before experimental animal modeling to examine cell connections, cell-matrix connections, and fibroblast activation. Cell-cell connections that play an active role in wound healing and cell ECM can be used in the examination of both 2D and in vivo studies as an alternative study; since activators and inhibitory substances to be used in wound healing can be used as preferable modeling in the study of cases in cell-cell interactions and ligaments, and alternative to 2D cell culture for the study of fibroblast and keratinocyte activation involved in wound healing, there may be an alternative preliminary study to experimental animal studies, both economically and recurrent before applying to the experimental animal study. Considering the study construction and evaluation stages, it is thought that the study can be expanded using different cell skeleton structures and using different cells. Studies can be carried out where complex phenomena can be examined by making more complex 3D cultures and showing cells and cellular interactions. When the materials used in the study were evaluated, it was observed that the matrix better mimics ECM in the cell and retains spheroid structures for longer when preferred as a cell skeleton. The formation continues for a long time. It is thought to be preferable as a working material when the chronic wound healing process is examined.

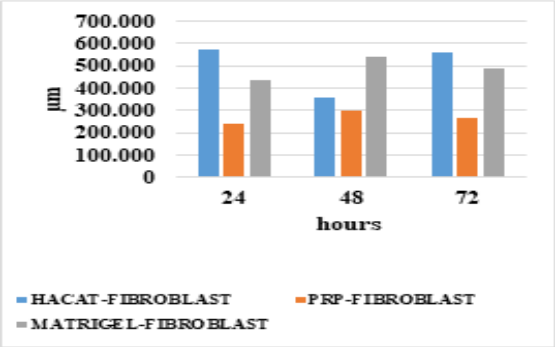

Figure 8. Comparison of Environmental Measurements of Spheroid Formation in Control Groups

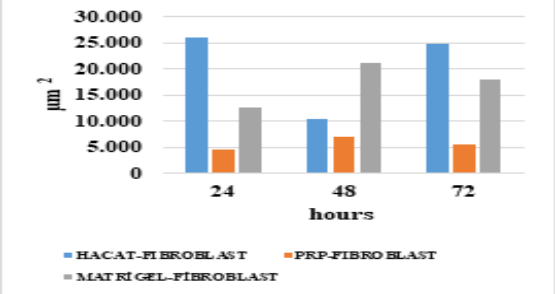

Figure 9. Comparison of The Formation of Spheroid Areas of Control Groups.

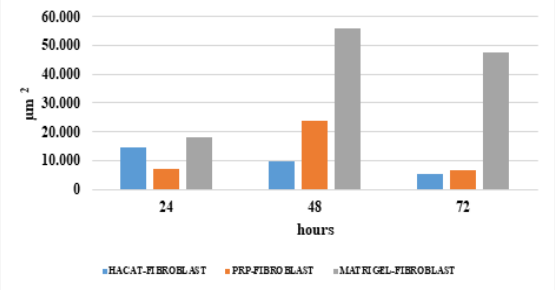

Figure 10. Comparison of The Formation of Spheroid Areas of Activator Groups

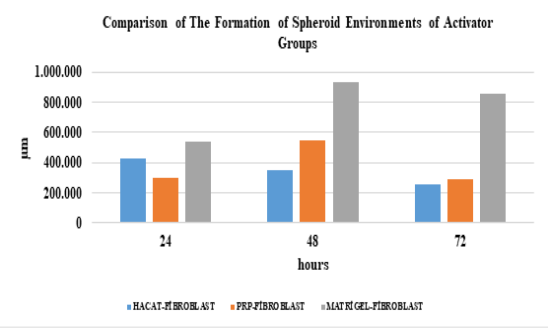

Figure 11. While there was an increase in the spheroid area and circumference measurements until the 48th hour due to the administration of a single dose of the drug to the groups to which the activator substance was added, there was a decrease in these results as the activator effect decreased at the 72nd hour measurements

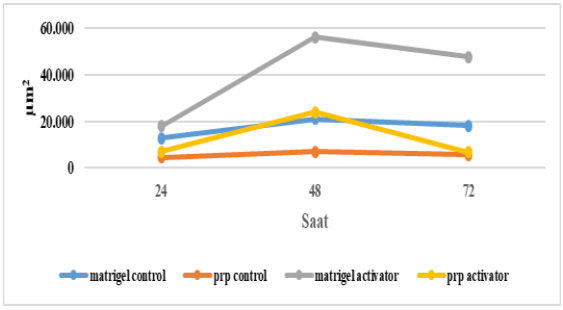

Figure 12. Fibroblast (PRP) Groups Comparison of The Formation of Areas of Matrigel Groups

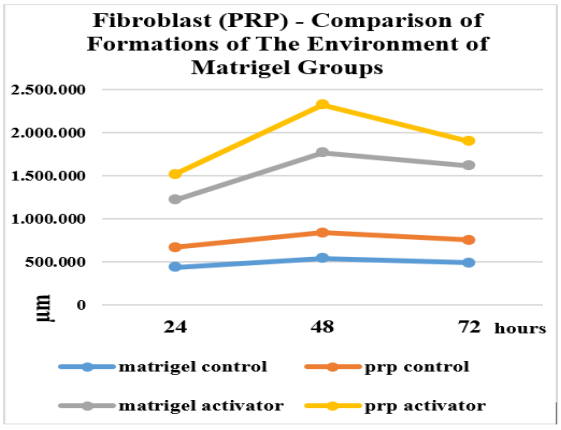

Figure 13. Matrigel fibroblast and prp fibroblast groups were compared because they contain the same cell lines. In the comparison of activator groups between the groups, the matrigel-fibroblast group stands out as the area, while the prp-fibroblast group stands out in the measurement of the circumference

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Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: E.E.A., O.K., Design: O.K., E.E.A, Data Collection or Processing: E.E.A., O.K., Analysis or Interpretation: O.K., E.E.A., Literature Search: E.E.A., O.K., Writing: E.E.A., O.K.

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Investigation of the Protective Feature of the Shell Part of Japanese Quail *(Coturnix Coturnix Japonica)* Eggs Against Ionizing Radiation

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ABSTRACT

In this study, we aimed to assess the protective capacity of the eggshell against radiation and the impact of ionizing radiation on the embryonic development process in quail eggs exposed to varying doses of radiation during incubation. A total of 740 quail eggs were divided into six groups, each exposed to different doses of ionizing radiation. Throughout the incubation period, we monitored hatching rates and examined hatched chicks for potential pathologies. Additionally, over six months, we conducted routine weekly examinations and observed the sexual activity of mature quails. At the study's conclusion, clinical pathology was not observed, and there were no mass mortalities. Quails whose laying activities were monitored exhibited normal sexual behavior. Statistical analysis revealed significant differences in hatching rates among the main study groups ($p<0.001$). Specifically, Group VI, exposed to the highest levels of X-rays, exhibited significantly lower hatching rates compared to Groups I and V ($p<0.001$). These findings suggest that the quail eggshell provides some protection against ionizing radiation, as evidenced by the absence of anomalies and the high hatching rates observed in the chicks.

INTRODUCTION

Imaging methods such as direct radiography, fluoroscopy, and computed tomography, commonly utilized for diagnostic purposes in both human and veterinary medicine, represent the primary sources of ionizing radiation exposure (Adalı and Adalı, 2008). Ionizing radiation is well-established to induce cell death or cytogenetic effects through either direct ionization or the indirect interaction of free radicals with crucial cellular components such as DNA (Mayr et al., 1998). The principal cellular mechanisms underlying these effects include fetal cell death, mitotic delay, and disruptions in cell migration (Brent RL, 1989; Mayr et al., 1998). The biological consequences of radiation on fetal tissues typically hinge upon the stage of pregnancy and the radiation dose administered (Mayr et al., 1998; Valentin, 2003; Adalı and Adalı, 2008; Bıçakçı BC, 2009; Shaw et al., 2011). Consequently, exposure to radiation during pregnancy can lead to a spectrum of effects encompassing lethality, teratogenicity, carcinogenicity, genetic mutations, as well as structural and functional abnormalities in the fetus (Mayr et al., 1998; Adalı and Adalı, 2008).

It is widely acknowledged that the most effective means of safeguarding against the deleterious effects of radiation is through the use of protective materials. Currently, lead stands as the predominant choice for such shielding; however, its high cost and density impose limitations on its widespread utilization (Singh et al., 2015; Sevinç and Durgun, 2021). Consequently, alternative materials suitable for radiation protection have been the focus of many studies (Singh et al., 2015; Cherkashina et al., 2020). Exploring unconventional

avenues, researchers have investigated the permeability of chicken eggshells, typically considered waste material, to ionizing radiation, with the aim of ascertaining its efficacy as a component in radiation shielding. Several studies have addressed this topic, reporting the eggshell's potential protective properties against radiation (Binici et al., 2013; Binici et al., 2015; Jasmine et al., 2020; Sevinç and Durgun, 2021; Azman et al., 2022). However, a notable gap exists in the literature regarding scientific investigations evaluating the protective capabilities of both chicken and quail eggshells during embryonic development (incubation).

In this study, inspired by previous research suggesting the protective potential of chicken eggshells against radiation, quail eggs were exposed to radiation during incubation to assess both the protective properties of the eggshell and the effects of ionizing radiation on hatching and subsequent embryonic development.

MATERIALS AND METHODS

Animal Material and Creation of Groups

This study, approved by Siirt University Animal Experiments Local Ethics Committee (HADYEK) under decision number 2016/20 and conducted at Siirt University Experimental Animals Application and Research Center (DEHAM), utilized 740 Japanese quail (*Coturnix Coturnix Japonica*) eggs sourced from Siirt University-Wildlife Center, which were divided into 6 main groups and placed in a T series Incubator (Çimuka, Ankara) to initiate the incubation process under controlled conditions (37.7°C temperature and 50-55% humidity). X-ray exposure, according to the predetermined groups, was carried out using a portable X-ray machine (FPX-F3200 portable X-ray, Fuji) at a film-focus distance of 90 cm, with settings of 50 kV and 2.5 mAs dose, with a maximum duration of 3 minutes for egg removal and reinsertion into the incubator for radiography. The X-rays were applied to the eggs within the same group from the same direction simultaneously.

Formation of Working Groups and Group-Based Radiological Applications

Group I (n=100): Served as the control group, with eggs not exposed to X-rays.
Group II (n=240): Divided into 15 subgroups with 16 eggs each, undergoing one radiographic exposure on a matching study day.
Group III (n=100): Received a total of 5 radiographs, initially on the first study day and subsequently every 3 days.
Group IV (n=100): Subjected to 15 daily radiographic imaging procedures.
Group V (n=100): Underwent radiographic imaging 5 times daily (75 images over 15 days), consecutively.
Group VI (n=100): Exposed to radiographic imaging 10 times daily (150 shots over 15 days), every other day.

Following the incubation period, hatched chicks were transferred to cages illuminated with white fluorescent light at a temperature of 35°C and humidity of 30%. Cage conditions were adjusted to maintain a temperature of 30°C during the second week. Post-hatching, chicks were provided with 5% sugar water every 4-6 hours until reaching 3 weeks of age, subsequently transitioning to ground broiler chick feed with ad libitum access to water for the duration of the study. At three weeks of age, quails were relocated to laying cages according to their respective study groups, irrespective of gender, and continued to be fed broiler chick feed until reaching 6 months of age. Throughout this 6-month period, all chicks underwent systematic weekly macroscopic examinations, evaluating for visual impairments, skin lesions, hair structure anomalies, as well as skull and limb abnormalities.

Statistical analysis

The variables in the study were quantified using both absolute numbers (n) and percentages (%). Statistical comparisons between groups were conducted using Fisher-Freeman-Halton and Pearson chi-square tests. Data analysis was performed using the IBM SPSS Statistics 20 software package. Statistical significance was defined as $p<0.001$.

RESULTS

Examination Findings

Throughout the 6-month period following their transfer from the hatching unit, weekly examinations of the chicks revealed no instances of clinical pathology. Additionally, no mass mortality events were observed during this timeframe. Quails, whose laying activities were monitored, exhibited normal sexual behavior throughout the study duration.

Statistical Findings

The data regarding the number of eggs, number of chicks hatched, and corresponding hatching rates for each group are outlined in Table 1.

Table 1. The number of eggs incubated, number of chicks hatched, and hatching rates by groups

Group	Number of Incubated Eggs (n)	Number of Hatches	Hatching Rate (%)
I	100	99	99[a, b, c, d, e]
II	240	225	95[a, f, g, h, i]
III	100	99	99[b, f, j, k, l]
IV	100	98	98[c, g, j, m, n]
V	100	94	94[d, h, k, m, o]
VI	100	83	83[e, i, l, n, o]

a: Significance of association (X^2) between Group I and II in terms of hatching rates (P= 0.037).
b: Significance of association (X2) between Group I and III in terms of hatching rates (P= 1).
c: Significance of association (X^2) between Group I and IV in terms of hatching rates (P= 1).
d: Significance of association (X^2) between Group I and V in terms of hatching rates (P=0.54).
e: Significance of association (X^2) between Group I and VI in terms of hatching rates (P<0.001).
f: Significance of association (X^2) between Group II and III in terms of hatching rates (P=0.037).
g: Significance of association (X^2) between Group II and IV in terms of hatching rates (P=0.010).
h: Significance of association (X^2) between Group II and V in terms of hatching rates (P=0.93).
i: Significance of association (X^2) between Group II and VI in terms of hatching rates (P=0.001).
j: Significance of association (X^2) between Group III and IV in terms of hatching rates (P=0.56).

k: Significance of association (X^2) between Group III and V in terms of hatching rates (P=0.54).
l: Significance of association (X^2) between Group III and VI in terms of hatching rates (P<0.001).
m: Significance of association (X^2) between Group IV and V in terms of hatching rates (P=0.14).
n: Significance of association (X^2) between Group IV and VI in terms of hatching rates (P<0.001).
O: Significance of association (X^2) between Group V and VI in terms of hatching rates (P=0.014).

The differences in hatching rates among the main study groups were statistically significant (p<0.001). Specifically, the hatching rate of eggs in Group VI, which received the highest X-ray exposure, was significantly lower (p<0.001) compared to both Group I, Group III and Group IV. Notably, there was no statistical difference in hatching rates between Group V, exposed to the second-highest X-ray dose after Group VI, and Group I. Furthermore, the hatching rate of Group I exceeded that of Group II (p<0.001), while no significant difference (p<0.001) was observed among the 15 subgroups within Group II regarding hatching rates.

DISCUSSION AND CONCLUSION

In recent years, the proliferation of radiation-related applications, particularly in fields such as medicine, the energy industry, and the military, has surged. In tandem with these advancements, research into radiation protection methods has become increasingly imperative (Binici et al., 2013; Sevinç and Durgun, 2021). Presently, available data on the subject primarily stem from two sources: experimental data derived from laboratory animals with short gestation periods and observational data gleaned from humans inadvertently exposed to radiation or survivors of nuclear incidents (Mayr et al., 1998; Shaw et al., 2011). When reviewing the existing literature, it becomes evident that studies have predominantly centered around the potential applications of various materials for radiation protection, particularly as building materials (More et al., 2021). Eggshells have emerged as one such material of interest, with investigations into their effectiveness. However, scientific inquiry into the protective capabilities of eggshells against ionizing radiation exposure during embryonic or fetal development is notably lacking. The present study endeavors to fill this gap by assessing the protective properties of quail eggshells against ionizing radiation, with a particular focus on hatchability rates and the observation of radiation-related pathologies post-hatching.

In this study, no macroscopic pathology was detected in the chicks during weekly examinations, and normal laying activities were observed in the quails that reached adulthood. It is well-established that ionizing radiation can directly impact fetal development, with the sensitivity of rapidly dividing and mitotic phase cells being particularly noteworthy. Consequently, the effects of radiation exposure on organisms may manifest as prenatal or postnatal mortality, congenital anomalies, growth disorders, and various structural and functional impairments (Mayr et al., 1998; Bıçakçı, 2009). Considering the absence of any pathology in chicks exposed to ionizing radiation in our study, two interpretations can be proposed. Firstly, it's possible that the eggshell offers comprehensive protection against radiation. Alternatively, it's plausible that the cumulative radiation dose necessary to induce anomaly formation during the embryonal process was not reached. Further research is warranted to elucidate the precise mechanisms underlying these observations and to ascertain the extent of eggshell protection against radiation during embryonic development.

In the present study, significant differences in hatching rates were observed among quail eggs exposed to different doses of ionizing radiation (Table 1). This finding suggests that hatching rates may indeed vary depending on the level of radiation exposure. Consequently, it is reasonable to infer that the eggshell provides protection against radiation to varying degrees, as evidenced by the differential hatching rates observed across the study groups. The observation that the hatching rate of eggs from Group VI, which received the highest radiation exposure, was lower than that of Group I, the control group (p<0.001), initially suggested an expected proportional decrease in hatching rates with increasing radiation exposure. However, the absence of a statistical difference (p<0.001) in the hatching rate between eggs from Group V, which received the second-highest radiation dose after Group VI, and eggs from Group I, challenges this expectation. The disparity in hatch rates between Group V and Group VI, both subjected to daily ionizing radiation throughout the study, underscores the significance of radiation dose as the sole variable distinguishing these groups, as well as the efficacy of the eggshell's permeability or protection against this dose. Existing literature on this topic is limited, as observed in previous studies (Binici et al., 2013; Binici et al., 2015; Jasmine et al., 2020; Sevinç and Durgun, 2021; Azman et al., 2022), which primarily focus on testing various materials for their radiation permeability as potential building materials. Indeed, previous scientific studies have reported that eggshells, including those from chicken eggs, possess the capability to absorb radiation, thereby exhibiting protective properties against it (Binici et al., 2013; Bani-Ahmad et al., 2022). Binici et al., (2013) determined that eggshell and waste battery charcoal could be utilized as radiation-shielding materials in composite materials produced using waste battery charcoal, barite, limestone, cement, sawdust, epoxy, and eggshells. Azman et al., (2022) proposed that bentonite clay blocks, enriched with bentonite clay and eggshell powder (ESP) and reinforced with Bi2O3, WO3, Bi2O3 + GO, or WO3 + GO layers, could serve as an effective radiation shielding material. Sevinç and Durgun, (2021) demonstrated that waste eggshells could be used as an additive to protect against gamma radiation, using composite materials made from waste sawdust, waste PVC sawdust, waste eggshells, and vermiculite. While the aforementioned studies predominantly focused on chicken eggshells, quail eggs were employed in the present study. The eggs were exposed to radiation throughout the incubation process, particularly during the embryonic stage. Despite the difference in study design, the consistent finding that eggshells provide radiation protection aligns with the existing literatüre.

In conclusion, the high hatch rate observed in quail eggs exposed to radiation, along with the absence of any abnormalities in the hatched chicks, provides evidence supporting the notion that quail eggshells may offer some degree of protection against ionizing radiation. Nevertheless, the inability to measure the radiation dose applied to the quail eggs using specialized dosimeters is acknowledged as a limiting factor of this study. The present study serves as a preliminary foundation for future investigations on this topic, and it is recommended that

subsequent research incorporate standardized radiation measurements in order to conclusively establish the protective properties of quail eggshells against radiation exposure.

Ethical Declaration

This study, approved by Siirt University Animal Experiments Local Ethics Committee (HADYEK) under decision number 2016/20

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Study and data collection: A.G., M.B.A., Analysis and interpretation: N.Ş., M.B.A., Statistical analysis: D.Ö., Literature search and writing: K.S., N.Ş.

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Investigation of the Contribution of Different Antiseptic Solutions to Clinical Recovery in Uroretropropulsion Technique in Cats

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ABSTRACT

The aim of this study was to report the contribution of different antiseptics to clinical improvement during intravesical lavage in cats with urethral obstruction problems. Urethral obstruction (UO) is a problem mostly encountered in male cats, requiring urgent intervention with a high success rate in the inferior urinary system. A total of 42 cats of different breeds and ages were evaluated, and the first medical treatment was applied, but no urine output was detected. All cases were randomly divided into 3 different groups and one-time intravesical lavage was performed with different fluids: GI (0.9% saline), GII (Dimethyl sulphoxide) and GIII (lugol's solution). The controls performed on the 7th, 14th, and 30th days of the study showed similar results in all cases, and no statistically significant difference was determined. As a result, it was concluded that lugol's solution can be used as a new alternative to DMSO antiseptic, which is the only agent approved for treatment by the US Food and Drug Administration, when performing intravesical lavage in cats with urethral obstruction.

INTRODUCTION

Urethral obstruction (UO) in cats is a mostly encountered lower urinary system problem that requires urgent intervention and has a high success rate when treated. However, recurrence rates vary between 22-57% and are mostly observed in male cats (Kim et al., 2011; Eisenberg et al., 2013; Reineke et al., 2017). The underlying causes of UO often cannot be determined, dietary factors such as urine pH, obesity, stress and other environmental factors, urethral plugs (proteinaceous debris in which crystals can become trapped), and urethral spasm are known to be involved. Feline idiopathic cystitis (FIC), also known as 'stress cystitis', causes urethral obstruction in 65-90% of cases (Breheny et al., 2022a).

Clinical symptoms of UO often include stranguria, pollakiuria, periuria (inappropriate urination), haematuria, lethargy, vomiting, abdominal pain symptoms and painful vocalisations. Urethral obstruction is a serious urinary tract problem that can lead to postrenal azotemia and severe metabolic abnormalities (Lee and Drobatz, 2006; Cooper et al., 2019). Emergency treatment of cats with UO includes anesthesia, retrograde urethral lavage to ensure clearance, compositions of fluid and electrolyte residues, permanent urethralization connected to a sterile closed collection system for a period of 24 to 48 hours, and the use of non-steroidal agents and α-1 adrenergic receptor antagonist systems (Segev et al., 2011; Hetrick and Davidow, 2013). However, it should be noted that indwelling urinary catheter placement in cats with UO has been shown to increase the risk of urinary tract infection due to bacterial biofilms colonising the catheter surface (Delcaru et al., 2016). Several agents are used during intravesical lavage application, including dimethyl sulphoxide (DMSO), hyaluronic acid, heparin, Bacillus

Calmette-Guerin, pentosan polysulphate sodium, and resinferatoxin (Fall et al., 2008; Dasgupta and Tincello, 2009). DMSO is the most widely used antiseptic and the only agent approved for intravesical treatment by the US Food and Drug Administration in 1978 (Hanno et al., 2010). Lugol's solution, also known as Lugol's iodine, has been used as an antiseptic in difirent medical care in cats since the 19th century. It was described by French physician JGA Lugol in 1829 and consists of 85 mL of distilled water with 5 g of iodine (I2) and 10 g of iodide (KI). Its antimicrobial effect is based on the degree to which free iodine remains bound to cell wall, oxidation and supplementation of the microbial content with free iodine (Grønseth et al., 2023).

The main aim of this study is to determine the clinical recurrence rates of 42 male cats with idiopathic UO of different breeds and ages at 7, 14 and 30 days after 3.5Fr indwelling catheterisation and one-time lavage with different antiseptics. Our hypothesis is that the use of Lugol's solution in one-time retrograde intravesical lavage will contribute to the clinical improvement in cats with UO undergoing indwelling catheterisation.

MATERIALS AND METHODS

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

The material of the study consisted of 42 male cats that presented to Dicle University, Faculty of Veterinary Medicine, Department of Surgery with the complaint of inability to urinate and were previously treated medically. In all cases, the first empirical treatment was applied but no urine output was detected. Male cats suspected of having feline idiopathic cystitis (FIC) were prospectively included in the study. The diagnosis of urethral obstruction was based on initial physical examination (hard, painful, swollen bladder) and a history of signs of feline lower urinary tract disease (pollakiuria, haematuria, stranguria and vocalisation). Exclusion criteria included cats with UO caused by other underlying disease rather than FIC, such as urinary tract infection, radiographic cystic calculi and neoplasia.

The anesthesia protocol applied to all groups during the application is; Midazolam + butorphanol + ketamine as 0.3 + 0.3 + 5 mg/kg IM determined.

All cats in the group underwent one-time intravesical lavage by applying an intravesical catheter after anesthesia (Fig 1). Medical empiric treatment in cats was continued for 5 more days.

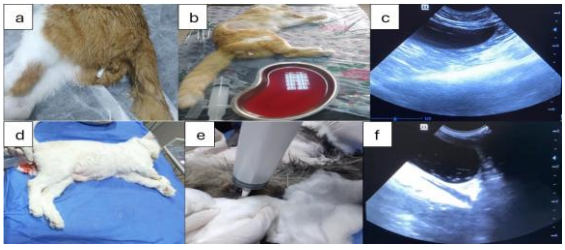

Figure 1. Images of some cases during the procedures performed
a) Intravesical catheter application and fixation
b) Haematuria in the tub as a result of intravesical ejaculation
c) Ultrasound view of the intravesical catheter
d) Intravesical catheter application and haematuria
e) Intravesical lavage application
f) Appearance of the sac during intravesical lavage

In the diagnostic examination; anamnesis, clinical examination, haematology, radiography, and ultrasonography were performed appropriately in each cat. The urinary catheter was removed at the 24th hour in order to relieve the patients from the stress factor, and the issues to be considered during the surveillance (especially ensuring that the patients drink plenty of water, cleaning of wet food and urine containers) were explained to the patients' relatives and they were warned to bring them back for follow-up if the problem persisted.

Follow-up evaluation was performed through clinical evaluation of the animals and telephone interviews with relatives. Data collected include the duration of hospital stay, emergency treatment, post-discharge treatment and time until symptoms reappear, nature of clinical signs (obstruction, other signs of urinary tract disease such as hematuria, pollakiuria, choking, urination in inappropriate places, and pain) was taking. Glasgow Feline Composite Measure Pain Scale was used in all cases and repeated analgesics were administered if necessary.

RESULTS

In this study, the average age range of 14 cats in GI was 28±5 months and 7 Scottish folds, 2 Iranian, 3 tabby, 2 crossbred, the average age range of 14 cats in GII was 28±8 months and 6 Scottish Folds, 1 Iranian, 4 tabby, 3 crossbred, the average age range of 14 cats in GIII was 28±7 months and 6 Scottish Folds, 2 Iranian, 3 tabby, 3 crossbred.

Since empirical treatment (amoxicillin+cluvanic acid, meloxicam, oxybutynin hydrochloride) was previously applied in all cases of this study, vesical bacterial load could not be defined in all included cases. Thus, differences between of groups in terms of clinical recovery levels in terms of 0.9% saline and different antiseptics (DMSO, Lugol's solution) of the lavage to be applied during intravesical catheter insertion were investigated. No complications were encountered during or after anaesthetic administration in all cases. The dose of anaesthesia administered was adequate and no maintenance dose was needed until the end of the procedure. All cases woke up after the procedure without complications. In terms of pain management, it was determined that all cases needed analgesic at 24 hours according to the results of Glasgow Feline Composite Measure Pain Scale. No significant difference was found between the groups in terms of pain.

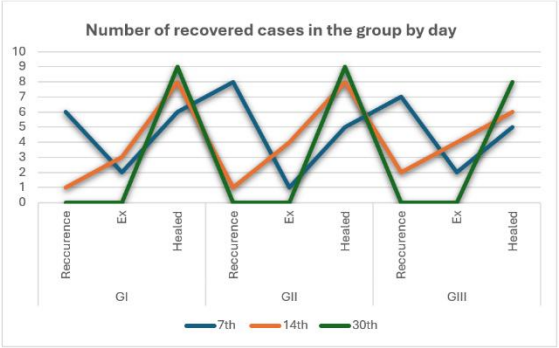

Figure 2. Survival rates by groups and recurrence numbers by days are given

The recovery, recurrence and ex rates according to the groups and the days of recovery are shown in the fig 2.

There was not statistically significant difference with antiseptic fluids used during lavage between the groups.

According to the results of the study, it was determined that the cats that died were caused by Iranian and Scottish Fold cats. It was determined that the recovery rates of tabby breed cats were better and the recurrence rates were lower. In all cases, it was aimed to maintain indwelling catheterisation for 24 hours, but the tabby breeds were uncomfortable with the catheter and removed it in the 9th hour with the help of tongue and teeth, although an Elizabethan collar was worn. No wound or infection was detected at the suture site. In all cases, the diet and toilet cleaning applied in the postoperative period were followed, 2 cases in GI and GII were reported to be stray animals and were released back to their place in the street since no complication occurred until the 18th day and they were followed up in the street until the 30th day. No signs of allergic reaction were found in any of the 14 cases as a result of lavage using Lugol's solution. The rate of haematuria was quite high in all groups of the study.
Recurrence and ex in all cases; 6 of 14 cases developed recurrence and 2 cases died until the 7th day in GI. In the continuation of the follow-up until the 14th day, the condition of 2 of the 6 cases with recurrence improved, while the recurrence of 1 case continued and 3 cases died. On the 30th day of the study, a total of 5 cases died and no clinical symptom was found in a total of 9 cases. Thus, the survival rate of GI on the 30th day was determined as 64%. When we evaluated this situation for GII; recurrence developed in 8 of 14 cases and died in 1 of 14 cases until the 7th day. In the continuation of the follow-up until the 14th day, the condition of 3 of the 8 cases who had previously developed recurrence improved, while the recurrence of 1 case continued and 4 cases died. On the 30th day of the study, a total of 5 cases exited and no clinical symptom was found in a total of 9 cases. In this case, the survival rate of GII on the 30th day was determined as 64%. In GIII in which Lugol's solution was used, 7 of 14 cases developed recurrence and 2 cases died until the 7th day. In the continuation of the follow-up, the condition of 1 of the 7 cases whıch developed recurrence until the 14th day improved, while the recurrence of 2 cases continued and 4 cases died. On the 30th day of the study, a total of 6 cases died and no clinical symptom was found in a total of 8 cases. In this case, the survival rate of GIII on the 30th day was determined as 57%. The rates of recurrence, ex and recovered cases at day 7 were determined as GI 42-14-42%, GII 57-7-35% and GIII 50-14-35%, respectively, and at day 14 as GI 7-21-57%, GII 7-28-57% and GIII 14-28-42%.

DISCUSSION AND CONCLUSION

The aim of this study was to examine the clinical differences between the use of different antiseptics (DMSO, Lugol's solution) and 0.9% saline in the lavage performed during indwelling catheterisation in addition to empirical treatment in cats with UO.

Studies have shown that UO has different causes, 60% have urethral obstruction, 20% have urethral calculi, <5% have stricture or neoplasia. In the remainder, there is no clear evidence of physical obstruction and the disease is supported by previous aetiological studies (Gerber et al., 2008).

In the present study, cats with urethral plugs, which are encountered with a high rate of 60%, were selected. Thus, it was aimed to prevent intravesical infections that may occur with catheter application with the help of lavage performed during indwelling catheterisation in urethral plugs. Cooper et al., (2019) reported that 13% of cats without intravesical lavage developed bacteriuria within 24 hours after urinary catheterisation. However, urine cultures were obtained through the catheter and therefore it is thought that the biofilm sample may have been taken rather than the actual colonisation in the lower urinary tract. Although one of the disadvantages of the present study was that urine culture results could not be obtained, they were not obtained because culture tests did not give reliable results since empirical treatment was initiated in all cases and all cases were cats presenting with persistent UO. However, since it was aimed to investigate the clinical efficacy of permanent catheterisation and lavage applications in addition to empirical treatment, urine culture tests were ignored and clinical improvements were evaluated.

It is known that clinical symptoms observed in the studies depend on the duration and intensity of the obstructive event, and haematuria, pollakiuria and stranguria are commonly reported clinical symptoms (Ferreira, 2013; Breheny et al., 2022a). In our study, similar results were encountered and it was determined that all cases had similar clinical symptoms and different antiseptics used during lavage did not make any difference in clinical recovery. The small number of cases in the present study necessitates the need for further studies on this subject.

The intravesical administration of DMSO used in the current study one of the primary treatment for IC/PBS patients and an FDA-approved treatment option for IC/PBS. This therapeutic modality has proven to be feasible and effective in the treatment of this painful urinary condition (Kim et al., 2011). Considering this situation, DMSOused in the present study has been used in many previous studies and the decrease observed in NGF, MCP-1 and IL-6 mRNAs in DMSO treated bladders may reveal the inhibitory effect of DMSO on the abnormal activation of sensory neurons, urothelial cells, mast cells and detrusor muscle cells. Therefore, the general anti-inflammatory effects of DMSO are thought to help inhibition of many inflammatory cell types in infected bladders. However, no difference was found between saline, Lugol's solution and DMSO groups used in the present study. The reason for this is thought to be that all of the treated cats were treated with antibiotics and NSAID agents in their empirical treatments. In addition, in an experimental mouse model by Soler et al., (2008), intravesical DMSO also showed a similar protamine sulphate-induced reduction of hyaluronic acid levels in urine, suggesting that it may regenerate the damaged glycosaminoglycan (GAG) layer (Soler et al., 2008).

However, the fact that there is no clinical difference in different fluids used during intravesical lavage does not support the opinion of Soler et al., (2008), in addition, there is various hypotheses regarding mechanism of action topical application in iodine solution. Several studies have shown that iodide is an anti-inflammatory effect resulting from its ability to down-regulate free oxygen radicals produced by polymorphonuclear cell activation. Another hypothesis for its anti-microbial role is based on the participation of iodide in halogenation reactions mediated by roxidase, which is essential for phagocyte function. Iodine solution used in topical treatment browns the skin and evaporates rapidly. It is argued that one application loses 50% of the free iodine in two hours, 80% in two days 88% evaporates on third day. Lugol's solution has proven

useful in inflammatory, infectious pathologies, immune-mediated therapies (Taranu et al., 2018). Likewise, the fact that intravesical lavage showed similar results to DMSO antiseptic in the present study and no allergic effect was encountered in clinical use, we believe that intravesical lavage may be useful in the use of intravesical lavage in cats and may be an alternative to DMSO antiseptic.

In all cases, empirical treatment and fluid therapy were generally provided with similar protocols. Fluid administration is needed to increase urine production and clear urine components. Higher fluid volumes may theoretically reduce the risk of UO recurrence. However, a recent study found no relationship between UO and the total volume of fluid administered during hospitalization or the duration of fluid administration after permanent removal of the urethral catheter. To our knowledge, there are no studies evaluating the effect of a single fluid dose in cats with UO; However, the 1996 study by Osborne et al. 1996 suggest that this type of treatment is not effective enough to show any benefit in reducing the clinical signs associated with feline idiopathic cystitis (Osborne et al., 1996).

In the present study, since classical empirical treatment was applied to all cases, similar results were found with the studies that there was no clinical relationship between fluid therapy and recurrence rate in UO cats.

This is because the cat's urethra is known to consist of smooth muscle only along the proximal 28% to 37% of its length, and the remainder of the urethral musculature is known to consist of non-relaxing muscle with α-1, so the second dose was not used other than spasmolytic agents used in empirical therapy due to adrenoreceptor blockade. Lulich et al. 2013 argue that the pharmacological activity and potential smooth muscle relaxant effect of prazosin make urethral muscle relaxation an ineffective management strategy in cats with UO (Lulich and Osborne, 2013). In the current study, Breheny et al., (2022b) argue that the likelihood of dehydration and re-occlusion in the post-catheterisation period is most likely to occur in the week following the first episode and the likelihood of feeding is low. He argues that NSAIDs should be avoided as renal perfusion is not guaranteed.

In our study, it was observed that a large proportion of recurrences occurred in the first week in all groups. However, NSAIDs were used in all cases due to the high pain scores used in our study. H-2 blockers were used in all cases because of the second dose used due to the presence of pain in the cases.

In the present study, there was no statistically significant difference between the recurrence, ex and recovery rates on the 7th, 14th, 30th days. The similarity of all results due to the similarities in markers such as gender and age of the cases in the group distribution, in fact, it was concluded that intravesical lavage contributes to clinical recovery when used for the removal of UO rather than the fluid used during intravesical lavage.

It was concluded that lugol solution can be used as an alternative to DMSO used as an antiseptic for intravesical lavage in addition to the medical treatment of UO which is frequently encountered in cats.

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Ethical Declaration

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees". (In this case, the "Ethics Statement Form" or "Informed Consent Form" must be filled in, signed by all authors and uploaded to the system.)

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Idea / Concept: NS, US, Supervision / Consultancy: NS, US, Data Collection and / or Processing: NS, Analysis and / or Interpretation: NS, US, Writing the Article: NS, US, Critical Review: NS, US.

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Investigation of the Antibacterial Effectiveness of Various Licensed Surface Disinfectants

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ABSTRACT

The concept of hygiene has become more important today due to pandemic infections. Many products on the market have been licensed, but whose antibacterial effectiveness has not been checked later. For this reason, the study aimed to determine the antibacterial activities of surface disinfectants available in the market for public and personal use, based on the international standard. Eight different surface disinfectants, licensed from the authority and for public and personal use, were collected and processed without any dilution (100%). In the study, standard strains *Staphylococcus aureus* (*S. aureus*) (ATCC 6538), *Escherichia coli* (*E. coli*) (ATCC 10536), and *Pseudomonas aeruginosa* (*P. aeroginosa*) (ATCC 15442) were used. Exposure conditions; it was done for 5 minutes and under clean conditions (0.3 g/L bovine albumin solution) at room temperature (22-24°C). It was determined that the eight surface disinfectants included in the study showed different levels of antibacterial activity. When the logarithm differences and antibacterial activities of surface disinfectants are examined as percentages; while the logarithm difference of three disinfectants against the three bacteria examined was ≥ 5; the logarithm difference of five disinfectants against two bacteria is ≥ 5; the percentage of those effective against all three bacteria was 37.5%; the percentage of those effective against both bacteria was found to be 62.5%. Due to the improprieties detected in the antibacterial activities of the surface disinfectants examined, when their antibacterial activities are examined, although they are licensed, it is seen that the products do not provide the specified antibacterial activity. The selection of disinfectants used to neutralize bacteria that cause infections, the determination of their antibacterial activities, and the sustainability of the determined antibacterial activity are of great importance. For this reason, it is necessary to check the antibacterial activities of disinfectants even after the registration stage and to select disinfectants according to their effectiveness to prevent infections.

INTRODUCTION

Nowadays, the concept of hygiene has become more important due to pandemic infections. Many disinfectants are used individually or institutionally for hygienic purposes. Especially due to the Covid-19 pandemic, the use of disinfectants has become much more common and many disinfectants with different properties have been used.

Chemical disinfection is used on various surfaces due to its wide usage area, cost-effectiveness, and lack of need for mechanical devices. Among the many chemical disinfectants authorized by the competent authorities, those generally used in hospitals and at home, include

formaldehyde, glutaraldehyde, hydrogen peroxide, ozone, peracetic acid, phenolics, and quaternary ammonium compounds. Long-term use of disinfectants may cause health risks, human toxicity, and eco-toxicity (Gessi Alessandro, 2023). During the Covid-19 epidemic, there was a shortage of masks and disinfectants. Therefore, instead of alcohol (76.9-81.4% ethanol solution), various disinfectants whose effectiveness was researched and received permission from authorized institutions were used (Kameda et al., 2022).

Aqueous hypochlorous acid (HOCl), which has broad effectiveness against pathogens and is environmentally safe, is actively used. The final product is water and salt; it has no ecotoxicological effects. Hypochlorous is routinely applied for environmental disinfection. HOCl is successful in neutralizing the most resistant infectious agents. HOCl is highly pure, reliable, and has consistent production capability in industrial quantities. It has the feature to meet the needs of pandemics; it is a disinfectant that is affordable in large quantities. HOCl is currently included in the World Health Organization's (WHO) list of biocides effective against coronavirus. In many different brands, aqueous HOCl formulations have been approved for topical use by the Food and Drug Administration (FDA) in the United States of America (USA), again in the European Union (EU) as a Class III medicinal product, and also in Japan by the Ministry of Health (WHO, 2021).

Sodium hypochlorite (SH), usually mixed with a strong alkali, is a disinfectant with a wide antibacterial effect for bacteria and viruses with or without many spores; however, it should not be contacted. High temperatures and ultraviolet light (UV), on the other hand, degrade hypochlorous acid. For these reasons, the effects of reagents are variable and inconsistent. Hypochlorous acid is used as an alternative to alcohol; however, it has a poor shelf life and storage difficulties. In the studies conducted, it is shown that hypochlorous acid is more practical, reliable, and comfortable than alcohol. For the ideal use of hypochlorous acid, it should not be stored and should be used immediately, stored in a cool and dark places (Kameda et al., 2022). With benzalkonium chloride, Choi et al., (2020) conducted a mammalian exposure study, which they did; they found that it causes lung damage even at fairly low levels.

HOCl is the strongest oxidant of the chlorine family, stronger than sodium. It is slightly acidic, at a neutral pH (5-7), and is located in the white blood cells of all mammals. In addition to being cheap, it is water-soluble, non-toxic, and attracts bacteria thanks to its neutral charge and affects the cell wall of bacteria, causing them to die quickly (Practice Guidance for Health Care Environmental Cleaning, 3rd eBook, 2022).

Disinfectants also have many disadvantages. Studies conducted show that the use of SH is responsible for a high rate of poisoning. Sodium hypochlorite accounted for 62.1% of the poisonings during the Covid-19 pandemic, followed by non-alcoholic disinfectants, 36.7%, and hand sanitizers, 36.7%. These products cause indoor air pollution, asthma, and allergies; 0.1% concentration of SH, 70-90% ethanol, or isopropyl alcohol irritates the respiratory tract, eyes, or skin. In addition, SH causes the formation of organic chlorinated compounds, becoming very toxic to humans and the environment. SH droplets that remain in the air for 30 minutes after spraying become quite harmful. Hand sanitizers containing quaternary compounds such as benzalkonium may irritate the skin and respiratory system, triggering asthma. Ozone also has harmful effects on health. It has been determined that its concentration, which is safe for humans, cannot provide adequate disinfection in indoor control. In a study, it was found that when used at a concentration of 0.3 ppm, the time to inactivate 90% of viruses is more than 100 minutes. Nanomaterials have been developed that will reduce some of the negative aspects of chemical disinfectants and differ in terms of harmfulness, abrasiveness, and bacterial resistance. The antibacterial effect of silver and silver nanoparticles has been studied, and it has been found that even safe doses for repeated exposures such as skin, inhalation, or ingestion can lead to health problems (Gessi Alessandro, 2023).

There are many licensed disinfectants on the market. However, the question of the adequacy of the antimicrobial activities of these disinfectants is in doubt. For this reason, the study aimed to determine the antibacterial activities of commercially available surface disinfectants for public and personal space use based on the International Standard determined by the "Biocidal Products Regulation".

MATERIALS AND METHODS

Sampling

In the study, eight different surface disinfectants (Active anionic oxygen natural water, quaternary ammonium, 5% anionic surfactant, didesil dimetil amonyum klorür (120 g/L), Ahp: Accelerated hydrogen peroxide, cationic polymer layer, octadecyl dimethyl ammonium chloride, hydrogen peroxide, deionized water, deodorizing, general surface cleaning liquid) licensed by competent authority, with different active ingredients on the market, were processed without any dilution (100%).

Analysis

S. aureus (ATCC 6538), *E. coli* (ATCC 10536), and *P. aeruginosa* (ATCC 15442) standard strains were used and exposed to disinfectants for 5 minutes. In the study *E. coli* ATCC 10536 was used instead of *Enterococcus hirae* ATCC 10541 specifed in the standard. Exposure conditions was conducted at room temperature (22-24°C) with 0.3 g/L bovine albumin solution under clean conditions. The standard strains were adjusted to be $1.5x10^8$-$5x10^8$cfu/mL with test suspension (N) diluent. One mL of bacterial suspension was added to the tube containing 1 mL of inhibitory substance (0.3 g/L bovine albumin solution). It was mixed and waited for 2 minutes. 8 mL of disinfectant to be tested (100%) was added to it. After mixing, it was waited for 5 minutes. From here, 1 mL of sample was taken and transferred to a tube with 8 mL of neutralizer (Bovine serum albumin) and 1 mL of sterile distilled water. It was mixed and waited for 5 minutes. Then, 1 mL of the neutralized mixture was taken and was inoculated to 2 Tripticase Soy Agar (TSA). Plates were incubated at 36±1°C for 24 hours. At the same time, for experimental control: validation suspension was prepared, experimental conditions were checked, neutralizer control was performed, and dilution-neutralization validation was determined. The obtained results, titer calculation, and logarithm reduction were calculated as specified in the relevant standard (BS EN 13727: 2012+A2: 2015).

RESULTS

The eight disinfectants included in the study were tested under clean conditions without any dilution and were

bactericidal at different levels within 5 minutes against *S. aureus*, *P. aeruginosa*, and *E. coli*, which are the microorganisms that must be used in the effectiveness test of standard disinfectants and antiseptics were found to have an effect. The logarithm differences of the disinfectants (those greater than and equal to ≥ 5) were determined; their bactericidal activity against the tested standard strains was determined.

In our study, active anionic oxygen natural water is 100% effective against *E. coli* and *P. aeruginosa*, 98.875% against *S. aureus*; quaternary qmmonium is 100% effective against *E. coli* and *S. aureus*, 98.875% against *P. aeruginosa*; 5% anionic surfactant, didecyl dimethyl ammonium chlorid (120 g/L), octadecyl dimethyl ammonium chloride, hydrogen peroxide, deionized water combination is 100% effective against three bacteria; Ahp: accelerated hydrogen peroxide is 100% effective against *E. coli* and *S. aureus*, 88.462% against *P. aeruginosa*; cationic polymer layer was found to be 100% effective against *E. coli* and *P. aeruginosa*, 99.625% effective against *S. aureus* and deodorizing, general surface cleaning liquid was found to be 100% effective against *E. coli* and *P. aeruginosa*, 98.875% effective against *S. aureus*.

However, when the logarithm differences and antibacterial properties of a total of eight different surface disinfectants were examined as percentages, the logarithm difference of three disinfectants against the three bacteria examined was ≥ 5; the logarithm difference of five disinfectants against two bacteria was ≥ 5; the percentage of those effective against all three bacteria was 37.5%; the percentage of those effective against both bacteria was found to be 62.5% (Table 1).

As a result of our study, it was determined that three surface disinfectants containing 5% anionic surfactant, didecyl dimethyl ammonium chloride (120 g/L), and octadecyl dimethyl ammonium chloride, hydrogen peroxide, and deionized water had logarithm differences ≥5 log after 5 minutes of exposure to the three bacteria used in the analysis, and therefore they were effective. In the studies conducted, the antimicrobial activities of many different disinfectants have been investigated.

Table 1. Logarithm differences and antibacterial properties of different surface disinfectants

Active ingredient	*E. coli*[a]	*E. coli*[b]	*S. aureus*[a]	*S. aureus*[b]	*P. aeruginosa*[a]	*P. aeruginosa*[b]	Antibacterial Property
Active Anionic Oxygen Natural Water	100	≥5 Log	98.875	<5 Log	100	≥5 Log	**
Quaternary Ammonium	100	≥5 Log	100	≥5 Log	98.875	<5 Log	**
%5 Anionic surfactant	100	≥5 Log	100	≥5 Log	100	≥5 Log	***
Didecyl dimethyl ammonium chlorid (120 g/L)	100	≥5 Log	100	≥5 Log	100	≥5 Log	***
Ahp: Accelerated hydrogen peroxide	100	≥5 Log	100	≥5 Log	88.462	<5 Log	**
Cationic polymer layer	100	≥5 Log	99.625	<5 Log	100	≥5 Log	**
Octadecyl Dimethyl Ammonium Chloride, Hydrogen Peroxide, Deionized Water	100	≥5 Log	100	≥5 Log	100	≥5 Log	***
Deodorizing, general surface cleaning liquid	100	≥5 Log	98.875	<5 Log	100	≥5 Log	**

[a]: % Antibacterial Property, [b]: Logarithm difference, **: two bacteria, ***: three bacteria

DISCUSSION AND CONCLUSION

There are few studies on the determiation of the antibacterial activities of the surface disinfectants we used in our study.

In the study of Mataracı and Gerçeker (2011) examined the minimum bactericidal concentrations (MBC values) of SH and benzalkonium chloride of the *P. aeruginosa* ATCC 15442 standard strain against planktonic and biofilm cell cultures by microdilution method under two different experimental conditions: clean and dirty. It has been determined that benzalkonium chloride does not show any significant bactericidal activity against *P. aeruginosa*. It was stated that the type, amount, and contact time of the disinfectant are effective when disinfecting a water system with suspicion of biofilm (Mataracı and Gerçeker, 2011). The three disinfectants examined in our study (5% anyonic surfactant, didecyl dimethyl ammonium chloride (120 g/L) and octadecyl dimethyl ammonium chloride, hydrogen peroxide, and deionized water combination) are stated to be effective. It was found to be effective against the three bacteria examined.

In another study, the activities of three different disinfectants containing sodium dichloroisocyanurate aldehyde, and didecyl dimethyl ammonium chloride were investigated with three different methods. In the study, it was determined that European Suspension Test (EST) was the most convenient and easy-to-apply method among the Modified Kelsey-Skyes, Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM), and EST methods. It has been observed that there is a variable agreement between the results of the two methods depending on the disinfectant used and the type of bacteria (Özbek, 1997). As a result of the study, disinfectant containing sodium dichloroisocyanurate was effective against *P. aeruginosa*; disinfectants containing aldehyde and didecyl dimethyl ammonium chloride were found to be ineffective against *P. aeruginosa*. On the other hand, in our study, it was

observed that the disinfectant containing didecyl dimethyl ammonium chloride was effective against *P. aeruginosa*.

Kaya and Altanlar, (2021) investigated the antimicrobial activities of disinfectants and antiseptics frequently used in hospitals. 2% glutaraldehyde, 6% hydrogen peroxide solution, and sodium hypochlorite solution (1000 ppm) were used as disinfectants; *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 15542, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, methicillin-sensitive *S. aureus* (MSSA) ATCC 25923, *P. aeruginosa* ATCC 27853. Antimicrobial activities were investigated by quantitative suspension test against *E. coli* ATCC 25922 reference strains. 2% glutaraldehyde and SH (1000 ppm) showed antimicrobial activity against *P. aeruginosa* ATCC 15542 strain at all contact times (5 minutes, 20 minutes) except 1 minute and against all other strains at all contact times (1 minute, 5 minutes, 20 minutes). 6% hydrogen peroxide solution was not effective against *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 15542, *S. aureus* ATCC 43300 (MRSA), and *S. aureus* ATCC 25923 (MRSA) strains at 1 minute contact time. In our study, it was determined that the surface disinfectant containing octadecyl dimethyl ammonium chloride, hydrogen peroxide, and deionized water was effective against the three bacteria used in the analysis, after 5 minutes of exposure, the logarithm difference was ≥5 log and therefore it was effective. Other chemicals used together with hydrogen peroxide may have increased antimicrobial effects.

The selection, effectiveness, reliability, and correct application of disinfectants used to neutralize bacteria that cause infections are very important. Therefore, determining the antibacterial effectiveness of disinfectants and using disinfectants according to these results is necessary to prevent infections. Appropriate disinfectants must be selected to protect consumers and/or healthcare workers from bacterial infections, especially to prevent hospital-acquired infections. The surface disinfectants we found in our study, which are licensed by competent authorities but do not have sufficient antimicrobial activities, will not be able to adequately protect healthcare workers and consumers in terms of hygiene. For this reason, it is necessary to check the antibacterial activities of disinfectants even after the registration stage and to select disinfectants according to their effectiveness to prevent infections.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: S.K, G.E. E.G, Design: S.K, G.E., E.G, Ş.D., Data Collection or Processing: S.K, G.E., E.G, Ş.D., B.G.G., Analysis or Interpretation: S.K., E.M.Ç., A.K., E.G., B.G.G., Literature Search: S.K., N.A., Writing: S.K., N.A.

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Development of a LAMP Assay Targeting the rfbE Gene for Rapid Detection of Escherichia coli O157:H7

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ABSTRACT

Infections with *Escherichia coli (E. coli)* O157:H7 can lead to severe health complications. This pathogen is commonly found in contaminated meat and fresh produce, posing significant public health risks. The Loop-Mediated Isothermal Amplification (LAMP) method offers a rapid and accessible alternative to conventional nucleic acid amplification techniques, making it particularly suitable for on-site diagnostic systems. This study aimed to design a quick method using LAMP to detect the rfbE gene of *E. coli* O157:H7. *E. coli* ATCC 43888 was used as the positive control, while *Candida albicans* ATCC 10231, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 served as negative controls. Positive and negative controls were tested 10 times each for both analyses. Following DNA isolation, real-time PCR and LAMP were performed and compared with culture methods. For the positive control strain (*E. coli* ATCC 43888) at 10 CFU/mL, positivity was detected in 8 out of 10 samples by real-time PCR and in 7 out of 10 samples by LAMP. The sensitivity, specificity, negative predictive value, and positive predictive value of LAMP and real-time PCR were 95.00%–96.67%, 100%–100%, 94.34%–96.15%, and 100%–100%, respectively. In conclusion, our study successfully developed a rfbE gene-specific LAMP kit for *E. coli* O157, demonstrating comparable sensitivity and specificity to real-time PCR and culture methods. This kit can be effectively utilized in resource-limited settings.

INTRODUCTION

Escherichia coli (E. coli) is a ubiquitous microorganism that resides in the gastrointestinal tracts of all living organisms. It plays a significant role in global morbidity and mortality in both humans and animals, with animal hosts being crucial in the epidemiology of infections. The adaptive and versatile characteristics of *E. coli* highlight the need for ongoing research within the One Health approach, which integrates human, animal, and environmental health (García and Fox, 2021).

E. coli is a highly adaptable pathogen capable of causing a wide range of diseases and is responsible for at least 2 million human fatalities annually. Its involvement in both intestinal and extraintestinal diseases was recognized shortly after its discovery (Foster-Nyarko and Pallen, 2022). Enteropathogenic *E. coli* strains are categorized based on serogroups, pathogenicity, and clinical symptoms. Enterohemorrhagic strains, in particular, spread through contaminated food and water, producing Shiga toxins. The most common strain, O157, has been associated with numerous foodborne outbreaks worldwide (Yinur et al., 2023).

E. coli O157:H7 is a significant public health concern, as it can cause severe illnesses such as hemorrhagic colitis and hemolytic uremic syndrome (HUS). Even a small number of bacteria can result in serious infections. Given its high risk, the development of rapid and accurate diagnostic methods for *E. coli* O157:H7 is a global priority.

Traditional methods for detecting *E. coli* O157:H7 are often inefficient and time-consuming. Although newer immunological and genetic methods have been developed, they frequently face limitations such as low specificity or sensitivity. Molecular methods like PCR are more

promising but require specialized equipment and expertise. Thus, there remains a need for a more efficient and accessible diagnostic method for detecting *E. coli* O157:H7 (Ranjbar et al., 2016). In 2000, a new nucleic acid amplification technique called Loop-Mediated Isothermal Amplification (LAMP) was introduced. LAMP provides a faster and more accessible alternative to conventional nucleic acid amplification techniques. Its simplicity and ease of use make it particularly suitable for on-site diagnostic systems, and it can be adapted for detecting various pathogens (Kirkoyun et al., 2024).

Notably, all *E. coli* O157 strains have tested positive for the rfbE gene, which is specific to the O157 antigen, using PCR analysis (Tóth et al., 2009). The aim of this study was to develop a quick and simple LAMP-based method for detecting the rfbE gene of *E. coli* O157:H7.

MATERIALS AND METHODS

Escherichia coli American Type Culture Collection (ATCC) 43888 was performed as positive control for *E. coli* O157:H7. Candida albicans ATCC 10231, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Pseudomonas aeruginosa ATCC 27853, and Staphylococcus aureus ATCC 29213 were served as negative control. Positive and negative control used for 10 times for both real-time PCR and LAMP PCR analysis. A fresh bacterial culture was prepared by inoculating 10 ml of TSB (Oxoid, USA) with 1 µl of each strain and incubating overnight at 37°C (Liao et al., 2022).

DNA isolation was revealed using the boiling method, and dilutions of the DNA were prepared for standardization in optimization (Kirkoyun et al., 2024).

To design the LAMP assay for *E. coli* O157, LAMP Primer Sets were designed using PrimerExplorer Ver.5 (http://primerexplorer.jp/lampv5e/index.html) (accessed on 24/10/2024) with FASTA sequence file of the rfbE gene (GenBank ID: AF163332.1) as the target. B3, F3 (outer) and BIP, FIP (inner) primers were identified, and LF and LB primers were created to target single-stranded loop regions, utilizing database of NCBI's primer. The rfbE gene (GenBank ID: AF163332.1), a conserved sequence in *E. coli* O157:H7, was chosen as the target and highly conserved regions within this gene were identified using the BLAST program. Table 1 showed these primers and sequences.

Table 1. Oligonucleotid primers and sequence informations for *E. coli* O157

Label	5′pos	3′pos	len	Sequence
F3	63	87	25	GGAAATAAAACTATTACTACAGGTG
B3	248	271	24	CGTGATATAAAATGATCAGCTTGT
FIP			44	TTGGCCTTTAAAATGTAAACAACGGAAGGTGGAATGGTTGTCAC
BIP			45	AGCTGTACATAGGCAATATTGGCATCTGGGCTAATCCTATAGCAG
F2	88	106	19	AAGGTGGAATGGTTGTCAC
F1c	128	152	25	TTGGCCTTTAAAATGTAAACAACGG
B2	223	242	20	CTGGGCTAATCCTATAGCAG
B1c	158	182	25	AGCTGTACATAGGCAATATTGGCAT
LF	109	127	18	TGACAAAACACTTTATGA
LB	202	220	18	ATAGGATGACAAATATCT

Protocols of LAMP analysis were applied to each tube using the T1 System (BioRad) at 65°C for 30 minutes. Amplified products were analyzed on the LightCycler 480 (Roche Diagnostics) using a melting curve protocol, and melting analysis was used to consider the LAMP reactions. Real-Time PCR was conducted in a 96-well microplate using the LightCycler FastStart SYBR Green kit (Roche Diagnostics) on the LightCycler 480 instrument. Each run included a negative control without template and a positive control. The optimized reaction mixture for the stx1 gene primers (Jothikumar and Griffiths, 2002) with SYBR Green assays and 2µl of template DNA in a 20-µl volume. The cycling profile involved an initial 10-minute denaturation at 95°C, followed by 45 cycles of 95°C for 15 seconds, 55°C for 10 seconds, and 72°C for 15 seconds, with fluorescence measured at the end of each extension step. After PCR melting curve protocol was used (Jothikumar and Griffiths, 2002).

Test performance indicators for LAMP and Real-Time PCR results were calculated using the classical culture method as a reference. Sensitivity was measured using the formula true positive / (true positive + false negative), specificity with true negative / (true negative + false positive), positive predictive value with true positive / (true positive + false positive), and negative predictive value with true negative / (true negative + false negative) (Kirkoyun et al., 2024).

RESULTS

Table 2 summarizes the results obtained for all samples analyzed in this study. Using the positive control strain *Escherichia coli* ATCC 43888, positivity was observed in 8 out of 10 samples when tested with real-time PCR at a detection limit of 10 CFU/mL. Similarly, the LAMP PCR method successfully detected positivity in 7 out of 10 samples under the same conditions. These results demonstrate that both methods exhibit high sensitivity and are effective in detecting *E. coli* at low concentrations.

Table 3 provides a comparative analysis of the LAMP PCR and real-time PCR methods in relation to the results obtained through classical culture techniques, which are considered the gold standard in bacterial identification. This comparison highlights the practical applicability and performance metrics of each molecular technique when validated against traditional culture methods.

Table 4 presents the calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the LAMP PCR and real-time PCR methods. While both methods demonstrated high accuracy, it is noteworthy that the LAMP PCR method

exhibited a slightly lower sensitivity compared to real-time PCR. Despite this slight difference, the specificity of the LAMP PCR method was found to be comparable to that of real-time PCR, indicating its reliability in accurately identifying *E. coli* O157:H7.

These findings collectively suggest that LAMP PCR, despite minor differences in sensitivity, is a robust and reliable alternative to real-time PCR for detecting *E. coli* O157:H7, especially in resource-limited settings where advanced equipment may not be readily available.

Table 2. Distribution of the number of positives obtained after culture, Real-time PCR and LAMP PCR

		Culture	Real-Time PCR	LAMP PCR
*Escherichia coli ATCC 43888 - 10*6*	*Positive Control*	10	10	10
*Escherichia coli ATCC 43888 - 10*5*	*Positive Control*	10	10	10
*Escherichia coli ATCC 43888 - 10*4*	*Positive Control*	10	10	10
*Escherichia coli ATCC 43888 - 10*3*	*Positive Control*	10	10	10
*Escherichia coli ATCC 43888 - 10*2*	*Positive Control*	10	10	10
*Escherichia coli ATCC 43888 - 10*1*	*Positive Control*	10	8	7
Escherichia coli ATCC 25922	*Negative Control*	10	0	0
Staphylococcus aureus ATCC 29213	*Negative Control*	10	0	0
Pseudomonas aeruginosa ATCC 27853	*Negative Control*	10	0	0
Klebsiella pneumoniae ATCC 13883	*Negative Control*	10	0	0
Candida albicans ATCC 10231	*Negative Control*	10	0	0

Table 3. Comparison of LAMP PCR and Real-time PCR assay with culture results

		Culture		
		Positive	Negative	
LAMP PCR	Positive	57	0	57
	Negative	3	50	53
		60	50	
		Culture		
		Positive	Negative	
Real-Time PCR	Positive	58	0	58
	Negative	2	50	52
		60	50	

Table 4. Comparison of diagnostic results for LAMP PCR and Real-Time PCR tests

	LAMP PCR	Real-Time PCR
Sensitivity	95.00%	96.67%
Specificity	100.00%	100.00%
Positive predictive value	100.00%	100.00%
Negative predictive value	94.34%	96.15%

DISCUSSION AND CONCLUSION

E.coli O157 is a major foodborne pathogen with significant implications for food safety and public health. Its rapid and precise detection is crucial to minimizing the risk of foodborne illnesses and preventing outbreaks (Yin et al., 2024). In this study, we utilized the LAMP PCR technique as a potential tool for rapid and reliable detection of EHEC O157:H7.

Previous studies have emphasized the advantages of the LAMP technique in detecting EHEC O157:H7. Yinur et al., (2023) demonstrated that LAMP has higher sensitivity and specificity compared to traditional PCR. Their results also showed a high correlation between LAMP and PCR outcomes, suggesting that LAMP is particularly useful in resource-limited settings where cost-effectiveness and simplicity are essential. Consistent with their findings, our study observed that the LAMP assay displayed strong diagnostic accuracy. However, real-time PCR outperformed LAMP PCR in terms of precision under laboratory conditions, likely due to the controlled environment that enhanced its performance. Despite this, the LAMP technique remains a viable option in settings where advanced laboratory infrastructure is unavailable.

Ranjbar et al., (2016) highlighted the utility of targeting the rfbE gene for EHEC O157:H7 detection using the LAMP assay. Their assay achieved remarkable sensitivity, detecting DNA concentrations as low as 78 pg per reaction, and exhibited high specificity, with no cross-reactivity with non-EHEC strains. Our findings align with these results, as our rfbE-targeted LAMP assay also demonstrated high sensitivity and specificity. In addition, Wang et al., (2023) developed two LAMP-based methods: a real-time fluorescent LAMP (RT-LAMP) and a visual LAMP assay using calcein as an indicator. These assays targeted the Ecs_2840 gene and demonstrated excellent performance in detecting EHEC O157:H7 from pure bacterial cultures and milk samples. The RT-LAMP method detected as few as 8.8×10^0 CFU/mL, while the visual LAMP assay achieved a detection limit of 2.35×10^0 CFU/mL. These findings underline the adaptability of LAMP for use in various sample types, supporting our assertion that LAMP assays are promising tools for rapid pathogen detection in food safety applications.

Cui et al., (2024) evaluated two complementary methods, colorimetric LAMP and immunochromatographic test strips (ICTs), for detecting EHEC O157:H7. Both methods showed high specificity, with detection limits of 5.7 CFU/mL. Similarly, our study found that the LAMP assay we developed had a detection limit of 10 CFU/mL, closely aligning with these results. This level of sensitivity confirms the capability of LAMP as a competitive alternative for pathogen detection.

Qin et al., (2018) further expanded on the potential of LAMP by combining it with immunomagnetic separation (IMS), targeting the rfbE gene. Their IMS-LAMP method demonstrated both high sensitivity and specificity, detecting 3×10^1 CFU/mL in meat samples. Our findings, with a detection limit of 10 CFU/mL, are consistent with their results, demonstrating that LAMP can provide reliable performance across different applications and sample types. Despite these promising results, our study had certain limitations. We did not incorporate colorimetric analysis, which could have broadened the applicability of the LAMP assay in non-laboratory settings. Additionally, we did not test the assay with a wide range of routine biological or environmental samples. Future studies should address these limitations to further validate and optimize the utility of the LAMP assay in diverse conditions.

In conclusion, our study successfully developed an rfbE-specific LAMP kit for detecting *E. coli* O157. The assay demonstrated sensitivity and specificity comparable to real-time PCR, and its simplicity and cost-effectiveness make it an excellent option for resource-limited settings. These findings contribute to the growing evidence that LAMP is a valuable tool for improving food safety and public health diagnostics, offering a robust alternative to traditional methods in various settings.

Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship contributions

Concept: M.D., S.E., Design: M.D., S.E., Data Collection or Processing: M.D., S.E., Analysis or Interpretation: M.D., S.E., Literature M.D., S.E., Writing: M.D., S.E.

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Intestinal Mucosal Damage and Intestinal Permeability In Non Infectious and Infectious Diarrheic Calves In Relation to Diamine Oxidase Activity

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ABSTRACT

This field study was designed as a cohort (prospective) and involved 71 calves within the borders of Aydın Province. Each calf in the study was from one of three different farms (Farm A with 12 non-infectious diarrheic, 15 infectious diarrheic, Farm B with 13 non-infectious diarrheic, 19 infectious diarrheic, and Farm C as the healthy control group n=12). Serum samples were separated into Eppendorf tubes after centrifugation and stored in a -80 °C freezer until analysis. The competitive enzyme immunoassay technique was applied using the Bovine Diamine Oxidase ELISA test. Diamine Oxidase (ng/mL) measurements in infectious, non-infectious, and healthy calves were determined to be 6.52 ± 2.85, 7.16 ± 3.40, and 17.05 ± 2.63 ($p< 0.001$), respectively, in terms of mean ± standard error. The data obtained suggest that determining circulating diamine oxidase enzyme levels under field conditions can support diagnosis as a biological marker of intestinal permeability, and more prominently, diamine oxidase activity may decrease in both non-infectious and infectious diarrheic calves in relation to small intestinal mucosal damage. This could represent a step closer to the diagnosis of leaky gut in calves.

INTRODUCTION

In the intestine, the monolayered epithelial cells that separate the lumen, where microorganisms are densely located, from the largest immune compartment in the body; It acts as a protective barrier by preventing microorganisms, toxins, inflammatory metabolites and antigens from entering the systemic circulation (Mani et al., 2012). When the intestinal barrier has been injured, infiltration of toxic luminal antigens and bacteria can elicit aggressive immune activation leading to persistent systemic inflammation. Such inflammation causes changes in tissue function that ultimately alter the metabolic priorities of the animal to support the increased energy demands of the immune system, which negatively affects growth and productivity (Kvidera et al., 2017; Liehr et al., 2017). Diamine oxidase (DAO) is a predominant antihistamine intracellular enzyme exhibited within the mucosal segments belonging small intestine in an attempt to prevent enterocytes through histamine (Kovacova-Hanuskova et al., 2015). It has been elucidated that DAO participated within the solidarity of intestinal barrier and the grade for mucosal villous injury (Fukudome et al., 2014). The latter enzyme has been positioned at edged closing of fully-gorwn villous cells, mainly with elevated swithced on position, and its occupation denotes the rectitude and full growth for small intestinal mucosae (Honzawa et al., 2011). Even if injured mucosae or impoverised integrity for intestinal wall, DAO ooze via jejunal villous era through the systemic circulation (Zhang et al., 2016). It has been also postulated previously that DAO has been denoted as a biomarker for intestinal permeability (Alizadeh et al., 2022). In addition, injured small intestinal mucosae might deduce DAO activity (Alizadeh et al., 2022). Increased DAO serum levels and decreased DAO activity are associated with increased intestinal permeability and therefore lower

intestinal development (Alizadeh et al., 2022; Song et al., 2017). Regarding the hypothesis of this study, diamin oxidase as a valuable biomarker for intestinal injury, was analyzed in an attempt to detect its exitance in calves with non-infectious diarrhea. In this study, DAO levels were investigated for the detection of intestinal permeability and intestinal mucosal damage related to non-infectious or infectious diarrhea in 71 calves from 3 different farms located within the borders of Aydın Province.

MATERIALS AND METHODS

This field study was planned as a cohort (prospective), and 71 calves within the borders of Aydın Province were included. The ages of the calves were ranged beteeen 26 days to 67 days. Veterinarians with ethics committee certificates were involved in the collection of blood samples. The calves were included in the study after obtaining written consent forms from all 3 farm owners within the Aydın Adnan Menderes University Experimental Animals Local Ethics Committee [HADYEK] document no: 64583101/2024/21.

Each calf included in the study was collected from 3 different farms (Farm a: 12 with non-infectious diarrhea, 15 with infectious diarrhea, farm b: 13 with non-infectious diarrhea, 19 with infectious diarrhea and farm c: n=12 from calves without any diarrhea, referred to as the healthy control group). The veterinarians with the above-mentioned ethics committee certificate obtained a 0.7 ml blood sample from each calf via V. jugularis and quickly shipped it in the cold chain to be taken to the laboratory for processing. At this stage, our doctoral and graduate students provided assistance and logistic support on a voluntary basis. After centrifugation in the laboratory, the serum samples were separated into Eppendorf tubes and stored in a -80 °C freezer no longer than one mont until analysis was performed. Then, the samples were analyzed with the Bovine Diamine Oxidase ELISA test. Diamine Oxidase ELISA kit, competitive enzyme immunoassay technique. In this method, polyclonal anti-DAO antibody and DAO -HRP conjugate were used. The detection range was between 0.312 ng/ml and 20 ng/ml. Serum samples and buffer were added to pre-coated plates and incubated with DAO -HRP conjugate for 60 minutes. Following incubation, the wells were emptied and washed on 5 sessions. Obtained wells forwarded to incubation by use of a substrate for the HRP enzyme. The enzyme-substrate reaction leads to the formation of a blue-colored complex. A stop solution was then administered for stopping, which turns the blue-colored enzyme-substrate complex into yellow. The color shading was analyzed spectrophotometrically at 450 nm in a microplate reader.

Statistical analysis

Nonparametric methodology based on ranking was preferred with Kruskal Wallis one-way ANOVA test. P value was established as =0.001. Even if data was not normally distributed, other relevant methodology was deemed available.

RESULTS

As was given in table 1 dAo (ng/mL) values were deemed detected as 6.52 ± 2.85 vs. 7.16 ± 3.40 and 17.05 ± 2.63, regardind infectious, non-infectious and healthy calves, respectively ($p<0.001$). Achieved ata and relevant results were shown on table 1 and figure 1. There was no anaytical ELISA error during testing. Entire samples were correctly virtualized. All sample interpretation was performed at RDA Group Facilities in Tekstilkent, Istanbul by a specialized ELISA technician. Mean DAO (ng/mL) values comperativel was shown on table 1 and figüre 1 below.

Table 1. Table showing descriptive statistical analysis of dAo DAO values

	Infectious	Non-Infectious	Healthy	p Value
DAO (ng/mL)	6.52 ± 2.85[a]	7.16 ± 3.40[a]	17.05 ± 2.63[b]	< 0.001

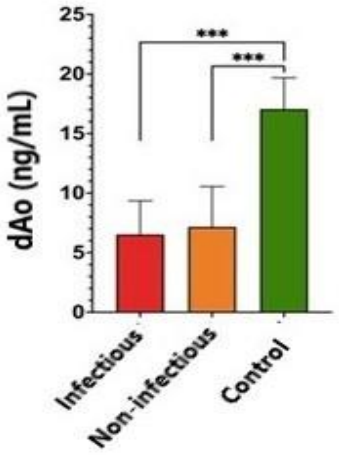

Figure 1. Comparative views of bar charts of dAo levels in infectious, non-infectious and healthy control group calves.

DISCUSSION AND CONCLUSION

As was given in table 1 dAo (ng/mL) values were deemed detected as 6.52 ± 2.85 vs. 7.16 ± 3.40 and 17.05 ± 2.63, regardind infectious, non-infectious and healthy calves, respectively ($p<0.001$). Intestinal permeability has been well recognized as a valuable biomarker for elucidating functioning of the intestinal barrier. Taking into account inflammatory bowel disease, permeability of the intestine has been a determinant component for interpretation of normal/abnormal conditions for gastrointestinal route (Usuda et al., 2021). Various studies in humans and animals have shown that increased intestinal permeability is positively correlated with plasma DAO concentration and negatively correlated with DAO activity. In this study, DAO concentration was measured in calves.

DAO, as an enzyme, catalyzes the oxidation of diamines histamine, putresin and cadaverine (Shakir, 1977). Regarding humanbeing and rodent models, DAO is uniquely located within the apical edges of mature villous cells and shows elevated activity. The latter venture denotes rectitude and full growth in the small

intestine. Many studies in humans and animals have shown that DAO activity is inversely correlated with increased permeability in the small intestine (Ayuso et al., 2007, Palacios et al., 2009). This study has the potential to be a cornerstone in the detection of intestinal mucosal damage in calves.

In a prior study in which calves with diarrhea were classified based on fecal consistency and blood pH, calves with severe or moderate diarrhea were compared to the control groupin which it was determined that plasma dAo DAO activity was significantly lower in the severe group compared to the moderate group. Quanz, (2022) reported that plasma DAO activity clearly decreased in the weeks when clinical signs coinciding with markers of dysbiosis indicated gastrointestinal distress in the cows in the study group. Increased dAo DAO and IL-6 levels are shown in a study examining the effect of early pathogenic *Escherichia coli* (*E. coli*) infection on the intestinal barrier and immune function of newborn calves (He etz al., 2022). In this study, DAO (ng/mL) measurements in healthy control calves compared to calves with both infectious and non-infectious diarrhea were determined as 6.52 ± 2.85, 7.16 ± 3.40 and 17.05 ± 2.63 ($p< 0.001$) in terms of mean ± standard error, respectively (table 1 and figure 1). The data obtained may show that the relevant enzyme may be of diagnostic benefit and that possible intestinal mucosal damage develops parallel to diarrhea. Obtained data is capable of updating veterinary surgeons at field conditions for their treatment protocoles.

Heat stress is also a factor that impairs intestinal function by inducing excessive production of reactive oxygen species (ROS) and proinflammatory cytokines along with increased intestinal permeability (Cheng et al., 2019; Song et al., 2017). It has been demonstrated that serum DAO activity increases in chickens induced with heat stress (Lan et al., 2020). In a mouse model, as a result of induction of leaky gut with *E. coli*, in addition to the observation of villus damage in histological examination; DAO and zonulin levels were found to be significantly higher than in control mice (Ren et al., 2022). Increased DAO and endotoxin levels after administration of methotrexate, which is used for its antitumoral activity but is also likely to have toxic effects on other cells, for chemotherapy in children are associated with increased intestinal permeability (Meng et al., 2016). In a study conducted on 69 humans with inflammatory bowel disease, increased levels of DAO associated with intestinal permeability and intestinal damage and levels of the intestinal bacterial metabolite D-Lactate were determined compared to after treatment (Song et al., 2009). In a pig model, decreased DAO activity following LPS-induced damage to the intestinal mucosa was increased due to increased villus height-crypt depth ratio when pigs were given fish oil (Liu et al., 2012). Decreased DAO activity in the intestine has been reported in Crohn's disease patients, which is associated with the severity of histological changes (Thompson et al., 1988). Takimoto et al., (2014) reported that decreased DAO activity in anorexia nervosa patients suggests the presence of intestinal structural disorder as one of the physical complications of malnutrition. In this study, it was thought that it would be useful to review hygiene and nutritional conditions under the supervision of a specialist veterinarian in all 3 farms.

In a previous study, 22 sick Japanese Black Calves were divided into equal groups and exposed to probiotics or antibiotics. Within 8 days of treatment, serum DAO activity increased significantly only in calves receiving probiotics ([64.4 ± 7.2 on day 1 vs. 76.3 ± 5.1 IU/ml on day 8)], indicating that probiotics were effective on serum DAO activity in calves with diarrhea (Fukuda et al., 2019b). In an important study conducted in our country, multi-strain probiotic treatment was applied rectally to calves with diarrhea; in terms of mean DAO levels (ng/mL), before and after values were determined as 8.48±1.67 vs. versus 28.06±3.51, respectively, with statistically significant changes ($p<0.001$). According to the results of the relevant study, it has been suggested that intestinal mucosal damage may develop in connection with diarrhea, and plasma DAO activity will increase sequentially. However, it has been thought that 10-day rectal enema probiotic treatment reverses this situation and mucosal healing is achieved with proportional feedback regulation (Alic Ural et al., 2023).

In this study, under field conditions, the determination of DAO enzyme activity with statistical significance ($p<0.001$) compared to the control group animals in calves with infectious and non-infectious diarrhea may be an indicator of intestinal mucosal damage. As a result, DAO enzyme maintains its potential to be a useful biomarker.

Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship contributions

Concept: D.A.U., Design: D.A.U., Data Collection or Processing: D.A.U., Analysis or Interpretation: D.A.U., Literature Search: D.A.U., Writing: D.A.U.

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Ethical Approval

The study was conducted with the approval of the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee under permit number 64583101/2024/21.

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In Vitro Evaluation of Genotoxicity of a Commercial Polyaxial Pedicle Screw for Spine Surgery

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ABSTRACT

Biocompatibility, which shows the compatibility between the host and the biomaterial, is very important for the reliability of a biomaterial. It is a must for a newly produced biomaterial to meet the biocompatibility criteria, which are bound to certain rules by international organizations. One of the criteria of biocompatibility is genotoxicity. In this study, it was aimed to evaluate the genotoxicity of a commercial polyaxial pedicle screw *in vitro*. For this purpose, *in vitro* micronucleus test and bacterial reverse mutation test were performed. Extraction method was used for both tests. When the obtained results were compared statistically, it was concluded that the tested biomaterial was not genotoxic.

INTRODUCTION

Biocompatibility is a multidimensional concept and refers to the physical, chemical, and biological compatibility of a biomaterial with its host. Biocompatibility is an indicator of the host-material interaction. It refers to the optimum adaptation of a biomaterial to the mechanics and physiology of the host tissue. To explain it in more detail, biocompatibility can be defined as "the functionality of a material in the medical treatment, that is, its suitability for the targeted purpose, having the most appropriate cellular or tissue interaction for the host's condition, without causing any local or systemic side effects on the host, and having optimum clinical performance in that treatment" (Williams, 2008). Therefore, biocompatibility tests are a must for the development and approval of materials for clinical use, and biomaterials must meet the biocompatibility criteria determined by the International Standards Organization (ISO 10993-1, 2018).

One of the criteria for biocompatibility is genotoxicity, and the evaluation of genotoxicity is addressed in ISO 10993 section 3 (2018). Genotoxicity is a term that affects the genetic material (DNA) in the cell and covers various changes in DNA (DNA breaks, gene mutations, chromosome abnormalities, etc.) (Mohamed et al., 2017). Cells have some mechanisms to prevent genetic damage, but in case of genetic damage, they can prevent it from being transmitted to future generations by apoptosis. The degradation of genetic material in the cell can induce carcinogenesis, and damage to germ cell DNA can negatively affect reproduction or cause hereditary mutations (Elshahawy, 2011; Huzum et al., 2021). Genotoxicity tests are designed to evaluate two important endpoints: gene mutations and chromosomal damage.

Because there is no single mechanism that includes the pathway of action of genotoxic substances and they may act through various genotoxic mechanisms. Therefore, a standard genotoxicity assessment requires at least one *in vitro* test on mammalian cells and one *in vitro* test on bacteria to be designed (Assad and Jackson, 2019).

Spine diseases, which are problem that can reduce people's quality of life, are medical problems that are tried to be solved with various tools and medical applications in parallel with the advancement of biomedical applications and the development of medical techniques. The extension of the average life expectancy by medical technology has caused an increase in the activities of the older generation, and the increase in activity has led to an increase in lumbar stenosis, disc herniation and degenerative spine diseases in these individuals. In young people, similar spine diseases occur in people with high activity or posture disorders (Kwon et al., 2020). Individuals suffering from various spine diseases are usually treated using surgical techniques such as spinal decompression or spinal fusion. Pedicle screws are also one of the biomaterials used in spinal surgeries (Albanese et al., 2017). When associated with the purpose of use, it is inevitable for pedicle screws to remain in the patient's body for a long time. In such a case, although it is an inert metallic material, its long-term stay in the body brings the living tissue-material interaction to the fore. Continuous contact of living tissues with the surface of metal implants can lead to slow but continuous release of metal ions and accumulation in surrounding tissues, which can lead to toxicity, carcinogenicity, or delayed-type hypersensitivity reactions, leading to failure of the implant material (Gotman, 1997; Latka et al., 2024). In ISO 10993 part 1 (2018), the biomaterial contact location and duration in the patient are taken into account in the assessment of biocompatibility. Devices with longer patient contact and/or a more invasive contact area are classified in a high-risk category. Accordingly, genotoxicity assessment is not required for all medical devices (ISO 10993-1, 2018). However, it is important to evaluate the genotoxicity of biomaterials that have long-term patient contact, such as pedicle screws.

This study aimed to evaluate the genotoxicity of a commercial polyaxial pedicle screw *in vitro*.

MATERIALS AND METHODS

In this study, *in vitro* micronucleus test and bacterial reverse mutation test (Ames) were used to evaluate genotoxicity. A commercial polyaxial pedicle screw brought to Kırıkkale University Scientific and Technological Research Laboratory for testing was used in the tests.

In vitro micronucleus test

In vitro micronucleus test was performed by ISO 10993-3 and OECD 487 standards. The specified Chinese Hamster ovary epithelium (CHO) cell line specified in the standard was used as the cell line. (CHO-K1/An1, 95122902, Foot and Mouth Institute). The extraction method was used in testing the sample. The extraction process was performed by the ISO 10993-12 standard. The obtained extract was applied to the cells. The application was done both in the presence and absence of metabolic activation. First, 15x103 cells were seeded in 48-well well plates. The cells were left for incubation (37°C, 5% CO2) for 24 hours. At the end of incubation, the medium in the wells were discarded and three different concentrations of the prepared sample extract were applied as 1:1, 1:2, 1:4. For short application (3-6 hours), application was performed in the presence and absence of metabolic activation (medium containing 2% S9 enzyme), and for long application (24 hours) only in the absence of metabolic activation. For all applications, the test was performed in the presence of Cytochalasin B (3 µg/ml). For positive control, Mitomycin C was used in the absence of metabolic activation in short application, cyclophosphamide was used in the presence of metabolic activation, and colchicine was used in long application. A fresh medium was used for negative control. At the end of the application periods, the medium in the wells were discarded and 75 mM KCl was dropped into each well. Then, methanol: glacial acetic acid (3:1) was added to fixation the cells. Finally, the cells were stained with propodium iodide and mononuclear, binuclear and multinuclear cells were counted under a fluorescence microscope. Binucleated cells containing micronuclei were counted to determine the micronucleus ratio. Then, the Cytokinesis Block Proliferation Index (CBPI), % Cytostasis and % micronucleus ratio were calculated as specified in the standard.

Bacterial reverse mutation test (Ames)

The experiment was carried out under the guidance of the OECD 471 standard. For the experiment, Salmonella typhimurium (S. typhimurium) TA97a, S. typhimurium TA98, S. typhimurium TA100, S. typhimurium TA102 and S. typhimurium TA1535 strains recommended in OECD 471 standard were used. The strains were incubated in 30 ml nutrient broth in 250 ml erlenmeyer flasks for 10-15 hours at 37ºC. After incubation, measurements were made at 600 nm on a spectrophotometer, and the experiment was started by measuring absorbance values as 0.08-0.1. For the experiment, 5 different concentrations of the sample extracted for 3 days at 37ºC according to the recommendations of OECD 471 and ISO 10993-12 standards were used (1/1, 1/2, 1/4, 1/8, and 1/16). Each concentration and solvent control, negative and positive controls were tested in 3 replicates. For each replicate; 2 ml of histidine-biotin supplemented semi-melted (at 43-48ºC) top agar was transferred to the tubes and 0.1 ml of the sample concentration to be tested/solvent/positive control solution (mutagen)/negative control solution (phosphate buffer or sterile distilled water); 0.1 ml of the bacterial strain with determined concentrations and 0.5 ml of S9 mix and phosphate buffer instead of S9 mix for the 2nd series were added and vortexed and spread on minimal glucose agar. After the agar solidified, the petri dishes were turned upside down and incubated at 37ºC for 2-3 days. After incubation, the number of colonies in all petri dishes was determined and statistical calculations were made.

Statistical analysis

Student t-test was used in comparisons between groups, the difference was considered statistically significant when $p<0.05$.

RESULTS

In vitro micronucleus test results

The calculations made as a result of the in vitro micronucleus test are shown in Table 1 and Table 2. Additionally, photographs representing cell counts of the test groups are shown in Figure 1. As a result of the calculations, in the statistical comparisons of the negative

control and sample extracts with different concentrations in terms of *in vitro* micronucleus ratios, it was seen that the difference between the negative control and sample extracts was not statistically significant. In contrast, the difference between the positive control and sample extracts was statistically significant (Table 3). Therefore, when the results of the *in vitro* micronucleus test were evaluated, it was concluded that the tested sample was not genotoxic.

Table 1. Cell and micronucleus numbers of the short application

	Cell numbers and calculations					
	BN	**MN**	**MNC**	**Total cell count**	**CBPI**	**% Cyt**
Sample extract (1/1)	511	7	115	1111	1.66	6.33
Sample extract (1/2)	507	6	112	1064	1.68	3.51
Sample extract (1/4)	522	6	138	1151	1.69	2.63
Negative	512	5	157	1160	1.71	
Positive	500	68	117	1252	1.58	17.66

BN; Number of binucleate cells, MN; Number of micronucleus in binucleated cells, MNC; Number of multinucleated cells, CBPI; Cytokinesis-Block Proliferation Index, Cyt; cytostasis rate.

Table 2. Cell and micronucleus numbers of the long application

	Cell numbers and calculations					
	BN	**MN**	**MNC**	**Total cell count**	**CBPI**	**% Cyt**
Sample extract (1/1)	505	8	125	1177	1.64	9.41
Sample extract (1/2)	510	6	118	1115	1.66	5.51
Sample extract (1/4)	519	6	133	1145	1.68	3.18
Negative	503	5	167	1182	1.70	
Positive	506	74	107	1298	1.55	21.66

BN; Number of binucleate cells, MN; Number of micronucleus in binucleated cells, MNC; Number of multinucleated cells, CBPI; Cytokinesis-Block Proliferation Index, Cyt; cytostasis rate.

Table 3. Statistical comparison of the calculated micronucleus ratios (%) in the *in vitro* micronucleus test

	Micronucleus rates (%) short application	**Micronucleus rates (%) long applicaion**
Sample extract (1/1)	1.36[a]	1.58[a]
Sample extract (1/2)	1.18[a]	1.18[a]
Sample extract (1/4)	1.15[a]	1.16[a]
Negative control	0.98[a]	0.99[a]
Positive control	13.6[b]	14.62[b]

[a,b] Different superscripts in the same column indicate statistically differences (p<0,05).

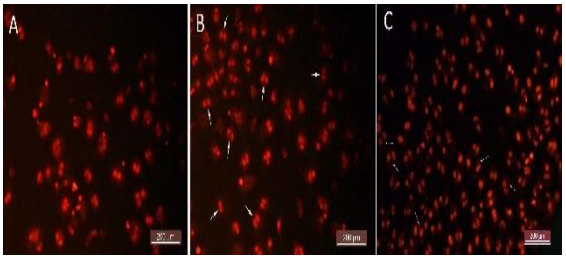

Figure 1. Representative photographs of test groups. A) negative control, B) sample extract applied, C) positive control. In B, arrows indicate binucleated cells, which lack micronuclei. In C, arrows indicate micronuclei in inucleated cells. Propidium iodide stain. Scale bar: 200 µm

Bacterial reverse mutation test (Ames) results

The application was carried out in 3 repetitions for each concentration. Negative and positive controls and sterility control petri dishes were interpreted. No incompatibility was found in positive and negative controls. Sterility control was successful. As a result of the Ames test scoring, no statistically significant genotoxic effect was determined for the dilutions of the sample subjected to the test (Table 4).

Table 4. Statistical comparison of Ames test results

Bacterial strains	With S9 mixture p value	Without S9 mixture p value	Result
S. typhimurium TA97a	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA98	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA100	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA102	p>0.05	p>0.05	Not significant (All concentrations)
S. typhimurium TA1535	p>0.05	p>0.05	Not significant (All concentrations)

DISCUSSION AND CONCLUSION

Genotoxins cause DNA damage and can disrupt chromosomal structure in various ways. Efforts are made to prevent potential risks through genotoxicity studies. Because genotoxicity studies give an idea about whether a drug or medical material causes mutation or genotoxicity and tell us whether a developed material or drug is dangerous at an early stage.

In this study, it was concluded that the tested product has no genotoxic potential. These tests, which are performed in terms of chromosome damage and gene mutations, which are two important points for genotoxicity, can be attributed to the natural non-genotoxic structure of the produced biomaterial and the quality control measures in the production process. The absence of evidence that could cause genetic damage as a result of both *in vitro* cell culture and bacteria tests indicates the reliability of the tested material. However, if it is considered that it will interact and contact with the tissue for a long time, the genotoxicity of the wear particles may need to be evaluated (George et al., 2023). On the other hand, the evaluation of the contact surface, which plays a key role in the biomaterial-tissue interaction, and the determination of the concentrations of metal ions in body fluids such as blood and interstitial fluid are valuable in terms of evaluating the genotoxicity of the new biomaterials produced and predicting potential risks. In the study by Schliephake et al., (1993), high metal concentrations were observed in the lungs 5 months after the placement of metallic implant material. These accumulations prove that particles from the implant surface to erode and be transported hematogenous to distant regions. The biological interactions of such accumulations, even in non-toxic concentrations, should not be ignored (Ribeiro et al., 2007). Studies have also shown, for example, that exposure to nickel compounds is associated with various cancers, and similarly, the possible carcinogenic effects of cobalt or cobalt compounds (Merk and Speit, 1999).

As a result, it was concluded that the polyaxial pedicle screw tested in this study did not have a genotoxic effect. However, the possible negative effects of host-material interaction in the long term should not be ignored and it should not be forgotten that additional tests may always be needed for the reliability of biomaterials.

Acknowledgement

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Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship contributions

Concept: Y.Ö., N.A.Ç., Design: Y.Ö., N.A.Ç., Data Collection or Processing: Y.Ö., N.A.Ç., Analysis or Interpretation: Y.Ö., N.A.Ç., Literature Search: Y.Ö, Writing: Y.Ö.

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Ruminal Acidosis: A Systematic Review

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ABSTRACT

Ruminal acidosis is a metabolic disorder that affects ruminants, including cattle, sheep, and goats, due to a pH imbalance in the rumen. The rumen has a diverse population of microorganisms involved in carbohydrate metabolism, with anaerobic microorganisms in the rumen and cecum playing a crucial role. During healthy rumen metabolism, microbial fermentation produces volatile fatty acids, including acetic acid, propionic acid, and butyric acid. Excessive intake of feeds with high soluble carbohydrate content can cause ruminal acidosis by altering the ratio of volatile fatty acids produced through microbial fermentation, which in turn changes the rumen pH. Acidosis is defined as a decrease in the alkalinity of body fluids relative to their acid content. The pH of body fluids may or may not decrease during acidosis, depending on the degree of bicarbonate compensation. Impaired central nervous system function can occur even if blood pH remains stable due to low bicarbonate concentrations, which are buffered by bicarbonate. While a blood pH below 7.35 is required for a clinical diagnosis of acidosis, other clinical signs such as ruminal pH, anorexia, variable feed intake, diarrhea, and lethargy are commonly used to diagnose acidosis in beef cattle.

INTRODUCTION

Carbohydrates are fermented by anaerobic microorganisms in the rumen and cecum, resulting in the production of volatile fatty acids (VFA) and lactate. Steers fed feedlot diets have been measured to produce more than 55 mol of VFA per day (Azizi et al., 2020). These organic acids are absorbed by ruminants from the rumen and/or cecum for metabolism by tissues. An increase in carbohydrate supply leads to an increase in total acid supply and the prevalence of lactate in the mixture.

Lactate is typically found in low concentrations in the digestive tract. However, sudden increases in carbohydrate supply can cause lactate to accumulate, resulting in ruminal concentrations that can sometimes reach 100 mM. This metabolic disorder has been referred to as 'D lactic acidosis' by Dunlop, (1972) and has been associated with overeating, acute impaction, grain blockage, founder, and grain overload. The term 'acidosis' refers to digestive disorders of the rumen and intestines in ruminants. It is typically categorized into acute, chronic (or subclinical), and subconscious types. Acute acidosis is an overt disease that occurs when easily fermented carbohydrates are consumed in quantities sufficient to lower digestive pH. Chronic acidosis, on the other hand, results in reduced feed intake and performance, but animals may not exhibit any symptoms. The clinical diagnosis of acidosis relies on measuring the acidity of ruminal or blood samples. A ruminal pH of 5.6 or lower is considered chronic acidosis, while a pH of 5.2 or lower is considered acute acidosis, according to Jennings et al., (2018). The variation in feed intake from one day to the next was used as an indicator of subclinical or chronic acidosis (Sanchez et al., 2021). This is based on the idea that increased day-to-day variability in feed intake by individual animals is associated with feeding acidic diets (DeClerk et al., 2020).

The lack of a comprehensive study on acidosis and the lack of a study in which general literature information was given in a collective manner constituted the subject of this study and a systematic review study on acidosis in cattle was carried out by utilizing the basic literatures. The aim of the study was to create a general review framework of the mechanism of acidosis.

MATERIALS AND METHODS

The study was prepared following the PRISMA 2020 Systematic Review Guidelines (Page et al., 2021). The words acidosis, nutritional diseases in ruminants, asidosis in ruminants, ruminal asidosis, etiology of asidosis were searched in Google Scholar, Elsevier and Wiley in Turkish and English. The information obtained from 1 printed

book chapter, 1 congress paper, 3 board reports, and 35 research articles and reviews were used in the study from 1991 to 2024. Related information is summarized in the flow diagram in Figure 1.

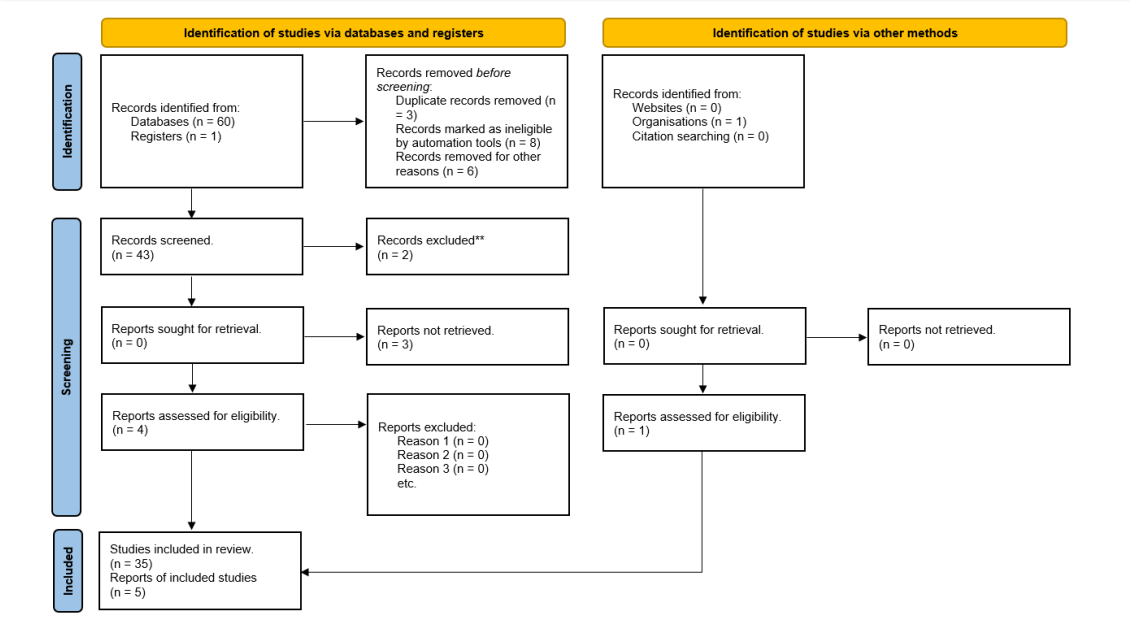

Figure 1. From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al., The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021; 372: n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/

Etiology of Acidosis

Acidosis in ruminants is usually caused by overconsumption of large amounts of starch or rapidly fermentable carbohydrates. This usually occurs when animals are first adapted to a high-concentrate ration and/or when animals switch from bulk feeding to a chemostatic intake arrangement. Additionally, acidosis can occur when grazing animals are given a large amount of a starch-rich supplement (Owens et al., 1998; Millen et al., 2016; Monteiro and Faciola, 2020).

Starch Concentration and Glucose Conversion: Depending on the grain source, processing and type of starch, the rate at which starch is converted to glucose varies. Some grain sources and cereal varieties extract starch more readily, which is preferred by distilleries, and are likely to be hydrolyzed to glucose faster than others. In the endosperm of milo and maize, protein-embedded starch granules are exposed to fewer surfaces for microbial attack. Heat and pressure treatment cause the starch granules to expand into thin sheets, which ferment rapidly. According to Vieria-Neto et al., (2021), heat and pressure treatment of grains, reduction of particle size, and storage at high moisture levels increase starch availability and the likelihood of acidosis. Certain strains of microbes bind to grain particles and release glucose from starch granules. Flake quality can be measured using various methods, such as test weight, birefringence, and rate of gas production during incubation with yeast or rumen contents, as well as glucose or maltose release during incubation with amyloglucosidase or amylase. These methods should reflect the degree of starch exposure and/or fermentation rate. To achieve maximum energy efficiency, it is desirable to have a high degree of fermentation. However, to prevent acidosis, a slow fermentation rate is preferred. Unfortunately, the digestion rate and extent are usually positively correlated across different grain sources and processing methods.

Conventionally, glucose has not been considered an important metabolic intermediate in the rumen because rumen concentrations are normally extremely low. However, many studies found that glucose concentrations in the rumen can exceed 160 mg/dL, which is higher than the concentration found in blood (Sommai et al., 2020; Klotz et al., 2023; Deimeters et al., 2024). In one of acidosis studies, rumen glucose levels exceeded 1,400 mg/dL. Glucose is released from starch by amylase. However, it is unclear whether the higher concentration of glucose is due to faster hydrolysis or a reduction in the rate of glucose utilization by rumen microorganisms (Deimeters et al., 2024). Ruminal pH changes are shown in Figure 2.

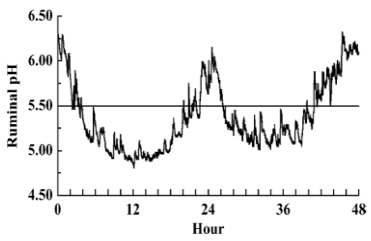

Figure 2. Ruminal pH over a 48-h period in a steer adapted to a 92.5% concentrate diet based on dry rolled maize fed once daily at 0 and 24 h. (Ruminal pH was monitored with a submersible pH electrode suspended from the plug of the ruminal cannula and recorded every minute (data from Cooper et al., (1998)

The presence of free glucose in the rumen can have at least three side effects. Firstly, non-competitive rumen bacteria can grow rapidly when high amounts of glucose are provided. Streptococcus bovis, which thrives only when free glucose is present, is the main cause of lactic acidosis (Chen et al., 2016). However, concentrations of this organism in the rumen of cattle fed high concentration feeds are very low (Semwogerere et al., 2020). Other bacteria that are directly involved in starch fermentation may be more significant sources of lactate. In fact, lactate accumulates faster from starch than from glucose in vitro. Additionally, other opportunistic microorganisms, such as coliforms and amino acid-decarboxylating bacteria, can thrive in the rumen of cattle fed concentrate feeds (Eger et al., 2018; Semwogerere et al., 2020) and produce or release endotoxins or amides (e.g., histamine) during lysis (Beauchemin, 2018). Thirdly, glucose that is released from starch increases the osmolality of the rumen contents. This increase in osmolality worsens acid accumulation in the rumen by inhibiting VFA absorption.

Limiting Starch and Glucose Intake: To prevent acidosis, two common management practices are diluting the ration with roughage or modulating starch intake. Ration-derived roughage reduces feed rate and meal size, while increasing the concentration of dry roughage increases chewing time and saliva production. Increasing the degree of chewing can reduce the size of grain particles entering the rumen, which in turn can increase the rate of fermentation. However, it is important to note that the increased buffer input from saliva due to longer chewing time or ruminating can neutralize and dilute rumen acids. To reduce the starch content of the ration, consider using starch-extracted concentrates such as distillation or fermentation by-products and intermediates instead of cereal grains (Figure 3). Total feed intake can also be restricted by using a limited maximum intake feeding scheme, as described by Lawrance et al., (1995).

For experimental purposes, researchers typically induce acute acidosis by withholding feed for 12 to 24 hours and then feeding (or ruminally dosing) 150% of the normal daily feed ration. This demonstrates that increasing meal size can accelerate acidosis and suggests that daily variation in feed intake between days in an animal will increase the potential for acidosis. Regularity of intake is also recognized as a sign of subclinical acidosis. Fulton et al., (1979) observed that animals' feed intake was typically low after a bout of acidosis, indicating a cyclic pattern of feed intake reflecting repeated bouts of acidosis. Detecting such fluctuations in intake is easy when animals are fed individually. However, when feeding 20 or more animals together, it may be difficult to detect daily fluctuations in intake unless all animals experience acidosis simultaneously, such as during feed changes or mishaps during processing or mixing.

The effects of feed intake regulation on acidosis were investigated in trials conducted in New Mexico, California, and Nebraska (Galyean and Goetsch, 1993; Zinn et al., 1995; Cooper et al., 1999). Although altering daily feed intake slightly decreased feed efficiency and reduced performance in the New Mexico trial, animal health was largely unaffected. Many studies reported that the inclusion of monensin and monensin-tylosin combinations in the diet of feedlot cattle reduced the daily variation in feed intake (Dunlop,1972; Cooper et al., 1999; Beauchemin 2018; Klotz et al., 2023; Kachhadia et al., 2023; Chu et al., 2023; Huot et al., 2023). The inclusion of monensin in the ration has reduced the incidence of digestive deaths in feedlot cattle, probably due to the inhibition of some lactate-producing bacteria and the reduction in daily feed intake variation (Cooper et al., 1997; Nagaraja and Titgemeyer, 2007; Millen et al., 2016; Neumann et al 2018; Huot et al., 2023).

Meal frequency can be as important as total feed intake as a cause of acidosis. For example, cannulated cattle typically have higher feed intake. Weather changes and processing cattle for cannulation or vaccination often disrupt feeding patterns and can lead to overconsumption and acidosis. Proper timing of processing can be beneficial so that cattle are not deprived of feed; limiting intake after work or weather changes can also be beneficial. Estrogen implants have been shown to increase meal frequency, which may reduce the potential for acidosis. The effects of meal frequency may also explain why more skittish animals and some breeds are more prone to acidosis. However, if meal frequency is important, one would expect the incidence of acidosis to be higher when cattle are restricted or programmed fed. To date, restricted feeding has not been reported to increase the incidence of acidosis, perhaps because the total amount of feed provided is not excessive. On the other hand, if excessive amounts of feed are given inadvertently or during the transition from restricted feeding to free choice, acidosis can be expected (Beauchemin 2018; Deimeters et al., 2024).

The role of ruminal protozoa in acidosis is not clear. By ingesting starch particles and storing glucose as polysaccharides, protozoa delay starch fermentation by bacteria, help delay acid production and stabilize ruminal fermentation (Cooper et al., 1997; Nagaraja and Titgemeyer, 2007; Millen et al., 2016; Perez et al., 2024). Given the large amounts of starch consumed by ruminants, the quantitative importance of starch consumption by protozoa seems questionable. However, the ruminal bacterial population is normally reduced when protozoa are present; this reduction may also delay fermentation. Protozoal numbers in the rumen are typically reduced when fed highly concentrated diets, possibly because the long ration fiber provides a fibrous cover in the rumen to which protozoa attach and remain long enough to multiply. Free fatty acids and detergents also reduce protozoal numbers, and a low pH can cause deafunition. However, in addition to stabilizing normal fermentation, protozoal presence in the rumen can be detrimental. Since bacteria have much higher amylase activity than protozoa, amylase activity is higher when degrading per unit protein (Castillo-González et al., 2014). Due to changes in acid or osmolality associated with acidosis, large amounts of amylase are secreted, which accelerates the production of glucose from starch and increases the likelihood of acidosis.

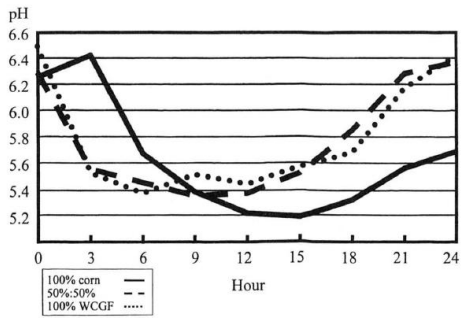

Figure 3. The role of moist corn starch in acidosis (Stock, 2000)

Protozoal stimulants or inhibitors may affect the tendency and severity of acidosis. High levels of dietary fat often led to ruminal instability as protozoal numbers are reduced by highly concentrated rations and removed by unsaturated fatty acids. Huffman et al., (1992), suggested that by coating the grain and reducing the rate of fermentation, added oil should reduce the incidence of acidosis. However, in vivo loading studies with corn and wheat found that the amount of oil had no effect on pH when it fell below 6.0, suggesting that oil was ineffective in preventing subacute acidosis (Krehbiel et al., 1995).

Inclusion of lactobacilli cultures in the diet may prolong ruminal retention of protozoa (Monteiro and Faciola, 2020), reduce fermentation and ruminal lactate production, and help maintain a higher ruminal pH (Cooper et al., 1999). Williams et al., (1991), observed that the mean and peak L-lactate concentration in the ruminal fluid of cattle fed a barley-straw ration indicated that ruminal pH was higher when a yeast culture was added to the ration.

Glycolysis: Anaerobic microorganisms typically thrive when free glucose is available. High concentrations Yet, high concentrations of free glucose in the rumen during acidosis suggest that glycolysis may be partially blocked. In rumen fluid incubation studies, less than half of the glucose incubated with rumen contents (1% wt/vol) was lost within 6 hours, supporting the concept that free glucose is not readily catabolized for unknown reasons.

Essential Fatty Acid and Lactate Production and Utilization: Bacteria in the rumen are typically categorized as either 'lactate producers' or 'lactate users'. The balance between these two groups determines whether lactate accumulates or not. The end products of bacterial strains can vary depending on substrate availability and culture conditions (Monteiro and Faciola, 2020). Most microorganisms that utilize lactate are sensitive to low pH, while most lactate producers are not. Under anaerobic conditions, pyruvate is converted to lactate to regenerate NAD used in glycolysis. Normally, lactate does not accumulate in the rumen at concentrations above 5 mM. Concentrations exceeding 40 mM are indicative of severe acidosis. Two forms of lactate are produced by ruminal and silage microorganisms: the D+ and L form. The L form, which is identical to that produced by exercising muscle from glucose, can be readily metabolized by liver and heart tissue. In contrast, D+ lactate, which typically accounts for 30 to 38% of the total lactate present in the rumen, is not produced by mammalian tissues. The accumulation of free lactate in silage serves to stop fermentation and stabilize the mass. Acidosis is often accompanied by the presence of other microbial products such as ethanol, methanol, histamine, tyramine, and endotoxins, which may have systemic effects (Monteiro and Faciola, 2020; Perez et al., 2024).

Control of Lactate Production and Utilization: Rumen acidosis can be caused by lactate-producing Streptococcus bovis and lactobacilli, coliforms that may lead to anaphylactic shock and sudden death, and amino acid-degrading microorganisms associated with tyramine and histamine production (Amin and Mao, 2021). These microorganisms can be controlled with antibiotics or bacteriophages. To prevent acid accumulation, inoculation with lactate-utilizing microorganisms that can tolerate low pH may be beneficial. Some examples of such microorganisms include Megasphaera elsdenii (Owens et al., 1998), Lactobacillus acidophilus (Monteiro and Faciola, 2020), and three species found in Active Rumen Microorganisms (ARM). Survival in the face of fierce competition from other microbial species makes long-term replacement of rumen microflora difficult. However, individual animals exhibit consistent differences in rumen metabolism. During the 6-month ad libitum feeding period, ruminal lactate production ranked 10th when feeding with corn starch. This suggests that the mix of microbial species within an animal remains constant, although some animals may be more prone to acidosis.

Ruminal pH Decline: Ruminal pH is determined by the relative concentrations of bases, acids, and buffers. Ammonia is the primary rumen base, while bicarbonate and phosphate are the two primary buffers under neutral pH conditions. When pH drops below 5, VFA and lactate also act as buffers (Beauchemin, 2018). During acidosis, when the pH drops to 5.0, the ionization of acids increases slightly. However, the primary cause of the increased hydrogen ion concentration is the added lactate (Figure 4). Lactate is responsible for lowering the pH more severely than similar amounts of other rumen acids due to its low pK (pH point of maximum buffering) of 3.8 compared to 4.8. The acidic pH leads to an increase in osmotic pressure due to greater ionization of acids and the presence of free glucose. Compared to normal concentrations, the change in osmolality during acidosis is much greater than the change in hydrogen ion concentration. Normally, absorption from the rumen prevents acid accumulation. However, the high osmolality of the rumen contents reduces the rate of acid absorption, exacerbating acidity and osmolality. This, in turn, increases the conversion of pyruvate to lactate by lactate dehydrogenase activity, making recovery from acidosis more difficult (Millen et al., 2016). The severe drop in rumen pH is difficult to reverse due to the increased pyruvate hydrogenase activity and promotion of pyruvate to lactate conversion caused by low pH (Perez et al., 2024).

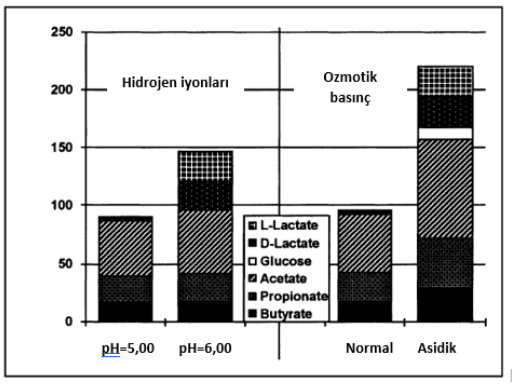

Figure 4. Relative contributions of various organic compounds to ruminal acidity and osmolality under normal or acidotic conditions. Bar heights indicate (from bottom to top) the relative contributions to hydrogen ion and osmolality from butyrate, propionate, acetate, glucose, D-lactate and L-lactate (Owens et al., 1998)

Ruminal Osmolality

The normal range for ruminal osmolality is 240-265 mOsm/L with roughages and 280-300 mOsm/L with concentrates (Owens et al., 1998). The main solutes in rumen fluid are minerals, urea, lactate, and glucose. Protein dissolved in blood significantly contributes to the osmotic pressure, which typically ranges from 285 to 310 mOsm. If rumen osmolality significantly exceeds blood

osmolality, water in the blood is rapidly drawn through the rumen wall. In a study has demonstrated in histological studies that rapid flow to neutralize osmotic pressure causes the ruminal papillae to swell, which can pull patches of rumen epithelium into the rumen, stripping the inner surface layers of the rumen wall from the underlying layers. This rapid water flow can result in damage to the rumen wall or small intestine, which can later be identified as abscess sites. When sepsis occurs, the microorganisms responsible for liver abscesses in the rumen can enter the bloodstream, causing hyperkeratosis or parakeratosis in the repaired tissues of the digestive tract (Eger et al., 2018). This can inhibit the absorption rate of VFA for months or even years after the damage has occurred (Huot et al., 2023). The absorption of VFA through the postruminal passage is possible, but its presence in the abomasum may hinder postruminal starch digestion by inhibiting acidification, protein, and mineral digestion. Therefore, a single episode of non-fatal acidosis can have long-lasting effects. An increase in osmotic pressure in the rumen is detected by the reticulorumen wall, which then inhibits feed intake (Nagaraja and Titgemeyer, 2007; Kachhadia et al., 2023). In addition, bacterial digestion of fiber and starch in the rumen is inhibited by osmotic pressures above 350 mOsm, leading to stagnation of rumen contents. High osmolality (>300 mOsm), coupled with obstruction of outflow causing swelling of the abomasal cavity, makes it challenging to remove fluid and acid from the rumen. While ruminal hypertonicity can reduce the frequency of rumen contractions, it is important to note that inhibited intestinal motility or hypertonicity in the abomasum can stop flow and worsen rumen acidification. Additionally, altered motility or tonicity can cause fluctuating feed intake, leading to chronic acidosis (Cooper et al., 1999; Trotta et al., 2021; Fu et al., 2022). It is crucial to maintain proper motility and tonicity in both the rumen and abomasum to prevent these issues.

Acid Absorption

Lactate and VFA are passively absorbed by the rumen and intestinal epithelium. The absorption rate is higher when concentrations are high, pH is low, and osmolality is normal (Snyder and Credille, 2017).

The percentage of each organic acid in the undissociated form increases with decreasing pH, resulting in a higher absorption rate. Butyrate is partially metabolized as an energy source for the rumen wall during absorption, and glucose is partially converted to D-lactate. Lactate is produced in the intestines and absorbed from them (Singh et al., 2022). Therefore, the total lactate load for the liver can greatly exceed lactate absorption from the rumen.

Blood Osmolality

Blood osmolality increases during acidosis due to two reasons. Firstly, the high osmotic pressure within the rumen draws fluid from the plasma within the rumen. Concentrates blood components and increases blood osmolarity, cell density, and water absorption. Additionally, if the rate of absorption of rumen acid or glucose exceeds the rate of metabolism or excretion, these compounds can accumulate in the blood and directly increase osmotic pressure. High histamine concentrations and vascular damage due to uncontrolled increases in blood pressure within the hoof are associated with acidosis-specific hoof disease and laminitis (Annatte et al., 2019). Osmoreceptors in the rumen sense food intake, rumination in the portal system or liver, and inhibition of saliva secretion (Jiang et al., 2017; Loncke et al., 2020). Fluctuations in blood osmolarity may be the cause of the short-term reduction in feed intake observed in subclinical acidosis. Therefore, it is recommended to consider acute acidosis and chronic acidosis as separate diseases, despite their similar rumen acid bases.

Acid Metabolism and Excretion

Metabolism of VFA results in the conversion of glucose for storage or carbon dioxide for energy. This process occurs rapidly, maintaining low blood concentrations. Previous studies, reviewed by Kachhadia et al., (2023), indicate that D-lactate is not metabolized as efficiently as L-lactate, suggesting that it is eliminated from the blood through renal excretion. Much research suggests that ruminant tissues can metabolize D-lactate. The conversion of L-lactate to glucose by calf hepatocytes is reduced in the presence of butyrate, indicating that interactions between absorbed acids may affect the metabolism rate (Annatte et al., 2019; Loncke et al., 2020; Trotta et al., 2021; Fu et al., 2022).

Classification Of Ruminal Acidosis

Ruminal acidosis is classified into two types according to the rate of occurrence. The first is acute ruminal acidosis, which occurs when large amounts of highly fermentable carbohydrates are consumed in a short period of time (Owens et al., 1998). Acute acidosis is characterized by a rapid decrease in ruminal pH, decreased fiber digestion and excessive lactic acid production. The accumulation of lactic acid in the rumen can be detrimental to the microbial population and cause a decline in rumen function (Monteiro and Faciola, 2020; Amin and Mao 2021; Perez et al., 2024). The other type of ruminal acidosis is subacute, a more chronic form of acidosis that occurs when animals consume moderately fermentable feedstuffs over an extended period of time. The ruminal pH drops, but not as drastically as in acute cases. Subacute rumen acidosis can be more difficult to diagnose, as symptoms may be less pronounced.

1. Subacute Ruminal Acidosis (SARA) is a milder form of ruminal acidosis characterized by recurrent or chronic episodes of low ruminal pH. It is often associated with high-grain diets, inadequate fiber intake, or improper feed management practices. Causes include reduced feed intake, inconsistent milk production, mild diarrhea, and occasional lameness (Fu et al., 2022).
2. Acute Ruminal Acidosis (ARA) is a severe and sudden form of ruminal acidosis, typically caused by a rapid overload of fermentable carbohydrates. This results in a drop in rumen pH to below 5.0, reaching highly acidic levels. ARA can occur due to nutritional errors, sudden changes in diet, or inadvertent consumption of large quantities of easily fermentable carbohydrates. Symptoms of the condition include deep depression, anorexia, diarrhea, dehydration, rapid and weak pulse, lying down, and laminitis. If left untreated, severe cases can lead to shock and death.
3. Chronic ruminal acidosis refers to a prolonged state of low ruminal pH that persists over a long period of time. The ruminal pH remains consistently below the normal range of 6.0 to 6.5. Chronic acidosis can occur as a result of long-term imbalanced diets, insufficient fiber, or constant exposure to stressors. Clinical symptoms include variable appetite, decreased milk production,

weight loss, poor body condition, and increased susceptibility to secondary health problems.

4. Subclinical ruminal acidosis (SARA) refers to a condition in which rumen pH is intermittently below optimal levels without obvious clinical signs. Rumen pH is between 5.6 and 5.2 for most of the day. Similar to SARA, subclinical acidosis is primarily caused by highly concentrated diets, low fiber intake, or improper nutritional management. Diagnosis of subclinical acidosis often requires pH monitoring devices such as rumenocentesis or permanent pH probes. Although there are no obvious clinical signs, subclinical acidosis can have a detrimental effect on rumen health, feed conversion and animal performance.

It is important to note that the classifications provided here are general categories. The severity and presentation of ruminal acidosis may vary depending on individual animal factors, management practices, and environmental conditions. Prompt diagnosis and appropriate management are critical to preventing and reducing the adverse effects of ruminal acidosis.

Symptoms of Ruminal Acidosis

Symptoms of ruminal acidosis can vary depending on the severity and duration of the condition. Some common symptoms included;

- Decreased feed intake,
- Decreased rumination,
- Diarrhea or abnormal stools
- Pain (abdominal kicking, etc.)
- Decreased milk production
- Lameness or reluctance to move
- Dehydration and
- Cachexia in severe cases

If ruminal acidosis is suspected, it is important to consult a veterinarian for accurate diagnosis and treatment. Treatment may include reducing the intake of fermentable carbohydrates, adjusting the diet to include more fiber, providing supportive care, and administering medications to restore rumen function and microbial balance (Stock, 2000; Millen et al., 2016; Kachhadia et al., 2023).

Prevention and Protection

Prevention of ruminal acidosis includes proper management practices such as gradual feed changes, providing a balanced and consistent ration, avoiding sudden feed changes, and ensuring adequate fiber intake to maintain a healthy rumen environment. Regular monitoring of rumen pH and observation of animal behavior can also help identify early signs of acidosis and allow for immediate corrective action (Dunlop, 1972; Stock, 2000; Millen et al., 2016; Kachhadia et al., 2023). Reduce lactate concentrations; control ruminal pH, glycolysis, osmolality, acid absorption, blood pH, and blood osmolality; increase acid metabolism and excretion are important to prevent ruminal acidosis.

Balanced Diet: Adequate fiber should be provided. Ensure that ruminants have access to high quality forages such as hay or pasture to maintain proper rumen function and encourage rumination and salivation. At least 40% of the ration should be roughage. When transitioning animals to a high-concentrate diet, a gradual transition should be made over a period of 7-10 days to allow rumen microorganisms to adapt to the new feed. The adaptation period helps prevent sudden changes in rumen pH. Consistent feeding times and amounts should be maintained to establish a routine for rumen fermentation and to minimize fluctuations in rumen pH.

Feed Management: Diets should be formulated to meet the nutritional requirements of the individual animal or herd. Care should be taken to provide adequate levels of carbohydrates, protein, minerals and vitamins in the diet. Avoid overfeeding grain and other highly fermentable carbohydrates. The amount of starch in the diet should be limited to prevent excessive acid production. Finally, grains should be processed appropriately to improve digestibility and reduce the risk of acidosis. Techniques such as grinding or crushing can enhance rumen fermentation (Galyean and Goetsch, 1993; Krehbiel et al., 1995; Zinn et al., 1995).

Dietary Practices: To promote more consistent rumen fermentation and minimize acid fluctuations, the number of daily feedings can be increased, and the daily feed ration can be divided into several small meals. Adequate feeding space must be provided to prevent aggressive competition between animals, which can lead to overconsumption and digestive disorders (Sommai et al., 2020; Trotta et al., 2021).

Water Management: Clean, fresh water should be always available. Adequate water intake is very important for rumen function and maintenance of rumen pH. Water quality should also be monitored to ensure that it does not contain any contaminants or substances that could affect the health of the rumens (Millen et al., 2016; Jiang et al., 2017; Huot et al., 2023; Kachhadia et al., 2023).

Environmental Factors: Stressful events such as sudden ration changes, transportation or extreme heat, which can disrupt rumen function and increase the risk of acidosis, should be kept to a minimum. Adequate ventilation should be provided in barns or housing facilities to provide a comfortable environment and prevent heat stress (Fu et al., 2022; Chu et al., 2023).

Regular Monitoring: Rumen pH monitoring techniques such as rumenocentesis or permanent pH probes can be used to assess rumen pH and detect early signs of acidosis. Similarly, animal health, body condition and production parameters should be monitored regularly to detect any changes or abnormalities that may indicate ruminal acidosis (Stock, 2000; Singh et al., 2022).

CONCLUSION

Ruminal acidosis is defined as an acid imbalance in the digestive system of ruminants. This condition usually occurs due to factors such as excessive consumption of high grain diets, inadequate fiber intake, or rapid changes in diet. High levels of fermentation, lactic acid accumulation and a decrease in pH are characteristic features of ruminal acidosis. In our review article, we thoroughly examined the available information in the literature and focused on understanding and addressing this important health problem by providing a comprehensive analysis of the etiology, clinical manifestations, diagnostic methods, and treatment strategies of ruminal acidosis. In doing so, we highlighted various feeding strategies to prevent and control ruminal acidosis, as well as methods to improve animal health and production efficiency. However, we also identified knowledge gaps in this area and potential opportunities for future research. Finally, we emphasize that ruminal acidosis requires a multidisciplinary approach and the importance of combining scientific and practical efforts to

improve animal welfare. The aim of this review is to provide a resource to guide future studies into the understanding and management of ruminal acidosis.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: S.K, G.E. E.G, Design: S.K, G.E., E.G, Ş.D., Data Collection or Processing: S.K, G.E., E.G, Ş.D., B.G.G., Analysis or Interpretation: S.K., E.M.Ç., A.K., E.G., B.G.G., Literature Search: S.K., N.A., Writing: S.K., N.A.

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