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Minimally invasive plate osteosynthesis (MIPO) in veterinary orthopedics

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ABSTRACT

The current increase in the numbers of fracture treatment by plate osteosynthesis in veterinary medicine is leading to the production of specific plates for different types of fractures. Recent studies about fracture healing show that MIPO procedure is superior for faster union and healing by decreased contamination risk, faster return of function, lower complication rates and blood supply preservation. By now, indirect reduction technics are more valuable in preservation of the biological structure of bone than full anatomic reduction techniques. Day by day, MIPO becomes more popular in veterinary orthopedics. Basically the method is applying a plate without opening the fractured area to make a bridging between the proximal and distal metaphysis/diaphysis of the fragments. The success of the procedure relies on the type of the fracture and the fracture area. The procedure can be applied especially diaphyseal segmental fractures with success but to be avoided in articular fractures. The procedure has been being used usually in the diaphyseal tibial and radial fractures of the cats and dogs. But nowadays it has started to be used in femoral and humeral fractures as well. The disadvantages of the procedure is the difficulty of the application and the need of the intraoperative radiography or fluoroscopy for the correct positioning of the fractures.

Keywords: mio, mipo, fractures, plate osteosynthesis

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Introduction

Plate osteosynthesis, in addition to other orthopaedic procedures, has been widely employed in the treatment of fractures since the 1800s. The Arbeitsgemeinschaft für Osteosynthesefragen / Osteosynthesis working group, or ASIF (Internal fixation working group), was founded in 1958. The primary goal of this study group was to develop a healthy fracture management procedure that reduced pain and loss of function. This study has facilitated the development of novel osteosynthesis procedures and improved osteosynthesis results throughout the years by offering standardization. Surgical procedures have changed as a result of this process. First and foremost, indirect reduction took the role of anatomical reduction. The plate designs were reconstructed with biological osteosynthesis concepts in mind (Hudson et al., 2009) The primary goal of this modification was to retain the vascularization essential for bone regeneration.

These advancements also made the less invasive surgical method useful in the treatment of fractures. The fracture location is damaged by soft tissue trauma during anatomical reduction with an open approach, and fracture hematoma and extra-osseous blood

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circulation of the bone are also affected. This condition has been proven to result in iatrogenic damage and a delay in bone healing. In reality, in some circumstances, iatrogenic stress promotes necrosis in fracture fragments. When fracture fragments do not sustain iatrogenic damage as a result of trauma during surgery, they preserve their vitality. According to the principles of biological osteosynthesis, the fracture hematoma and vascularization of the area are preserved in the minimally intrusive technique. Fracture pieces are not anatomically firmly positioned. Hulse's connections with soft tissue, on the other hand, are kept without being damaged by the indirect reduction approach based on the idea of "open but don't touch." (Aron 1995; Perione et al.2020). As a result, while environmental tissue damage is reduced, bone fragment vascularization is conserved (Johnson, 2003).

Another advantage of biological osteosynthesis is that it reduces the time required for surgery when compared to open reduction treatments (Johnson, 1998). This minimizes the possibility of infection (Eugster, 2004). By avoiding often observed difficulties, it is assured that the fracture heals before infection arises, reducing the requirement for grafts and the danger of non-union. Internal fixations based on biological osteosynthesis principles eliminate any difficulties associated with external fixation. Because the plates are applied from a more distant region, rather than immediately over the fracture area, the fracture area is not exposed, and tissue damage is decreased. Simultaneously, the fracture hematoma is maintained. This procedure is known as wig plate application or minimally invasive plate osteosynthesis (MIPO) (Perione et al.2020; Witsberger et al., 2010).

Basic principles of minimally invasive plate osteosyntesis (MIPO): Stabilization is performed using a plate implanted through a soft tissue tunnel in the form of a bridge between two minor incision lines far from the fracture line in MIPO, which has recently been employed in veterinary medicine. MIPO is used in a wide range of fracture types. The use is successful with an indirect reduction without the necessity for anatomical reduction, especially in diaphyseal fractures. The creation of calluses, which develops quickly as a result of the adhesion of the fracture components to the neighboring soft tissues, is essential for bone repair. The "blind" stability of bone fragments is the most difficult aspect of indirect reduction employed in MIPO applications. Fracture fragments are attempted to be aligned by manipulating from the outside prior to and during the surgery. Fluoroscopy is also employed to regulate this positioning. It is advanced by regulating with fluoroscopy at every step before stabilizing in place of the plate in order to preserve bone length throughout the surgery and to manage the right placement of bone fragments. The purpose of this entire procedure is not to expose the fracture line and so protect the soft tissue and expedite recovery (Perione et al., 2020; Chao et al., 2012).

Surgical approach: Because the critical neurovascular structures in the area cannot be fully visualised, the surgical approach must be undertaken with extreme caution. The incision line is 2 to 4 cm long and extends to the proximal and distal regions of the area where the plate will be put (Wagner and Frigg, 2006). In extreme instances, a third observation portal with MIPO and IM pin combinations can be used (Perione e al., 2020). Following the formation of the incision line, an epiperiosteal tunnel is opened between the proximal and distal incisions using a periosteum elevator or blunt-tipped soft-tissue scissors.

Figure 1. Application of MIPO in a comminuted femoral fracture of a dog; application of an epiperiosteal tunnel for the insertion of a compression plate locked from the distal to the proximal (Hudson et al., 2009).

Plates of several varieties can be used. The plate is applied to the fracture region by changing the epiperiostal tube from distal to proximal. Fluoroscopy is then used to ensure proper positioning. Screws are put on the proximal and distal sections of the plate using the proximal and distal incision lines. The screws are stabilized once they have been checked for accurate insertion. The surgeon's knowledge of MIPO procedures significantly reduces the surgery time (Schmokel et al., 2007). Since open reduction cannot be used for placement, fluoroscopy is used to confirm the precision of the location at each stage of the application. After fluoroscopy confirms proper implantation, the MIPO plate is fully adhered to the bone.

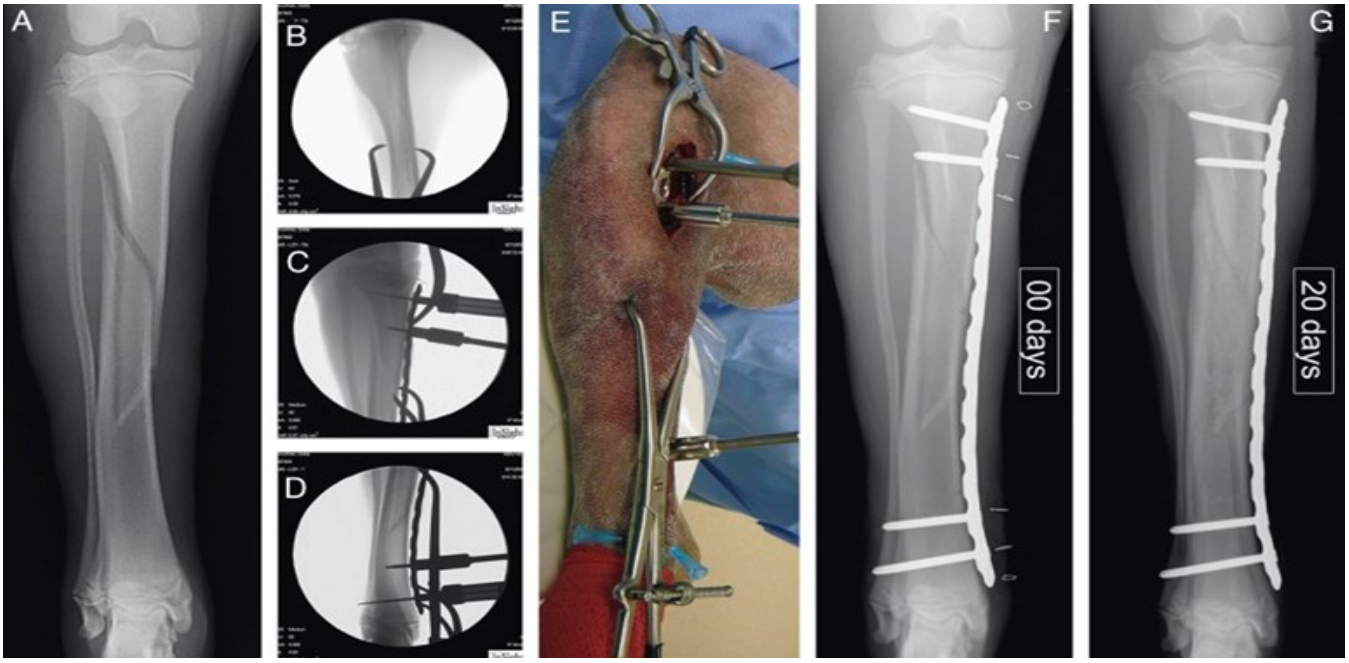

Figure 2- A spiral diaphyseal tibia fracture in a 6-month-old German Shepherd weighing 28 kg. During the operation, alignment was achieved by using bone reduction forceps (B-E). 13-hole 3.5mm LCP plate is placed. (C-E). A small number of screws were used to encourage secondary bone healing, cause minimum harm to the fracture site, and to provide flexible fixation (D-E). Hypodermic needles were placed to see and protect the growth plates of the tibia (E). At the end of 20 days, postoperative imaging showed continued growth (G). (Guiot and Dejarden, 2011)

Pre-operative planning: Adequate preoperative planning is essential in MIPO applications, as it is in any surgical method (Hudson et al., 2009; Hudson et al., 2020) A thorough preoperative planning is very crucial in this approach, where the application differs from conventional procedures, in order to decrease the surgery time and accomplish indirect reduction without any complications. MIPO can be utilized on a wide range of bones and fractures. It does, however, need a more careful approach in neuro-vascularly hazardous locations. It is critical to plan the implant selection and placement in detail during the preoperative phase, based on the patient's radiological results. Despite the fact that long plates aid in fracture stabilisation in implants, smaller plates are preferable owing to mechanical limitations (Hudson et al., 2020).

Implant selection: Preoperative planning must pay special attention to implant selection (Hudson et al., 2020). The weight of the patient, the position of the fracture fragments, the kind of fracture, and the stress to be imposed on the fracture are the primary parameters in this respect (Hudson et al., 2009). MIPO is frequently used in conjunction with normal locked bone plates (Hudson et al., 2020). Locking plates offer effective fixing because they prevent fracture section location from being altered during screw tightening (Hudson et al., 2009). Conventional plates may also be selected to assure alignment on the sagittal axis depending on the state of the case. This decision is largely dependent on the type of fracture (Hudson et al., 2020). Although locking plates are favoured because they give stability without touching the bone surface, they are not appropriate for every MIPO situation. Dynamic compression plates (DCP), limited contact dynamic compression plates (LC-DCP), and locking compression plates are utilized in MIPO applications (Johnson, 2003; Wagner and Frigg, 2006). MIPO applications can also be combined with IM pins or ESFs (Hudson et al., 2009).

Traction application or hanging the extremity: Since the bone fragments cannot be relocated with an open reduction, it is critical in MIPO applications to restore the decreased limb length caused by the patient's present muscle contraction in the preoperative period. Traction applications are chosen for this purpose. During the preoperative planning of the procedure, the traction process is initiated to both stretch the reduced limb length and assure proper posture. Traction tables designed specifically for veterinary medical applications can be utilised. In the absence of them, adequate pressure can be applied by suspending the extremities from a point during preparations. The stretching force created by the present weight of the patient's leg also assists to arrange the fracture pieces appropriately in tractions done by hanging this sort of extremity. Before the procedure, fluoroscopy or portable radiography can be used to manage preoperative and intraoperative placement (Perione et al., 2020).

Case selection for MIPO

Not every fracture method is appropriate for every patient. As a result, selecting the appropriate treatment strategy for the condition becomes increasingly important in each case. MIPO is not appropriate for every fracture. They are particularly useful in metaphyseal fractures and, in instances when anatomical reduction is not required, in fractures of the long bones of the extremity (Hudson et al., 2009; Wagner and Frigg, 2006). MIPO plates operate as a bridge over the fracture line, reducing strain on the fracture fragments (Wagner and Frigg, 2006).As a consequence of the research, we now aware that MIPO is effective in the stabilization of tibial fractures in people, cats, and dogs (Schmokel et al., 2007; Guiot and Dejardin 2011). However, in recent years, it has also been employed in fractures of the radius, humerus, and femur, as well as in certain investigations of metacarpal bone fractures. MIPO is effectively used in diaphyseal fractures, especially because the fracture pieces allow enough margin for fixing both distally and proximally (Hudson et al., 2009). As a result, fractures in the joint area are not desired in joint fractures because they do not give adequate room for the proximal and distal halves of the plate to be placed. Another concern is the presence of essential nerve and circulatory structures along fracture lines. When the neurovascular anatomy in the area must be maintained, open reduction is a better option. In such cases, MIPO is not recommended.

Another key consideration is the amount of time that has passed after the fracture. MIPO's success rate is unquestionably higher in emergency situations (Hudson et al., 2020).

In MIPO applications, the approach to the surgical site is critical for minimizing soft tissue injury. Although radius-ulna and tibia fractures are anatomically suited for MIPO applications, the approach in humerus and femur fractures is fairly problematic due to the anatomical nature of the bone and the neurovascular organization of the region. The method's main drawback is its difficulty in applying to humeral and femoral fractures, both neurovascularly and owing to the anatomical features of the bones and the bonding point of several muscle groups. Nonetheless, studies show that the procedure is effective in treating humeral fractures and has several advantages (Maritato and Rovesti, 2020).

Advantages and disadvantages of the method

This procedure, like any other surgical approach, has advantages and disadvantages. Many comparison research on ORIF and MIPO have been conducted. Internal fixation (ORIF) and MIPO treatments with open reduction were evaluated in a retrospective investigation on diaphyseal tibia fractures in dogs. As a consequence, whereas dogs treated with MIPO recovered quickly and without difficulties, dogs treated with ORIF recovered slowly and with repeated issues (Baroncelli et al., 2012).

MIPO treatments have been effectively employed in humeral, radial, ulnar, and femoral fractures in cats, according to research (Aron et al., 1995; Perione et al., 2020; Hudson et al., 2019; Schmierer and Pozzi, 2017; Maritato and Rovesti, 2020). In practise, anatomical approach challenges, as well as anatomical and neurovascular structural obstacles for the humerus, should be taken into account.

The most prominent advantages of MIPO treatments are the decrease of microbial contamination and tissue damage, the great acceleration of healing as a result of these, and the reduction of the time to restore the function of the broken limb. The method's main drawback is that visual control of the area can only be performed by fluoroscopy. Fluoroscopy is an imaging method that exposes the entire place to radiation exposure. During MIPO application, it is required to employ highly intense fluoroscopy. At each level of the application, imaging should be undertaken for control reasons. The fluoroscope is both costly and dangerous owing to significant radiation emissions. Simultaneously, visual control of the region's neuro-vascular anatomy cannot be accomplished as clearly as in open reduction throughout the surgery. As a result, despite its many advantages, MIPO is a tough treatment to use and necessitates extensive surgical knowledge with technological equipment.

Conclusion

MIPO has begun to be used in our country in recent years, but it is still a procedure that is not widely used in clinical practise. The utilization of this approach, which is often used in veterinary colleges and animal hospitals, is expanding in orthopaedic problems on a daily basis. In practice, it was discovered that the use of MIPO greatly reduced the operation time in tibial fractures, but in femur and humeral fractures, the abundant muscle tissue anatomically produced some application issues. Because of this structure, it has been difficult to access the periosteum and decrease fractures (İstim and Arıcan, 2020)

Minimally invasive procedures are becoming more popular all around the world. This circumstance has also resulted in the recent proliferation of MIPO

practices. Especially in certain research comparing ORIF with MIPO, it is a striking outcome that MIPO achieves an improvement without problem (Baroncelli et al., 2012). A survey of veterinary surgeons, members and diplomats of the American College of Veterinary Surgery, the European College of Veterinary Surgery, and the veterinary orthopaedics community was conducted in 2018. 62 percent of the 256 veterinary surgeons who took part in this study said they wished to do more MIO and MIPO in the near future (Robinson et al. 2020).

Despite its technical problems MIPO (İstim and Arıcan, 2020; Bedizci and Kurum, 2020) which has been effectively applied in our country, is projected to be a favored approach among orthopaedists in the next years by finding more space in practice.

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Clues for zoonotic potential and transmission of Sars-CoV-2 via food and water

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ABSTRACT

As a result of the COVID-19 pandemic caused by SARS-CoV-2 virus, emerged from Wuhan, China in 2020, economic, social, and psychological problems occurred all over the world, mandating implementation of strict curfew, quarantine, travel restriction measures, and vaccinations against the virus. Initially, the source of the virus was not clearly revealed, and case reports from a market in Huanan selling animal products, coupled with sequence analyses of the isolates, revealed close similarity to coronavirus isolated from bats (RatG13) and pangolins, questioning the suspect source of SARS-CoV-2 as zoonotic. Additional epidemiological and experimental studies indicated the presence of SARS-CoV-2, and its specific antibodies in many animals such as cats, dogs, ferrets, calves, and deer. Besides, by the detection of the virus in treated waters of wastewater treatment plants, faecal shedding, and possible faecal-oral transmission of the virus gained importance. Accordingly, vegetables and fruits irrigated with contaminated water, and foods such as shellfish grown in contaminated waters had the risk of carrying the virus. Although one of the most effective ways for protection against SARS-CoV-2 is mass and booster vaccinations, emergence of new variants raises concerns on vaccines' effectiveness. Thus, urgent implementation of one health concept addressing human, animal, and environmental health as a whole is required to overcome this and other possible future pandemics. In this article, emergence, spread, zoonotic potential, faecal-oral transmission risk, and the possible role of food and water in the transmission of the SARS-CoV-2 virus were reviewed based on up-to-date published data.

Keywords: Covid-19, faecal-oral transmission, zoonotic suspicions

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Introduction

Coronavirus (CoV) is an enveloped, single-stranded (ss) RNA virus belonging to the *Coronaviridae* family. CoVs are divided into four genera: Alphacoronavirus (alpha-CoV), betacoronavirus (beta-CoV), gammacoronavirus (gamma-CoV), and deltacoronavirus (delta-CoV). As of today, there are seven types of coronoviruses that can infect humans. Two of them are CoV-229E and CoV-NL63 belonging to the alpha-CoV genus. Others are in beta-CoV genus, including CoV-OC43, CoV-HKU1, severe acute respiratory syndrome (SARS-CoV), Middle East respiratory syndrome (MERS-CoV), and SARS-CoV-2, which is newly discovered as the causative agent of the current pandemic. CoVs were first discovered in 1968, and named 'corona' meaning 'crown' in Latin, based on their crown-like appearance under electron microscopy (Masters, 2006; Ye et al., 2020).

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Until the end of 2002, CoVs were known as viruses that cause mild diseases, such as mild upper respiratory diseases and colds. However, with the outbreaks of SARS-CoV between 2002-2003, and MERS -CoV of 2012, the perspective on the virus changed, due to concerns about the pathogenicity and increased pandemic potential of CoVs (Harypursat and Chen, 2020). CoVs circulate among many species in nature affecting gastrointestinal, respiratory, central nervous system, and liver, thus causing infections in humans, cattle, birds, bats, rodents and wild animals. After SARS-CoV and MERS-CoV epidemics, CoVs gained importance, and wild animals such as bats were indicated as natural hosts of the virus in both epidemics, where various intermediate hosts as camels and civets had roles in transmission to humans. Likewise, in SARS-CoV-2 epidemic of December 2019, initial source and intermediate hosts of infection is thought as bats, and wild animals such as pangolins sold in wild animal market in Wuhan, respectively (Lam et al., 2020; Rodriguez-Morales et al., 2020).

SARS-CoV infection progressed with malaise, headache, myalgia, fever, cough, dyspnea and eventually respiratory failure, causing 8096 cases and 774 deaths (9.5% mortality rate). SARS-CoV was also isolated from bats, which was shown as evidence that the virus is zoonotic and transmitted to humans via intermediate hosts (Kiulia et al., 2015; Wassenaar and Zou, 2020; Ye et al., 2020).

MERS-CoV infection had symptoms as myalgia, diarrhoea, fever, sore throat, cough, respiratory and multi-organ failure, causing 2494 cases and 858 deaths (34.4% mortality rate) to date. In seroepidemiological studies, MERS-CoV antibodies were found in dromedary camels suspected as the zoonotic source of MERS infection by direct contact and processing / consumption of camel meat and milk (Ay, 2020).

SARS-CoV-2 Virus

Origin: COVID-19 emerged in Wuhan, China, towards the end of December 2019, and was declared 'public health emergency' by World Health Organization (WHO) on January 30, 2020. On March 11, 2020, COVID-19 infection caused by SARS-CoV-2 virus was confirmed as a pandemic by WHO. The first case of COVID-19 disease was reported in China on December 31, 2019, and the first death occurred on January 11, 2020. The first case was detected in Turkey on the day the disease was announced as a pandemic by WHO on March 11, 2020, and the first death occurred on March 15, 2020 in our country (Göncüoğlu et al., 2020).

Spread: SARS-CoV-2 infects humans by direct contact with infected or asymptomatic people, such as contact with mouth, nose or eyes, after contact with infected surfaces or inhalation of droplets after coughing or sneezing. Infection through contaminated environmental routes can also occur, as supported by isolation of SARS-CoV-2 from faecal samples. Depending on environmental factors, there is also a risk of nosocomial transmission. In addition, the virus can hang in the air for at least 30 minutes without losing its infectivity in closed and poorly ventilated environments such as buses (Mohan et al., 2021; Ren et al., 2020). Also, SARS-CoV-2 is more stable on plastic and stainless steel surfaces than copper and cardboard, and can maintain its viability on these surfaces for up to 72 hours (van Doremalen et al., 2020). Still, a study from Bangladesh reported 7.29% SARS-CoV-2 RNA carriage by RT-PCR in 425 banknotes collected from the market, emphasizing application of appropriate hygiene measures to the circulating banknotes (Akter et al., 2021).

Course of COVID-19: COVID-19 can progress asymptomatically, as well as with one or more symptoms such as fever, cough, chills, dyspnea, chest pain, pneumonia, myalgia, headache, fatigue, sputum formation, haemoptysis, nausea, vomiting and diarrhea (Huang et al., 2020; Li et al., 2020). In addition, disorders in taste (dysgeusia, hypogeusia, ageusia) and smell (anosmia, hyposmia) are also observed (Giacomelli et al., 2020). A study from China reported these clinical features (number of patients) in decreasing order as fever (82), cough (81), respiratory distress (31), muscle pain (11), confusion (9), headache (8), sore throat (5), rhinorrhoea (4), chest pain (2), diarrhea (2), vomiting (1) in 99 (67 male and 32 female) COVID-19 patients treated at a hospital in Wuhan, with 11 deaths (Chen et al., 2020). Another study from Bangladesh, psychological effects of COVID -19, such as depression, anxiety, insomnia, and suicidal thoughts were investigated on 10,067 participants, and indicated that community psychology was adversely affected by the pandemic (Pakpour et al., 2020).

SARS-CoV-2 and Its Zoonotic Potential: In China, domestic and wild exotic animals (chicken, pheasant, rabbit, snake, civet, pangolin, mouse, salamander, dog, wolf, marmot, beaver, porcupine, crocodile) are frequently consumed for their supposedly health benefits. These animals are kept together in cages alive in close contact to each other, and slaughtered immediately for their meat after purchase. Carrier animals kept under these conditions may well lead to rapid transmission of the virus to other animals or even to humans (Duda-Chodak et al., 2020). In initial emergence of COVID-19, first identified cases were epidemiologically related to a market, where live animals and animal products were sold in Wuhan, Hubei region of China (Lu et al., 2020a). Thus, a systematic review of recent epidemiological, genetic and experimental studies investigating the primary source of the disease, and questioning its zoonotic potential are presented below in detail.

Epidemiological studies: The fact that SARS-CoV-2 emerged from Huanan seafood market, where live animals and animal products are sold, and the first cases appeared in

people residing very close to this market or working / shopping in this market show that this location may be the source of the virus, and this situation increased the possibility of the virus being of zoonotic origin. For this reason, this market was temporarily closed by the Chinese government on January 1, 2020 (Duda-Chodak et al., 2020). In an epidemiological study conducted in a hospital in Wuhan, 27 (66%) of 41 COVID-19 patients with symptoms were reported to have direct contact with the Huanan seafood market (Huang et al., 2020), while in another study, 49 (49.5%) of 99 patients reported to have contacts to this market (Chen et al., 2020).

Among studies examining the role of animals, namely cats and dogs in transmission from animal to human in different countries; in Italy, oropharyngeal, nasal and rectal swabs of 603 dogs and 316 cats in regions, where COVID-19 disease was epidemic, were all negative by RT-PCR. In the same study, serum samples taken from 451 dogs and 191 cats within the same group were examined by plaque reduction neutralization test (PRNT), and antibodies were detected in 15 dogs (3.3%) in the titer range of 1/20 – 1/160, and in 11 cats (5.8%) in the titer range of 1/20 – 1/1280, indicating that cats were more susceptible to the disease than dogs, and although the risk of virus transmission from pet animals to humans is not high, this risk should not be ignored in natural environments with highly populated animals (Patterson et al., 2020). Similarly, in a study conducted in China, presence of antibodies against SARS-CoV-2 was investigated in 102 serum samples collected from cats of Wuhan region after COVID-19 pandemic, and antibodies were detected in titers ranging from 1/20 to 1/1080 in 11 samples (10.78%), showing that SARS-CoV-2 could also infect cats (Zhang et al., 2020a). In France, a serological study was carried out on 47 pets (34 cats, 13 dogs), whose owners had COVID-19, and 38 pets (16 cats, 22 dogs) with owners of unknown disease status. Antibodies against SARS-CoV-2 were detected in 8 (23.5%) cats and in 2 (15.4%) dogs with infected owners, and only in one pet of an owner with unknown disease status. Study results showed higher seropositivity in pets of COVID-19 patients, suggesting owners could play a role in the transmission of the virus to animals rather than other animals (Fritz et al., 2020).

Genetic studies: Whole genome sequence results, indicating close similarity between Sars-Cov-2 and viruses isolated from animals such as pangolin and bat; and recent studies showing reverse zoonotic events occurring between humans and animals such as mink, pet animals (cat and dog) and mouse, have led to a conclusion that there is a tight relation between these viruses of different hosts (Wu et al., 2020a). For instance, a study from China, SARS-CoV-2 whole-genome sequences of 9 patients, 8 of whom had visited the Huanan seafood market in Wuhan, had higher (87.99% and 87.23%) similarity to 2 SARS-like bat CoVs (bat-SL-CoVZC45 and bat-SL-CoVZXC21), respectively, than to the SARS-CoV (79%) and MERS-CoV (50%) (Lu et al., 2020b). In another study, whole genome comparisons of SARS-CoV-2-related coronavirus isolates from two Rhinolophus shameli bats in Cambodia in 2010 (RshSTT200 and RshSTT182) were found to share 92.6% genetic similarity to SARS-CoV-2 (Hul et al., 2021). Another recent genome sequence analyses of SARS-CoV-2 virus results revealed 96.2% similarity to the coronavirus isolated from the bat (RaTG13), indicating that SARS-CoV-2 virus originated from bats (Zhou et al., 2020). Similarly, another study, which reported 91.02% and 90.55% similarity between coronavirus isolates from pangolin and bat, respectively to SARS-CoV-2, suggested that pangolins, like bats, could be natural reservoirs of this virus circulating among animals (Lam et al., 2020; Zhang et al., 2020c). A study from Hong Kong investigated genetic similarities of human and animal SARS-CoV-2 isolates from 15 dogs with SARS-CoV-2-infected owners, where 2 dogs were found infected with the virus with no signs of the disease. Based on the results of genetic sequence analysis, similarity was detected between viruses isolated from dogs and viruses in human cases, and it was stated that this could be an indicator of transmission from humans to animals (Sit et al., 2020). In another study carried out in Netherlands, genome sequence analysis of the virus isolated from 16 mink farms, where SARS-CoV-2 virus emerged, showed that the virus was first introduced to animals by humans, then evolved and circulated among minks, and then transmitted back to humans. Results indicated that virus can be transmitted from animal to human in mink farms (Munnik et al., 2021). A parallel study by Wei et al. (2021) suggested that the progenitor of Omicron variant jumped from humans to mice and mutated in the host, then jumped back into humans, and indicated a reverse zoonotic event from humans to mice.

Experimental studies: Palmer et al. (2021) emphasized the importance of detecting the animal species susceptible to SARS-CoV-2 in nature to determine the origin, potential reservoir, and intermediate hosts of the virus. In their study, white-tailed deer (Odocoileus virginianus), a species with high similarity to humans in terms of angiotensin-converting enzyme 2 (ACE2), was intranasally

inoculated with SARS-CoV-2, and infectious virus shedding was detected in nasal secretions, and faeces of this animal with subclinical infection. In addition, deer fawns, which did not receive viral inoculation, but were in contact with infected animals were also infected, and virus shedding was detected in their nasal secretions and faeces, as well. Serological examinations from both virus inoculated and from contact animals revealed that antibodies against the virus started to observe at the end of day 7, and that white-tailed deer was a highly susceptible species to this infection. In another study from China, the SARS-CoV-2 virus was inoculated intranasally to a group of animals consisting of cats, dogs, pigs, chickens, ferrets and ducks to determine their susceptibility to the virus. Study results indicated that ferrets and cats were highly susceptible to the virus, and the virus can replicate in these animals. Contrarily, dogs had lower, pigs, chickens, and ducks had no susceptibility to the virus. The same study, which also found that transmission occurs by air in cats, reported that surveillance studies on these animals in the future would be important in terms of providing helpful information in the elimination of COVID-19 in humans (Shi et al., 2020). In South Korea, ferrets, which were experimentally infected with SARS-CoV-2, developed symptoms such as fever, bronchiolitis and cough in two days. Also, viral RNA was detected in nasal fluid, saliva, stool, and urine samples obtained from animals, which spread the virus for 8 days after the onset of infection. Sars-Cov-2 RNA, detected in some ferrets sharing the same environment with the infected animals, indicated that airborne infection could also occur among ferrets. Authors suggested that ferrets can be used as a model for the development of vaccines and drugs against COVID-19 due to the resemblance of the symptoms developed in ferrets and humans (Kim et al., 2020). A study from Germany experimentally infected 6 calves with SARS-CoV-2 through intranasal inoculation, while 3 calves were left uninoculated but in close contact with the inoculated calves. SARS-CoV-2 RNA was detected in nasal swabs of 2 calves in the days of 2 and 3 following inoculation, and antibodies were detected in both calves, while no viral RNA was found in uninoculated calves. Authors concluded that although cattle have lower susceptibility to the disease, anthropo-zoonotic infections can occur in large farms, where infected animal owners or animal keepers work in close contact with cattle (Ulrich et al., 2020). Naturally and experimental occurrence of the disease in many domestic and wild animals, and high sequence similarity between viruses isolated from bats and pangolins, and to SARS-CoV-2 isolates of humans, increase and support the possibility that the source of the disease is zoonotic. Besides bats and pangolins, emergence of the virus in cats, dogs, tigers, lions, and minks increases the concerns of reverse zoonotic transmission (Dhama et al., 2020). However, there are no official declarations either from World Animal Health Organization (OIE) or WHO on the zoonotic transmission of SARS-CoV-2 from pet animals as cats and dogs to humans (Acter et al., 2020). On the other hand, on April 6, 2020, the SARS-CoV-2 s was first detected in a tiger, and later in lions at the Bronx Zoo in New York, USA, where the virus transmission to these animals by an asymptomatic zoo worker was suspected (USDA, 2020).

The role of faecal-oral transmission, food, and water in SARS-CoV-2 infection

Faecal-oral transmission of Sars-Cov-2: In 2020, live detection of SARS-CoV-2 in human excreta, water and sewage suggested the possibility of the virus transmission via faecal-oral route, and food and water. This is a particularly important risk factor in developing countries, which apply agricultural irrigation with contaminated water, with inadequate wastewater treatment systems (Aboubakr et al., 2021). In a study conducted in the same year, SARS-CoV-2 RNA was also detected in urine samples, although not as frequently as in stool samples. Results indicated that the viral load in urine (102-105 gc/ml) and in faeces (102-107 gc/ml) was lower than those in nasopharyngeal fluid samples (105-1011 gc/ml). For this reason, contaminated water with sewage, plants irrigated with this type of contaminated water, or food produced from shellfish grown in these waters can serve for the transmission of the virus (Jones et al., 2020). Since enterocytes are rich in ACE2 receptors, they can easily be infected with the SARS-CoV-2, and replication of the virus in intestinal epithelium supports the possibility of the virus' faecal spread (Lamers et al., 2020). In a data-based analysis study on the transmission of the SARS-CoV-2 to the environment with faeces, average viral concentration, and duration of shedding was 3.4 log virus copies/g, and 20-32 days after the onset of symptoms, respectively (Miura et al., 2021). In another study, 15 (25.4%) of 59 patients treated for COVID-19 in Hong Kong showed gastrointestinal symptoms, and viral RNA was detected in 9 (15.3%) stool samples. When similar studies were examined, 17.6% of a total of 4243 COVID-19 patients showed gastrointestinal symptoms, where 48.1% had SARS-CoV-2 RNA in their stools. Study reports indicated the importance of faecal

shedding of the virus even in asymptomatic patients (Cheung et al., 2020). In 8 out of 10 paediatric patients infected with SARS-CoV-2, viral RNA was detected in rectal swabs even after nasopharyngeal swab specimens turned negative, indicating the possibility of both longer duration of faecal shedding, and faecal-oral transmission (Xu et al., 2020). Also, patients with viral RNA detected in stool samples carried SARS-CoV-2 RNA in their stools for at least two weeks after the symptoms of the disease subsided (Pan et al., 2020). In a patient treated for COVID-19, viral RNA was detected in stool on day 33, despite no detection in respiratory tract samples. Another patient with viral RNA in the stool for 47 days after the onset of symptoms indicated the persistence of the virus in stool for longer periods than the respiratory system (Wu et al., 2020b). Similarly, in a study conducted on 3 children, who were treated for COVID-19 in China, virus shedding in stool for 10 days despite negative throat swab samples proved the possibility of faecal-oral transmission of SARS-CoV-2 infection (Zhang et al., 2020b). Another study from China, 93 (36%) of 258 COVID-19 patients' stool samples were found to have SARS-CoV-2 RNA, and although some patients had negative results from oropharyngeal swabs, viral RNA was detected in stool samples. For this reason, authors emphasized that oral swabs, which could not be sufficient for virus detection from infected individuals, should be accompanied by faecal samples (Zhang et al., 2020d).

SARS-CoV-2 virus has been stated to acquire the potential for fecal-oral transmission due to its capacity for multiplication in the human gastrointestinal tract and fecal shedding, and surviving on surfaces for a long time. Based on epidemiological study results showing viral RNA detection in wastewater, occurrence of faecal-oral transmission through the consumption of contaminated drinking water, raw or undercooked saltwater or freshwater products harvested from contaminated sources, and vegetables irrigated with contaminated waters could be expected. There is additional risk for this type of infection in developing countries due to inadequacy of drinking water supplies, improper hygiene applications in food chains, and insufficiencies in health services (Gwenzi, 2021).

Sars-Cov-2 in food and food industry: Today, although there is no clear data on direct food-borne transmission in COVID-19, one cannot assume that contact with food is entirely safe. After determination of high resistance of the virus against environmental conditions, and its detection in the stool in recent studies, Yekta et al. (2021) reported that food should be cooked at 60°C for at least 15 minutes for inactivation of the virus against possible faecal-oral transmission.

Another study from France, conducted to determine the role of shellfish as indicators of SARS-CoV-2, seawater and shellfish (oysters, mussels, clams) were collected between April and August 2020 from the coasts of the touristic beaches. Detection of only human norovirus, but not SARS-CoV-2 RNA in the samples, directed the researchers to conclude that shellfish are good indicators for microbial quality of their environment (Desdouits et al., 2021).

Food facilities, where SARS-CoV-2 virus infected personnel are allowed to work pose a great risk to consumers. In order to reduce this risk, effective food safety control systems together with hygiene applications for personnel, food contact surfaces, and packaging materials must be implemented in the facilities. Utmost care must be taken for: 1 - cleaning and disinfection of the premise, 2 - correct use of protective equipment such as masks and gloves, 3 - social distance between personnel, and 4 - placement of hand disinfectants at easily accessible points within premises (Duda-Chodak et al., 2020). German Federal Institute for Risk Assessment (BfR) report indicated that although there is no definitive proof of transmission of the SARS-CoV-2 virus via food, washing of the textile products as clothes and towels at a temperature of at least 60°C should not be ignored. The same report emphasized contaminated frozen foods as a risk factor, and compliance with hygiene rules in food facilities due to the resistance of viruses in the Coronaviridae family to cold (BfR, 2021). In a study by Shahbaz et al. (2020), washing hands with soap under hot water for at least 20 seconds, sanitation and disinfection of surfaces in contact/non-contact with food, complying with the rule of at least two meters social distance between employees, placing hand disinfectants at the entrances and all accessible places within the enterprise, using appropriate protective clothing for personnel, and taking routine body temperature controls were stated as measures to prevent COVID-19 outbreaks in food facilities. In addition, the importance of reducing contact between personnel by dividing employees into small groups during meal breaks and other breaks, and on-line meetings instead of face-to-face was emphasized. Authors once more pointed out the use hand disinfectants would not replace but only be adopted as an additional preventive measure to hand washing. In a study examining SARS-CoV-2 outbreaks in meat processing facilities in Germany, Günther et al. (2020) stated that environmental conditions such as

low ambient temperature and air exchange/refreshing rates, constant air circulation, insufficient social distance between personnel, and overwhelming working load could cause further virus spreading. In the same study, two meters of social distance was reported as insufficient in facilities with these environmental conditions, and implementation of a social distance of at least eight meters between people was advised. Efficient ventilation and air filtering, in addition to the use of high protective masks were also indicated as important factors to reduce infection, particularly in meat and fish processing facilities. Based on United States Centers for Disease Prevention and Control (CDC) report, 4,913 of 130,578 employees from 115 poultry and red meat processing facilities in 19 states had COVID-19 infection with 20 deaths, where physical conditions and working environment were stated as important risk factors in such food businesses (Dyal et al., 2020).

Sars-Cov-2 in water and wastewater: Recent studies from Netherlands (Medema et al., 2020), Spain (Balboa et al., 2021; Randazzo et al., 2020), Italy (La Rosa et al., 2020), Japan (Haramoto et al., 2020; Hata, Hara-Yamamura et al., 2021), United States (Gonzalez et al., 2020; Sherchan et al, 2020), England (Hillary et al., 2021), Serbia (Kolarevic et al., 2021), Hungary (Róka et al., 2021) and Australia (Ahmed et al., 2020) reported the detection of SARS-CoV-2 RNA in wastewater, and indicated the possibility of faecal-oral transmission of the virus should not be overlooked. In this context, focus was given to review the studies below on Wastewater Based Epidemiology (WBE) of municipalities, hospitals, and reports on the detection of viral RNA in various water samples, which were conducted in different continents and countries.

In studies from different continents related to WBE, a study from USA by Weidhaas et al. (2021), detection of SARS-CoV-2 RNA in 61% of 126 wastewater treatment plant samples collected between April and May 2020 was linked to the viral RNA increase in wastewater in parallel to the rise in the cases in the community, emphasizing the importance of WBE studies for public health during the pandemic. Similarly, in a study conducted in Australia, 21 out of 63 (33.3%) wastewater treatment plant water samples collected between February 24 and May 1, 2020, were positive for SARS-CoV-2 RNA. Detection of viral RNA in samples taken up to three weeks before the first clinical case notification in South Brisbane suggested the use of WBE as an early warning system against COVID-19 by the authors (Ahmed et al., 2021b). Parallel to this, findings from wastewater samples collected between June and August 2020 in Canada, where presence of the virus in samples increased 48 hours before new COVID-19 cases, and 96 hours before hospitalizations, implied WBE as more useful than clinical tests in determining the fluctuations of COVID-19 cases in the community (D'Aoust et al., 2021). In South Africa, SARS-CoV-2 presence and load was examined in samples collected from 4 wastewater treatment plants in Central, Isipingo, Darvil, and Howick, suggesting WBE based studies could be used to predict the prevalence of the virus in the population. In the report, viral RNA was detected in all (14/14) Central and Isipingo, and 12 (86%) and 13 (93%) of Darvil and Howick samples. Viral loads of the plants were 3 (x) 104 - 7.32 (x) 105 gc/100 ml, 1.55 (x) 104 - 4.12 (x) 105 gc/100 ml, 0 - 2.73 (x) 105 gc/100 ml, and 0 - 1.52 (x) 105 gc/100 ml, respectively (Pillay et al., 2021).

In one study from Asia, untreated and treated water samples of 11 different wastewater treatment plants in the United Arab Emirates between May and June 2020 were examined, and only 85% of untreated water samples were reported positive for the presence of SARS-CoV-2 (Hasan et al., 2021).

In studies from Europe on WBE, Baldovin et al. (2021) from Italy, presence of viral RNA in 4 of 9 untreated and both of the 2 treated wastewater samples indicated that viral RNA could still be detected in wastewater even after treatment, and the importance of WBE studies was once more emphasized. A second study conducted in Italy in 2020 by Rimoldi et al. (2020), untreated and treated water samples from 3 wastewater treatment plants, and water samples taken from 3 rivers from Milan region were examined for SARS-CoV-2 by RT-PCR. SARS-CoV-2 was present in untreated waters, whereas viral RNA was not detected in treated waters. Sequence analysis of the virus isolates indicated that virus was the most common strain in Europe and was not infective in the cell culture test. Sars-CoV-2 RNA in river samples were suspected as a contamination of sewage or discharge of insufficiently treated waters into the rivers and isolate from this source had no potential infectivity. A study from Sweden, SARS-CoV-2 was detected by RT-PCR method in both treated and untreated samples collected weekly from 5 treatment plants in and around Gothenburg between February and June 2020, where a decrease of 4 log10/l in the viral genome in treated waters, and an increase in viral load in wastewater during peak periods of the epidemic in the community was reported (Saguti et al., 2021). In a study conducted in 9 wastewater treatment plants in Germany, SARS-CoV-2 RNA was detected both in untreated and treated samples, where isolated viral

RNAs' sequences showed significant similarity to human isolates. However, Caco-2 cell culture results indicated that viruses detected in treated wastewater had no infectious potentials to cell culture (Westhaus et al., 2021). A WBE study on hospital related samples from Slovenia, 10 out of 15 SARS-CoV-2 RNA positive hospital wastewater samples (66.7%), where COVID-19 patients were treated, indicated the importance of WBE in determining the social prevalence of COVID-19 disease (Gonçalves et al., 2021).

There are also 'first time' detection reports of SARS-CoV-2 RNA from different water sample types. In one of those reports, Ahmed et al. (2021a) from Australia identified SARS-CoV-2 RNA from cruise and aircraft wastewater tank samples. One other 'first time report' from Mexico examined viral RNA and load in groundwater, dam, and river water samples, which were intentionally collected between October and January 2020 to coincide the SARS-CoV-2 virus peaks, and found viral RNA at the rates of 44%, 12%, and 13% and the viral loads 2.6-38.3 gc/ml, 3.3-3.8 gc/ml, and 2.5-7.0 gc/ml of the samples, respectively (Mahlknecht et al., 2021).

Additionally, the presence of SARS-CoV-2 virus in aquatic environment was investigated in several studies. In 2021, de Oliveira et al. examined the T90 (time required for 1 log reduction) and T99 (time required for 2 log reduction) in viral load over time in two different temperature values (24°C and 4°C) in river water (RW) and wastewater (WW) samples, where from each water type, one sub-group was unfiltered (U, untreated), while another sub-group was filtered (T, treated). At 24°C, T90 and T99 of URW and UWW were 1.9 and 1.2 days, and 6.4 and 4.0 days, respectively. At the same temperature, T90 and T99 of TRW and TWW were 3.3 and 1.5 days, and 8.5 and 4.5 days, respectively. The detection period of the virus increased at 4°C to T90 of 7.7 and 5.5 days for RW and WW, while T99 was reported as 18.7 and 17.5 days. Similarly, in another study by Sala-Comorera et al. (2021), T90 of SARS-CoV-2 virus was measured in river (R) and seawater (S) at 4°C and 20°C. The T90 of the virus in R and S were 3.8 and 2.2 days at 4°C, and 2.3 and 1.1 days at 20°C, respectively, indicating that the virus was more stable in cold conditions and in river water. In a study from Finland, Hokajärvi et al. (2021) collected wastewater plant samples in Helsinki, and stored aliquots at 4°C, -20°C, and -75°C for 84 days to determine the effect of storage temperature on the virus stability. By day 84, viral RNA was detected in all samples, indicating its stability especially in frozen storage conditions. Results suggested that samples from wastewater facilities, which could not be processed immediately should be frozen.

Conclusion

COVID-19 pandemic and similar outbreaks are increasing threats and concerns to humankind mainly as a result of worldwide intentional damage given to forests, thus disturbing natural balance of the ecosystem. Poaching, game meat trade, uncontrolled animal-derived food production and consumption, and increased international trade and travel play vital roles for the occurrence of these problems. Thus, as animal and human health are intermingled, greater concerns for recurrence of zoonotic diseases require strategies to develop and sustain new prevention, diagnosis, and treatment approaches (Contini et al., 2020).

For safe and wholesome food, companies establish quality assurance systems such as Hazard Analysis and Critical Control Points (HACCP), Good Hygiene Practices (GHP), Good Manufacturing Practices (GMP) and standards such as British Retailers Association (BRC), ISO 9000 and ISO 22000. However, these approaches remained inadequate during COVID-19 pandemic, as stated by Jawed et al. (2020), mainly because the agent was then an 'unidentified' hazard, food facilities required a broader system, spanning food fraud and food defense besides HACCP. In the current pandemic, although wild animal trade was thought important in the spread of the SARS-CoV-2, measures taken to prevent the wild animal trade were not sufficient, and that countries should take more effective measures in this regard (Morcatty et al., 2021).

The fact that the origin of SARS-CoV-2 virus is still not clearly revealed, possibility and concerns of faecal-oral transmission have increased consumer interest in food safety and affected consumption habits. A study from Germany indicated an increase in food safety concerns and anxiety during the pandemic, with a negative perception towards game meat consumption in the society (Yang, 2020). Similarly, a Chinese study revealed that the pandemic had a significant positive effect on the food safety knowledge and behavior of the consumers (Min et al., 2020).

There is no specific cure against the SARS-CoV-2 virus, thus strict adherence to up-to-date protection and control measures maintain their importance. Today, vaccination is the most effective approach to keep the pandemic in control, and vaccines produced by various companies are already in use as well as there are vaccine candidates in the approval phase (WHO, 2021). In addition, due to continuous mutations, many new variants such as England, South Africa, Brazil, United States, Japan and India keep emerging (Mahase, 2021; Tao et al., 2021). Also recently, a variant has emerged in South Africa called

Omicron, which is rapidly increasing COVID-19 cases and is likely to be the most contagious variant so far (He et al., 2021). Therefore, in addition to the increase in the contagiousness of the virus, there are also concerns on the effectivity of the currently administered vaccines against new variant viruses.

Apart from vaccination, based on the phylogenetic relatedness, and cross immunity between Bovine coronavirus (BCoV) and SARS-CoV-2 virus, consumption of immune milk containing anti-BCoV antibodies (Bovine coronavirus immune milk-BIM) obtained from cows vaccinated with BCoV was reported to completely or partially inactivate the SARS -CoV-2 virus with a vaccine-like and immunostimulant effect, which could help activate intestinal immune system (Gut-associated lymphoid tissue - GALT) and boost passive immunity (Arenas et al., 2021). Another passive immunity approach reported was immunoglobulin G (IgG) collection from people, who have had the COVID-19, and transferring it to new patients to stimulate the immune system. Similarly, vaccination of cows against SARS-CoV-2 before collecting their milk or colostrum to increase the specificity of IgG in the milk or colostrum against virus, and consumption of this hyperimmune milk was thought to provide short-term protection in individuals (Jawhara, 2020). Another study by Campione et al. (2020), lactoferrin was reported to protect against the coronavirus infection by acting either as natural protector of both respiratory and intestinal mucosa or reverting the iron disorders occurring during viral colonization, suggesting that it could be used to prevent worsening of the course of the disease in mildly symptomatic and asymptomatic patients. A study by Pace et al. (2021), milk produced by SARS-CoV -2 infected mothers, a rich source of specific antibodies as IgA and IgG against the SARS-CoV-2, was suggested for consumption to help neutralize the SARS -CoV-2 activity. In another study by Baird et al. (2021), human milk after vaccination showeding high levels of SARS-CoV-2 specific IgA and IgG antibodies were recommended to protect infants against the virus. Considering the tight link between human, animal and environmental health, one health approach has gained even more importance in COVID-19 pandemic, indicating health programs should keep highlighting this issue in the future (Bonilla-Aldana et al., 2020), in combination with antimicrobial resistance and food safety (Kanamori et al., 2021).

Another huge negative impact of the pandemic is on world's economy, and social psychology. Although there is no official statement about the origin of the virus, zoonotic suspicions are growing after the virus first appeared near the wild animal market in Huanan, and recent studies have shown a great similarity between the SARS-CoV-2 virus and coronaviruses isolated from wild animals such as bats and pangolins. At the same time, the risk of reverse zoonotic infection should not be ignored, as the virus can naturally infect various animals, including pets, and many animals can be experimentally infected. However, the possibility of faecal-oral transmission of the virus is also considered after WBE studies detect the presence of SARS-CoV-2 virus in wastewater, groundwater and rivers, and after detection of the virus being shed in feces and urine, including in asymptomatic patients. In developing countries, the risk of faecal-oral transmission increases further due to inadequate water treatment systems, uncontrolled animal food production and trade. For this reason, precautions should be taken by considering the possibility of faecal-oral contamination in food facilities.

COVID-19 pandemic explicitly indicated how close human, animal and environmental health are, and that a problem in one affects the others as a chain reaction. All measures taken until now and in the future seriously requires immediate action to implement 'one health approach', where veterinary medicine, human medicine and environmental experts work in a harmony to develop control strategies on public health protection to prevent possible future epidemics.

Conflict of interest

The authors declare no conflict of interest for the present study.

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Silicone plastination of spinal cord of cat: as an alternative specimen for neuroanatomy education

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ABSTRACT

Plastination is a technique that aims to preserve biological materials for education, training, and research. Plastinated models increase knowledge and skill, make students easily understand the complex anatomical parts of the central nervous system, meanwhile can reduce the use of animals in research and education. The study aimed to produce a silicone plastinated model of the spinal cord of a cat for practical teaching of neuroanatomy. The spinal cord of a stray cat that died of natural causes was plastinated using silicone plastination method. The cervical spinal nerves (1-8) and brachial plexus were demonstrated. The thoracic region of the spinal cord was also well preserved, but the demonstration of thoracic spinal nerves became very difficult because of too much thinness of the nerves. The lumbosacral plexus was preserved well. In this region cranial iliohypogastric nerve, caudal iliohypogastric nerve, ilioinguinal nerve, femoral nerve, gluteal nerve, ischiadic nerve, obturator nerve, pudendal nerve and cauda equina were visible. The spinal cord of cats prepared by silicone plastination methods can be used as an alternative sample to formalin preserved specimens.

Keywords: cat, neuroanatomy, silicone plastination, spinal cord

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Introduction

Plastination, invented by Gunther von Hagens in 1977, has produced ideal training models for education, research, and display (von Hagens and Tiedeman, 1987). Plastination is a technique that aims to preserve biological materials for education, training, and research. It is one of the most recent and efficient preservation methods used in departments of anatomy for preserving bodies, body parts, and organs in various forms (Pashaei 2010). This technique offers a unique way of preserving body parts or the entire body of animals and humans (Lahunta et al. 2008, Schoefert 2019).

The spinal cord is a tubular structure composed of nervous tissue that extends from the brainstem and continuing through vertebral canal and anchored caudally by the filum terminale, a fibrous extension of the pia mater anchoring the spinal cord to the coccyx (Bican, 2013; König and Liebich, 2020). It has certain regional variations in form and diameter: at two locations, where nerves to the limbs arise, the relative diameter of the spinal cord is increased. The cervical enlargement or intumescence (intumescentia cervicalis) involves the caudal part of the cervical spine and the initial part of the thoracic spine and gives rise to the spinal nerves that form the brachial plexus that innervates the thoracic limb. The lumbar enlargement (intumescentia lumbalis) gives rise to the spinal nerves, which innervate the pelvic cavity and the pelvic limb (Bican, 2013; Toossi et al., 2021, König and Liebich, 2020).

Plastinated models increase knowledge and skill, make students easily understand the complex anatomical parts of the central nervous system, meanwhile reduce the use of animals in research and

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education. Detailed knowledge of the neuroanatomy of the spinal cord is critical for veterinary students to understand its pathologies, for diagnoses and finding possible treatment for the common disorder of a nervous system (Lahunta et al. 2008, Schoefert 2019, Toossi et al., 2021). The most common disease that brings about spinal cord problems in cats is neoplasia of the vertebral column (Marioni-Henry et al. 2004), most commonly spinal lymphosarcoma (LSA) (Vail and Macewen 2000, Forterre et al. 2007, Marioni-Henry et al. 2008), feline infectious peritonitis (FIP) (Baroni et al. 1995, Legendre et al. 1995), and intervertebral disc disease (Knipe et al. 2001, Munana et al. 2001, Lu et al. 2002).

Teaching tools like the plastinated model have paramount importance for the anatomical diagnosis of neurologic disorders. The use of plastinated tissues in the neurosciences greatly facilitates teaching neuroanatomy (Holladay and Hudson, 1989). The is no documented previous study that focused on plastination of the spinal cord of cat. Therefore, the study aimed to produce a silicone plastinated model of cat spinal cord for practical teaching of neuroanatomy.

Materials and Methods

A stray cat that died of natural causes was taken from department of veterinary pathology, Faculty of Veterinary Medicine, Ankara University and used for the study. This study was approved by the Ethical Committee of Ankara University (2021-9-56). The specimen was fixed in a 10% formalin solution for 4 weeks before dissection. Dissection and demonstration of the spinal cord were performed by dorsal laminectomy (Figure 1).

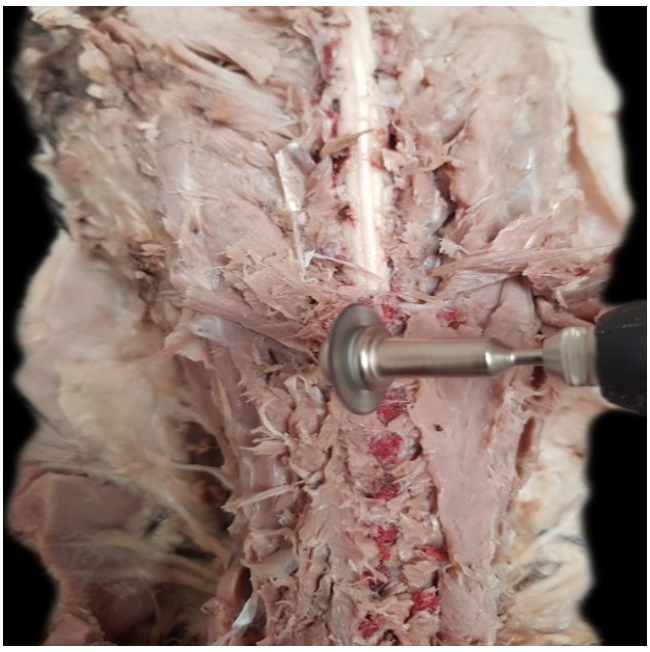

Figure 1. Dissection and laminectomy step

After removing skin and muscles gently, dorsal osseous parts of the vertebral column were removed with an oscillating saw to obtain a clean area and undamaged spinal cord. The dorsal part of the spine was removed starting from the atlas to the end of the sacral region. Then dissection of the spinal nerves was performed. Even though the objective of the study was plastination of spinal cord, from the relative importance point of view, the authors focused on spinal nerves raised from the cervical, lumbar sacral regions, and plexus formation. Following dissection, the cadaver is subjected to fixation. Fixation converts proteins of the body to a longer-lasting substance by forming cross-linkages between adjacent protein molecules. After the fixation, the dehydration step continued. For dehydration three consecutive acetone changes were made at -20 °C. The mass ratio of the acetone to the organs was 10:1. The acetone concentration of the last bath was 99%. Dehydration process take three weeks. After the complete dehydration step, Specimens are removed from the acetone, excess fluid was shake off, and the specimens are submerged in the impregnation mixture. Vacuum pump is applied and bubbles formation was totally ceased at 20 days of forced impregnation. Forced impregnation was carried out in a vacuum tank at -20 °C using S10B silicone polymer and S3 catalyzer. Completion of impregnation was monitored by observing the acetone bubbles on the surface of the silicone-filled vacuum tank. Finally, the gas curing was done with S6 in air-tight bags to harden the specimens (Henry et al., 1997; Henry et al., 2007; Henry et al., 2019). Nomina Anatomica Veterinaria was used for anatomical nomenclature (NAV, 2017).

Results

The vertebral column of the cat was opened and dissected, and then it was plastinated by the silicon plastination method (Figure 2). The cervical nerves were well demonstrated. Due to the chosen dissection technique and the objectives of the study, the evaluation of the existence and course dorsal branch of spinal nerve were not demonstrated. Cervical spinal nerves (1-8) and right and left brachial plexus were demonstrated (Figure 3, Figure 4, Figure 5). They are somewhat flexible and easy to handle. In the thoracic region, the spinal cord was also preserved well but, the demonstration of course of thoracic spinal nerves became very difficult because of too much thinness of spinal nerves. The lumbar, sacral region, and lumbosacral plexus (both left and right) were demonstrated in a way that enables students to a better understanding of the origins and formation of plexuses in the lumbosacral. In this region cranial

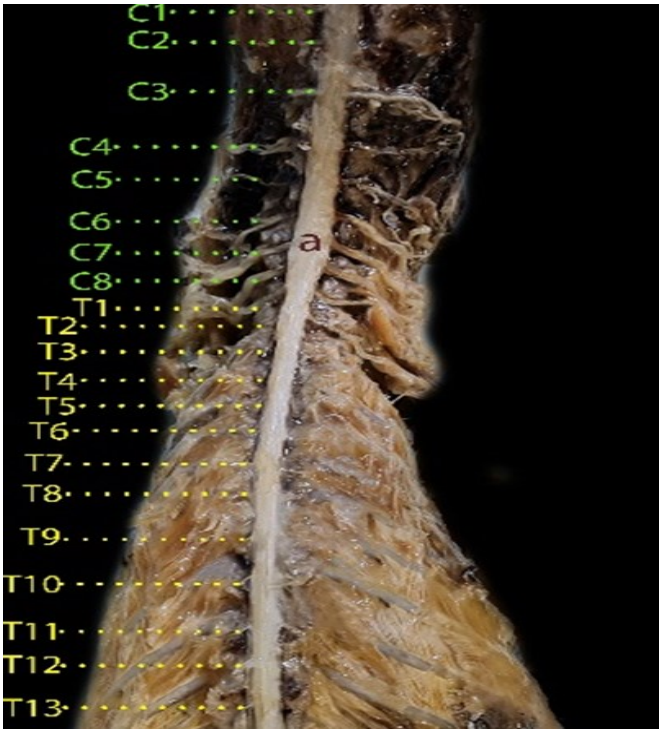

Figure 2. Plastinated anatomical specimen of cervical and thoracic regions of the spinal cord. C1, 1st cervical spinal nerve, C2, 2nd cervical spinal nerve, C3, 3rd cervical spinal nerve, C4, 4th cervical spinal nerve, C5, 5th cervical spinal nerve, (a), cervical intumescence, C6, 6th cervical spinal nerve, C7, 7th cervical spinal nerve, C8, 8th cervical spinal nerve, T1, 1st thoracic spinal nerve, T2, 2nd thoracic spinal nerve, T3, 3rd thoracic spinal nerve, T4, 4th thoracic spinal nerve, T5, 5th thoracic spinal nerve, T6, 6th thoracic spinal nerve, T7, 7th thoracic spinal nerve, T8, 8th thoracic spinal nerve, T9, 9th thoracic spinal nerve, T10, 10th thoracic spinal nerve, T11, 11th thoracic spinal nerve, T12, 12th thoracic spinal nerve, T13, 13th thoracic spinal nerve.

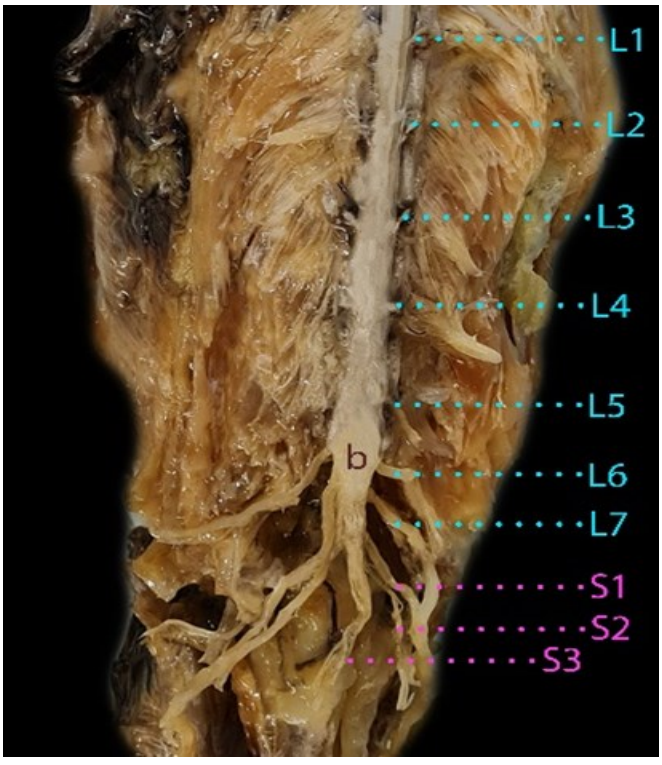

Figure 3. Plastinated anatomical specimen of cervical and thoracic regions of the spinal cord. L1, 1st lumbar spinal nerve (cranial iliohypogastric nerve), L2, 2nd lumbar spinal nerve (caudal cranial iliohypogastric nerve), L3, 3rd lumbar spinal nerve (ilioinguinal nerve), L4, 4th lumbar spinal nerve (genitofemoral nerve), L5, 5th lumbar spinal nerve, (b), lumbosacral intumescence, L6, 6th lumbar spinal nerve, L7, 7th lumbar spinal nerve, S1, 1st sacral spinal nerve, S2, 2nd sacral spinal nerve, S3, 3rd sacral spinal nerve.

iliohypogastric nerve, caudal iliohypogastric nerve, ilioinguinal nerve, femoral nerve, gluteal nerve, ischiadic nerve, obturator nerve, pudendal nerve and cauda equina were visible (Figure 1, Figure 6). The present investigation showed an excellent specimen for display in practical anatomical sessions.

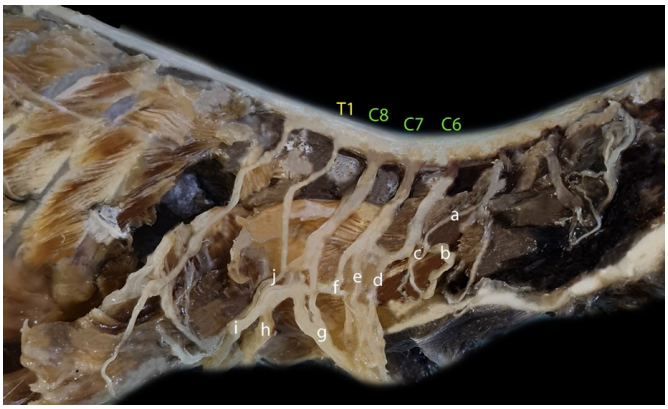

Figure 4. Formation of plexus brachialis and their branches. C6, 6th cervical spinal nerve. C7, 7th cervical spinal nerve, C8, 8th cervical spinal nerve, T1, 1st thoracic spinal nerve. Phrenic nerve (a), dorsal scapular nerve (b), suprascapular nerve (c), subscapular nerve (d), musculocutaneous nerve (e), Abxillary nerve (f), Radial nerve (g), median nerve (h), ulnar nerve (i), lateral thoracic nerve (j).

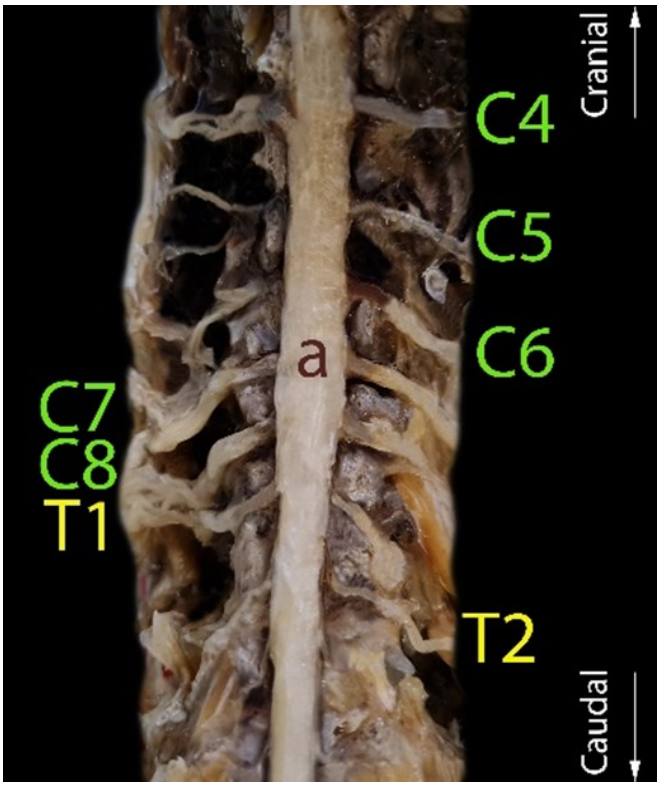

Figure 5. Formation of plexus brachialis. C4, 4th cervical spinal nerve, C5, 5th cervical spinal nerve, C6, 6th cervical spinal nerve, C7, 7th cervical spinal nerve, C8, 8th cervical spinal nerve, T1, 1st thoracic spinal nerve, T2, 2nd thoracic spinal nerve, a, cervical intumescence.

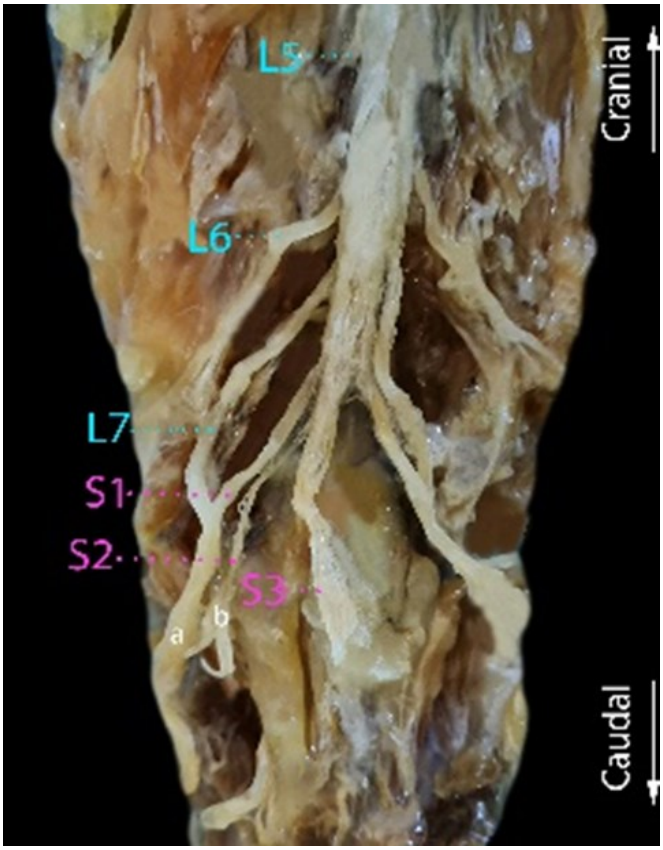

Figure 6. Formation of plexus lumbosacralis. L5, 5th lumbal spinal nerve, b, lumbosacral intumescence, L6, 6th lumbal spinal nerve, L7, 7th lumbal spinal nerve, S1, 1st sacral spinal nerve, S2, 2nd sacral spinal nerve, S3, 3rd sacral spinal nerve, (a) ischiadic nerve, (b) pudendal nerve.

Discussion

Disturbance in the gait of animals is a common neurological problem in veterinary medicine (Garosi 2004). Besides, results of neurological related studies in cats indicate that lymphosarcoma is most common in affecting the spinal cord of cats (Vail and Macewen, 2000, Forterre et al. 2007, Marioni-Henry et al. 2008). For the appropriate treatment of neurological problems, understanding their anatomy is crucial. Therefore, the plastinated specimen in our research will probably provide an efficient preliminary information not only for the clinicians but also for the pathologists. The present investigation showed spinal nerves in the cervical and lumbar region with their ventral roots. In the cervical region, the ventral root spinal nerves (1-5) were visible and the formation of plexus cervical was also demonstrated. The ventral roots of the last three cervical spinal nerves (6-8) and the first thoracic spinal nerve (T1) were well demonstrated and how they formed plexuses brachialis was visible. Ventral branches of plexuses brachialis were also well preserved. Suprascapular nerve, subscapular nerve, musculocutaneous nerve, axillary nerve, radial nerve, median nerve, ulnar nerve, and lateral thoracic nerve were among well-preserved plexuses brachialis. Produced specimens were dry, non -sticky, odorless, with detectable morphological structure, and almost retain their natural form. Central nervous system preserved in formalin solution has been using for practical teaching of neuroanatomy. Long term storage of Central nervous system in formalin solution has strikingly noticeable influence on its lipids content. Central nervous system preserved in formalin can be easily tear or break down while students use it (Jain et al., 2014; Tomalty, 2019, Heslnga and. Delerkauf, 1962).

Our study showed an excellent specimen to display samples in practical anatomical sessions which enable students to better understand the origins and formation of brachial plexus. In the lumbar and sacral region, the spinal cord with its roots (ventral rami) and cauda equina was well demonstrated. In the lumbar region the first three ventral rami (cranial iliohypogastric nerve, caudal iliohypogastric nerve, ilioinguinal nerve) after leaving the vertebral column, didn't form any plexus in both sides (left and right). The last four ramus ventralis of the spinal cord in the lumbar region was attached and forms the lumbar plexus. Lumbar plexus further attached to the sacral plexus and forms lumbosacral plexus. The sacral plexus was formed by joining of the ramus ventral rami of the spinal cord in the sacral region. In comparison to the formalin preserved specimen of the spinal cord and its roots, the specimen preserved with plastination technique was visible, unbroken but they were much firmer. The same finding was also reported by Basset et al. (2014). The skill and knowledge of regional anatomy in the nerve system had an important part in general surgical practice. Having good knowledge and skill of spinal cord regional anatomy could be useful in studying and determining an area for epidural anesthesia during surgical practices.

Conclusion

Spinal cord in cat prepared by silicone plastination method can be used as the best alternative to formalin preserved specimens in the teaching of neuroanatomy, but it needs very careful dissection and specimen preparation for plastination.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Effects of waiting time between trials and water temperature on cognitive functions, body temperature and body weight in rats in Morris water maze

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ABSTRACT

The Morris water maze (MWM) is a widely used test among neurobiologists to measure spatial memory. The implementation of this test carries the risk of hypothermia periods in animals. The level of hypothermia may affect age-related memory processes as a significant factor. The occurrence of hypothermia throughout the MWM protocol should be better understood as hypothermia may impair memory performance. Ensuring the standardization of the experiments and minimizing side effects require a detailed examination of the hypothermia-related processes. Our study aims to replicate and extend the data of previous studies in terms of determining the possible species-specific variations and provide data for reorganizing the time intervals. In this study, rats (Wistar Hannover) were used and grouped according to the differences in the inter-trial interval (ITI) (30-s and 13-min) and water temperatures (20 °C and 24 °C). The effects of ITI and water temperature on probe performance were analysed statistically (mixed two-way ANOVA). Results showed that the 13 minute waiting group of animals performed statistically better in the MWM probe phase compared to the 30 second waiting group. The prolongation of ITI between the tests was found to have a positive impact on the memory performance. Longer ITI should be preferred instead of the frequently used 30-60 second test intervals. Thus, animals will be exposed to less stress and more reliable results can be obtained, also possible side effects of hypothermia can be minimized while performing the MWM test.

Keywords: comorbidity, hypothermia, memory, spatial, probe performance

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Introduction

The Morris water maze (MWM) test is one of the most preferred cognitive tests in the field of age-related learning and memory loss (Ecevitoglu et al., 2020; Pezük et al., 2006; Iivonen et al., 2003; Morris, 1984). It is also a reliable spatial learning and reference memory test and is widely used in neurobiological studies. However, this test has strengths and weaknesses that arise from various causes. During the administration protocol of the MWM test on the animals, different levels of hypothermia can occur when animals remain in the water for certain periods. Iivonen (2003) has performed different MWM protocols and reported that female mice which were exposed to short inter-trial interval (ITI) and the lower water temperature had up to 9 centigrade lower body temperature. Therefore, to ensure the standardization and reliability of the experiments and to minimize side effects for the animal welfare a detailed examination

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of the hypothermia-related process of the test and its optimization according to certain criteria is required (age, sex, body weight, disease model, etc.). Especially in experimental studies where variables such as stress level, body temperature, and body weight need to be stable, standard protocols can be re-adjusted in a species-specific manner.

Studies in both mice and rats have reported that hypersensitivity to stress negatively affects the performance on the MWM test (D'Hooge & De Deyn, 2001). Additionally, studies have shown that extending the ITI eliminates the "net cooling effect of water" which causes a drop in body temperature. (Iivonen et al., 2003). It has been reported that this effect may also be age-related (Iivonen et al., 2003; Lindner & Gribkoff, 1991; Hamm, 1981). Therefore, the level of hypothermia may affect age-related learning and memory performance as a significant factor. Moreover, hypothermia may also cause comorbidities including neurodegeneration. For example, in studies of disease models such as traumatic brain injury (TBI) accompanied by many comorbidities such as progressive neuronal damage, neurodegeneration, and inflammation (Golub & Reddy, 2022), test-induced hypothermia in addition to these comorbidities is considered an undesirable condition.

Unlike in previous studies, our study investigates the effects of ITI and water temperature values on memory performance, body weight, and body temperature in rats to determine the existence of possible species-specific variations in these variables. In addition, it is aimed to determine the optimal ITI that will enable accurate assessment of memory performance, improve animal welfare, and minimize potential side effects by examining the range of the waiting time values used in standard MWM protocols. Thus, our study will replicate and extend the data from previous studies by revealing the effect of different water temperature values at different intervals during the MWM protocol on experimental rats.

Materials and Methods

Subjects: In the study, 32 female rats of 6 months old Wistar Hannover strain (with an average weight of 220-260 g) were used. In our reference study, the greatest difference in body temperatures was observed between female mice (Iivonen et al., 2003). For this reason, female rats were chosen for the study. Water and food were provided as ad libitum. Animals were housed in room conditions with 50-60% humidity, 18-22 °C temperature, 15-17 cycles per hour ventilation, and a lighting cycle of 12 hours light and 12 hours dark. Groups of animals were formed according to the differences in waiting times between swimming trials (30-s and 13-min) and different temperatures of the water (20 ± 0.5°C and 24 ± 0.5°C). 32 rats were randomly divided into groups as follows:
Group: 30-s ITI / 20 °C WT (n=8)
Group: 30-s ITI /24 °C WT (n=8)
Group: 13-min ITI / 20 °C WT (n=8)
Group: 13-min ITI / 24 °C WT (n=8)

Experimental design. Animal experiments were carried out after approval (No:2022-04) from the Animal Experiments Ethics Committee of Istanbul Bagcilar Training and Education Hospital on 28.02.2022. The MWM test was applied to all animals to evaluate hippocampus-dependent memory performance. Before the protocol, the tails of all animals were painted and individually numbered. Each animal was weighed one day before starting the protocol. In addition, body temperatures were measured rectally twice a day, before and after the MWM test (as soon as 4 trials were completed for each animal). Basal body temperature is the first measurement of each day. The difference in body temperature (Δt) was calculated by subtracting the body temperature before the first trial from the body temperature after the last trial (Δt=initial measurement - last measurement). After the trials were done, animals were dried in cages with a heating source.

Morris water maze. The MWM was conducted in a standard water-filled pool with a diameter of 150 cm and a depth of 60 cm. It was filled with water to rise 1.5 cm above the 15 cm wide platform placed inside the pool. Every object in the room was kept in the same place from the beginning to the end of the experiment. The water temperature of the pool was fixed at 24 °C and 20 °C in accordance with the experimental groups, and the water has been blurred with black food dye. The pool was kept blurred throughout all learning trials and probe trials. In learning trials, rats were put into the water from the farthest point (South) to the quadrant (North) where the platform is located. Rats were allowed to learn the location of the platform by swimming for 60 seconds in a 150 cm diameter pool for four consecutive days and four times a day. The starting quadrants of the groups were counterbalanced between the 3 quadrants, except for the quadrant where the platform is located. Escape latency is defined as the time for the rats to find the platform. Escape latencies for all rats were recorded. After the learning trials were completed, to assess memory function, the platform was lifted and the duration the rat swam in the target quadrant, where the platform was located during the learning trials, was recorded

(day 5). Probe performance was defined as the time the rats spent in target quadrant after the platform was removed. All trials were recorded with a camera.

Statistical analysis: Statistical analyses were performed using SPSS software ver. 25.0 2 (water temperature effect: 20 °C and 24 °C × 2 (time between learning trials: 30-s-13-min) factorial design was applied. First, it was determined whether the data showed a normal distribution by considering the Shapiro-Wilk and histogram graphs. In the measurements in which the assumption of normality was met, two-way and mixed-design ANOVA tests were applied. In accordance with the results of Levene's test, Tukey or Games-Howell's post hoc test was used to identify the cause of the difference between the groups. The differences at the level of $p < 0.05$ were accepted as significant.

Results

Although the number of animals in the study was first determined as 32, 29 animals were included in the statistical analysis, as three had swimming problems. 2×2 ANOVA analysis was used to determine the effects of ITI and the water temperature on probe performance. The analysis yielded a statistically significant ITI main effect F (1, 25) = 7.191, $p = 0.013$, partial η2 = 0.223). The 13-min ITI group's (M = 21.5, SEM = 1.53) probe performance was better than 30-s ITI group's (M = 15.79, SEM = 1.48). The water temperature main effect and the interaction between ITI and the water temperature were not significant, $p > 0.05$.

Table 1. The effects of ITI and WT on MWM probe performance.

	MWM Probe Performance		
	n	x̄	SEM
Main effect of ITI			
30-s group	15	15.79[a]	1.48
13-min group	14	21.5[b]	1.53
Main effect of WT			
20˚C	15	19.65	1.48
24˚C	14	17.65	1.53
*ITI*WT interaction*			
30s-20˚C	8	16.88	2.02
30-s-24˚C	7	14.71	2.16
13-min-20˚C	7	22.43	2.16
13-min-24˚C	7	20.57	2.16
		p- value	
ITI		0.013	
WT		0.354	
ITI*WT interaction		0.944	

a, b = Values with different superscripts within each column are significantly different. ITI = Inter-trial interval; S.E.M = Standard Error of Mean; WT = water temperature. Probe performance was defined as the time spent in the target quadrant.

Table 2. Change in the body weight of the WT groups during the days

		Body Weight		
		n	x̄	SEM
WT	*Time*			
20˚C	1	15	246.8[a,d]	3.34
	2	15	243.8[b]	3.71
	3	15	244.7[c,b]	3.67
	4	15	245.9[c,a,d]	3.71
	5	14	247.3[d]	3.85
24˚C	1	12	237.9[a]	3.72
	2	12	236.2[a,b]	4.14
	3	12	234.5[b,c]	4.09
	4	12	233.0[c]	4.14
	5	12	233.8[c]	4.29
			p-value	
WT*Time interaction			<0.001***	

Note. *$p < 0.05$, **$p < 0.01$, $p < 0.001$, ***; a, b, c, d = Values with different superscripts within each column are significantly different. SEM = Standard Error of Mean; WT = Water temperature.

A mixed-design ANOVA was performed with body temperature, as the dependent variable, body temperature (day1-pre/post, day2-pre/post, day3-pre/post, day4-pre/post, and day5-pre/post) as the within-subjects, and ITI and the water temperatures as the between-subjects independent variables. As a result of the analysis, less decrease in the body temperature was observed in 13-min ITI group (M = 37.64, SEM = 0.11), compared to 30-s ITI group (M = 37,28 SEM = 0.11) (F (1, 25) = 5.114, $p = 0.033$, η2 =0.17). The interaction between time and the water temperature was significant (F (9, 225) = 2.842, $p = 0.043$, η2 = 0.102) (Table 3).

A 2×2 ANOVA was conducted to evaluate the effects of ITI and the water temperature on Δt. Results showed that 30-s ITI group (M = 1.13, SEM = 0.11) had a statistically significant decrease in Δt value compared to 13-min ITI group (M = 0.53 SEM = 0.12) (F (1, 25) = 13.528, $P = 0.001$, η2 = 0.351). Similarly, a statistically significant decrease in Δt value was observed in the 20 °C WT group (M = 1.06, SEM = 0.11) compared to 24 °C WT group (M = 0.59, SEM = 0.12) (F (1, 25) = 8.154, $P = 0.009$, η2 = 0.246). The interaction between ITI and the water temperature were not significant, $P > 0.05$.

Table 3. Change in the body temperature of the WT groups during the eight trials (day 1, day 2, day 3 and day 4) and probe phase (day 5)

		Body Temperature		
		n	x̄	SEM
WT	*Time*			
20˚C	1	15	38.4[a]	0.16
	2	15	35.92[b,f]	0.23
	3	15	37.87[c,d,e,g,i]	0.15
	4	15	37.51[d,c,e,g,i]	0.26
	5	15	37.9[e,c,d,g,l]	0.15
	6	15	36.38[f,b,h]	0.51
	7	15	37.67[g,c,d,e,j]	0.10
	8	15	37.29[h,d,f,j]	0.17
	9	15	38.01i[,c,d,e]	0.12
	10	15	37.43[j,d,g,h]	0.16
24˚C	1	14	37.87[a]	0.16
	2	14	35.93[b]	0.24
	3	14	37.97[c,a]	0.16
	4	14	37.01[d]	0.27
	5	14	37.96[e,a,c,h,j]	0.15
	6	14	37.56[f,a,c,d,e,g,h,i,j]	0.52
	7	14	37.63[g,a,c,d,e,f,g,i,j]	0.11
	8	14	37.61[h,a,cd,e,f,g,i,j]	0.18
	9	14	37.52[i,a,d,f,g,h]	0.12
	10	14	37.84[j,a,c,e,f,g,h]	0.17
			p-value	
WT * Time interaction			0.043	

a, b, c, d, e, f, g, h, i = Values with different superscripts within each column are significantly different. SEM = Standard Error of Mean; WT = water temperature. 1, 3, 5, 7, and 9th body temperature measurements were taken before the MWM trials, 2, 4, 6, 8, and 10th body temperature measurements were taken after the MWM trials.

Discussion

There have been many well-studied procedures for the optimal use of the MWM test or present new methods for behavioral analysis, such as providing the appropriate interval training and probe trials, and control procedures to evaluate non-spatial features (Vorhees & Williams, 2006) or the assessment of age-related cognitive deficit (Gallagher et al., 2015). However, the hypothermia-related issues of MWM have not been sufficiently discussed in the previous research. Our study revealed that this issue may play an important role in experimental studies. Therefore, we provide a protocol that minimizes hypothermia-related issues during the assessment of spatial memory. This study was designed specifically for the more effective implementation of serially applied MWM protocols. The results of our study show that differences in ITI affect memory performance. Particularly, longer ITI has been found to improve memory performance compared to shorter ITI. In addition, it has been observed that long-term exposure to differences in water temperature has an effect on body weight over time. Another finding is that a greater decrease in body temperature is observed in shorter ITI and 20 °C water temperature. The last finding is that the longer ITI compensates for the decrease in body temperature. As a result, the prolongation of the time between trials in young rats positively affects memory processes and body temperature remains more stable. In studies, the 30-s ITI in the MWM is preferred for both young and old rats for learning and memory assessment (Conn, 2011; Bizon et al., 2001). The results of our study reveal that a longer ITI should be preferred instead of the frequently used 30-60 second ITI in the assessment of the memory with MWM. However, only young animals were used in our study. It is thought that working in different age groups and different waiting times (5-10 minutes) will contribute to the literature.

The MWM test is a reliable test correlated with hippocampal synaptic plasticity (Vorhees & Williams, 2006). Studies have shown that intraoperative

Table 4. The effects of ITI and WT on Δt

	Δt		
	n	x̄	SEM
Main Effect of ITI			
30-s group	15	1.13[a]	0.11
13-min group	14	0.53[b]	0.12
Main effect of WT			
20˚C	15	1.06[a]	0.11
24˚C	14	0.59[b]	0.12
*ITI*WT interaction*			
30s-20˚C	8	1.47	0.47
30-s-24˚C	7	0.79	0.44
13-min-20˚C	7	0.66	0.47
13-min-24˚C	7	0.41	0.37
		P- value	
ITI		0.001	
WT		0.009	
ITI*WT interaction		0.2	

a, b = Values with different superscripts within each column are significantly different. ITI = Inter-trial interval; SEM = Standard Error of mean; WT = water temperature; Δt = Difference between body temperatures (initial body temperature measurement - last body temperature measurement).

hypothermia negatively affects spatial memory (Xu et al., 2022). The hippocampus is a brain region that is responsible for spatial memory functions (Bellmund et al., 2018) and is quite responsive to stress (Eichenbaum, 2000; O'Keefe & Nadel, 1979; Scoville & Milner, 1957). Synaptic plasticity, the ability of synapses to modulate their strength or efficacy of synaptic transmission, underlies learning, memory, and information processing in the brain (Mansvelder et al., 2019). A decrease in rat body temperature down to 28–30 °C is known to impair MWM spatial memory performance (Rauch et al., 1989). In audiogenic stress studies, MWM can be used to measure spatial memory, especially after the electrodes are placed in the hippocampus regions of the animal stereotactically (Kim et al., 2007). In stress-related studies mentioned, the addition of hypothermia to MWM may affect the stress-related reflections of learning abilities. Therefore, maintaining core temperature in a normal range is essential for avoiding MWM-related comorbidities.

Conclusion

Our study will provide valuable data for experiments using the MWM test. Especially in some disease model studies, increasing the resting time between swimming times will both reduce stress and be effective in reducing comorbidities. The minimum fluctuation in body temperature with increasing water temperature will provide an advantage for manipulations that may cause changes in body temperature, such as stress-related or intraoperative studies to be made in addition to behavioral experiments. In addition, the parameters studied in postoperative cognitive function studies that require minimal changes in body weights may benefit from these data in terms of the standardization of metabolic changes. Moreover, the standard protocol may be reorganized in a species-specific manner and ITI values determined with this perspective may be considered as a hypothermia-related comfort zone for animal welfare.

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A Preventive herb against bone loss in diabetic rats: *Zingiber officinale*

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ABSTRACT

The study aims to determine and compare bone mechanical and material properties in experimentally diabetic rats treated with ginger extract. Forty female, healthy Wistar albino rats were used in the study. Rats were divided into five groups; Control (C), Sham (S), Ginger (G), Diabetic (D), and Diabetic rats treated with Ginger (DG). Diabetes mellitus was induced by a single intraperitoneal injection of 50 mg/kg streptozotocin. Ginger-treated rats received 200 mg/kg ginger extract by oral gavage in a 30-day-trial. At the end of the study, tibiae were harvested and subjected to a three-point bending test. Plasma samples were also analyzed for calcium and phosphorus concentrations. It was observed that the bending strength significantly decreased in the groups Ginger (234.78 ± 16.79; P = 0.019) and the Diabetic (223.90 ± 29.90; P = 0.028) compared to group Control (275.75 ± 33.47). In addition, the bending strength of the diabetic rats treated with ginger (DG group; 251.92 ± 15.90) was also significantly higher than the rats in the Ginger and Diabetic groups (P = 0.032 and P = 0.037, respectively). Although the plasma calcium concentrations showed no differences among any of the groups, the plasma phosphorus levels decreased significantly in group Diabetic (3.47 ± 0.28; P = 0.05) compared to Control (5.11 ± 0.21). However, there was a significant increase in plasma phosphorus in group DG (4.32 ± 0.12; P = 0.05) compared to Diabetic. In conclusion, ginger extract treatment of diabetic rats improves bone material properties. The adverse effects of diabetes on the mechanical properties of the bone were prevented by using ginger extract in diabetic rats.

Keywords: biomechanics, diabetes mellitus, ginger, *Zingiber officinale,* rats.

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Introduction

Diabetes is caused by deficiencies in insulin production or insufficient insulin production along with insufficient insulin resistance. Diabetes mellitus (DM) is a chronic metabolic disease with high blood glucose levels (Gong, 2012; Moseley, 2012; Yan and Li, 2013). DM frequently results in crucial complications affecting the heart, blood vessels, eyes, nerves, and kidneys. In addition, adverse effects of diabetes on bone health have been progressively recognized and reduced and delayed bone formation were shown in diabetic animals (Follak et al., 2005).

Bone homeostasis is balanced between bone resorption by osteoclasts and bone formation by osteoblasts (Ckarke, 2008). The failure in the balance

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between these two important cell functions leads to bone loss (Karsenty and Wagner, 2002; William et al., 2003). Insulin stimulates anabolic effects on bone by binding receptors on osteoblasts (Thomas et al., 1996). Thus, decreased insulin levels or reduced insulin signaling in osteoblasts may cause inhibition of bone formation in diabetes (Thrailkill et al., 2005; Gandhi et al., 2006). Diabetes also leads to imbalance in osteoclast/osteoblast function, and thereby decreases bone quality (William et al., 2003; Nyman et al., 2011).

Minerals are important for structural components of body as well as for regulation of chemical reactions of body processes. The alteration of bone structure, bone metabolism rate, endocrine system and calcium-phosphorus are balanced by nutrients and natural supplements. Researchers observed that especially herbal foods can affect bone resorption and have protective roles against bone loss (Hwang et al., 2018; Zammel et al., 2018). It is possible to treat bone loss pharmacologically by inhibiting osteoclastogenesis and/or by inducing osteoblast activity. Several drugs such as bisphosphonates, are commonly used for the treatment of bone loss (Rogers et al., 2011; Das and Crockett, 2013). However, the effects of plants on human health have been utilized for thousands of years (Koehn and Carter, 2005; Jones et al., 2006; Newman and Cragg, 2014). Especially the traditional herbal medicines have been preferred by clinicians because of their fewer side effects. These medicines are also more appropriate for long term treatments compared to chemically synthesized ones (Hidaka et al., 1999).

Zingiber officinale (ginger) is a flowering plant whose rhizome, ginger root, or ginger, has been commonly used throughout the world as a cooking spice and has also been used as a natural medicine due to its anti-inflammatory and pain relief agent for musculoskeletal diseases in traditional medicines (Ali et al., 2008; Baliga et al., 2011). There are more than 50 types of antioxidants have been extracted from ginger. The principal pharmacological activity of ginger is related to its active compounds such as 2- and 6-Gingerol (Shukla and Singh, 2007), especially anti-inflammatory effects of ginger have come from 6-gingerol (Semwal et al., 2015). The 6-gingerol has anti-oxidant, anti-tumoral, anti-obesity and anti-diabetic activities besides its anti-inflammatory properties (Semwal et al., 2015). In DM patients, ginger consumption improves glycemic status (Bhandari et al., 2005; Thomson et al., 2007; Shanmugam et al., 2011; Mahluji et al., 2013), insulin sensitivity, lipid profiles (Shanmugam et al., 2011; Huang et al., 2011) and other metabolic disorders by reducing inflammatory factors. Moreover, ginger may inhibit bone loss and contribute bone formation. Zammel et al., (2018) indicated that ginger extract could depress osteoclast activation and decrease their number throughout inhibiting the osteoclastogenesis. Also, ginger extracts caused improvements in the vertebral microarchitecture. These researchers also suggested that the positive effects of ginger may be related to increasing osteoprotegerin (OPG) and/or decreasing the receptor activator of nuclear factor-κB ligand (RANKL) expression by osteoblast. Another study (Hwang et al., 2018) suggested that 6-gingerol inhibited IL-1-induced osteoclast differentiation, and 6 -gingerol might be useful for inflammatory bone loss treatments.

According to the knowledge from the previous studies, ginger seems to be useful to decrease fracture risk, to inhibit bone loss and enhance bone strength. The aim of the study is to determine and compare bone mechanical and material properties in experimentally diabetic rats treated with ginger extract.

Materials and Methods

Animals: The experimental protocols were approved by the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Animal Care and Use Committee of Tekirdag Namik Kemal University, Turkey (Approval No: T2019-232).

In this study, forty female, healthy Wistar albino rats weighing 150-250 g and aged 4 months old were used. The animals were housed under standard laboratory conditions (22±1 oC; 55±10% humidity) in clear, plastic cages, with stainless steel feed hoppers. Wood shavings were used as bedding material. Rats were given tap water and ad libitum access to commercial rodent diet.

Plant supplementation: Fresh ginger rhizomes were purchased from a local store and authenticated at department of Botany in University. The ethanolic extract of ginger and the experimental design was prepared using the method described by Shanmugam et al., (2011). The rhizomes were washed and air-dried. Then the air-dried rhizomes were transformed into powder mechanically and prepared extract 95% ethanol for 24 hours. After that, the extract was filtered and 95% ethanol was added. The process was repeated three times. The three extracts were pooled together then filtered and evaporated to dry. As a result, a dark brown and gelatinous extract was obtained. Before the onset of the experiment, 200 mg/kg gelatinous extract was dissolved in 2% Tween 80 solution.

Experimental design and treatment process (Protocol): The rats were divided into five groups with eight animals in each group based on previous mechanobiological studies (Main and Biewener, 2004; Main et al., 2010; Lynch et al., 2010). The total experiment protocol was maintained for thirty days. The experimental groups as follows: Control (C); Sham (S) (2% Tween 80 was applied); Ginger (G) (Oral gavage 200 mg/kg Ginger extract); Diabetic (D) (50 mg/kg STZ i.p.); Diabetic + Ginger (DG) (Oral gavage 200 mg/kg Ginger extract).

Diabetes mellitus was induced by a single intraperitoneal injection (i.p.) of 50 mg/kg streptozotocin (STZ) (Sigma, St. Louis, MO, USA) dissolved in 50 ml citric acid + 40 ml of disodium hydrogen phosphate buffer (pH 4.5) was administered after an overnight fasting. Three days after STZ administration, fasting blood glucose levels of tail vein blood of rats were measured by glucometer (Accu-Chek Instant, Roche, Switzerland). The animals were labeled diabetic with fasting blood glucose of 250 mg/dl and above.

Blood analysis: The blood samples were collected into Ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes by heart puncture of the overnight-fasted rats under isoflurane anesthesia at the end of the trial. The samples were centrifuged in the same day at 3000 rpm for 10 minutes to separate the plasma, transferred the plasma into microtubes and samples were stored at -80 °C until the analysis day. The changes of plasma calcium and phosphorus levels were determined with commercial kits (Calcium Ref No: 90004, and phosphorus Ref No: 80015, Biolabo, France) using the spectrophotometric method, and the parameters were measured by microplate reader (Biotek, Epoch, USA).

Bone preparation and mechanical testing: Tibiae were harvested and wrapped with Phosphate-Buffered Saline (PBS) soaked gauze and frozen at-20°C until further analyses were conducted (Van Haaren et al., 2008). Prior to tests, bones were thawed at room temperature. Then, tibiae were subjected to mechanical compression test. For three-point bending tests a custom-made, low strength material testing machine was used designed and manufactured by Tufekci et al. (2014). To measure force and corresponding displacement, the loading machine had a load-cell (100 N, Tedea Huntleigh Malvern, USA) and a Linear Variable Differential Transformer (LVDT) (10-mm stroke, Novotechnik Tr10, Germany). Force and displacement measurements were recorded by an oscilloscope (Nicholet-Oddysey XE, USA) at the rate of 50 data/sec. The span length was set to 21mm and the load was applied at the mid-shaft with a constant loading speed of 10mm/min (Jast, 2011). Loading was applied until the bones were broken. Maximum bone breaking force (Fmax) was obtained from the load-displacement curve which was the highest value of the force. After biomechanical test, 2mm sections of mid-shaft of tibia were taken from the fracture site and the section were photographed under a stereomicroscope (Motic, Model: SMZ-168, Hong Kong). Pictures were transferred to Solidworks R17 3D CAD software (Dassault Systèmes, Waltham, MA; USA) and cortical area (Acort) and the minimum principal moment of inertia (Imin) was calculated. Furthermore, flexural (bending) strength (σf), and bending modulus of elasticity (E) were calculated by using the equations below respectively:

$$\sigma_f = \frac{My_{max}}{I_{min}} \qquad E = \frac{FL^3}{48\delta I_{min}}$$

Where M is the ultimate moment at the middle of the specimen, Y_{max} is the maximum vertical distance between neutral axis and the outer edge of the specimens, I_{min} is the minimum moment of inertia related the neutral axis, F is the applied force, L is the length of the support span, δ is the deflection due to corresponding force.

Statistical analysis: Data for the measured parameters were checked for normally distribution and assumptions for homogeneity of variance (Shapiro-Wilk test). If data normally distributed, One-way ANOVA test was applied, and the differences between groups were analyzed by the post hoc Tukey test. If data did not provide normality and homogeneity assumptions, data were subjected to Kruskall-Wallis test followed by post-hoc Mann-Whitney U multiple comparison test of significance using SPSS (IBM SPSS, Version 23.0, Chicago, IL). The differences were considered significant at $P < 0.05$.

Results

Mechanical test measurements for the tibiae of the rats were presented in the Table I. According to these results, F_{max}, I_{min}, A_{cort}, and elastic modulus showed no significant differences among experimental groups (P>0.05). However, changes were observed between experimental groups in terms of bending strength of the tibia. It was seen that the bending strength value significantly decreased in the Ginger and the Diabetic groups compared to the Control (P=0.019 and P=0.028, respectively group G and D). In addition, the bending strength value of the DG (Diabetic+Ginger) group was also significantly higher than the rats in the

Table I. Mechanical test measurements for the tibiae of the rats in experimental groups

Groups	F_{max} (N)	σ_f (MPa)	I_{min} (mm^4)	A_{cort} (mm^2)	*E* (GPa)
Control (C)	62.6 ± 8.73	275.7 ± 33.47^{ab}	1.30 ± 0. 25	3.30 ± 0.32	14.9 ± 2.12
Sham (S)	60.1 ± 16.84	272.3 ± 50.80	1.12 ± 0.26	3.43 ± 0.50	13.9 ± 3.01
Ginger (G)	65.7 ± 5.01	234.8 ± 16.79^{ac}	1.54 ± 0.23	3.76 ± 0.20	14.7 ± 4.75
Diabetic (D)	64.5 ± 15.80	223.9 ± 29.90^{bd}	1.57 ± 0. 53	3.68 ± 0.44	11.3 ± 3.36
Diabetic+Ginger (DG)	67.2 ± 9.03	251.9 ± 15.90^{cd}	1.56 ± 0.30	3.61 ± 0.37	16.3 ± 4.26

*n = 40, Data was presented as mean±standard deviation of the mean. Fmax: Maximum breaking force, σf: Bending strength, Imin: Minimum principal moment of inertia, Acort: Cortical area, E: Elastic modulus. The groups: Control, Sham, rats supplied with ginger (Ginger), diabetic rats (Diabetic) and diabetic rats treated with ginger (Diabetic+Ginger). a, b, c, d Superscripts show that there is a difference between the groups indicated with the same letters.

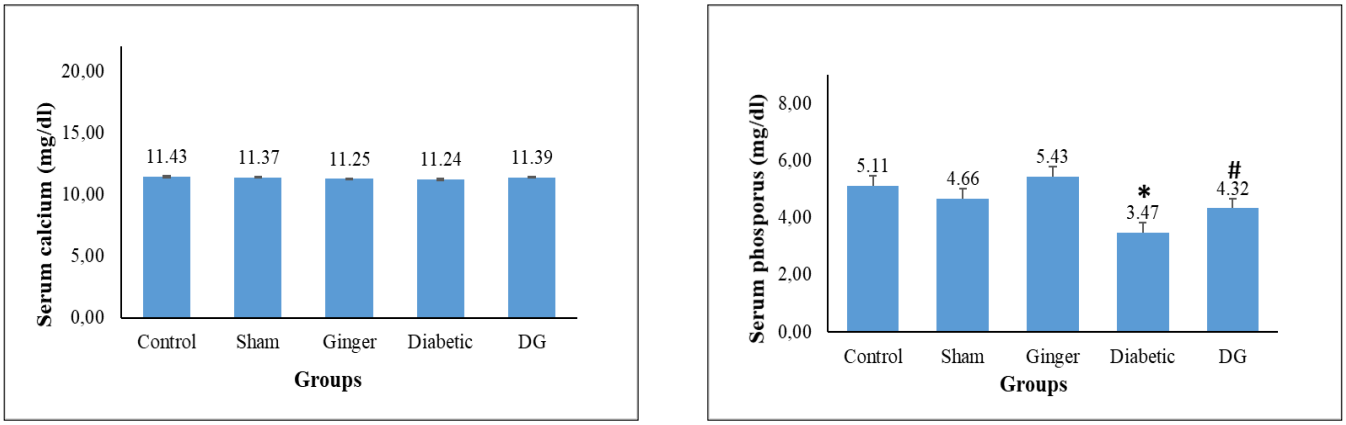

Figure I. The serum indicators of the rats in the control and experimental groups.
(A), The serum calcium concentration of the rats. (B), The serum phosphorus concentration of the rats. The groups: Control, Sham, rats supplied with ginger (Ginger, G), diabetic rats (Diabetic, D) and diabetic rats treated with ginger (Diabetic+Ginger, DG). * $P < 0.05$; Diabetic rats versus Control group. # $P < 0.05$; Diabetic rats treated with ginger versus Diabetic group.

higher than the rats in the G and the D groups (P=0.032 and P=0.037, respectively). However, no significant difference was observed between the Control and the Sham groups (P>0.05).

The plasma calcium and phosphorus for all the groups are shown in Figure I. There were no differences in plasma calcium among any of the groups (P > 0.05). However, the plasma phosphorus value in group D was lower than Control (P = 0.05; 5.11 ± 0.21 and 3.47 ± 0.28, respectively group Control and D), and a significant increase in group DG group than group D (P = 0.05; 3.47 ± 0.28 and 4.32 ± 0.12, respectively group D and DG).

Discussion

Diabetes causes changes and deterioration in bone metabolism and microarchitecture through various mechanisms at the bone's structural and molecular levels. These changes cause detrimental effects on bone biomechanical properties and increase the risk of fractures. Furthermore, these changes also cause impaired healing of the bone tissue (Funk et al., 2000; Janghorbani et al., 2006; Mashadi et al., 2013).

Ginger (*Zingiber officinale*) is a perennial tropical plant belonging to the Zingiberaceae family. Ginger has been widely used as a food flavoring and herbal medicine for many years (Mashadi et al., 2013). The clinical usage of ginger as a medicinal or alternative therapy is of great interest due to its diverse influences such as antibacterial, anti-inflammatory, antihyperglycemic, antitumor, renal protection, and cardiotonic effect in different systems (Danwilai et al., 2017; Marx et al., 2017). However, few studies have assessed on bone strength and bone fracture risk. So, further research is needed to identify the efficiencies of ginger intakes for bone health. Therefore, in the

present study, we focused on some biomechanical properties of the tibia in diabetic rats treated with ginger to investigate the potential effects of ginger to prevent fracture risk.

The bone tissue is a model that has a lifelong cycle of construction and destruction. Calcium and phosphorus are the most important factors for the cycles. Xiao et al., (2015) showed that in diabetic rats, plasma calcium and phosphorus levels were found lower than the control ones. It was reported that decreasing in plasma calcium and phosphorus could be due to impaired renal reabsorption and osmotic diuresis by hyperglycemia in diabetic rats. Also, it was suggested that alteration of calcium may correlate with abnormality of fasting serum glucose and insulin levels (Sun et al., 2005). In the present study, a significant decrease in plasma phosphorus in D group compared to Control (Control: 5.11±0.21 and D: 3.47±0.28, P=0.05). Ginger treatment improved the phosphorus levels in group DG as to D significantly (D: 3.47±0.28 and DG: 4.32±0.12, P=0.05). However, there was a slight decrease in plasma calcium in D group compared to Control, and an increase in DG group compared to D group. These results may be due to ginger ingredients which have roles on enhance of bone phosphorus mobilization. It was reported that Ginger contains calcium, phosphorus and magnesium which have important roles on bio-functions, especially bone formation and curbing muscle spasms (Kikuzaki and Nakatani, 1993). Also El-Mottaleb et al., (2016) reported that aqueous extract of raw ginger has potential hypoglycemic properties that reflected on bone formation in diabetic rats treated with ginger.

A_{cort} and I_{min} values, which are the geometric properties of the bone and calculated from the broken bone section, were not statistically significant. However, when the mean maximum bone breaking force (F_{max}) values are examined, a higher F_{max} was observed in the G, D and DG groups than in the Control group. A similar increase was also observed in I_{min} and A_{cort} values. Therefore, it can be said that the high strength value in these groups (G, D and DG groups) was an increase not due to the superiority in the microstructure of the bone, but rather due to the geometry. To better evaluate the situation in the microstructure, the elastic modulus and bending strength values should be considered. In this study, the mentioned values were determined by calculating the relevant values (A_{cort} and I_{min}) of the fracture section of the bone in the computer environment. When the sham group was compared with the Control group, it was observed that there was no significant difference in mechanical and geometric properties. These findings suggested that operational stress had no adverse effects on the mechanical and geometrical properties of the bone in the present study.

The mineral and collagen structure of the bone is effective on bending strength. Even if the collagen totally normal, alterations in collagen features may change the amount and arrangement of the mineral, which would affect the bone mechanics (Currey, 2003). The modulus of elasticity is mostly influenced by the mineral structure and depends largely on the degree of mineralization. Because, the elastic modulus of collagen is so low (Hamed et al., 2010). Changes in mineralization have an intense effect on the elastic modulus of the bone. High mineralization of the bone cause high elastic modulus values and this causes low work to fracture (Currey, 1984). In the comparison of the Control and G groups, it was observed that the elasticity modulus was barely unchanged and the bending strength values were lower in the G group. This result suggested that ginger has an adverse effect on collagen tissue of the bone in healthy individuals. To our knowledge, there is only one study mentioned the detrimental effects of ginger on trabecular bone. Therefore, our result can be explained with the research by Khan et al., (2012), that 6-gingerol has harmful effects on cancellous bone and consequently has a negative effect on the mechanical properties of the bone.

The fact that bending strength was significantly lower in the D group than the Control group, and the average value of the elasticity modulus was lower, although not significant. This result indicates that diabetes has adverse effects on both collagen tissue and mineral tissue. Diabetic fractures occur as a result of decreased bone quality and changes in bone microarchitecture (Jiao et al., 2015). Insulin induces osteoblast proliferation and collagen synthesis (Nyman et al., 2011). The deficiency of insulin hormone due to diabetes causes disruptions in collagen production and may causes a decrease in the bending strength of the bone in diabetic rats. Katayama et al. (1996) also suggested that AGE (Advanced glycation end-products)-modified collagen inhibits osteoblastic differentiation and function in diabetic animals. In addition, diabetes may negatively affect the microvascular environment of the bone and increase bone loss and fracture risk (Shanbhogue et al., 2017). As mentioned above, in the present study, the bending strength was lower in the D group than in the Control group, and the fracture force (F_{max}) values were almost the same as in the Control group. This may occur due to the bone adaptation mechanism; the bone adapted to balance its deteriorated

deteriorated microstructure and lost mechanical properties by increasing volumetrically. However, there was a significant difference in bending strength values between the DG group and the D group. The DG group had higher bending strength and elastic modulus values compared to the diabetic group, although there was no significant difference. It can be concluded that the negative effects of diabetes on the mechanical properties of the bone were prevented by using ginger in diabetic rats. In addition, Zammel et al. (2018) also reported that ginger showed a potential protective effect by reducing changes in vertebral microarchitecture and preserving the mineral composition of the spine.

Conclusion

Despite its mild adverse effects on the collagen and phosphorus mechanism in healthy rats, ginger treatment of diabetic rats indicated that it could be used as supplementary food in diabetic patients due to the possibility of restoring the mechanical and material properties and preventing potential fractures of the bone. Ginger may serve as a potent medicinal agent for the treatment of reducing fracture susceptibility in diabetic patients. Moreover, we hope the current study encourages the researchers to investigate the mechanisms of ginger extract on bone biomechanics.

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Electron microscopy and histopathological examination of canine papilomavirus

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ABSTRACT

Diagnosis of canine papillomavirus (CPV) infection by histopathology, transmission and scanning electron microscopy is presented. The study is based on data obtained by examining nonregressing papillomas (warts) from naturally infected dogs with clinical manifestations of CPV infection. Papules on the mouth and lips were common bilaterally in 6 dogs. Confirmatory diagnosis of sick dogs was made by clinical findings, histopathology, transmission and scanning electron microscopy. Histopathological examination of hematoxylin and eosin stained papillomas revealed lymphoplasmocytic cell infiltration and fibrosis, parakeratosis in the dermis, papillary proliferation and intranuclear vacuole degeneration in the stratum spinosum. Electron microscopy demonstrated viral icosahedral capsid formation and non-enveloped viral structure of CPV. Transmission electron microscopy demonstrated viral particles and virions in the nuclei of infected cells, viral crystal mode formation in the nucleus. Scanning electron microscopy demonstrated virions and virus-like particles budding in the infected tissue. The findings of the study reveal that electron microscopy and histopathology are effective and sensitive methods in the diagnosis of CPV infection. Electron microscopy is the only imaging technique that allows direct visualization of viruses, along with affected tissues and cells, due to its nanometer-scale resolution. This study reveals the intracellular and extracellular viral pathogenesis, viral ultra structure and structural components of CPV. Present findings indicate canine papillomavirus causes canine papillomatosis, inclusion bodies are common in nonregressive infection, papillomavirus induces cytopathic effect and pathogenesis, viral particles are located in the cell and form crystal mode in nuclear space.

Keywords: Canine papillomavirus, scanning electron microcopy, transmission electron microscopy, histopathology, viral particles.

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Introduction

Papillomaviruses are small, non-enveloped viruses with circular double-stranded DNA genome size of 5748 to 8,607 bp. and belongs to Papillomaviridae family. Taxonomically have two subfamilies, in more than 50 genera and 130 species. The virions have a diameter of 52-55 nm and icosahedral capsid assembly (Doorslaer et al., 2018; ICTV, 2018). The virus genome consists of three main regions: the early region and late regions encode proteins E1 to E7 and L1 and L2, respectively, while the non-coding "long control region" regulates viral gene transcription and replication (Bernard et al., 2010; Doorslaer et al., 2018; ICTV, 2018). Papillomaviruses have been isolated from mammals, birds, fish and reptiles to date and are highly species

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specific (Murphy et al., 1999; Ogawa et al., 2004; Sterling et al., 2014; Tan et al., 2012; Tekelioglu et al., 2017). The papillomaviruses has a high tropism to the mucosal and keratinized epithelium. Cutaneous and mucocutaneous infection occurs and has been associated with squamous cell carcinomas. Papillomaviruses have been classified into different genotypes according to the degree of sequence variation (Bernard et al., 2010; Lange and Favrout, 2011; Doorslaer et al., 2018; ICTV, 2018).

CPV often causes benign skin tumors in dogs called warts, which are typically small, cauliflower or solid-shaped and rough and irregular growths. Warts contain large amounts of infectious virus that are relatively stable in the environment. Transmission between animals occurs through direct or indirect contact with contaminated fence posts or halters. Tattooing or tagging with equipment containing the virus is another common source of infection (Lange et al., 2011; Matheus et al., 2011; Murphy et al., 1999; Nichols et al., 1999; Tekelioglu et al., 2017). CPV warts usually appear on the lips, mouth and skin of dogs, and less frequently on the eyelids and even on the surface of the eyes or between the toes. Warts are usually found in groups rather than single growths (Matheus et al., 2011). The duration of the infection is very variable, from one month to one year, and recurrence is possible (Lange et al., 2011; Nichols et al., 1999; Tekelioglu et al., 2017).

The aim of the present study was to confirm the diagnosis of the viral disease by imaging the virions and virus-like particles of CPV. The use of electron microscopy and histopathology are among the recommended gold standards for diagnosis and were used in this study in accordance with the catch-all principle (Martins et al., 2008; Goldsmith and Miller, 2009, Platter and Hosttetter, 2009; Gentile and Gelderblom, 2014; Roingeard et al., 2018; Gelderblom and Madeley, 2018; Cheng et al, 2020). This study is designed to reveal the intracellular and extracellular viral pathogenesis, viral ultra structure and structural components of CPV.

Materials and Method

Sampling: Aggressive nonregressing papillomatosis of naturally infected six dogs presenting clinical symptoms of CPV in 2017-2021 were investigated with the consent of their owners. These are the dogs referred to Çukurova University Ceyhan Veterinary Faculty Virology Department for consultation. Papillomas were excised surgically and removed forhistopathological diagnosis and further investigations. After excision of papillomas, the sick dogs were provided with the good veterinary practice and care.

Histopathology: Biopsy samples from papillomas were immediately fixed in 10% neutral buffered formalin for histopathological examination. Definitive diagnosis was done at a private veterinary pathology diagnostic laboratory. Following the fixation process, the samples were embedded in paraffin blocks and 4-5 µm sections were cut, stained with hematoxylin and eosin (HE), and examined with a light microscope and their images were recorded.

Electron Microscopy: Scanning and transmission electron microscopy were done byfixation of excised papillomas in 5% glutaraldehyde in Millonig's phosphate buffer at pH 7,4 followed by post fixation step with 1% osmium tetroxide in the same phosphate buffer at 4 °C according to the procedures described by the Electron Microscopy unit of the Çukurova University Central Research Laboratory and Faculty of Medicine Department of Histology and Embryology. Following preparation, specimens were examined and photographed under scanning electron microscopy (SEM FEI, Quanta 650 Field Emission SEM, USA), at 20KV. Sections were also examined and photographed under transmission electron microscopy (JEOL JEM-1400, Japan). The exposed surface was tracked by quadrant, and then photographed at increasing magnification**.**

Results

Papillomas (warts)of different sizes were observed extensively around the mouth, nose and lips in all dogs and were indicated by arrows (Figure 1).Findings were recorded and detailed information about sick dogs is given in Table 1. One of the dogs was 16 years old and the other 5 dogs were younger than 8 months old and two of the sick dogs were female and four were male. The breeds of the dogs were; Cane Corsa (n:1), Çatalburun / Germanpointer mix (n:1) and mixed (n:4), respectively.

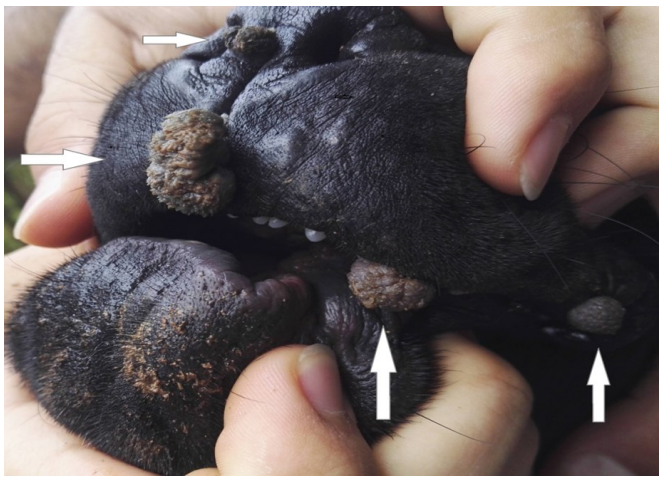

Figure 1. Different sizes of multiple papillomas (warts) at the mouth, nose and lips of the dog (arrows).

Table 1. Data table of sick dogs

Breed	Gender	Age (year)	PapillomLocalisation
Çatalburun/German Pointer mix	Male	16	Mouth + Nose
Mıx	Male	0.6	Mouth + Nose
Mıx	Female	0.5	Mouth
Mıx	Male	0.8	Mouth
Mıx	Female	0.6	Mouth
CaneCorsa	Male	0.5	Mouth + Nose

Macroscopically, gray-brown, moderately firm to hard polypoid growth with skin on its surface was observed. When scanned with serial sections, the incision sections were observed to be gray brown and yellow brown. Histopathological examination of the serial sections of six cases revealed common findings including parakeratotic hyperkeratosis on the surface, increase in keratocytes and papillary proliferations in the squamous epithelium. In the nuclei of keratocyte and spinosum cells, anionucleosis, vesicle formations of varying diameters, marginal chromasia, and two to three nucleolus were observed. Eosinophilic, intranuclear inclusion-like materials were seen in some cells. Disruption of the basement membrane and diffuse lymphplasmocytic cell infiltration was evident in the stroma (Figure 2).

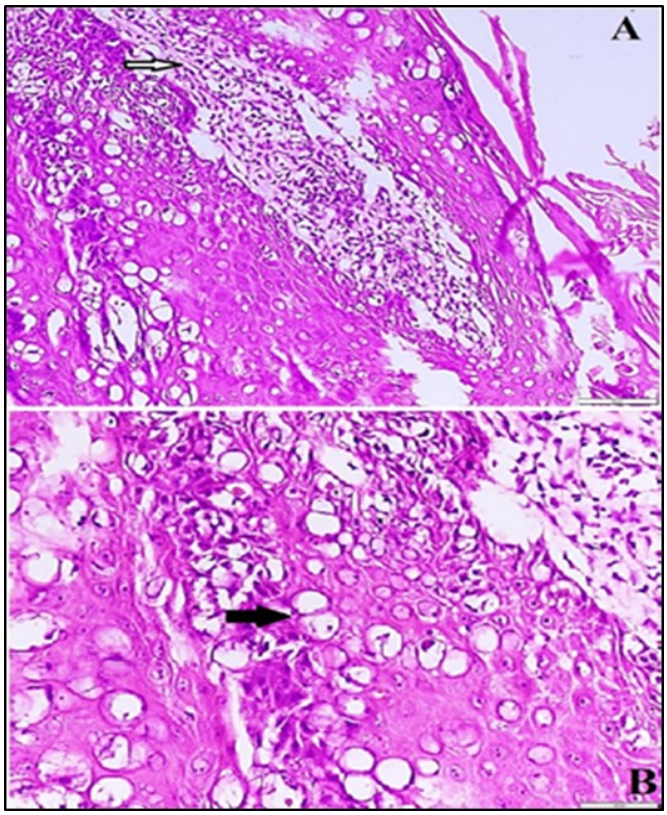

Figure 2. HE stained histopathological images. A; Lymphoplasmacytic infiltrates and fibrosis in dermis (white arrow), B; intranuclear vacuolar degeneration in spinosum cells (black arrow), (bar=100µm).

Scanning electron microscopy (SEM) revealed the damage to mucosal tissues infected by CPV. Characteristic numerous epithelial papillomatous protruding growths of different sizes with diffused exfoliated superficial cells were observed from the inner layers of the infected mucosal tissue. Areas of cellular desquamation were observed more intensely in certain regions, and characteristic papillamatous growths of variable size were observed, and smaller growths of similar nature appeared among larger ones (Figure 3). Due to CPV infection, deterioration was detected in the inner layers of the epidermis, stratum spinosum and stratum basale (Figure 3 a-500X; b-15.000X; c-30.000X).

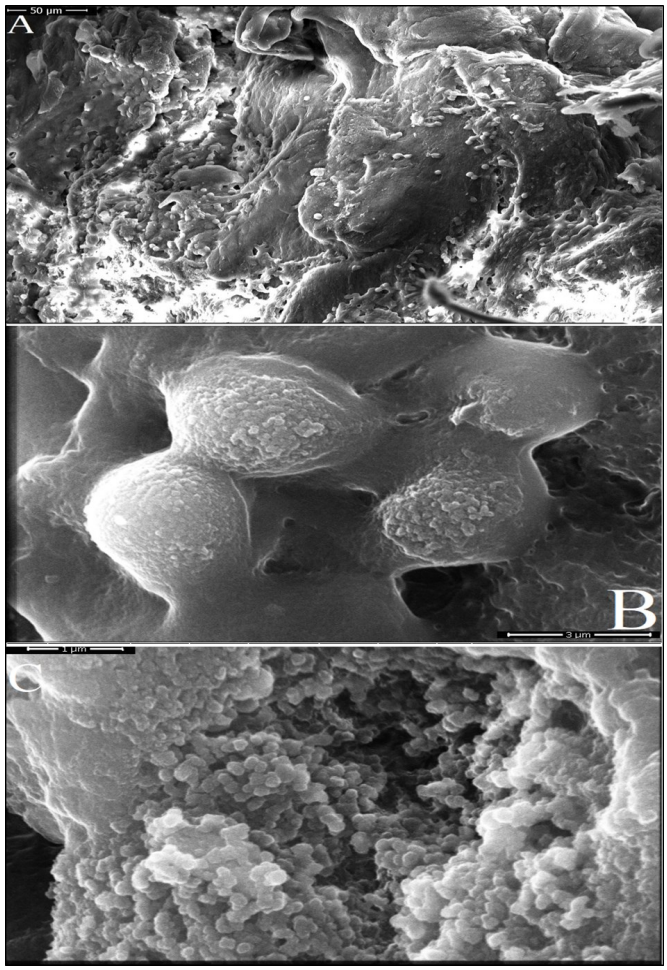

Figure 3. Scanning electron microscopy images of the inner surface of the infected oral mucosa. Observation of disruption of the inner layers of the epidermis, stratum spinosum and stratum basale and protruding growths and scattered exfoliated cells (images and magnification; at 20.00 kV; A; 500X, B; 15.000X, C;30.000X).

Transmission electron microscopy (TEM) revealed the damages to infected cells by CPV. Presence of perinuclear vacuolization was evident in both light and transmission electron microscopy. In CPV-infected cells, nuclei were observed to contain decondensed chromatin and evident nucleoli. Viruses and virus-like particles were observed in the nucleus with the mode of crystal aggregation mode in nuclear space. Various organelles were found in the cytoplasm, especially the mitochondria, which suggests intense cell metabolism. In the examined tissues, it was determined that the spaces between the interdigitated intercellular cells were widened, and it was concluded that this situation had an effect of disrupting the desmosomal structure (Figure 4 a,b,c).

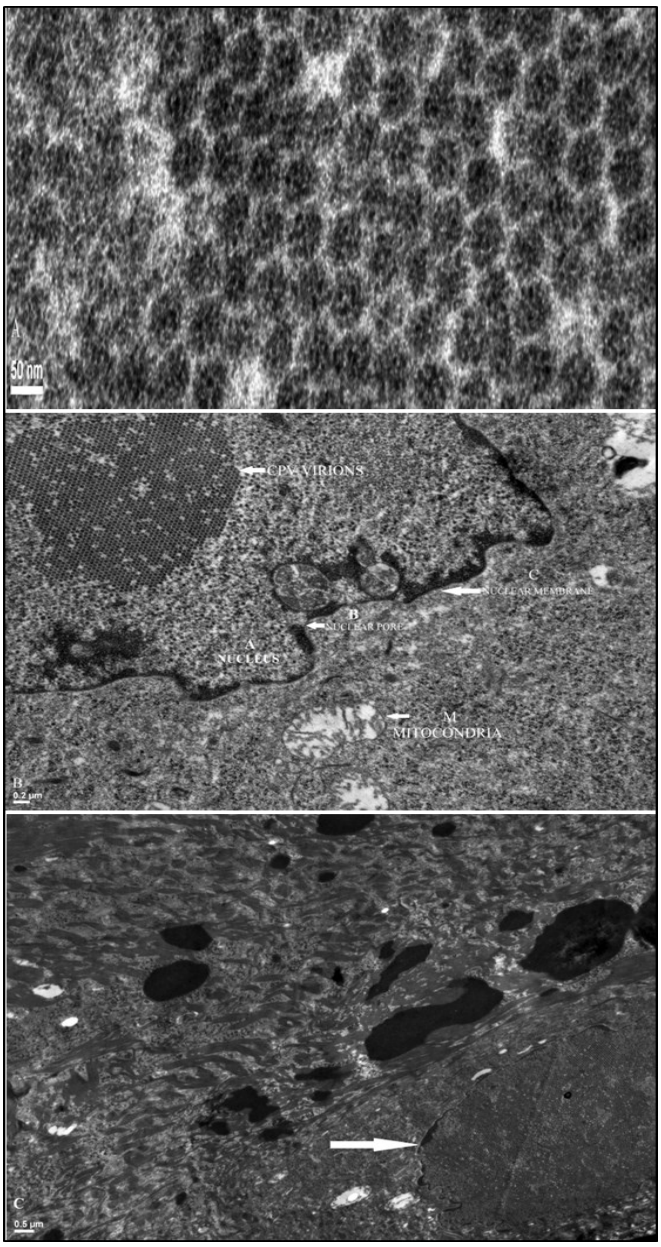

Figure 4. Transmission electron microscopy images of CPV infected cells. A; Aggregation of numerous CPV virions (aprox. 50 nm and icosahedral shaped) as a result of dense clustering within an epithelial cell nucleus. A closely packed array of crystalline virus particles with a packing arrangement. B; Arrows; Nucleus (A), Nuclear Pore (B), Nuclear Membrane (C), CPV virions (CPV); Crystalline formations of virus particles remain in a disintegrated nucleus. Mitocondria (M). C; An intranuclear inclusion body consisting of numerous close-packed aggregates of virus particles with decondensed chromatin.

Discussion

CPV is the etiological agent of canine papillomatosis, which is characterized by benign neoplasms, commonly known as warts, localized mostly in the oro-nasal region, which can spread to the tongue, pharynx and skin (Figure 1). Moreover, in dogs, several canine papillomaviruses (CPVs) have been identified in malignant lesions and are suggested as one of the risk factors for the development of squamous cell carcinomas (SCCs)recently (Gürgen et al, 2020; Chang et al, 2021). The host range of papillomaviruses are very narrow and highly species specific.

Five of the dogs examined in the study were younger than 8 months, and one dog was 16 years old.CPV infection is more common in young dogs and no breed or gender related susceptibility has been previously reported by most (Christian and Claude, 2011; Lange et al, 2011; Sykes and Luff, 2014) accept Bianchi et al. (2012). Interestingly, five dogs were mixed breed and younger than 8 months old in the present study (Table1). These are the dogs referred to Çukurova University Ceyhan Veterinary Faculty Virology Department for consultation.

Results of the histopathological differential diagnosis examination, characteristic superficial vacuolated (koilocytic) keratinocytes specific to the pathogenesis of CPV infection were observed as a typical finding in productive papillomavirus infections. Histopathological examination revealed hyperpigmentation and proliferation in the ephitelia, eosinophilic intra-nuclear inclusion bodies in the stratum granulosum, invasion in stroma, keratinization and polymorphism in the nucleus, as described previously by others (Nicholis et al, 1999; Martins et al, 2008; Platter and Holstetter, 2009; Tekelioglu et al,2017; Chang et al, 2020).In contrast with the findings of Bianchi et al. (2012), intranuclear inclusion bodies were observed in all cases.

It was demonstrated in this study that CPV has a high affinity for the nuclei of epithelial cells and the method of identifying the virus in these tissues by both light and scanning and transmission electron microscopy. The virus-like particles observed in infected cells were found morphologically similar to the findings caused by canine papilloma viruses such as dimension ranging as ~45- 50 nm, replication in the nucleus with the formation of nuclear inclusion bodies

and crystal aggregation mode in nuclear space. As reported by others (Watrach 1969, Nicholis et al, 1999; Martins et al, 2008;Platter and Holstetter, 2009), papillomaviruses have a tendency to collect in the host cell in a crystal structure, which is consistent with the CPV findings of this study (Figure 4).

Some differences can be observed during imaging in the estimated sizes of the same viruses in electron microscopy, these differences may be due to the effects of fixation and electron density differences of viral components. This phenomenon was previously described by Watrach (1969). Clinical, histopathological, and both SEM and TEM electron microscopy findings of this study indicate that the etiologic agent is canine papillomavirus. Similar to our findings explaining the various imaging differences of the same viruses in the cell by electron microscopy, it was also published by other researchers in previous years (Watrach, 1969; Nicholls and Stanley, 1999; Martins et al, 2008).

In the literature, few canine papillomavirus studies based on scanning electron microscopy were reported so far. Nicholls and Stanley, (1999) and Martins et al., (2008) reported epithelial protrusions and exfoliation of cells in affected tissues, similar to the findings of this study.

In the TEM findings, it was observed that the nuclei of the infected cells containing the CPV virion and virus like particles contained decondensed chromatin and the nucleoli were prominent. Various organelles were seen in the cytoplasm and especially the presence of mitochondria, suggesting intense cell metabolism. Additionally, CPVs in crystalline form were observed in the nuclei of the examined infected cells. The formation of the crystal structure requires the viral particles to be similar in shape and structure, the surface arrangement to be equivalent, and the purity of the aggregating particles. The pathological activity, structural and capsid properties of the virus were investigated by examining the crystallization structure formed by the CPV particles in the core cavity. The structural findings regarding the pathogenesis and crystal mode formation occurring in the infected cell and its nucleus are similar to the reports published in previous years by others (Watrach, 1969; Nicholls and Stanley, 1999; Narama et al., 2005; Martins et al, 2008; Platter and Hosttetter, 2009; Wang et al, 2010).

Upon the literature, deeper structures such as the basement membrane and the adjacent chorion are preserved in papillomavirus infections and are not affected by the infection. In this study, it was observed that an aggressive nonregressive pathogenesis occurred in the tissues examined and the basement membrane was also affected by the infection. It has been stated that similar aggressive pathogenesis may occur in infections caused by HPV 11, 16 and 18 subtypes, which are associated with airway malignancies, especially in humans (Martins et al, 2008;Chang et al, 2021).Particular types of papillomaviruses are associated with squamous cell carcinomas in dogs and cancer of cervix, anus and pharynx in humans (Muphy et al, 1999; Martins et al, 2008; Wang et al, 2010; Chang et al, 2020; Gürgen et al, 2021).The relationship of canine papillomaviruses with squamous cell carcinoma and other malignancies in the field of veterinary medicine is still not fully elucidated and studies are ongoing.

Present findings indicate papillomavirus causes canine papillomatosis, inclusion bodies are common in nonregressive CPV infection, papillomavirus induces cytopathic effect and pathogenesis, viral particles are located in the cell and form crystal mode in nuclear space.

Conclusion

The present study reveals the intracellular and extracellular viral pathogenesis, viral ultra structure and structural components of CPV. TEM and SEM electron microscopy provides an immediate overview of the virus replication, true state, distinctive amount and shape of the CPV in a detailed examination of the pathogenesis of current infection. Histopathologically, characteristic findings for CPV were determined and a confirmatory diagnosis was provided with clinical findings and electron microscopy.

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How do viruses use oxidative stress?

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ABSTRACT

Oxygen is a vital element for all living beings to continue their life activities and is the main component of oxidant–antioxidant metabolism, which should be in balance. The free radicals formed as a result of this metabolic process in the organism constitute a source of oxidants; external factors (radiation, exposure to sunlight, environmental pollution, cigarettes, etc.), inflammation and microbial agents also cause the formation of oxidants. Oxidative stress occurs when the balance between free radicals and antioxidants (which have an eliminating effect against them) shifts in favour of free radicals. Many studies have reported that oxidative stress may affect the virulence of pathogens during infection. Viruses use a pathological pathway that causes the production of reactive oxygen species (ROS) and the consumption of antioxidants. Thus, after viral infections, higher levels of ROS are often formed. Not only DNA-containing but also RNA-containing viruses were found to be associated with severe oxidative stress supporting DNA damage, high mutagenicity, initiation and/or progression of neoplasia. This review focuses on the relationship between oxidative stress and viruses.

Keywords: oxidative stress, viruses, virus infections, oxidative stress in infections

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Introduction

All living things need oxygen and, as such, it is an essential element to sustain vital activities. However, antioxidants are also needed for the organism to maintain a sensitive balance for neutralizing oxygen and oxygen-containing products. Any deterioration of this balance in favour of oxidants leads to “oxidative stress”; in other words, insufficient antioxidant metabolism initiates a chain of events resulting in tissue damage.

Free radicals are a primary source of oxidants and they initiate the oxidation of many structural components of cells. This abnormal event leads to pathological changes and initiates a process leading to collapse of the organism.

This review focuses on the oxidative stress caused by oxidation reactions of reactive oxygen species (ROS) in structural components, the damage that results from this process, the antioxidant system that effectively defends against this damage and also how viruses make use of this event.

History: In the 19th century, Paul Bert proposed that high oxygen concentrations are harmful to many organs, especially brain and lungs, therefore it became important to examine the damage caused by excess oxygen in organisms (Donald, 1947; Kliszczewska et al., 2018). The curiosity rised about free radicals, which are toxic agents caused by x-radiation , pollution, alcohol, etc. (Phaniendra et al., 2015), brought along an increase in studies on this matter to understand the role of viruses on oxidants release (Peterhans, 1979).

Peterhans (1979) published the first evidence that viruses can cause oxidative stress by increasing the

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number of ROS (Peterhans, 1979). He showed that a relationship exists between cell activation and ROS formation as a result of the virucidal effects of viruses (Peterhans, 1997).

Other studies have shown that influenza and paramyxoviruses cause an increase in phagocytic cells by infecting respiratory system cells and activating monocytes and polymorphonuclear leukocytes in vitro to form ROS (Schwarz et al., 1996; He et al., 2013).

Reactive species in oxidative stress: ROS, as a term, describes free radicals and moleculess, containing one or more unpaired electrons in the outer electronic shell (Turrens,2003; Lobo, 2010). They are essential products for physiological functions of cell but have also been proven by researchers to contribute to the pathogenesis of many diseases (Phaniendra et al., 2015). In amounts beyond physiological limits, it is possible that they can damage many structural components. There are three types of oxygen in the atmosphere: atomic oxygen (O), molecular oxygen (O2) and ozone (O3) (Donald, 1947). In physiological conditions, it is reported that every cell in the human body is exposed to 1010 O2 molecules per day (Donald, 1947; Phaniendra et al, 2015) and oxygen-derived free radicals (ROS) are a series of metabolites derived from O2 (Phaniendra et al., 2015). It has been known that mitochondria produce ROS (Zorov et al., 2014). Mitochondria consume 90% of the oxygen in as cell and only 3–5% of oxygen taken into the body is converted to products expressed as ROS (Phaniendra et al., 1979).

Although the majority of free radicals are formed in the respiratory tract, ROS are also produced during phagocytosis, redox reactions of xenobiotics and enzymatic reactions catalyzed by lipoxygenases, cyclooxygenases, oxidases and dehydrogenases (Di Meo et al., 2016; Kliszczewska et al., 2018). They are also secreted from dendritic cells, neutrophils and macrophages in response to inflammatory agents (Di Meo et al., 2016).

The role of ROS in physiological cell function was investigated by lots of researchers (Dröge, 2002), so it has been proven that reactive species contribute to many positive events, such as regulation of cytokines, growth factors, transcription, immunomodulation and apoptosis (Di Meo et al., 2016). Loss of enzyme activity, inhibition of protein synthesis, DNA damage as well as tissue damage are examples of negative events caused by excessive ROS production that can lead to disorders of cell integrity, functional losses and also cell death (Camini et al., 2017).

Levels of ROS are also affected by physical factors such as ionizing radiation, ultraviolet radiation, high or low temperature and environmental pollution (Phaniendra et al., 2015). Mitochondria, cytochrome P450 mechanism, peroxisomes and activation of inflammatory cells are examples of endogenous sources of ROS whereas environmental factors such as non-genotoxic carcinogens, xenobiotics, ultrasound and microwave radiation, air pollution and drug toxicities such as carbon tetrachloride and paracetamol are exogenous sources (Kliszczewska et al., 2018; Żukowski et al., 2018). It has been proven that besides stress, forest fires, using alcohol and smoking can also trigger oxidative stress by contributing to free radical formation (Phaniendra et al., 2015; Sebastiano et al., 2016). Hydroxyl radicals are the most reactive products known and their lifetime is concise (10-9 seconds) or the peroxyl radical (ROO-) has a long lifetime of 7 seconds, this knowledge proves that the lifetimes of ROS are variable (Camini et al., 2017).

Oxidative stress: There is a delicate balance between free radicals and antioxidants that has a sweeping effect in the biological system. The deterioration of this balance in favour of free radicals is called ‘oxidative stress’ (Özcan et al., 2015) and because it occurs in multiple systems such as redox signaling pathways it is known as a disorder of redox control and signaling (Camini et al., 2017). In tissues, the continuous flow of single electrons into oxygen causes endogenous oxidative stress (Phaniendra et al., 2015; Özcan et al., 2015).

Excessive increase in the number of free radicals causes damage to many structural components of cells, especially membrane, proteins and nucleic acids. Cytoplasm can be released by rupture of cell membranes, resulting in cell damage or even cell death (Özcan et al., 2015). Migraine triggers also seem to have the capacity to increase oxidative stress (Borkum et al., 2016).

Antioxidant defence: Antioxidants are substances that prevent or delay the damage of substances prone to oxidation, such as proteins, lipids, carbohydrates and DNA in living cells (Lobo et al., 2010). The process that uses these substances is called ‘antioxidant defence’ (Surai et al., 2019).

Antioxidants can also be classified as endogenous (enzymatic and non-enzymatic) and exogenous (Roehrs et al., 2011; Sen et al., 2011). Enzymatic antioxidants are enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Camini et al., 2017). SOD is an important enzymes because it forms the first line of defence against ROS

and also plays a role in killing phagocytosed bacteria in the intracellular environment (Ihara et al., 2005). It catalyzes the superoxide radical (O2.-) to hydrogen peroxide (H2O2) and O2 with H2O2 being removed by CAT or GPx (Lushchak et al., 2006). GPx is one of the most important antioxidants found in the cytoplasm and is responsible for protecting cells against oxidative damage caused by H2O2 (Sen et al., 2011). The majority of non-enzymatic antioxidants are present in food: glutathione, melatonin, uric acid, bilirubin, albumin, coenzyme Q10, selenium, α-lipoic acid, ceruloplasmin and transferrin (Sen et al., 2011; Camini et al., 2017). Glutathione plays a vital role in effectively sustaining the antioxidant defence system and removing ROS (Sen et al., 2011).

Exogenous antioxidants can be classified as vitamins and also some chemical substances taken from foods. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), sodium benzoate, ethoxyquin, propylgalate and Fe-SOD are examples of antioxidants taken from foods (Shahidi et al., 2000). Vitamin E (α-tocopherol) is one of the critical exogenous antioxidants and is a fat-soluble vitamin (Shahidi et al., 2000; Sen et al., 2011). Vitamin C is a member of anti-oxidant species capable of reducing ROS formation (Shahidi et al., 2000), it plays role as electron donor (Padayatty et al., 2003). In vitro experiments have shown that , as well as protection, vitamin Ccan damage different components of the cell (Padayatty et al., 2003). Antioxidants seems as a potential therapeutic option in the fight against influenza and many other viral infections and it is important to develop new compounds that block oxidative stress (Fulda et al., 2010).

While antioxidant defense network, existed inside the host cell, reduce the oxidative damage; at the same time they control ROS levels to allow beneficial functions (Sgarbanti et al., 2014).

Relationship between apoptosis and oxidative stress: The cell behaviors that occur in stressful medium is variable from survival to eliminate damaged cells (Fulda et al., 2010). When the cell choices the death, different pathways activate and process begin. There are different criteria used in the classification of cell death. For example; morphological appearance, enzymological criteria, functional aspects or immunological characteristics are some of them (Kroemer et al., 2009). Apoptosis, autophagy, necrosis, cornification, paraptosis, ferroptosis, methuosis and pyroptosis are among the examples of this (Yan et al., 2020). In maintaining physiological cellular homeostasis, a successful apoptosis process is a essential step (Fulda et al., 2010). In this context, it has been known that cell signaling and the regulation of the main pathways of apoptosis is depend on ROS (Di Meo et al., 2016). Apoptosis, which is one of cell death types, is defined as programmed cell death, where tissues surrounding the cells are not damaged, whereas necrosis is characterized by spillage of intracellular components and lysis of the plasma membrane (Schweizer et al., 1999; Liu et al., 2017). Apoptosis is triggered by various signaling pathways: receptors activated by stimulation of exogenous signaling molecules control the extracellular pathway; intracellular pathways are regulated by mitochondrial-mediated signaling pathways (Liu et al., 2017). Some research have revealed that ROS, such as O2•−and H2O2, can induce autophagy also (Yun et al., 2020).

One of the defence mechanisms used against virus infections is the destruction of infected cells by apoptosis; however, numerous viruses have developed strategies to prevent apoptosis (Schweizer et al., 1999). Oxidative stress is a mediator with an essential role in the induction of apoptosis (Liu et al., 2017) It is believed that oxidants contribute to the loss of CD4 T cells by way of apoptosis (Peterhan, 1979) and that intracellular levels of ROS, an indicator of oxidative stress, increase in the early part of apoptosis (Schweizer & Peterhans, 1999).

DNA mutations caused by oxidation: Oxidative DNA damage is one of the significant event observed as a result of oxidation (Cadet et al., 2017). Lots of damage occur and repaired by some mechanism, about 105 lesions per cell each day (Mehta et al., 2014). It is known that lesions caused by various mechanisms, such as base and sugar modifications, single- and double-chain fractures, non-basic regions and DNA–protein crosslinking, can all cause damage (Cooke et al., 2003). This damage is the starting point of a series of processes leading to mutagenicity (Lee et al., 2006), carcinogenicity (Klaunig et al., 2010) and aging (Junqueira et al., 2004). Guanine is the base that is most susceptible to oxidation and DNA damage caused by ROS was most frequently encountered on this base (Mc Dorman et al., 2005). Unlike other DNA damage, when guanine is oxidized its response will be a mutation but not a stop in development. It is one of the best-described mutations and can lead to more significant problems because the 8-oxoG level is important as a biomarker for measuring oxidative stress in cells. In physiological conditions 103 8-oxoG is produced in a cell, but this increases to 105 in cancer cells (Phaniendra et al., 2015; Markkanen, 2017). The reaction of nucleic acids with free radicals and also mutations in DNA are shown to be the main causes of cell death (Süleyman et al., 2018).

Determination of increased level of 8-hydroxydeoxyguanosine (8-OH-dG) is indicator of oxidative damage in DNA so it has important role as a biochemical marker in tissue, plasma and urine (Markkanen et al., 2017; Rehman et al., 2018).

Relationship between viral infections and oxidative stress: Plant and animal immune systems rapidly begin to secrete ROS in the presence of pathogens (Novaes et al., 2019). This is defined as the first line of defence and is called 'oxidative explosion' (Gambino et al., 2015; Di Meo et al., 2016). Examples of reactive species are: superoxide radical (O2.-), hydroxyl radical (OH), nitric oxide (NO), hydrogen peroxide (H2O2) and peroxynitrite (ONOO-).The most important mediators of reactives species that are induced by inflammatory processes, particularly microbial infections, are oxygen and nitric oxide (Borkum et al., 2016). The electrical charges of the reactive species can be positive, negative and also neutral (He et al., 2013; Phaniendra et al., 2015; Di Meo et al., 2016). Hydroxyl and superoxide radicals are examples of the most active species (Kliszczewska et al., 2018), with hydroxyl being the most reactive to attack biological molecules. Although this radical is synthesized from H2O2 via Fenton and Haber–Weiss reactions (in the presence of Fe2+ and Cu+), it also occurs when water is exposed to high-energy radiation. However, H2O2 does not have toxic effects unless converted to other free radicals (Kliszczewska et al., 2018). It should be noted that not all ROS are free radicals. For example, O2 and H2O2 are not radicals (Süleyman et al., 2018).

After viral infections, the formation of reactive species is frequently observed. Viruses are known to alter the balance between the host cell and the antioxidant system by causing an increase in the number of cellular pro-oxidants, such as iron and nitric acid, or by inhibiting the synthesis of important products for the antioxidant system, such as SOD (Durgur et al., 2013; Phaniendra et al., 2015). This situation contributes to viral evolution and facilitates viral replication. Viruses have also been shown to promote the synthesis of oxidants, such as superoxide and nitric acid (Camini et al., 2017). It is known that some DNA viruses have proteins that may show an antioxidant effect that depends on the oncogenic potential of the virus (Panda et al., 2008; Durgut et al., 2013). Regarding the role of cells in activation, it is showed that reactive species facilitates or promote viral replication, but this situation is depending on the cell type and the virus (Camini et al.,2017).

The following are examples of virus infections that use oxidative stress:

RNA viruses: Oxidative stress is one of the important factors that causes some disabilities associated with metabolic and physiological, also various diseases (Novaes et al., 2019). Many studies have revealed the relationship between oxidative stress and RNA viruses (Camini et al.,2017).

In addition to respiratory symptoms such as fever and pneumonia; the pandemic infection caused by Sars-CoV-2 (Severe acute respiratory syndrome coronavirus-2), which binds to the ACE receptors (Yang et al., 2020), is a big health problem nowadays due to high ratio of death (Delgado-Roche et al., 2020).

Infections of Sars-CoVs seems also one of the infections associated with oxidative stress (Delgado-Roche et al., 2020). The ''cytokine storm'' that is a result of overreaction of immune system causes severe tissue damage (Huang et al., 2020). The connection between inflammation and oxidative stress has been determined (Sies, 2015). Some researchers found that excessive production of ROS and a insufficient antioxidant system can play a major role in the pathogenesis of SARS-CoV infections (Delgado-Roche et al., 2020).

It has been showed that SARS-CoV 3CLpro (a viral protease) caused a significant increase in ROS production in HL-CZ cells and plays important role in 3CLpro-induced cell apoptosis (Lin et al., 2006).

Bovine viral diarrhoea virus (BVDV) infection has been reported frequently in Turkey (Oguzoglu et al., 2010; Oguzoglu et al., 2012). There are two different biotypes, cytopathic and non-cytopathic, but it is the cytopathic biotype that induces apoptosis. Early in the process of apoptosis the cells show a rise in intracellular ROS, indicative of oxidative stress (Schweizer & Peterhans, 1999).

Research to determine the effects of Bluetongue virus infection on oxidative stress parameters has detected an increase in some parameters related to oxidative stress and a decrease in antioxidant system parameters. However, the serum albumin, cholesterol, creatinine, total protein and GGT (γ-glutamyltransferase) values did not differ significantly between the two groups (Aytekin et al., 2015).

Distemper virus has been shown to have a pathology associated with ROS accumulation. Research has reported that plasma concentrations of methylenedioxyamphetamine (MDA), nitrate and nitrite were significantly increased in the infected group compared to the control group. Also, a significant decrease in plasma concentrations of antioxidants such as glutathione, ascorbic acid, retinol and β-carotene was found in the infected group (Karadeniz et al., 2008).

Levels of NO and MDA, which are indicative of lipid peroxidation and oxidative stress, were found to be high in animals with foot-and-mouth disease. As a result of the study, compared to healthy animals there was a significant decrease in total protein, albumin, globulin, calcium and cholesterol in infected animals (Mousa & Galal, 2013).

Studies on Chandipura infection have demonstrated that this can cause neuronal apoptosis by stimulating oxidative stress. ROS induced by oxidative stress are a critical factor for apoptosis following Chandipura infection. Calcium release or an increase in calcium in cells is one of the agents that causes oxidative stress and consequently ROS production (Verma et al., 2018).

In influenza infection, the signaling pathways associated with oxidative stress have been investigated in more detail. After infection of the respiratory epithelium, ROS levels have been found to be above threshold values (Liu et al., 2017). In H5N1 infected mice, it was observed that the SOD level was lower and the ROS concentration and lung destruction were higher than the control group. Superoxide anion (O2-) detected in the lungs of influenza-infected mice was considered to be a potential pathogenic agent for this infection (He et al., 2013).

In oxidative stress associated with influenza infection, three signaling pathways have been described. As a result of these three signaling pathways being triggered by oxidative stress, the immune response to influenza infection is suppressed. The mechanisms for these pathways are outlined as follows (Liu et al., 2017).

NF-E2-related factor 2 (Nrf2) is a highly sensitive transcription factor that regulates the cellular antioxidant response. Inactive Nrf2 needs cytosolic protein Keap1 in order to enter the cell. Phosphorylated Nrf2 enters the cell, where oxidative stress leads to the dissociation of Keap1 and NrF2, suppressing antioxidant formation (Liu et al., 2017).

The p38 MAPK signaling pathway participates in cellular responses due to its wide stimulus range, both in vivo and in vitro, and mediates a variety of processes: growth, development, differentiation and death of cells. Phosphorylated p38 enters the nucleus of host cells and plays a role in the expression of cytokine genes under oxidative stress stimuli (Liu et al., 2017).

NF-kB is a transcription factor that has a critical regulatory role in the immune response. The activity of NF-kB is induced by various stimuli, such as tumour necrosis factor alpha (TNF-α), para-methoxyamphetamine (PMA), cigarette smoke stress, lipopolysaccharides (LPS), antioxidants and viral infections. In general, the detection of NF-kB is considered to be a biomarker of oxidative stress (Liu et al., 2017).

Retroviruses: The observation of multiple pathogenetic interactions between ROS and the retrovirus HIV (human immunodeficiency virus) infers that such interactions may also play a role in the pathogenesis of other virus infections (Schwarz, 1999; Wang et al., 2018). The HIV infection process contributes to the disruption of the balance between the formation of free radicals and antioxidant defence. Oxidative stress contributes to the pathogenesis of HIV infection by stimulating the proliferation of the virus, decreasing the proliferation of immune cells and increasing the susceptibility to drug toxicity (Wang et al., 2018).

DNA viruses: Researchers have shown that oxidative stress, known as one of the suggested mechanisms to facilitate the replication of herpesviruses, changes the oxidative balance by increasing the formation of free radicals or inhibiting the enzymes involved in oxidative defence in the host cell (Kavouras et al., 2007) The biomarkers of oxidative stress may vary between tissues. When the effect sizes of infections are evaluated, it is known that virus strains cause significant changes (Sebastiano et al., 2016).

The latent membrane protein of Epstein-Barr virus and adenovirus 19K E1B transforming protein can inhibit apoptosis and thus increase oncogenic transformation. All of these viral proteins can inhibit apoptosis by using antioxidant pathways (Schwarz, 1996).

Ecthyma contagiosum is a zoonotic disease (Karakas et al., 2013). In animals diagnosed with this disease, elevated PON1 activity, TSA (total sialic acid), HDL (high-density lipoprotein), NO and glutathione levels have been measured in blood samples (Deveci et al., 2017). Besides playing an essential role in antioxidant defence against lipid peroxidation in the cell membrane, PON1 is believed to play a role in the anti-inflammatory process and in the protection of LDL (low-density lipoprotein) and HDL from oxidation (Çakırca, 2013).

In one study, oxidative stress parameters have been evaluated by taking blood and cerebrospinal fluid from animals infected with coryza gangrenosa bovum. Significant changes in the blood samples were found due to the inflammatory process associated with oxidative stress (Erkiliç et al., 2017).

Reductions in MDA level and SOD and CAT activities have been seen in dogs infected with

parvovirus in oxidative stress. In the haemogram obtained from the infected dogs, zinc levels also were decreased (Panda et al., 2008; Süleyman et al., 2018).

Conclusions

Although ROS are the basic products for cell function under physiological conditions (Chawla et al., 2001), they have been shown to play important roles at different stages of many diseases. ROS also cause or contribute to the development of head and neck cancer (Qian et al., 2018). DNA-containing and RNA-containing viruses have been reported to increase the production of oxidants such as superoxide and nitric oxide, affecting the cellular redox balance and inhibiting the synthesis of antioxidant enzymes. Thus, given the discussions so far, it is concluded that oxidative stress is associated with several aspects of the pathogenesis of various viral aetiological agents. Oxidative stress can both help and also inhibit viral replication (Camini et al., 2017). As a treatment approach, the use of antioxidants is an option. However, further studies on the use of antioxidants for the treatment of viral infections are needed. The extent to which the, as yet, unknown mechanisms may affect viral evolution should also be investigated further.

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The effect of capsaicin on TBARS and TAS levels in rats with hypothyroidism

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ABSTRACT

In this study, capsaicin was administered to rats with experimental hypothyroidism. İt was aimed to determine the changes in plasma levels of thiobarbituric acid reactive substances (TBARS), which are indicators of oxidative stress, and total antioxidant capacity (TAS), which is one of the components of antioxidant defence mechanisms. A total of 32 healthy male Wistar Albino rats weighing 300-350 g, approximately 12 weeks old, were used as animal material in the study. Rats were divided into four equal groups control (K), Capsaicin (C), Hypothyroid (H) and capsaicin + Hypothyroid (CH). During the 30-day trial period, (10mg / kg / day) capsaicin was administered to the rats in group C by oral gavage per animal. In group H, 6-n-propyl-2-thiouracil (PTU) was added daily to their drinking water at 0.05% weight/volume (W/V). In the CH group, 10 mg/kg/day of capsaicin was administered by oral gavage method and 0.05% weight / volume (W / V) of PTU was added to drinking water. At the end of the application, we obtained plasma and serum samples from the subjects in the groups under general anaesthesia (thiopental anaesthesia, 40 mg/kg) and by taking sufficient amount of blood from the heart by cardiac puncture. We determined thyroid-stimulating hormone (TSH), total triiodothyronine (TT3), free T3 (fT3), total thyroxine (TT4) and free T4 (fT4) levels from serum samples, and TBARS and TAS levels from plasma samples. In conclusion, in the light of the data obtained in this study, we determined that lipid peroxidation and oxidative stress occur in hypothyroidism. However, we concluded that the application of capsaicin is partially sufficient to maintain the oxidant/antioxidant balance.

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Introduction

Thyroid hormone secreted by the thyroid gland regulates the metabolic rate necessary for the normal function of tissues. Although thyroid hormone is not absolutely necessary for life, physical and mental regression is observed in its absence (Noyan, 2011).

Hypothyroidism is a term used to describe physiological disorders caused by suboptimal circulating levels of thyroid hormones. Hypothyroidism is associated with hypometabolism characterized by decreased blood thyroid hormone levels, decreased resting energy expenditure, weight gain, increased blood cholesterol levels, decreased lipolysis, and decreased gluconeogenesis (Brent 2012).

Oxidative stress parameters have been examined in different studies in patients with high (hyperthyroidism) and low (hypothyroidism) thyroid hormone levels in the blood, and it has been reported that there are changes in the body's oxidant and antioxidant systems in both hyperthyroidism and hypothyroidism (Torun et al., 2009; Santi et al., 2010).

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In hypothyroidism, a decrease in free radical production is expected due to metabolic suppression brought about by the decrease in thyroid hormone levels (Pereira et al., 1994). The effect of hypothyroidism on antioxidant enzymes has been investigated in several tissues, but the results are highly controversial. In some cases, the change in antioxidant enzyme activity appears to be tissue-specific (Venditti et al., 1997). On the other hand, within a single tissue, the response of antioxidant enzymes to hypothyroidism is not always similar (Das and Chainy 2001). Conversely, Duntas (2005) reported that hypothyroidism is associated with increased ROS production.

The hot component of cayenne pepper is capsaicin, and its active derivatives are called capsaicinoids. Capsaicinoids have been found to exert multiple pharmacological and physiological effects, including analgesia, anticancer, anti-inflammatory, antioxidant, and anti-obesity activities (Luo et al., 2011). Therefore, capsaicinoids may have potential clinical value for pain relief, cancer prevention and weight loss (Sun et al., 2016). In addition, capsaicinoids show benefits on the cardiovascular and gastrointestinal systems. The beneficial effects of capsaicin on cardiovascular function and metabolic regulation have been confirmed in experimental and population studies (Li et al., 2019).

Reactive free radicals produced during oxidative attacks lead to architectural changes in the cellular dimension, resulting in abnormal changes in macromolecules. These toxic changes are responsible for the pathogenesis of various diseases. The studies show that capsaicin as a dietary supplement may be beneficial in combating oxidative stress (Chaudhary et al., 2019).

Capsaicin, the main component of red pepper, has attracted increasing attention due to its multiple biological activities. Therefore it has been the subject of many studies in recent years (Antonious, 2018). On the other hand, it has been one of the most studied subjects hypothyroidism, especially in our country and in the world, because of the endemic regions in terms of iodine deficiency (Gluvic et al., 2020). However, we concluded that studies on the effects of capsaicin on thyroid hormones and metabolisms in humans and animals, and thus examine the protective effects of capsaicin in hypothyroidism, are quite insufficient. Therefore, this study planned to examine whether capsaicin affects TBARS and TAS in mice with experimental hypothyroidism, with the thought that it would contribute significantly to the knowledge on the subject.

In this study, it was aimed to determine the protective effect of capsaicin, whose antioxidant properties are well known, to reduce the amount of ROS increased Because of oxidative stress in experimentally induced hypothyroidism, to increase the amount of decreased antioxidants and to keep the inflammatory response in balance.

Material and Methods

In the research, S.U. Obtained from the Experimental Medicine Research and Application Center, 32 healthy male Wistar Albino rats, approximately 12 weeks old, weighing 300-350 g, were used. In the trial covering a 10-day adaptation period and a 30-day main research period; Appropriate living conditions were provided for the rats in the form of 22 ± 2 °C room temperature, 50 ± 10 % relative humidity and 12/12 day and night light periods. In the study, the average amount of water that the rats could drink daily was determined (average 50 ml/day/rat), and their water was refreshed daily. Animals were fed with standard rat chow (Bil-Yem) ad libitum. Chemically, PTU (Trademark; Sigma P3755) and capsaicin (Trademark; Tokyo Chemical Industry TCI M1149) were used.

During the trial, in the control group; standard rat feed and drinking water were given ad libitum and no application was made. To the capsaicin group; Standard rat food and drinking water were given ad libitum. Capsaicin was administered orally at a daily amount of 10 mg/kg/day (Joo et al., 2010). To the hypothyroidism group; Standard rat food and drinking water were given ad libitum. During the adaptation period, the daily average amount of water consumed by the rats was determined and 0.05 % PTU (Moulakasis et al., 2008; Tousson et al., 2012; Kandır, 2015) was added to the drinking water daily throughout the experiment in order to create hypothyroidism. Capsaicin+Hypothyroidism group; Standard rat food and drinking water were given *ad libitum*. A daily amount of 10 mg/kg/day capsaicin and 0.05 % PTU were added to drinking water.

Blood analysis: At the end of the experiment, animals were decapitated after taking sufficient amount of blood (approximately 10-12 ml) from the heart under general anesthesia (thiopental anesthesia, 40 mg/kg) and cardiac puncture at the end of the experiment. Collected blood was put into tubes with or without ethylene diamine tetra acetic acid (EDTA) to obtain plasma and serum. Plasma samples were obtained by centrifuging the blood at 3500 rpm at +4°C (HettichRotina 35 R). Plasma and serum samples were stored at -80 °C until analysis.

Determination of hormone levels in serum samples: TT3, TT4, fT3, fT4 and TSH hormone levels in serum

samples were determined by the chemiluminescence method using the immunoassay system on Abbottarchitech i2000 analyzer and commercial kits (Abbott) as specified in their package inserts (Sarandol et al., 2005; Kandır, 2015).

Determination of oxidative stress and antioxidant levels in plasma samples: Plasma TBARS and TAS levels were determined by colourimetric method by reading the absorbance values with the Biotek brand ELX800 model ELISA device such as by the package inserts of the commercial kits (Messarah et al., 2010; Kandır, 2015).

Statistical analysis: SPSS 25 statistical package program was used to evaluate the data. Variables mean ± standard error values were used. Kruskal Wallis test was used to analyze the statistical difference between the groups. P<0.05 value was accepted for the significance level of the tests.

Results

Thyroid Hormone Levels: In the research, considering TSH, TT4, TT3, Free T3 and Free T4 values, it can be said that PTU administration causes hypothyroidism in hypothyroid (H) and capsaicin+hypothyroid (HC) groups. As a matter of fact, the serum TSH level in the H and CH groups was higher (p<0.05) than in control (K) and capsaicin (C) groups, on the other hand, serum TT4 and TT3 values, which are the main thyroid hormones effective on tissues, and serum-free thyroxine and triiodothyronine levels were both The fact that it was found in lower amounts in rats in both H and CH groups compared to the same values in other groups confirms this (Table 1).

Antioxidant and free radical parameters: It was determined that plasma TBARS level in the hypothyroidism+capsaicin (CH) group was higher than the TBARS levels in the other 3 groups (K, C and H) (p<0.05). It was noted that there was no significant difference (p>0.05) between the same parameter levels among the K, C and H groups (Table 2).

When the plasma TAS variable of the groups was examined, a significantly higher value was determined only in the hypothyroidism group (H) compared to the other groups (F, C, CH) (p<0.05), and there were a significant difference between the TAS levels of the K, C and CH groups. İt is seen that there is no difference (p>0.05) (Table 2).

Table 1. Blood serum mean TSH (thyroid stimulating hormone), TT4 (total thyroxine), TT3 (total triiodothyronine), fT3 (free T3) and fT4 (free T4) levels of the groups used in the study, given capsaicin and/or hypothyroidism (x ± standard error).

Group	Control (n = 8)	Capsaicin (n = 8)	Hypothyroidism (n = 8)	Capsaicin + Hypothyroidism (n = 8)
TSH (mIU/L)	1.51 ± 0.74 [a]	4.64 ± 1.76 [a]	43.24 ± 16.04 [b]	49.36 ± 12.4 [b]
TT_3 (ng/mL)	1.21 ± 0.13[a]	1.19 ± 0.12[a]	0.51 ± 0.03[b]	0,51 ± 0.03[b]
TT_4 (ug/dL)	7.46 ± 1.51 [a]	8.39 ± 0.93 [a]	0.53 ± 0.09 [b]	0.63 ± 0.1 [b]
fT_3 (ng/L)	3.12 ± 0.6 [a]	2.62 ± 1.23 [a]	0.02 ± 0.02 [b]	0.05 ± 0.05 [b]
fT_4 (ng/dL)	2.88 ± 0.19 [a]	3.15 ± 0.31 [a]	0.21 ± 0.22 [b]	0.19 ± 0.05 [b]

a, b; The difference between the mean values of the same parameter displayed with different letters on the same line is important (p<0.05). There was no significant difference between groups containing the same letter ($P > 0.05$).

Table 2. TBARS and TAS levels measured from the blood plasma of the groups used in the study, given capsaicin and/or hypothyroidism (x ± standard error).

Group	Control (n = 8)	Capsaicin (n = 8)	Hypothyroidism (n = 8)	Capsaicin + Hypothyroidism (n = 8)
Tbars (nmol/mL)	4.63 ± 2 [a]	3.38 ± 0.94 [a]	4.46 ± 1.83 [a]	6.01 ± 0.73[b]
TAS (mmol/L)	9.31 ± 2.16 [a]	11.01 ± 2.5 [a]	14.09 ± 1.72 [b]	11.59 ± 1.84 [a]

a, b; The difference between the mean values of the same parameter displayed with different letters on the same line is important ($P < 0.05$). There was no significant difference between groups containing the same letter ($P < 0.05$). TBARS = Thiobarbituric acid reactive substances, TAS = Total antioxidant status

Discussion and Conclusion

In the study, when the obtained findings were compared, the increase in serum TSH level ($p<0.05$), the decrease in TT4, TT3, fT4 and fT3 levels ($p<0.05$) in the H and CH groups, which were applied PTU, were experimentally determined when compared to the K and C groups shows that hypothyroidism is formed (Table 1). Rondeel et al. (1992), Kandır (2015) and Yazıcı (2019) also reported that hypothyroidism was induced by adding PTU to the drinking water of rats.

Thiobarbituric acid reactive substances (TBARS) is a lipid peroxidation index and is one of the important indicators of oxygen reactive species (ROS) activities and is associated with membrane lipid degradation (Ottaviano et al., 2008). Messarah et al. (2007) and Coria et al. (2009) report that lipid peroxidation products such as malondialdehyde and hydroperoxide concentrations do not change in clinical hypothyroidism and subclinical hypothyroidism compared to euthyroidism. However, some researchers have observed that the level of TBARS in the blood is increased in clinical hypothyroidism compared to euthyroidism (Nanda et al., 2007; Erdamar et al., 2008; Santi et al., 2010). Reports also state that oxidative stress is reduced in experimental hypothyroid animal models (Brzezińska-Slebodzińska, 2003; Mogulkoc et al., 2005; Tenorio-Velázquez et al., 2005). The results obtained from this study, in accordance with the studies of Messarah et al(2007) and Coria et al (2009), show that the TBARS concentration did not change in rats in the hypothyroidism group compared to the rats in the control group ($p>0.05$). In the case of hypothyroidism, the reasons for opposing views in the literature can be attributed to tissue and organ sensitivity, lipid peroxidation measurement methods, animal species, and application method differences (Messarah et al., 2011; Cano-Europa et al., 2012).

Although the antioxidant property of capsaicin is well known (Chaudhary et al., 2019), it is reported that it can increase oxidative stress in some cases (Abdel-Salam, 2006; Baek et al., 2008; Schwartz et al., 2008), as observed in this study. It has been stated that when capsaicin is injected intradermally, the central transmission of nociceptive impulses induced by capsaicin may increase spinal reactive oxygen species due to increased mitochondrial superoxide production in dorsal horn neurons (Schwartz et al., 2008). It has been reported that capsaicin induces a complex expression pattern of both oxidative stress genes and antioxidant defence genes, and during apoptosis in response to capsaicin, reactive oxygen species levels increase or decrease depending on the cancerous cell type (Baek et al., 2008). Another study reported that oxidative stress created by endotoxin lipopolysaccharide application, liver MDA increased significantly after capsaicin application (Abdel-Salam et al., 2012). In this study, although the plasma TBARS level decreased in the capsaicin-administered group compared to the control group, this decrease was not statistically significant ($p>0.05$). When capsaicin was applied to the hypothyroidism group, the TBARS level increased ($p<0.05$), and its results are similar to the last reports mentioned above (Baek et al., 2008; Schwartz et al., 2008).

The mechanism of increased oxidative stress in hypothyroidism is controversial. It can be thought that An insufficient antioxidant defence system in hypothyroidism may be one of the factors (Torun et al., 2009). Venditti and Di Meo (2006) showed that antioxidants were not affected in the same way in different tissues of hypothyroid rats; They reported that they increased in some tissues, decreased in others or remained unchanged. Konukoglu et al. (2002) found that antioxidant plasma protein thiol levels decreased in patients with hypothyroidism and returned to normal with thyroxin treatment. This suggests that the deficiency of the antioxidant defence system may be a leading factor in the increase of oxidative stress in hypothyroidism (Santi et al., 2010). In another study, the antioxidant ceruloplasmin was decreased in hypothyroid patients compared to normal controls. While most of these studies evaluate different and only one or a few antioxidants, it may also be important to measure total antioxidant capacity, which may be more informative about overall antioxidant defences. Total antioxidant capacity (TAS) gives information about all antioxidants in the organism (Torun et al., 2009). In the study of Salama et al. (2013), in which they experimentally induced hypothyroidism in rats with PTU, it was reported that rats with hypothyroidism had higher plasma and tissue MDA, plasma NO2, NO3 and tissue total antioxidant levels compared to the control group rats. Cebeci et al. (2012) also recorded that they found the plasma total antioxidant capacity to be significantly higher in patients with hypothyroidism in their measurements, while Torun et al. (2009) found that the total antioxidant levels of SH and OH patients were lower than the control group. Similarly, Mancini et al. (2010), Gomathi et al. (2012), Deraz et al. (2016), and also report that plasma TAS levels are significantly lower in patients with hypothyroidism compared to healthy subjects. In their study, Kumari et al. (2011) reported that hypothyroidism did not cause a change in NO, SOD and total antioxidant levels compared NO, SOD

and total antioxidant values in hyperthyroid and hypothyroid patients. The data obtained in this study, in line with the findings of Cebeci et al. (2012) and Salama et al. (2013), were found to have a higher plasma TAS level in the hypothyroidism group than in the other groups ($p < 0.05$) (Table 2). In our study, the fact that plasma TAS levels were found to be higher in rats in the H group with hypothyroidism than in the rats in the K, C and CH groups ($p<0.05$) may indicate that the total antioxidant capacity of the body may have increased due to the increased oxidative stress in hypothyroidism (Table 2). Different findings among some studies: it may be caused by differences in tissue and organ sensitivity, measurement methods, animal species and application method (Messarah et al., 2010; Cana-Europa et al., 2012).

Capsaicin has potent antioxidative effects in vivo through a non-receptor-mediated mechanism. As a matter of fact, Chaudhary et al. (2019), in their study on erythrocytes without TRPV1 channels, reported that the plasma antioxidant capacity increased significantly ($p<0.05$) in capsaicin supplemented rats. This observation shows that capsaicin administration can protect cells from oxidative damage (Chaudhary et al., 2019). In a study examining the hypolipidemic and antioxidant effects of dietary capsaicin in hypercholesterolemic rats, it was found that while the levels of ascorbic acid and α-tocopherol, which are antioxidant molecules in the serum, did not change in the capsaicin diet compared to the control group, the total amount of thiol decreased (Manjunatha and Srinivasan, 2007). On the other hand, it was reported that the total thiol, α-tocopherol and intracellular total thiol levels in the basement membrane of erythrocytes in rats fed a high-fat diet did not change with the application of capsaicin, and in the same study, it was reported that the total thiol amount in the cell decreased with capsaicin in hypercholesterolemic animals (Kempaiah and Srinivasan, 2004b). The data obtained in this study show parallelism with the work of Manjunatha and Srinivasan (2007). TAS levels, which increased significantly ($p<0.05$) due to oxidative stress in the hypothyroidism group, decreased with capsaicin administration (Table 2). This may show that the use of antioxidants is increased to reduce oxidative stress.

In conclusion, the data obtained in this study suggest that lipid peroxidation and oxidative stress occur in hypothyroidism, but capsaicin administration is partially sufficient to provide oxidant/antioxidant balance. On the other hand, it has been concluded that the study's findings can contribute to the information that can be considered insufficient on the subject and can be a source for other research that can be done in this direction.

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Ethical Statement

This study was approved by Selcuk University Animal Experiments Ethics Committee with the decision dated 29.04.2019 and numbered 2019-20.

Conflict of Interest

The authors declared that there is no conflict of interest.

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