A Microbiologic and Histopathologic Evaluation of Anachoresis in Rats Heavy Infected with *Thiobacillus* Species

Thiobacillus Türleri ile Ağır Olarak Enfekte Edilen Ratlarda Anakorezis Varlığının Mikrobiyolojik ve Histopatolojik Değerlendirilmesi

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ABSTRACT Objective: The aim of this study was to evaluate the risk of Anachoresis in heavy infected rats with *Thiobacillus* species. **Material and Methods:** 20 of the 28 male Wistar albino rats were infected with *T. thiooxident, T. ferrooxidant, T. thioporus* and remaining 8 rats were not infected and served as control group. 3.5 ml containing 1x106 CFU/ml *Thiobacillus* species was used to infect the rats during 20 days. Following sacrification of 28 animals after 20 days, their mandibles were extracted. Each of the mandibles was divided into two halves from their median sutures. The first halves were evaluated microbiologically, and the other halves were selected for the histopathological investigation. **Results:** According to the microbiological investigation, the control group and the infected group showed no microbial growth. The results of the histopathologic evaluation showed the dental pulps did not have any inflammation signs in infected group (p>0.01). **Conclusion:** Although the rats were heavy infected with the *Thiobacillus* species which we are under the risk of contamination daily, Anachoresis was not observed in rat dental pulps.

Key Words: Blood-borne pathogens; pulpitis; gram-negative bacterial infections

ÖZET Amaç: Bu çalışmanın amacı, *Thiobacillus* türleri ile ağır olarak enfekte edilen ratlarda Anakorezis riskini değerlendirmek. **Gereç ve Yöntemler:** 28 erkek Wistar albino ratın 20'si *T. thiooxident, T. ferrooxidant, T. thioporus* ile ağır olarak enfekte edildi ve kalan 8 rat ise enfekte edilmeyerek kontrol grubunu oluşturdu. 1x106 CFU/ml *Thiobacillus* spp. içeren 3.5 ml doz 20 gün boyunca ratları enfekte etmede kullanıldı. 20 gün sonrasında 28 hayvanın sakrifikasyonunu takiben, ratların mandibulaları çıkartıldı. Her bir mandibula median sütürden ikiye ayrıldı. İlk yarısı mikrobiyolojik olarak değerlendirilirken, diğer yarısı da histopatolojik inceleme için seçildi. **Bulgular:** Mikrobiyolojik inceleme sonucunda kontrol ve enfekte grup arasında herhangi bir mikroorganizma varlığı gözlenmedi. Histopatolojik değerlendirmede ise enfekte gruptaki diş pulpalarında herhangi bir enflamasyon bulgusuna rastlanmadı (p>0,01). **Sonuç:** Her gün çeşitli yollarla kontaminasyon riski altında olduğumuz *Thiobacillus* türleri ile ağır olarak enfekte edilen ratlarda, Anakorezis gözlenmedi.

Anahtar Kelimeler: Kan kaynaklı patojenler; pulpit; gram negatif bakteriyel enfeksiyonlar

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Dental caries, trauma and dental procedures may cause the primary pulp infection. Moreover when the microorganisms enter *via* dentinal tubules, a lateral/accessory/furcation canal originated from the intact tissues without coronal infiltration is called secondary pulp infection. If the blood born microorganisms are entered and infected to the pulp by the circulation pathways, this is called a phenomena of the "Anachoresis" or "Anachoretic pulpitis".[1,2]

Inflammation is a fundamental biological process following tissue injury, and teeth and supporting tissues are susceptible to various forms of injury, intentionally or accidentally. During the inflammatory process cells may undergo reversible or irreversible changes. The result may be an altered or new steady state of some cells, whilst others are lethally injured. A lethal injury may lead to a series of changes: (i) prelethal, often reversible changes; (ii) cell death as the 'point-of-no-return'; (iii) postmortem autolytic and degenerative changes, i.e. necrosis that can either be oncotic or apoptotic. Such injury-induced changes in the tissue may cause an imbalance in the local homeostasis, susceptible to blood-borne pathogens.[3]

The genus *Thiobacillus* is also known under the name of *Acidithiobacillus*. *Thiobacillus* are colorless, rod-shaped, Gram-negative proteobacteria with polar flagella. *Thiobacillus* do not form spores. *Thiobacillus* are strictly aerobic bacteria. All species are respiratory organisms. Their life cycle is typical of bacteria, with reproduction by cell fission. *Thiobacillus* are obligate autotrophic organisms, meaning they require inorganic molecules as an electron donor and inorganic carbon (such as carbon dioxide) as a source. They obtain nutrients by oxidizing iron and sulphur with O_2. They possess an iron oxidase, which allows them to metabolize metal ions such as ferrous iron:[4,5]

$$Fe^{+2} + ½O_2 + 2H^+ \longrightarrow Fe^{+3} + H_2O.$$

Thiobacillus ferrooxidans is the most common type of bacteria in mine waste piles. This organism is acidophilic (acid loving), and increases the rate of pyrite oxidation in mine tailings piles and coal deposits. It oxidises iron and inorganic sulphur compounds. The oxidation process can be harmful, as it produces sulphuric acid, which is a major pollutant. However, it can also be beneficial in recovering materials such as copper and uranium. It has been suggested that *T. ferrooxidans* forms a symbiotic relationship with members of the genus *Acidiphilium*, a bacterial capable of iron reduction. Other species of *Thiobacillus* grow in water and sediment; there are both freshwater and marine strains. *Thiobacillus species* are used in mining, agriculture and in medicine for dissolution of urinary stones. Although the medical applications have been done under the aseptic or sterile conditions, industrial bioleaching processes and agriculture products have not been carried out under such sterile conditions yet. So humans may be infected with these species of *Thiobacillus* species.[6-10]

Cropaid Natural Plant Antifreeze® (Cropaid NPA®) contains *Thiobacillus subspecies* and minerals used by these bacteria. For this reason, it is ecological and natural. Freezing point of Cropaid NPA® is very low. It has being recorded in lab tests that at -17 centigrate degrees Cropaid NPA® was not frozen 2.5 hours and kept it's temperatures first 90 minutes +2.5 °C and remaining time did not drop below +1.5 °C. This shows that Cropaid NPA® can have some protection from frost at least 3 hours without any bio-chemical growth.[11]

The purpose of this study was that to investigate the existence of "*Anachoretic pulpitis*" and the *Anachoretic* effects of Cropaid NPA®, a natural plant antifreeze contained *Thiobacillus species*, on rat dental pulps. This project has been a part of multidisciplinary investigation of effect *Thiobacillus species* on the different systems of rats such as urogenital, cardiovascular, pulmonary, endocrinal and neurologic systems.

MATERIAL AND METHODS

This project was approved by the local ethical committee of animal experiments. The male Wistar albino rats weighed between 150-210 g were grouped. Group 1; the study group (n=20) was infected with 0.2 cc Cropaid NPA® (Cropaid Int. Ltd. Essex, England) *via* injected intravenously every day, at same hour (at 10 o'clock). Group 2: The control group (n=8) was fed with 0.2 cc water *via* orogastric gavage just. The experimental group of 20 rats were infected during 20 days with Cropaid NPA® contained "*Thiobacillus thiooxident, Thiobacillus ferrooxident, and Thiobacillus thioporus*. The intakes of the *Thiobacillus species* consist of 3.5ml containing $1x10^6$ CFU/ml bacillus were given to rats. Weights of the rats were scaled five times during 20 days. After taking the blood from heart, the animals were sacrificed under deep anaesthesia via intra-cardiac perfusion with 4%

paraformaldehyde (PFA) (Sigma-Aldrich Co., St. Louis, MO, USA) in a phosphate-buffered saline (PBS) solution at 0.1 mol L-1 (pH=7.4) through the left ventricle, using a sterile syringe. In blood samples were examined for other disciplines. The statistical analyzes were done in SPSS 15 programme. $p<0.01$ was accepted as significant.

Following sacrification, their mandibles of animals were extracted. Each of the mandibles was divided into two halves from their median sutures. The first halves were evaluated microbiologically, and the other halves were selected for the histopathological investigation. Samples were placed in sterile containers with 8cc of freshly prepared 10% phosphate buffered formaldehyde (Sigma-Aldrich Co., St. Louis, MO, USA) solutions for the evaluations.

Incisor teeth of group of the microbiological evaluation were perforated pulps with a carbide bur ISO 004 (Mani Dia-Burs, Mani Inc. Tochigiken, Japan) until the pulp was visible under sterile saline irrigation. Following the perforation, the pulps were extirpated with Headstroem file size #6(MANI Inc., Utsunomiya, Japan). The H-files were cut off 3 mm from these tips and placed in the sterile Eppendorf tubes within the 100 µl steril saline. All the Eppendorf tubes were vortexed for 2 minutes. 200 µl medium of *Thiobacillus species* prepared and added each of the Eppendorf tube. After all samples incubated in 48 hours at 37°C, the test tubes were centrifugated. 10 µl solutions were taken from the buttom of the test tubes and placed on Thomma Slide. The quantity of the bacteria was counted under the light microscope (Nikon Eclipse E-600, Tokyo, Japan).

For the histopathological evaluation the specimens fixed in 10% neutral buffered formalin, and decalcified in buffered 10% formic acid. After decalcification, the specimens were rinsed under running water for 4 hours followed by dehydration with ascending concentrations of alcohol and then embedded in paraffin blocks. 5 µm sections were prepared for histological analysis. All sections stained with Hematoxylin and Eosin (H&E). Van Gieson's Tricrome and Gomori's Reticulin staining were performed to evaluate pulp tissue organization while Brown&Brenn staining was used for determining bacterial presence in all specimens. Sections of the control (Figure 1, Figure 2, Figure 3 and Figure 4) and research group (Figure 5, Figure 6, Figure 7, Figure 8, Figure 9 and Figure 10) were examined under the light microscope (Nikon Eclipse E-600, Tokyo, Japan) The evaluation criteria for histological findings, inflammation degree and organization of pulp tissue, were given in Table 1 and Table 2.[12,13]

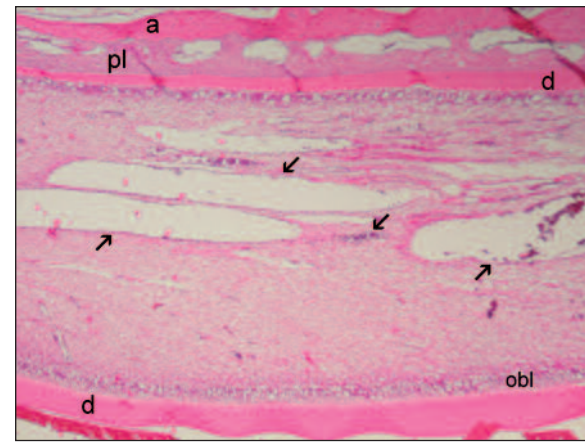

FIGURE 1: The panoramic view of anterior tooth in the control group (group K). Pulp connective tissue constituted cellular form and there were seen extensive congested and dilated capillary structures a: alveol bone, pl: periodontal ligament, d: dentin, obl: odontoblast layer, arrows: showing capillary forms. (HE, x40).
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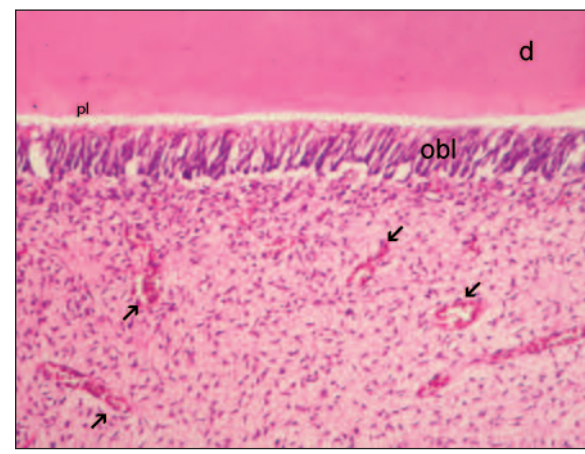

FIGURE 2: The arrows indicate that cellular form of a pulp connective tissue is contained mesenchymal cells without inflammatory cells under the odontoblastic layer (obl). d: dentin, pl: periodontal ligament. (HE, x200).
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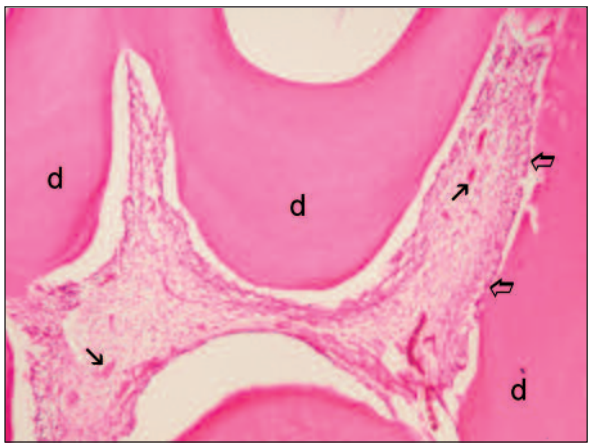

FIGURE 3: A panoramic view of pulp of molar teeth (HE, x100). d: dentin, arrows: Showing capillary forms, blank arrows: presenting odontoblastic layer.
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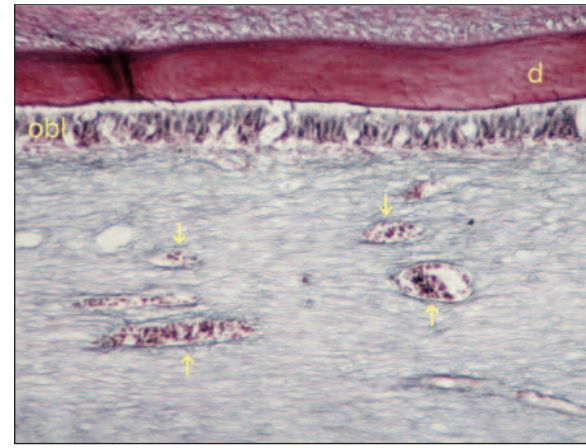

FIGURE 4: The arrows indicate that congested capillary within the pulp connective tissue which is built up thin reticular fibrils under the continuously odontoblast layer (obl) d: dentin, (Gomori's reticulin x200).
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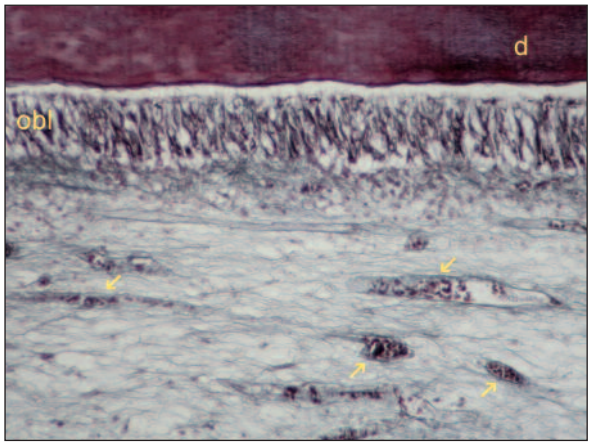

FIGURE 5: Congested capillary within the pulp connective tissue built up the thin reticular fibrils under the odontoblastic layer (obl), d: dentin, (arrows: Gomori's reticulin x200).
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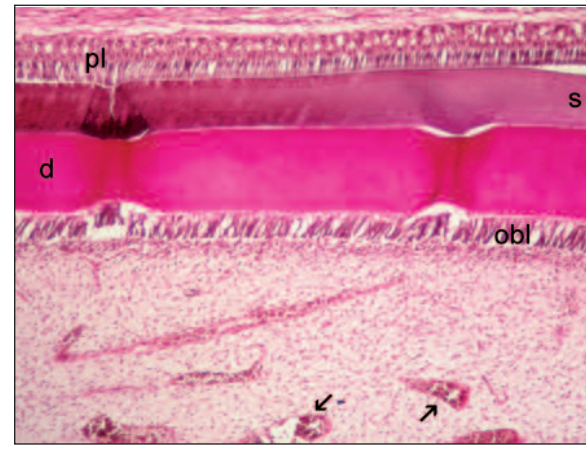

FIGURE 6: Congested capillary within the cellular type pulp connective tissue built up the mesenchymal cells under the odontoblastic layer (obl). (S: cement, pl: periodontal ligament) arrowss: Van Gieson's Tricrom x100).
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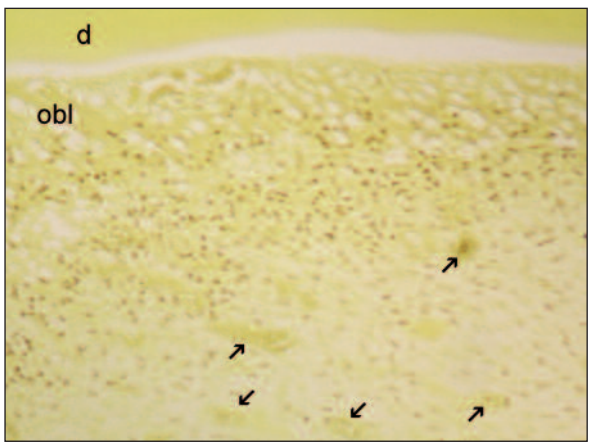

FIGURE 7: Any microorganism was not determined pulp connective tissue samples of group E (While arrows indicated capillary, red stained cell showed pulp mesenchymal cells. d: dentin, obl: odontoblast layer). Brown & Brenn x200).
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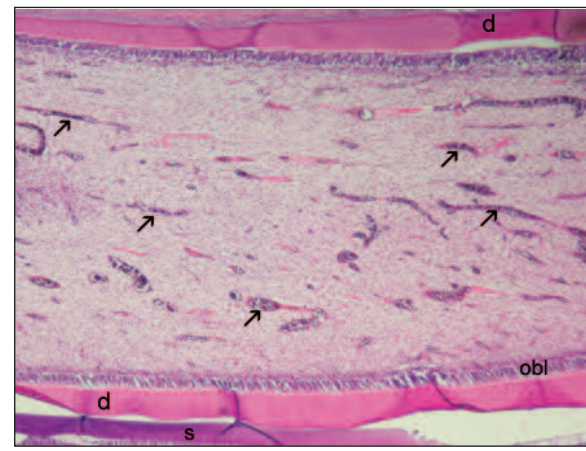

FIGURE 8: A panoramic view of anterior tooth in the group E. Pulp connective tissue constituted cellular form and seen a extensive congested capillary. d: dentin, obl: odontoblast layer. s: cement, (HE, x40).
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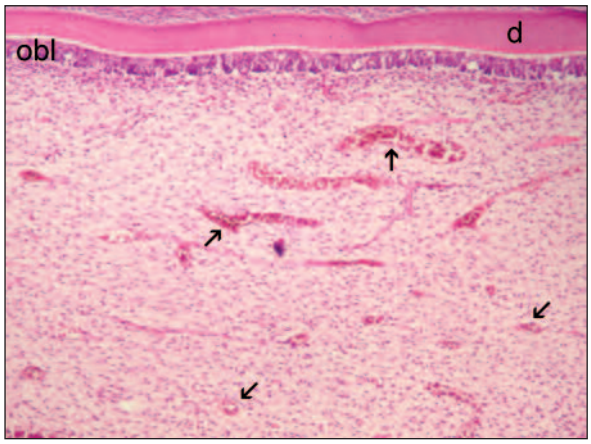

FIGURE 9: More magnification of picture 4. While odontoblastic layer (obl) within the cellular formed pulp connective tissue was observing normally, any inflammatory cells couldn't be seen in the pulp. d: dentin, arrows: showing capillary forms. (HE, x100).
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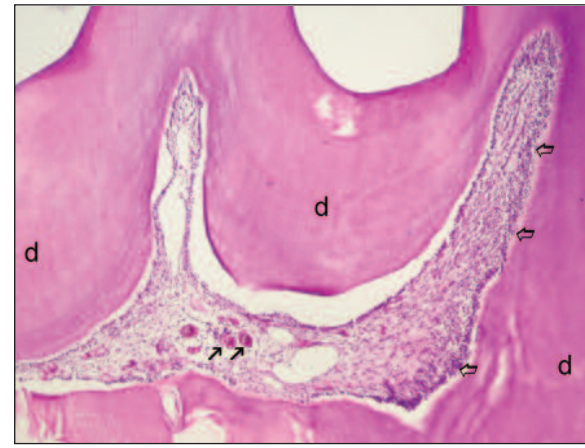

FIGURE 10: The panoramic view of a pulp of molar teeth. A number of congested capillary were seen in the pulp connective tissue contains continuously odontoblastic layer (HE, x100).
d: dentin, arrows: showing capillary forms, blank arrows: presenting odontoblastic layer.
(See color figure at
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TABLE 1: Inflammation degrees.

Scores	Inflammation (Inflamatory Tissue Responce)
0	No inflammation
1	No inflamatory cell or a few scattered inflammatory cell in the pulp connective tissue
2a	Acute inflammatory cell infiltration consist of polymorphonuclear leukocytes(from the neutrophills)
2b	Chronic inflammatory cell inflamation consist od mononuclear lymphocyte
3	Widespread inflammatory cell infiltration characterize with abscess formation or dens infiltration of polymorphonuclear leukocyte in the pulp connective tissue
4	Necrotic pulp

TABLE 2: Pulp tissue organization.

Scores	Pulp Tissue Organization
0	Pulp tissue views as a normal
1	Odontoblastic layer organization was spoiled but central pulp tissue views normal
2	Pulp tissue organization completely spoiled
3	Necrosis of pulp

RESULTS

Statistical evaluations were done with Kruskall-Wallis test and the differences between the groups were evaluated with Mann Whitney U test for the microbiological and histopathological investigation (Table 3, Table 4).

In the experimental group and control group samples showed similar pulpal condition such as neither inflammation nor bacterial invasion. Couple layers of columnar *paleosatic* arrangement of the odontoblast layer were seen intact to the dentin. Most of samples showed that mesenchymal cells with angle-shaped nucleus and increased collagen fibres scattered in the connective tissue served as normal histological findings. In contrary no bacterial invasion were detected in samples of the Brown-Brenn staining.

TABLE 3: The statistical evaluation for the inflammation dates. According to the Mann Whitney-U test there wasn't any statistical significant difference between the control and research group (p>0.01).

Groups	Number of specimens	Number of observations	Mean	Standard deviation
Control group	8	90	0	0
Experimental Group	20	300	0	0

TABLE 4: The statistical evaluation seen for the pulp tissue organization. According to the Mann Whitney-U test there wasn't any statistical significant difference between the control and research group (p>0.01).

Groups	Number of specimens	Number of observations	Mean	Standard deviation
Control group	8	90	1	0
Experimental Group	20	300	1	0

The results of the study showed that, there was no significant difference between control and experimental groups (p>0.01).

DISCUSSION

This project has been multidisciplinary studied as the department of Cardiology, Neurology, Nephrology, Endocrinology and Pulmonary & Critical Care. Their purposes of the investigation were that the effect of *Thiobacillus species* on the different systems of rats such as Urogenital, Cardiovascular, Pulmonary, Endocrinal and Neurologic systems.

Thiobacillus species is using treatment of leather industry wastewater by aerobic biological and Fenton oxidation process. The *Thiobacillus ferrooxidans* has ability of selective adhesion to pyrite so modelling and analysis of biooxidation of gold bearing pyrite-arsenopyrite concentrates. Moreover, *Thiobacillus ferrooxidans* involved in the biohydrometallurgical extraction processes; consequently it has been used bacterial leaching: oxidation of sulphidic minerals and novel mineral processing by flotation.[14-20] Humans are under the risk of contamination to the *Thiobacillus species.*

Despite its occurrence in experimental animals, the contribution of *Anachoresis* responsible for the pulpal infection has not been totally elucidated in humans.[2] The results of this study indicate that there wasn't any evidence for *Anachoresis* on rats infected with *Thiobacillus species*. Although the counting procedure was repeated five times for every test tube, no bacillus was detected under the stereo microscope.

Pulp necrosis is an excellent growth culture for microorganism, which may reach the pulp after an additional injury through the ruptured periodontal ligament, enamel-dentin cracks or from the blood stream by *Anachoresis.*[21]

The joint between the two halves of the lower jaw (mandibular symphysis, or symphysis mentis) is not fused, but is formed of fibrous tissue - fibrocartilage and intercrossing ligaments. This fibrous tissue allows each side of the lower jaw to rotate slightly along its long axis, thus separating the lower incisors. The widest angle obtainable is about 40º. The ability to separate the lower incisors is important in mastication: as a rat gnaws and bites, it adjusts the separation of its lower incisors.[22,23] The rat's incisors are open-rooted, which means they grow throughout life and microvascularisation of the rat's incisors are more complicated than molar teeth[23]. Rat mandible incisors were selected to investigate of existence *Anachoresis* in present study.

Dacre et al. have been identified anachorectic infection as no physical entry route for oral bacteria into the endodontic system *via* blood or lymph borne infection.[1] *Anachorectic infection* was considered to be the most likely cause of infection in 54/79 (68%) of apically infected Cheek Teeth. Although 19/54 CT (35%) had pulpal exposure, there was no evidence to suggest that this pulpal expo-

sure was the cause of infections, and all occlusal exposure was deemed to be secondary to pulp necrosis as also assessed.[2]

Gier and Mitchell were studied the *Anachoresis* on 115 Mongrel dog teeth. They injected 4 ml of *Escherichia coli* and beta-hemolytic *Streptococcus*. They extirpated pulps and a few drops of blood from the pulp canal were cultured. Bacterial smears were made and stained by the Brown and Brenn method. The pulps of the 23 control teeth were histologically normal. Bacteria detected in 58 samples of the Brown and Brenn stained sections were clearly seen in the centre of abscesses and as small colonies in the necrotic debris and the neighbouring inflamed tissue.[24] They speculated that it would be seemed that any dental preparation that caused pulp inflammation may be through the *Anachoretic effect*, attract microorganisms to the pulp during transient bacteremia.

The Gram negative microorganisms caused sepsis and bacteraemia mostly *Staphylococcus aureus,* coagulase negative *staphylococcus, Escherichia coli* and other *Enterobacteriaceae spp*, *Pseudomonas aureuginosa, Acinetobacter spp.* isolated from the blood cultures.[25] However, *Thiobacillus spp.* Gram negative bacteria, the blood borne infections has not been caused by these bacteria.

Both microbiological and histopathological examinations showed that the risk of *Anachoresis* with *Thiobacillus species* was almost impossible. All the infected and control groups were examined under the same conditions but no bacillus contamination was obtained according the microbiological investigation. During the histopathological examination the preparations "Brown-Brenn" staining was used to determine the bacteria presence in all specimens. The result of the microbiological investigation that showed no bacterial invasion was also proved with the Brown-Brenn staining by the histopathological examination. According to the histopathological investigation, there was not observed any pathological view on the pulp tissue of all rats in both experimental and control groups.

CONCLUSİON

The hypothesis was that *Anachoretic pulpitis* in rat dental pulp could not be confirmed. We suggest further investigations to understand the phenomenon of the *Anachoretic pulpitis* by using various microorganisms.

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