Histol Histopathol (2024) 39: 1587-1595

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Comparison of epoprostenol and *Viscum album* efficiencies in the treatment of avascular necrosis of the femoral head: An experimental animal study

Mert Kumbaraci[1], Banu Sarsik Kumbaraci[2], Basak Doganavsargil Yakut[2], Cenk Can[3], Gulcin Basdemir[4] and Semih Aydogdu[5]

[1]Tepecik Training and Research Hospital, Orthopedics and Traumatology Department, [2]Ege University, Faculty of Medicine, Pathology Department, [3]Ege University, Faculty of Medicine, Pharmacology Department, Izmir [4]Istanbul Galata University, Faculty of Dentistry, Pathology Department, Istanbul and [5]Ege University, Faculty of Medicine, Orthopedics and Traumatology Department, Izmir, Turkey

Summary. Background. The aim of our study is to compare the efficacy of epoprostenol and *Viscum album* in the treatment of femoral head avascular necrosis with an experimental study. Our hypothesis is that *Viscum album*, which has similar properties to epoprostenol on the vascular system, is as effective as epoprostenol in the treatment of avascular necrosis.

Methods. Avascular necrosis was created on the femoral heads of 45 New Zealand type rabbits by surgical vascular deprivation method. The rabbits were divided into 3 groups. Group 1 was designed as a control group, in group 2 Ilomedin (epoprostenol analogue) was administrated to subjects and in group 3, Helixor (*Viscum album* extract) was administrated. At the end of the study, there were nine subjects in each group. Osteocyte necrosis, bone marrow necrosis, new bone formation and cartilage degeneration were evaluated microscopically. The extent of bone necrosis and repair and involvement of epiphysis, the bone marrow cellularity ratio and trabecular bone volume were investigated.

Results. Subchondral necrosis was seen in more animals in the control group (p=0.03). Osteoblastic and osteoclastic activity were more prominent in the Ilomedin group (p=0.25 and 0.07, respectively). It was seen that the cartilages of the subjects in the Helixor and Ilomedin groups were less damaged. In the Ilomedin group, more animals were seen in the chronic phase of the repair process than in the other groups (p=0.07). Bone marrow cellularity was higher in treatment groups (22% and 20,6% for Ilomedin and Helixor, respectively, p=0,04). Trabecular volume was found to be increased in damaged femoral heads in the treatment groups, the highest increased observed in the Helixor group (p=0.01).

Conclusion. *Viscum album* seems to be effective in decreasing the extention of necrosis and protecting the articular cartilage, and epoprostenol in increasing repair and regeneration.

Key words: Avascular necrosis, Experimental model, Vascular deprivation, Epoprostenol, *Viscum album*

Introduction

Avascular necrosis of femoral head (ANFH) develops due to decreased blood flow in the femoral head arteries. It can be a result of traumatic or non-traumatic conditions and mostly affects young adults in the third and fourth decades of their lives (Zalavras and Lieberman, 2014; Moya-Angeler et al., 2015). The main causes of non-traumatic ANFH include corticosteroid usage, alcohol abuse, hemoglobinopathies, Gaucher disease, hyperlipidemia, coagulapathies as well as idiopathic diseases (Andriolo et al., 2018). Any of these etiologic factors causes ischemia in the femoral head and ischemia triggers the destructive process in osteocyte, adipocyte and hematopoietic marrow cells. The destruction results in new bone formation and repetitive cycles of construction and destruction often causing resorption and progressive collapse in the subchondral bone. As a result of these processes, development of osteoarthritis can be expected (Guerado and Caso, 2016;

Corresponding Author: Banu Sarsik Kumbaraci MD, Ege University, Faculty of Medicine, Pathology Department, Izmir, Turkey. e-mail: banu.sarsik.kumbaraci@ege.edu.tr
www.hh.um.es. DOI: 10.14670/HH-18-745

Abbrevation. ANFH, Avascular necrosis of femoral head; VA, *Viscum album*; TBV: Trabecular bone volume; FHH, Femoral head height; FHW, Femoral head width; HWR, Height to width ratio; OFH, Operated femoral heads; UFH, Unoperated femoral heads.

Andriolo et al., 2018).

Spontaneous regression of avascular necrosis is rarely seen. Femoral head collapse may develop in 2/3 of asymptomatic onset patients, whereas this rate is seen as 85% in symptomatic patients (Larson et al., 2018). If possible, early treatment before collapse is critical in protecting the femoral head. However, there is no established treatment method that can be used in patients with the disease detected at an early stage. Both surgical methods and pharmacological agents have been used in the treatment of early stage of ANFH. Core decompression is the most commonly used surgical procedure in early stage avascular necrosis, however success rates are only around 60% (Mont et al., 2015; Larson et al., 2018). T Pharmacological agents such as anticoagulants, biphosphonates, growth factors and vasoactive agents have been used in the treatment of this disease (Marker et al., 2008; Rajpura et al., 2011; Zalavras and Lieberman, 2014; Mont et al., 2015; Liu et al., 2022) Ilomedin (Schering AG, Germany) is a epoprostenol (prostaglandin I2) analogue administered intravenously and it prevents platelet aggregation, causes vasodilation and decreases vascular permeability (Aigner et al., 2001). It is used in the treatment of peripheral arteriosclerotic obliterative disease and pulmonary hypertension (Aigner et al., 2001; Disch et al., 2005). It can be used successfully in the treatment of bone marrow edema induced avascular necrosis (Pilge et al., 2016; Hörterer et al., 2018; Pountos and Giannoudis, 2018). *Viscum album* (VA) is a semi-parasitic shrub that grows on various trees in woodland. VA includes glucoprotein (lectin) and protein (viscotoxin) which have cytotoxic effects on cancer cells, and they also show an immunostimulant effect (Staupe et al., 2023). Helixor (Heilmittel GmbH & Co. KG, Germany) is produced from VA extracts and is used in cancer treatment (Kienle and Kiene, 2010; Sunjic et al., 2015; Ostermann et al., 2020). VA extracts also have different properties which have been demonstrated to cause vasodilation and prevent platelet aggregation in *in vitro* studies (Deliorman et al., 2000; Tenorio et al., 2005). Observing positive clinical results after the use of VA extracts as a complementary medicine therapy in some patients with ANFH, led us to investigate the effectiveness of this substance. Our hypothesis was that Helixor (VA extract) which has similar properties to Ilomedin on the vascular system, is as effective as Ilomedin in the treatment of osteonecrosis. To our knowledge, there is no experimental study investigating the effect of epoprostenol on necrotic bone and no literature knowledge about the use of viscum album in the treatment of ANFH. The aim of this study is to evaluate and compare the efficacy of epoprostenol and VA in the treatment of ANFH with an experimental animal study.

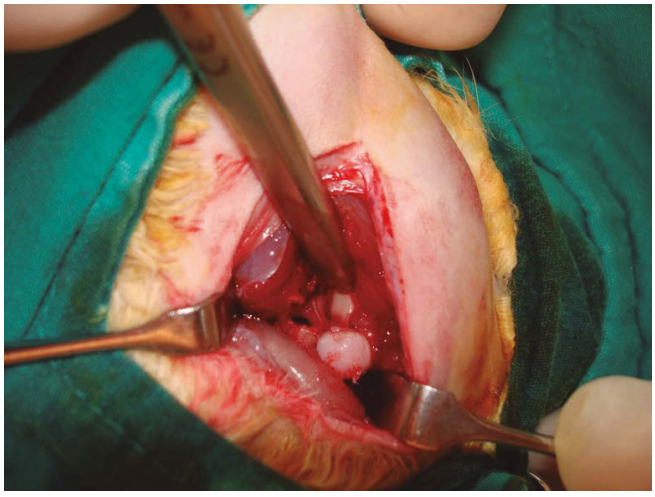

Fig. 1. Femoral head was dislocated after ligamentum teres was cut and the arteries on the femoral neck were damaged by rugine.

Materials and methods

Local ethical committee approval was obtained prior to start of this experimental study (2005-32). Forty-five New Zealand type six-month old rabbits (weighted between 3500-4000 grams) were separated into three groups (group 1: Control, group 2: Ilomedin, group 3: Helixor). We preferred surgical vascular deprivation method in creating femoral head avascular necrosis described by Norman et al. (1998). Before starting the experiment, a pilot study was conducted and this method was tested on five subjects (2 from group 1, 2 from group 2 and 1 from group 3). Subjects were sacrificed at postoperative different days and the study was initiated after the avascular necrosis was observed histopathologically beginning from the 10th day. The rabbits were anaesthetized with ketamine (Alfamine10% injectable, Alfasan,Turkey) (35 mg/kg,intramuscular) and xylazine hydrochloride (Ksilazol, Provet, Turkey) (8 mg/kg, intramuscular). After skin shaving and cleaning a longitudinal incision over the greater trochanter was performed. Gluteus maximus muscle was split in the direction of its bundles and anterior fibrils of gluteus medius muscle were detached from bone. Then, joint capsule was transected, allowing the joint to be visible. Once ligamentum teres was cut, femoral head was dislocated anteriorly. Femoral neck was stripped with a rugine both from anterior and posterior and capsular remnants were cleaned (Fig. 1). Femoral neck and intertrochanteric region were incised using a number 11 blade to damage the nutritional arteries of femoral head. After the femoral head was relocated, gluteal muscles and skin were closed. The rabbits were placed in spacious cages without restriction of their activities. For analgesia, meloxicam (Metacam, Boehringer, Germany 0.2 mg/kg) was applied subcutaneously for three days. Their health conditions were checked regularly every day, they received standard laboratory diet and care was taken for them to have easy access to food and water at all times.

Group 1 was designed as a control group and no

additional medication for avascular necrosis was given during recovery period. In Group 2, Ilomedin (2 ng/kg/min) (administration dose in human) was administered through the ear veins, via perfusor, started on the 10th day in which histopathologically avascular necrosis was detected in the pilot study and continued for the next five days. The rabbits were anaesthetized with ketamine (35 mg/kg, intramuscular) and xylazine hydrochloride (8 mg/kg, intramuscular) during infusion. In Group 3, Helixor treatment was started on the tenth day. It was administered subcutaneously 0.1 mg/day in the first three days and 1 mg/day during the next two days as in the administration dose in pediatric patients. The rabbits were observed in their cages and 13 rabbits died due to several reasons during the observation period (4 from group 1, 4 from group 2 and 5 from group 3) and the data of these animals were not used in the study. On the 30th day, each group consisted of nine rabbits and all subjects were sacrificed by giving a high dose of anesthetic substance (xylazine 10 mg/kg and ketamine 90 mg/kg) intramuscularly. Both femurs were removed for histopathological evaluation.

Histopathological evaluation

Bilateral femurs were cut along a line 1 cm inferior of the femoral neck in horizontal plane. Then femoral heads were split into two, along a visionary line in the middle of the insertion of ligamentum teres in coronal plane (Fig. 2). Following routine fixation, decalcification and tissue processing, sections of 5µm were stained with hematoxylin and eosin. Histopathological and histomorphometric evaluation were done by three pathologists specialized in bone diseases and blind to the experimental data, using an Olympus BX50 (Olympus Corp. Shinyukuku, Tokyo, Japan) light microscope.

Osteocyte necrosis, bone marrow necrosis, new bone formation and cartilage degeneration were evaluated microscopically (Fig. 3). The presence of empty osteocyte lacunae and/or bone trabeculae containing pyknotic nuclei were considered as "necrotic". Necrosis and repair staging were done according to the criteria proposed by Arlet (1992) (Table 1).

The extent of necrosis and repair in the proximal femoral epiphysis and joint cartilage degeneration were evaluated individually using Levin et al. (1999) criteria (Table 2).

All the parameters investigated regarding inflammation, necrosis, regeneration and articular cartilage damage are given qualitatively in Table 3.

Morphometric evaluation for bone volume was performed using a personal computer-based program, AxioVision LE, Rel.4.6 (Carl Zeiss microimaging Inc., North America). The epiphysis was divided into two parts by drawing an imaginary vertical line from the ligamentum teres to the physis, and bone volume measurements were made on this half epiphysis. In the selected area, the x10 magnification area where primary spongiosis was least observed was digitally photographed. The trabecular areas and overall tissue area were calculated in pixels on the digitally transferred image and the ratio of these was recorded as "trabecular bone volume (TBV) (%)". The ratio of bone marrow cells to fat cells in the inter-trabecular area of the epiphysis was determined as the "bone marrow cellularity ratio".

Femoral head height (FHH) and femoral head width (FHW) were measured using a millimetric grid with

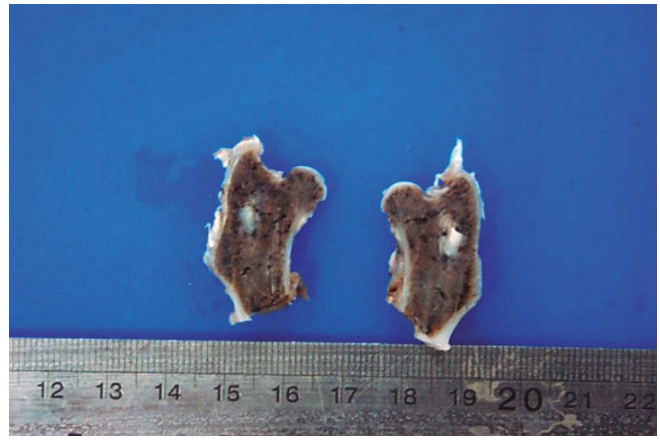

Fig. 2. Femoral head was split into two, along a visionary line in the middle of the insertion of ligamentum teres in coronal plane.

Table 1. Histopathological osteonecrosis and repair staging according to the criteria proposed by Arlet, 1992.

Osteonecrosis staging
Stage 1: Hematopoietic cell loss in bone marrow. Stage 2: Presence of fatty bone marrow necrosis. Stage 3: Presence of medullary and trabecular bone necrosis. Stage 4: Presence of medullary fibrosis and new bone formation accompanying necrosis.
Bone repair phases
Stage 1: Presence of acute inflammatory reaction. Stage 2: Presence of macrophage infiltration, granulation tissue and increase in vascularization. Stage 3: Presence of osteoclastic bone resorption, increase of osteoblastic activity and new bone formation.

microscope to demonstrate femoral head deformation which is the advanced stage evidence of avascular necrosis. Femoral head height was defined as the length between joint cartilage and superior epiphysis cartilage and FHW was defined as the distance between the corners which connects joint cartilage and epiphyseal cartilage and height to width ratio (HWR) was recorded for all femoral heads.

All measurements were performed both for operated femoral heads (OFH) and unoperated femoral heads (UFH). Also, changes in OFH to changes in UFH ratio was calculated and recorded as "adjusted ratio".

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences version 17.0 Windows (SPSS Inc. Chicago, IL, USA). The qualitative differences between groups were compared using χ^2 tests. The quantitative parameters were initially analyzed for normality using Shapiro Wilk test and accordingly analyzed with analysis of variance (ANOVA) or Kruskal-Wallis tests. Tukey and Dunnett tests were used for multiple comparisons. p value less than 0.05 was accepted as statistically significant.

Results

Osteonecrosis and repair findings were observed in the operated femoral heads in all subjects, and none of these findings were found in the non-operated femoral heads. Bone marrow necrosis was found in all operated subjects. Fatty bone marrow necrosis was seen in fewer subjects in Helixor group compared to other groups (p=0.01) (Fig. 4A). Subchondral necrosis was seen in more animals in the control group (p=0.03) (Fig. 4B). Fibrosis and new bone formation accompanying bone necrosis were seen in more subjects in the Ilomedin group (p=0.35). Osteoblastic and osteoclastic activity were more prominent in the Ilomedin group (Table 3) (p=0.25, p=0.07, respectively) (Fig. 4C). All investigated histological findings and their distribution by groups are shown in Table 3.

Chondrocyte irregularity, cartilage thinning, chondrolysis and pannus formation were evaluated individually to determine cartilage degeneration. It was

Table 2. Histopathological extent of necrosis and repair in the proximal femoral epiphysis and cartilage degeneration (Levin et al.,1999).

Extension of necrosis and repair
0: necrosis or repair is not observed 1+: Less than one third of femoral head epiphysis is involved 2+: One to two thirds of femoral head epiphysis is involved 3+: More than two thirds of femoral head epiphysis is involved.
Joint cartilage degeneration
Stage 1: Loss of basophilic staining in matrix Stage 2: Cartilage thinning, irregularly distributed chondrocytes and presence of a thin pannus at the surface Stage 3: Focal hypocellular-acellular areas and presence of a thick pannus

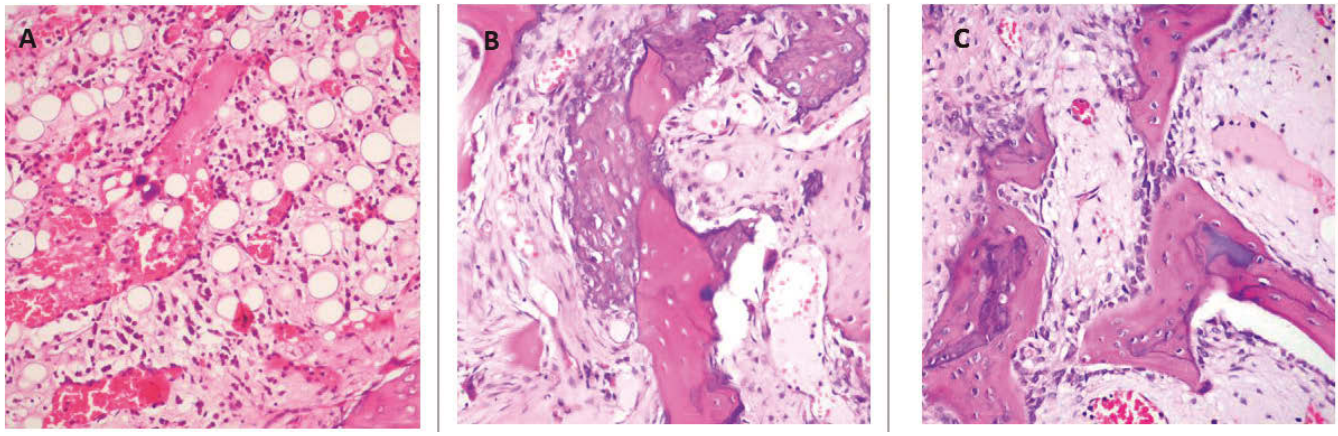

Fig. 3. Histological features of osteonecrosis and reparative process. **A.** Necrosis and acute inflammatory cell infiltration in the bone marrow, bone trabecula with empty lacunae visible in the lower right corner of the figure. **B.** New bone formation around necrotic bone. **C.** Fibrosis, granulation tissue and new bone formation in the bone marrow. Hematoxylin-eosine, x 200.

seen that the articular cartilage of the subjects in the Helixor and Ilomedin groups was less damaged (Table 3). To examine this difference thoroughly, 1 point was given for each aforementioned feature and a "total cartilage change score (TCCS)" was obtained for each subject. According to this score, Helixor group showed less cartilage degeneration (TCCS was calculated 26, 20 and 15 in control group, Ilomedin group and Helixor group, respectively).

Osteonecrosis is composed of histopathological stages such as hematopoetic cell loss, trabecular bone necrosis and new bone formation. There is no clear distinction between stages, on the contrary, there are transitions into each other. None of the subjects showed only bone marrow necrosis (Stage 1) and/or only fatty marrow necrosis (Stage 2), as Stage 3 and/or Stage 4 necrosis was found in all samples (Table 4).

In evaluation repair stages of all rabbits, 26% were in stage 1, 30% were in stage 2 and 37% were in stage 3. In Ilomedin group, more animals were seen in the chronic phase of the repair process than in the other groups (Table 4) (p=0.07). Moreover, repair extended to complete epiphysis was only seen in the Ilomedin group (Table 5) (p=0.01).

In the Helixor group, the extension of osteonecrosis in the epiphysis was less than the other groups (p=0.04). Histological findings of osteonecrosis in the entire epiphysis was not seen in any subject in the Helixor group (Table 4).

Bone marrow cellularity in normal femurs ranged between 10-70% (average: 34.63+ 18) and no significant difference was observed among the groups (p=0.35). In damaged femoral heads, in the control group, bone marrow cellularity was 2,33%, while it was higher in treatment groups (22% and 20,6% for Ilomedin and Helixor groups, respectively, p=0,04). Adjusted ratios (OH/NOH) were calculated as 0.087, 1.01 and 0.65 for

Table 3. All investigated histological findings.

	Control	Ilomedin (n)	Helixor (n)	p
Bone marrow necrosis	9/9	9/9	9/9	
Edema, eosinophil, amorphous substance	7/9	6/9	4/9	0.32
Fatty marrow necrosis	8/9	9/9	4/9	**0.01**
Subchondral necrosis	9/9	5/9	4/9	0.03
Trabecular bone necrosis	8/9	7/9	7/9	0.78
Cortical bone necrosis	5/9	2/9	2/9	0.22
Necrosis + Fibrosis + Newbone formation	3/9	6/9	4/9	0.35
Acute inflammation	4/9	0/9	2/9	0.07
Macrophage	5/9	3/9	3/9	0.54
Increased,vascularization, congestion	5/9	3/9	4/9	0.63
Granulation tissue	4/9	4/9	5/9	0.86
Fibrosis	3/9	3/9	5/9	0.54
Osteoclast, resorption	0/9	4/9	2/9	0.07
Increased osteoblastic activity	3/9	6/9	3/9	0.25
New bone formation	4/9	6/9	5/9	0.63
Cartilage basophil loss	7/9	7/9	9/9	0.30
Cartilage thinning	4/9	3/9	0/9	0.08
Chondrocyte irregularity	6/9	3/9	4/9	0.35
Thin pannus	5/9	4/9	2/9	0.34
Fibrilation	0/9	0/9	1/9	0.35
Chondrolysis	2/9	1/9	0/9	0.32
Thick pannus	3/9	0/9	0/9	0.03
Callus-like formation	6/9	6/9	2/9	0.09
Periosteal new bone formation	9/9	7/9	7/9	0.30
Cortical resorption	7/9	6/9	5/9	0.60
Endosteal new bone formation	1/9	3/9	1/9	0.37

Table 4. Distribution of osteonecrosis, repair and cartilage degeneration stages by groups.

	Stage	Control(n)	Ilomedin(n)	Helixor(n)	p
Osteonecrosis	I	0	0	0	0.35
	II	0	0	0	
	III	6	3	5	
	IV	3	6	4	
Repair Phase	I	5	0	2	0.07
	II	3	2	3	
	III	1	7	2	
Chondral degeneration	I	4	4	7	0.09
	II	2	5	1	
	III	3	0	1	

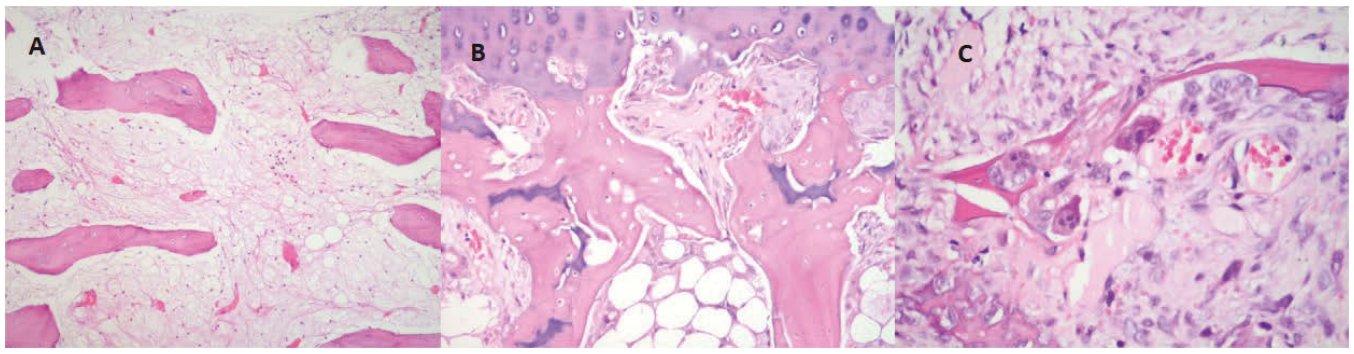

Fig. 4. Different histopathological findings of the subjects in the Ilomedine, Helixor and control groups. **A.** Appearance of bone marrow necrosis of the subject in the Helixor group. **B.** Extent of subchondral necrosis at the femoral head in the control group subject. **C.** The view of increased osteoblastic and osteoclastic activity in the subject treated with Ilomedine. Hematoxylin-eosine, x 200.

control, Ilomedin and Helixor groups, respectively (p=0.18).

Macroscopically, no remarkable deformity was seen at the damaged femoral heads. Although collapse of the damaged femoral heads was detected in the measurements made by using microscope and millimeter grid, there was no difference in FHH/FHW ratios between the groups (Table 6).

In the control group, mean trabecular volumes were similar in damaged and undamaged femoral heads. Trabecular volume was found to be increased in damaged femoral heads in the treatment groups, the highest increase observed in Helixor group (Table 6).

Discussion

In our study, VA appeared more efficient than epoprostenol in several parameters reflecting bone necrosis and repair. In the Helixor group, osteonecrosis and fatty bone marrow necrosis were seen in fewer subjects and the extension of these findings in the femoral head was also lower in this group. Increased osteoblastic and osteoclastic activity and new bone formation were more frequently observed in the Ilomedin group. VA appears to be effective in reducing necrosis and epoprostenol in increasing repair and regeneration. VA (mistletoe) is a hemiparasite living on trees in tropical and temperate climates. Currently mistletoe extracts produced in laboratory conditions are used for complementary treatment of several medical conditions (Ostermann et al., 2020; Staupe et al., 2023).

Table 5. Distribution of necrosis and repair extention in the femoral head by groups.

	Control(n)	Ilomedin(n)	Helixor(n)	p
Necrosis				0.04
1+	2	3	8	
2+	5	3	1	
3+	2	3	0	
Repair				0.01
1+	4	3	9	
2+	5	4	0	
3+	0	2	0	

Those extracts are composed of glycoprotein (lectin), protein (viscotoxin), polysaccharide (galacturonan) and alkaloids. Lectin inhibits protein synthesis at ribosomal level, activates macrophages and facilitates release of lymphokines from lymphocytes. It also inhibits serotonin secretion from platelets and histamine secretion from leucocytes (Deliorman et al., 2000; Tenorio et al., 2005). VA extracts have immunoadjuvant and antitumoral effects and Helixor is produced from viscum album extracts and is used in cancer treatment in various European countries (Kienle and Kiene, 2010; Sunjic et al., 2015; Ostermann et al., 2020). In Ostermann et al's meta-analysis examining 32 studies in which VA extracts were used as adjuvant therapy in the treatment of different cancer types, they found that this drug was more effective than the other treatment modalities especially in pancreatic cancers and osteosarcoma (Ostermann et al., 2020). In their meta-analysis, Kienle and Kiene (2010) investigated the effects of VA extracts on quality of life (QoL) in patients treated for cancer. VA treatment seems to have an impact on QoL and reduces side effects of conventional therapies (chemotherapy, radiation) in experimental trials as well as in daily routine application. However, there are *in vitro* studies in which other features of VA extracts are also investigated. Deliorman et al. (2000) and Tenorio et al. (2005) showed a vasodilation effect of aqueous VA extracts. Sener et al. (1996) demonstrated that VA extracts inhibit platelet aggregation. We designed our study in the light of the *in vitro* results proving epoprostenol-like effects of VA extracts such as vasodilation, preventing platelet aggregation and decreasing capillary permeability. To our knowledge, there is no experimental or clinical trial available in the literature on the efficacy of VA extracts in the treatment of avascular necrosis.

In the late 1990s epoprostenol analogs began to be tried in the treatment of avascular necrosis associated with bone marrow edema syndrome without having any use in experimental studies (Aigner et al., 2001; Disch et al., 2005; Meizer et al., 2005; Pilge et al., 2016; Hörterer et al., 2018; Pountos and Giannoudis, 2018). Disch et al., treated 33 patients with bone marrow edema related to osteonecrosis in proximal femur using epoprostenol and four months after the treatment, they reported that an increase in Harris hip score, significant improvement in

Table 6. Quantitative microscopic findings and their distribution by groups.

	Undamaged				Damaged		
	Control	Ilomedin	Helixor	p	Control	Ilomedine	Helixor
FHH(cm)	2.75+0.25	2.83+0.43	3.10+0.41	0.17	2.36+0.25	2.48+0.40	2.75+0.33
FHW(cm)	6.90+0.41	6.72+0.45	7.02+0.26	0.27	7.02+0.53	6.89+0.70	7.45+0.43
FHH/FHW	0.40+0.04	0.42+0.06	0.43+0.06	0.38	0.34+0.04	0.35+0.05	0.36+0.04
TV(mm^2)	0.22+0.09	0.18+0.04	0.15+0.04	0.15	0.23+0.08	0.23+0.06	0.33+0.04

FHH, Femoral head height; FHW, Femoral head width; TV, Trabecular volume.

hip range of motion and stage 4 edema in MRI resolved to stage 1 (Disch et al., 2005). Meizer et al. used epoprostenol treatment in 104 patients with bone marrow edema and found decreased pain levels and significant improvement of edema on the MRI (Meizer et al., 2005). In our study, we aimed to constitute an animal treatment model in rabbits similar to human models and we used Ilomedin at the same doses (2 ng/kg/min) and durations (1 h/day infusion, 5 days) used in humans in previous studies (Aigner et al., 2001; Disch et al., 2005).

In the present study, in the Helixor group, the extension of osteonecrosis in the epiphysis was less than the other groups. Shi et al. created ANFH in rabbits by injecting lipopolysaccaride and methylprednisolone. One group was fed by an icaiirin (Epimedium-prenylated flavonol) solution once a day for 6 weeks. They reported that the rate of empty lacunae of osteonecrotic femoral heads in the experiment group was higher than control group (Shi et al., 2020). Erken et al. created a steroid-induced osteonecrosis in the femoral heads of chickens and tested the effectiveness of pentoxifylline, which regulates blood circulation in peripheral vascular diseases (Erken et al., 2012). They found no pathological change in 13 out of the 20 femoral heads (grade 0). The agents used in both studies appear to be effective in the treatment of osteonecrosis.

It should be kept in mind that the method of creating steroid induced ANFH is not always 100% successful. Zhao et al. and Kang et al. tried to create ANFH by intramuscular administration of methyl prednisolone (20 mg /kg) in rabbits (Zhao et al., 2013; Kang et al., 2015). The incidence of ANFH was 75% and 70%, respectively. Therefore, if the steroid induced osteonecrosis method was used, it may be confused whether the absence of osteonecrosis was the success of the drug or the failure of the initial system. In our study, avascular necrosis was observed in all rabbits after surgical vascular deprivation of femoral head.

In our study, it was observed that there was less cartilage degeneration in subjects applied with VA extract, and even this substance was thought to protect the cartilage tissue and it may be considered as a superior to the VA treatment over epoprostenol. The effectiveness of enoxaparin was investigated in rats with surgically induced osteonecrosis, and it was also observed that articular cartilage degeneration was less common in subjects treated with this drug. The authors suggested the reason for this was that the remodeling process can be found in both osteochondral junction and cartilage and the treatment may have increased remodeling (Norman et al., 2002).

Little et al. (2003) administrated zoledronic acid treatment after inducing ANFH in rats and observed an increase of trabecular volume at the femoral head. Also in our study, the mean trabecular volume was higher in the treated groups and the highest increase was in the Helixor group. This finding can be explained by the fact that bone formation starts earlier in the Helixor group, so more bone tissue may have been made at the same time compared to the other groups. In addition, lower osteoblastic activity and osteoclastic resorption but higher trabecular bone volume in Helixor group suggest that the repair process is almost complete, and bone is in a quiet period in this group.

Although Ilomedin and Helixor were thought to decrease necrosis and increase bone formation with increased vasodilation and neovascularization, no increase in congestion or increased vascularity was observed in the subjects administrated these treatments compared to the control group. It is possible to attribute this result to the fact that the subjects in the Ilomedin and Helixor groups were in a more advanced repair period when they were sacrificed compared to the control group. In this period, new bone formation is seen more than congestion and vascularization.

In rodents, as well as many animal species, the anastomoses between both epiphyseal and metaphyseal circulation are functionally ineffective and destructing the retinacular vessels arround the cervical periosteum and cutting the ligamentum teres produce femoral head epiphyseal avascular necrosis (Fan et al., 2011). Norman et al. severed the blood supply of 30 rats' femoral heads by surgically induced vascular deprivation and they showed osteonecrosis in all of the rats and suggested that their method is a reliable method for experimental avascular femoral head necrosis (Norman et al., 1998). Our study confirmed the success of this technique. Boss et al. reported that, in the surgical vascular deprivation avascular necrosis model in rats, at the second week capillaries formed first, and then these structures transformed into arteries and veins to restore effective circulation (Boss and Misselevich, 2003). In the light of this knowledge, we decided to perform Norman's method and administer the drugs after the 10th day.

Our study has some strengths and weaknesses. Although the effectiveness of epoprostenol in the treatment of avascular necrosis associated with bone marrow edema has been demonstrated in many studies to date, there is no experimental study investigating the effect of this drug on necrotic bone (Meizer et al., 2005; Pilge et al., 2016; Hörterer et al., 2018; Pountos and Giannoudis, 2018)

In addition, since there is no study in which VA was used in the treatment of avascular necrosis, our study can be considered as a pioneering study in both fields. In future studies, better results can be obtained by increasing the dose of Helixor gradually as in human treatment modalities and increasing the treatment duration. We could not find any other experimental study that takes the selection of the VA dose as an example. In long-term studies, it may be more appropriate to use subjects with greater similarity to human femurs such as pigs or ostriches, rather than rodents which have rapid bone regeneration. The steps of osteonecrosis repair can be examined more clearly by sacrificing the subjects at certain time intervals.

When the results were evaluated, it was observed

that the study hypothesis was confirmed. VA seems to be effective in decreasing the extent of necrosis and protecting the articular cartilage, and epoprostenol in increasing the repair and regeneration. Both epoprostenol and VA appear to be promising agents in the treatment of femoral head avascular necrosis.

Declaration of Conflicting Interests. The authors declare that there is no conflict of interest.

Funding. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

Aigner N., Petje G., Steinboeck G., Schneider W., Krasny C. and Landsiedl F. (2001). Treatment of bone-marrow oedema of the talus with the prostacyclin analogue iloprost. An MRI-controlled investigation of a new method. J. Bone Joint Surg. Am. 83, 855-858.

Andriolo L., Merli G., Tobar C., Altamura S.A., Kon E. and Filardo G. (2018). Regenerative therapies increase survivorship of avascular necrosis of the femoral head: A systematic review and meta-analysis. Int. Orthop. 42, 1689-1704.

Arlet J. (1992). Nontraumatic avascular necrosis of the femoral head. Past, present, and future. Clin. Orthop. Relat. Res. 277, 12-21.

Boss J.H. and Misselevich I. (2003). Osteonecrosis of the femoral head of laboratory animals: The lessons learned from a comparative study of osteonecrosis in man and experimental animals. Vet. Pathol. 40, 345-354.

Deliorman D., Caliş I., Ergun F., Doğan B.C, Buharalioğlu C.K and Kanzik I. (2000). Studies on the vascular effects of the fractions and phenolic compounds isolated from *Viscum album* Ssp. Album. J. Ethnopharmacol. 72, 323-329.

Disch A.C, Matziolis G. and Perka C. (2005). The management of necrosis-associated and idiopathic bone-marrow oedema of the proximal femur by intravenous iloprost. J. Bone Joint Surg. Br. 87, 560-564.

Erken H.Y., Ofluoglu O., Aktas M., Topal C. and Yildiz M. (2012). Effect of pentoxifylline on histopathological changes in steroid-induced osteonecrosis of femoral head: Experimental study in chicken. Int. Orthop. 36, 1523-1528.

Fan M., Peng J., Qin L. and Lu S. (2011). Experimental animal models of osteonecrosis. Rheumatol. Int. 31, 983-994.

Guerado E. and Caso E. (2016). The physiopathology of avascular necrosis of the femoral Head: An update. Injury 47, S16-26.

Hörterer H., Baumbach S.F., Gregersen J, Kriegelstein S., Gottschalk O., Szeimies U. and Walther M. (2018). Treatment of bone marrow edema of the foot and ankle with the prostacyclin analog iloprost. Foot Ankle Int. 39,1183-1191.

Kang P., Xie X., Tan Z., Yang J., Shen B., Zhou Z. and Pei F. (2015). Repairing defect and preventing collapse of femoral head in a steroid-Induced osteonecrotic of femoral head animal model using strontium-doped calcium polyphosphate combined BM-MNCs. J. Mater Sci. Mater Med. 26, 80.

Kienle G.S. and Kiene H. (2010). Review article: Influence of *Viscum album* L (European Mistletoe) extracts on quality of life in cancer patients: A systematic review of controlled clinical studies. Integr. Cancer Ther. 9, 142-157.

Larson E., Jones L.C., Goodman S.B., Koo K.-H. and Cui Q. (2018). Early-stage osteonecrosis of the femoral head: Where are we and where are we going in year 2018? Int. Orthop. 42, 1723-1728.

Levin D., Norman D., Zinman C., Rubinstein L., Sabo E., Misselevich I., Reis D. and Boss J.H. (1999). Treatment of experimental avascular necrosis of the femoral head with hyperbaric oxygen in rats: Histological evaluation of the femoral heads during the early phase of the reparative process. Exp. Mol. Pathol. 67, 99-108.

Little D.G., Peat R.A., Mcevoy A., Williams P.R., Smith E.J. and Baldock P.A. (2003). Zoledronic acid treatment results in retention of femoral head structure after traumatic osteonecrosis in young Wistar rats. J. Bone Miner. Res. 18, 2016-2022.

Liu N., Changming Z., Qinglong W. and Zhipeng H. (2022). Treatment of non traumatic avascular necrosis of the femoral head (Review). Exp. Ther. Med. 23, 321.

Marker D.R., Seyler T.M., Ulrich S.D., Srivastava S. and Mont M.A. (2008). Do modern techniques improve core decompression outcomes for hip osteonecrosis? Clin. Orthop. Relat. Res. 466, 1093-1103.

Meizer R., Radda C., Stolz G., Kotsaris S., Petje G., Krasny C., Wlk M., Mayerhöfer M., Landsiedl F. and Aigner N. (2005). MRI-controlled analysis of 104 patients with painful bone marrow edema in different joint localizations treated with the prostacyclin analogue iloprost. Wien. Klin. Wochenschr. 117, 278-286.

Mont M.A., Cherian J.J., Sierra R.J, Jones L.C. and Lieberman J.R. (2015). Nontraumatic osteonecrosis of the femoral head: Where do we stand today? J. Bone Joint Surg. Am. 97, 1604-1627.

Moya-Angeler J., Gianakos A.L., Villa J.C., Ni A. and Lane J.M. (2015). Current concepts on osteonecrosis of the femoral head. World J. Orthop. 18, 590-601.

Norman D., Reis D., Zinman C., Misselevich I. and Boss J.H. (1998). Vascular deprivation-induced necrosis of the femoral head of the rat. An experimental model of avascular osteonecrosis in the skeletally immature individual or Legg-Perthes disease. Int. J. Exp. Pathol. 79,173-181.

Norman D., Miller Y., Sabo E., Misselevich I., Peskin B., Zinman C., Levin D., Reis D.N. and Boss J.H. (2002). The effects of enoxaparin on the reparative processes in experimental osteonecrosis of the femoral head of the rat. APMIS 10, 221-228.

Ostermann T., Appelbaum S., Poier D., Boehm K., Raak C. and Büssing A. (2020). A systematic review and meta-analysis on the survival of cancer patients treated with a fermented *Viscum album* L. extract (Iscador): An update of findings. Complement. Med. Res. 27, 260-271.

Pilge H., Bittersohl B., Schneppendahl J., Hesper T., Zilkens C., Ruppert M., Krauspe R. and Jäger M. (2016). Bone marrow aspirate concentrate in combination with intravenous iloprost increases bone healing in patients with avascular necrosis of the femoral head: A matched pair analysis. Orthop. Rev. 8, 111-116.

Pountos I. and Giannoudis P.V. (2018). The role of iloprost on bone edema and osteonecrosis: Safety and clinical results. Expert Opin. Drug Saf. 17, 225-233.

Rajpura A., Wright A.C. and Board T.N. (2011). Medical management of osteonecrosis of the Hip: A Review. Hip Int. 21, 385-392.

Sener B., Deliorman D. and Ergun F. (1996). Antiplatalet aggregation potencies of the extracts of *Viscum album*. J. Fac. Pharm. Gazi. 3, 143-146.

Shi L., Mao T., Luo P., Li T., Gao F., Sun W. and Li Z. (2020). Effect of icariin on early Steroid-Induced osteonecrosis of the femoral head in

rabbits. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 34, 206-212 (in Chinese).

Staupe H., Buentzel J., Keinki C., Buentzel J. and Huebner J. (2023). Systematic analysis of mistletoe prescriptions in clinical studies. J. Cancer Res. Clin. Oncol. 149, 5559-5571.

Sunjic, S.B., Gasparovic A.C., Vukovic T., Weiss T., Weiss E.S., Soldo I., Djakovic N., Zarkovic T. and Zarkovic N. (2015). Adjuvant cancer biotherapy by viscum album extract isorel: Overview of evidence based medicine findings. Coll. Antropol. 39, 701-708.

Tenorio F.A., Valle L., González A. and Pastelín G.(2005). Vasodilator activity of the aqueous extract of *Viscum album*. Fitoterapia 76, 204-209.

Zalavras C.G. and Lieberman J.R. (2014). Osteonecrosis of the femoral head. J. Am. Acad. Orthop. Surg. 22, 455-464.

Zhao G., Yamamoto T., Motomura G., Yamaguchi R., Ikemura S., Iwasaki K. and Iwamoto Y. (2013). Cholesterol- and lanolin-rich diets may protect against steroid-Induced osteonecrosis in rabbits. Acta Orthop. 84, 593-597.

Accepted April 9, 2024