Critical diaphyseal bone defect healing in hens by autologous free transplant of adipose tissue

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Abstract

Treatment of large bone defects and incurable fractures is a challenging clinical problem. A novel approaches in bone engineering is the use of adipose-derived stem cells and adipose tissue. This study aimed to analyse the impact of adipose autograft on the process of bone regeneration in a surgically created critical size defect (CSD) in the hen ulna. In this study, 30 laying hens at the age of 14 months were randomly divided into three groups: control (C, *n*=10), subcutaneous adipose tissue graft (SC, *n*=10) and abdominal adipose tissue graft (ABD, *n*=10). In all 30 hens, a CSD was made in the ulna. Hens from the SC and ABD groups underwent surgery to explant subcutaneous and adipose tissue graft, respectively, and those grafts were then implanted in the ulnar CSD. The first radiographic and histological analysis were performed 3.5 weeks after surgery on four hens from the C group and five hens from the SC and ABD groups. The second analysis on the remaining 15 hens was performed after 7 weeks. Data were analysed using the Freeman-Halton extension of Fischer's exact test at the level of statistical significance $P<0.05$. Statistically significant differences regarding the presence of bridging at 3.5 and 7 weeks after surgery were observed between the C and SC groups, and between the C and ABD groups. Formation of a callus, regardless of the time of analysis, was not observed in the C group, while it was present in the SC and ABD groups ($P<0.05$). More hens from the ABD group (3) than the SC group (1) developed a bony callus 7 weeks after surgery, though these differences were not significant. Autologous adipose tissue graft positively affected healing of the hens' ulna at 3.5 and 7 weeks postoperatively. Abdominal adipose tissue appears to have better healing properties than subcutaneous adipose tissue.

Key words: *adipose tissue; bone healing; critical size defects; hens*

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Introduction

Treatment of large bone defects and incurable fractures is a challenging clinical problem. Options for tissue coverage and restoration of bone defects include autologous bone grafts, cadaveric bone grafts, pedicle or free-tissue transfer, and allotransplantation (Bohnenblust et al., 2009). To date, autologous and allogenic bone grafts have been the most preferred options for bone regeneration in humans (Shafaei and Kalarestaghi, 2020).

A relatively novel method for the bone tissue regeneration is the use of the stem cells (Iaquinta et al., 2019). In humans, several types of stem cells have been investigated as a source of osteoblast progenitors (Mushahary et al., 2018). Adipose-derived stem cells (ASC) are one of the most used stem cells since they are able to self-renew, commit and to multiply cell lineages (Gimble et al., 2007) and can be easily harvested in larger amounts with minimal invasiveness (Fraser et al., 2006; Fitzsimmons et al., 2018). Ishikawa et al. (2010) compared ASCs with other types of stem cells and concluded that ASCs are a better, simpler and safer source of cells, and thus more suitable for clinical application. ASCs have been identified as a source of multipotent cells that have osteogenic differentiation potential *in vitro* and *in vivo* (Wang et al., 2009). Lendeckel et al. (2004) first reported the use of ASCs to augment cancellous bone for the treatment of a difficult reconstructive calvarial defect. Despite recent advances in the use of ASC, the use of adipose tissue graft has not been extensively evaluated *in vivo* as an alternative treatment for bone repair. Use of adipose tissue in bone engineering could be an excellent method of choice because it is abundant, easily accessible by liposuction or resection, and contains a large number of stem cells (Halvorsen et al., 2001). Also, adipose tissue has the higher percentage of adult stem cells compared to bone marrow (Raposio et al., 2008).

In bird bone engineering, there is no viable source of autologous cortical bone, and cancellous bone is only found in minimal quantities because of the pneumatic nature of the avian skeleton (Jones and Redig, 2001). Because of insufficient research in avian bone grafting, and given the different biology and structure of avian bone, there are no clear options currently available to the avian orthopaedist. The importance of further investigation is underscored by the fact that fractures are among the most common conditions affecting a large proportion of avian patients, particularly raptors (Bedrosian and Pierre, 2007). In this context, the use of adipose tissue and ASCs in bird bone engineering seems to be a viable option.

Taking into consideration the many biological advantages of adipose tissue in bone tissue regeneration, this study analysed the impact of adipose autograft on the process of bone regeneration in a surgically created critical size defect in the hen ulna.

Material and Methods

This study was approved by the Ethics Committee of Faculty of Veterinary Medicine, University of Zagreb by decision number 251-61-01/139-11-2 of 13 April 2011.

Hens and treatment groups. Thirty laying hens, hybrids of light breeds, weaned from production, aged 14 months were used. For the purpose of this study, hens were randomly allocated into three groups: one control (C) and two experimental: subcutaneous adipose tissue graft (SC) group and abdominal graft (ABD) group. Each group contained 10 animals.

Surgical procedures for the adipose tissue graft explantation, resection of the

ulna and adipose tissue graft implantation. Preoperatively for preventive measures and later postoperatively as a therapeutic measure, antibiotic therapy with enrofloxacin 10 mg/kg BM (Vetoflok® 5 %, Veterina d.o.o., Croatia) was applied for 5 to 8 days, respectively. Diazepam (Apaurin®, Krka, Slovenia) 0.5 mg/kg and ketamine hydrochloride (Narketan®, Vetoquinol, Switzerland) 20 mg/kg were administered to hens for surgical treatment.

a) Adipose tissue graft explantation. 20 hens from the experimental groups (SC and ABD) underwent surgery to explant the adipose tissue graft. The surgical field on the left side of the abdomen was freed of feathers, aseptically prepared and a 3 cm incision was made for explantation of a 10x15 mm adipose tissue graft. The subcutaneous adipose tissue graft was explanted in 10 hens from the SC group, and abdominal adipose tissue graft was explanted in 10 hens from the ABD group. The explanted adipose tissue was then placed in sterile saline. The wound was closed in layers, using glycolic acid suture 3-0 (Dexon S, Medtronic, USA) and polyamide suture 4-0 (Seralon®, Serag-Wiessner GmbH & Co.).

b) Resection of the ulna and adipose tissue graft implantation. The second part of the surgical procedure was undertaken on all 30 hens. In the area of the middle third of the right ulna, a 3 cm long skin incision was made. By releasing the surrounding structures of ulna, the bone prominated and a 15 mm long bone cylinder was resected with an oscillating orthopaedic saw (Aesculap® Surgical Instruments, Braun, Germany). In group SC, an autologous native adipose graft was then implanted in the bone defect, in group an ABD abdominal graft was implanted, while in the control group C, the phase of adipose tissue implantation was omitted. The soft tissues were closed in layers and thus fixed the adipose tissue graft within the critical size defect, without additional fixation. Muscles were sutured with a simple interrupted pattern with glycolic acid suture 4-0 (Dexon S, Medtronic, USA) and skin with a simple interrupted pattern with polyamide suture 4-0 (Seralon®, Serag-Wiessner GmbH & Co.), Germany. As the *os radius* remained intact, it served as the backbone for mechanical stability, so there was no need to use internal or external fixation devices, ultimately contributing to less stress for the hens. After the surgical procedures, one hen from the C group died from a gastrointestinal disorder.

Surgery for right forearm removal. 3.5 weeks after ulnar resection, the right forearm was surgically removed in four hens from group C and five hens from each of the experimental groups (SC and ABD). Surgery for the removal of the right forearm in the remaining 15 hens (five hens in each group C, SC and ABD) was performed 7 weeks after resection. Diazepam (Apaurin®, Krka, Slovenia) 0.5 mg/kg and ketamine hydrochloride (Narketan®, Vetoquinol, Switzerland) 20 mg/kg were administered to hens for surgical treatment. The operative field of the right olecranon was cleaned of feathers and prepared, and the right forearm was surgically removed. Animals were postoperatively placed under antibiotic therapy with Enrofloxacin 10 mg/kg BM (Vetoflok® 5%, Veterina d.o.o., Croatia) for 8 days. The removed right ulna was further processed for the preparation of histological samples, so the ulna was preserved in 10% formalin solution after removal of the surrounding soft tissue.

Radiographic controls. The first X-ray scan to evaluate the healing of the critical ulnar defect was performed immediately after ulnar resection in all 30 hens. The second control was performed 3.5 weeks after surgery on all surviving 29 hens, while the third control was 7 weeks after ulnar surgery in the remaining 15

hens (five hens in each group, C, SC and ABD). X-ray evaluation of changes in the macrostructure of the studied area of the ulna of all groups of hens was performed by visual analysis of radiographs. The observed variable was the presence of the bone bridging the critical size defect (marked as present (+) or absent (-)). Bridging was considered present if bone callus formation was observable. All radiographs of the studied area of ulnas were recorded by an X-ray machine (Siemens®, Germany) of 40 kV exposure and 6 mAs and processed with a digital reader Agfa CR30-X. Radiography of all operated animals was performed in sagittal projection.

Histological analysis. All bone samples were subjected to histological sample procedure. They were first cut at a 45-degree angle to the longitudinal axis of the bone with a dental circular saw to obtain histological specimens. They were then placed in 10% formaldehyde solution for fixation. After that they were demineralised in a mixture of 15% aqueous formic solution and 4% hydrochloric acid for 14 days. These obtained samples were then embedded in paraffin blocks, which were cut on a microtome (Microm) to a thickness of 3 micrometres (Lillie et al., 1976). After that, fixation on slides for further staining and analysis under a light microscope (Nikon, Eclipse E600) followed. Samples were analysed at different magnifications (40x, 100x, 200x, 400x) to determine the type of callus. Callus type was assessed by the following scale: 1. no callus formed, 2. fibrous (only fibrous callus observed), 3. mixed (fibrous and bony callus observed) and 4. (bony callus observed).

Statistical analysis. Statistical data processing was performed using the statistical program Statistica v.13.5.1. (STATISTICA, 2020). Differences between groups for the variables presence of bridging and type of callus were analysed using the Freeman-Halton extension of Fischer's exact test. This test is used as an alternative to the $\chi 2$ test in cases where the number of measurements is low and when the contingency table is greater than 2 x 2. All differences were observed at the level of statistical significance $P<0.05$.

Results

In the C group of hens, no bridging (Table 1) or callus formation (Table 2) was observed at 3.5 or 7 weeks. Only a sparse callus was formed at the edges of bone margins, but without bridging (Figure 1A).

Within the experimental groups, (SC and ABD) bridging (Table 1) and callus formation was present. Bridging was observed in four hens from the SC group at both 3.5 (Figure 1B) and 7 weeks, and in all five hens from the ABD group at both 3.5 (Figure 1D) and 7 weeks (Figure 1E). At 3.5 weeks, a fibrous callus was observed in all five hens from the SC group (Figure 2E), while fibrous (four hens) and mixed (one hen) callus was observed in the ABD group (Figure 2B). At 7 weeks, mixed callus was observed in the SC (Figure 2C) and ABD groups of hens, while bony callus was observed in one hen from the SC group and in three hens from the ABD group (Figure 2D).

Statistically significant ($P<0.05$) differences regarding bridging and the type of callus were observed between the C and SC groups, and between the C and ABD groups observed at 3.5 weeks and 7 weeks.

Table 1. Number of hens with an observable presence of bridging of the critical bone defect

Presence of bridging	Time point					
	3.5 weeks			7 weeks		
	C	SC	ABD	C	SC	ABD
+	0	4	5	0	4	5
-	4	1	0	5	1	0

C – control group, SC – subcutaneous adipose tissue graft group, ABD – abdominal adipose tissue graft group

Table 2. Number of hens according to the type of callus formed in critical bone defect

Type of callus	Time point					
	3.5 weeks			7 weeks		
	C	SC	ABD	C	SC	ABD
No callus formed	4	0	0	5	0	0
Fibrous	0	5	4	0	2	1
Mixed	0	0	1	0	2	1
Bony	0	0	0	0	1	3

C – control group, SC – subcutaneous adipose tissue graft group, ABD – abdominal adipose tissue graft group

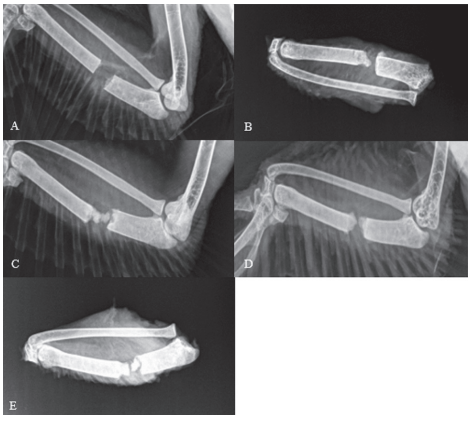

Figure 1. A: Control group without implantation of adipose tissue graft. X-ray shows sharp edges of bone defect with weak signs of periostal reaction 3.5 weeks after ulnar ostectomy. **B**: Implanted autologous subcutaneous adipose tissue graft 3.5 weeks after ulnar ostectomy. There are visible signs of bone healing. Periostal reaction is present with bone callus formation. Fracture line is still visible. **C**: Implanted autologous abdominal adipose tissue graft. X-ray of ulnar defect 3.5 weeks post ostectomy. Bone callus formation covers nearly the entire defect with endosteal callus formation. Periostal reactions are not visible. **D**: Implanted autologous subcutaneous adipose tissue graft seven weeks post ulnar ostectomy. Bone fragments are in position with round edges. Fracture line is visible but narrow. **E**: Implanted autologous abdominal adipose graft seven weeks post ulnar ostectomy. Nearly complete healing of the bone defect with abundant endosteal bone callus connecting both bone fragments is visible. Fracture line is still present.

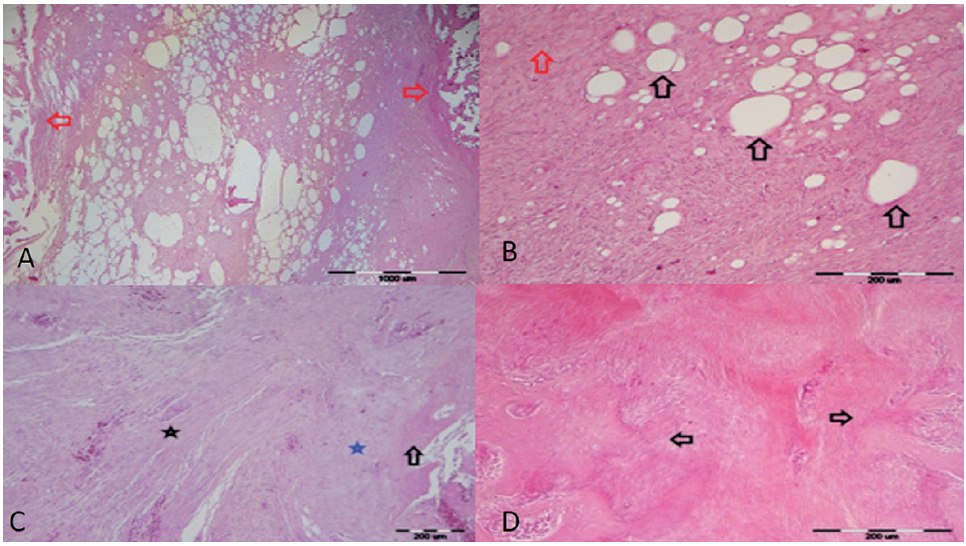

Figure 2. A: Critical defect healing with autologous subcutaneous adipose tissue graft 3.5 weeks post op. Some stages of fibrous tissue are present. Early stages of connective tissue regrowth within adipose tissue is seen between the edges of the bone defect (red arrow). **B:** Healing of the critical size defect using autologous abdominal adipose tissue graft 3.5 weeks post op. Fibrous callus surrounding adipose tissue cells (black arrows). Fibrous callus is present with uniform oriented cells and collagen fibres (red arrow). **C:** Healing of the critical defect using autologous subcutaneous adipose tissue graft 7 weeks post op. Ostectomy site is shown (black arrow) and bony tissue transfers into condensed fibroblast area with signs of ossification (blue star). Rest of callus is formed from mature cellular fibrous tissue (black star). **D:** Critical defect healing using autologous abdominal adipose tissue graft 7 weeks post op. Bony callus formation is present with signs of ossification starting to form edges of lamelar bone (black arrows).

Discussion

In this study, the effect of healing of a critical size ulnar defect supported by free grafts of subcutaneous and abdominal adipose tissue was observed in hens. Healing was defined as the visible presence of bridging of the critical size ulnar defect through radiological monitoring and as the observable presence of the callus and newly formed bone through histological monitoring.

A critical size defect (CSD) is used frequently in research, especially when assessing the effect of bone graft or the effect of other biological agents that trigger the healing of the bone gap. CSD is defined as the smallest intra-bone wound that does not heal during the lifetime of the organism (Leunig et al., 2000). To date, bone marrow has been the tissue of choice in bone engineering (Shafei and Kalarestaghi, 2020). However, the use of adipose tissue in bone engineering is becoming increasingly popular. The main advantages of adipose tissue over bone marrow are minimal morbidity after graft removal, higher rate of cell proliferation and larger yield of harvested stem cells (Strem and Hendrick, 2005; Ciuffi et al.,

2017; Shafei and Kalarestaghi, 2020). ASCs have shown the potential for osteogenic differentiation in studies on rabbits (Dudas et al., 2006), dogs (Cui et al., 2007) and rats (Bohnenblust et al., 2009). However, studies aimed at evaluating the effect of adipose tissue on bone healing are less frequent. Godoy Zanicotti et al. (2011) conducted the first pilot study to investigate the effect of non-processed adipose tissue graft on the healing of a rabbit tibia and concluded that the implanted graft interfered negatively with bone formation.

In this study, subcutaneous and abdominal adipose tissue autotransplants showed the potential for regeneration through the quantitative and qualitative results of the synthesis of new bone in a critical size defect of the ulna in a hen model. Bone bridging and formation of callus was observed in experimental (SC and ABD) groups of hens at 3.5 and 7 weeks, while it was absent in the control group. Our results are in agreement with the findings of Oliveira et al. (2013) who found a positive effect of a fragmented autologous adipose graft on bone healing on a surgically performed CSD of the rabbit calvaria. Their results, 40 days postoperatively, showed that the autologous adipose graft gave significantly better results in bone healing compared to the control group. Moreover, they observed that adipose tissue was absorbed and replaced by bone tissue without dehiscence or loss of tissue volume. Thus, they suspected that adipose cells transdifferentiated into osteoblasts.

Adipose tissue differs according to the different anatomical locations. Abdominal adipose tissue contains more blood vessels and less fibrous encapsulation compared to subcutaneous adipose tissue, which may contribute to the differences in osteogenic potential due to possible differences in the progenitor population (Peptan et al., 2006). In this study, no significant differences were observed between the SC and ABD groups of hens at both 3.5 and 7 weeks timepoints for the presence of bridging and type of callus formed. However, 3.5 weeks after surgery, only fibrous callus was observed in hens from the SC group, while one hen from the ABD group had a mixed (fibrous and bony) callus. Seven weeks after surgery, bony callus was observed in 3/5 hens from the ABD group and in 1/5 hens from the SC group. This might indicate that in hens, abdominal adipose tissue has better healing properties than subcutaneous adipose tissue.

During this study, no bone regeneration occurred, suggesting the importance of further research of the effect of adipose tissue graft on bone healing over a longer time period (more than 7 weeks). The biomechanical properties of newly formed bone in the critical size defect, which healed with the support of an adipose tissue graft, also remains to be explored in future studies.

Conclusions

Within the limits of the present study, the general conclusion is that an autologous adipose tissue graft positively affected healing of the hen ulna at 3.5 and 7 weeks postoperatively. Moreover, abdominal adipose tissue seems to have better healing properties than subcutaneous adipose tissue. Further research with a longer observation time is necessary to determine the effect of adipose tissue graft in bone regeneration in hens.

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Učinak autolognog presatka adipoznog tkiva na proces cijeljenja kritičnog defekta dijafizne kosti kokoši

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Liječenje opsežnih koštanih defekata i neizlječivih prijeloma kostiju predstavlja velik izazov u kliničkoj praksi. Jedan od novih pristupa u inženjerstvu koštanog tkiva je korištenje matičnih stanica iz masnog tkiva i samog masnog tkiva. U ovom smo istraživanju analizirali utjecaj autotransplantata masnog tkiva na proces regeneracije kosti u kirurški učinjenom kritičnom koštanom defektu (KKD) ulne kokoši. 30 kokoši nesilica u dobi od 14 mjeseci nasumično je podijeljeno u tri skupine: kontrolna skupina (C, n=10), potkožno masno tkivo (SC, n=10) i abdominalno masno tkivo (ABD, n=10). U svih 30 kokoši napravljen je KKD na ulni. Kokošima iz skupine SC eksplantirano je potkožno masno tkivo, a kokošima iz ABD skupine eksplantirano je abdominalno masno tkivo. Graftovi masnog tkiva su zatim implantirani u ulnarni KKD. Prva radiografska i histološka analiza obavljena je 3,5 tjedna nakon operacije na 4 kokoši iz C i 5 kokoši iz SC i ABD skupina. Druga analiza na preostalih 15 kokoši izvršena je nakon 7 tjedana. Podatci su analizirani korištenjem Freeman-Haltonove ekstenzije Fischerova egzaktnog testa na razini statističke značajnosti P<0,05. Statistički značajne (P<0,05) razlike u pogledu prisutnosti premošćavanja 3,5 i 7 tjedana nakon operacije uočene su između C i SC skupina te između C i ABD skupina. Formiranje kalusa, bez obzira na vrijeme analize, u skupini C nije uočeno, dok je bilo prisutno u skupini SC i ABD (P<0,05). Više kokoši iz ABD skupine (3) nego iz skupine SC (1) razvilo je koštani kalus 7 tjedana nakon operacije, ali razlike nisu bile značajne (P>0,05). Autologni transplantat masnog tkiva pozitivno je utjecao na cijeljenje ulne kokoši 3,5 i 7 tjedana nakon operacije. Abdominalno masno tkivo moglo bi imati bolji učinak na zacijeljivanje kosti od potkožnog masnog tkiva.

Ključne riječi: *masno tkivo, zarastavanje kostiju, kritični koštani defekt, kokoš*