COMPUTER ASSISTED MICROSCOPIC ANALYSIS OF BONE TISSUE DEVELOPED INSIDE A POLYMER, POLYACIVE, IMPLANTED TO EQUINE ARTICULAR SURFACE.

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SUMMARY

One of the most promising application of small or moderate sized focal articular lesions restauration is the mosaicplasty (MP). Although recurrent hemarthrosis is a rare complication after MP, recently different strategies designed to find an effective filling material to prevent the postoperative bleeding from the donor site. The porosus biodegradable polymer Polyactive (polyethylene oxide terephthalate - polybutylene terephthalate copolymer; PA) represents a promising solution in this respect. Histological evaluation of long-term PA filled donor sites obtained from 10 experimental horses was performed. In this paper, attention was primarily focused to the bone tissue developed in the plug. The computer assisted image analysis and quantitative polarized light microscopic measurement of the decalcified, longitudinally sectioned dimethylmethylene blue (DMMB) and picrosirius red (PS) stained sections delivered that the engaged area of the bone trabecules of the PA filled donor tunnels is substantially (25%) increased compared to the neighbouring cancellous bone. For this quantification, identical ROI-s (region of interest) were used and compared. The retardation values of birefringence were also measured in polarized light microscope using monochromatic light. The same retardation values could be recoded from the bone trabeculae developed in the PA and in the neighbouring bone indicating that the collagen orientation pattern does not differ significantly in these bone trabeculaes. Based on our de novo data we may speculate that PA may promote bone formation, and some of the not yet identified degradation products of PA may enhance osteoconduction and osteoinduction inside the donor canal.

INTRODUCTION

Articular cartilage has a poor capacity of repair and healing (Horas et al., 2003; Bedi et al., 2010). Due to its necessary function, that uniformly transfer and decrease the level of load on underlying bone (Shirazi and Shirazi-Adl, 2009), failing to do so can initiate further joint abnormalities (Ding et al., 2008). Therefore increasing number of theoretical and clinical research is designed to achieve the regeneration of organized articular cartilage.

One of the major innovative and biological approach to restore function of articular cartilage for more than a decade is mosaicplasty (MP), focusing on reconstructing the joint articular surfaces via transplanting some small autolog osteochondral grafts (OCGs) in a rosetta pattern to form a stable clot that fills the lesion (Hangody et al., 1997; Hangody et al., 2001a; Hangody et al., 2001b; Hangody and Fules, 2003; Hangody and Modis, 2006; Hangody et al., 2008). The theoretical advantage of it is based on Pap and Krompecher's study published in 1961 (Pap and Krompecher, 1961), in which they proved that the OCG builds in the recipient surrounding in a functional situation, even in lack of compatibility; on the other hand the graft's size and ratio have a significant influence on its survival. Although in the case of autologous osteochondral mosaicplasty serology and tissues typifying are redundant, it also have significant restrictions: limited size of useable donor area, donor site morbidity, differences in orientation and thickness, mechanical properties between donor and recipient cartilage (Bedi et al., 2010).

In contrast to many articles deal with graft-host integration or post-operative function just a few is engaged in donor site events. Normally the tunnels after removing the OCGs, get filled with spongy bones within four weeks – similarly to the Pridie's drilling method – through mesenchymal stem-cells' invasion due to bleeding from the subchondral area (Hangody and Modis, 2006). Surface of tunnels tend to be covered with a so-called primer repair tissue which

becomes fibrocartilage during appropriate loading (Bodo et al., 2000). Unfortunately, excessive bleeding may start spontaneously from the donor tunnels remained empty just in a few cases, which can lead to haemarthros (Feczko et al., 2003). Ferric ions (Fe^{3+}) derived from the degradated normocytes of blood getting into the joint cavity may irreversibly destroy the proteoglycan structure of hyaline cartilage (Sokoloff, 1963).

Some clinical and pre-trial experimental studies had been made to exclude the possibility of post operative haemarthros to fill the donor tunnels such as hydroxylapatite (Litvinov et al., 2000), carbon fiber (Meister et al., 1998), polyglyconate (Freed et al., 1994) and compressed collagen (Nixon et al., 1993) which resulted in scar tissue or poor fibrocartilage formation on the surface. In our earlier study we investigated a copolymer material, polyethylene glycol terephtalate - polybutylene terephthalate (PEGT/PBT) – Polyactive, PA, (IsoTis OrtoBiologics, Bilthoven, the Netherlands), where we provided clear evidences that the PA is one of the most appropriate filling material in this context (Módis et al., unpublished data). The histological examinations on the human samples, which studied the influence of the PA, have shown that the PA helps the fibrocartilage (in few cases hyaline-like cartilage) formation on the joint surface as well as the bone and the connective tissue generation inside (Módis et al., 2005a).

To the best of our knowledge there are no publications about the quantitative and qualitative analysis on bone trabecules evolved in the donor tunnels filled with any material, among PA, therefore in this present study we aimed to perform the quantitative and qualitative analysis on them.

MATERIALS AND METHODS

Mosaicplasty of horses

All procedures using animals were approved by the Ethical Committee for animal trialsof Szent István University, Faculty of Veterinary Sciences and tissues were obtained in accordance with the guidelines. The osteochondral autograft transplantations were carried out on 10 horses. The donor area was the femoral medial trochlea and the recipient region was the medial or lateral trochlea of the animal's forelimb's distal third metacarpal bone. The applied method has been described earlier (Bodo et al., 2000). Briefly, horses were positioned in lateral recumbency in general anesthesia. Four pieces of OCG – each of 6.5 mm diameter and about 20 mm length – containing healthy joint cartilage and spongiosa were collected from the same wound. The grafts were kept in sterile isotonic solution (0.9% NaCl) until implantation. 22 mm deep tunnels were drilled on the recipient area than the OCGs were planted into them. Half of the donor areas were filled with biodegradable polymers (PolyActive, PA, IsoTis OrtoBiologics, Bilthoven, the Netherlands) (Figure 1), others were left empty.

Tissue processing for histological analysis

After a long (2 years) follow up the horses were sacrificed in accordance with the study guidelines and the donor tunnels filled with PA were obtained with their intact surrounding. Samples were immediately transferred into Sainte-Marie's solution a (SAINTE-MARIE, 1962). After fixation tissues were put into 10% ethylenediaminetetraacetate (EDTA) (Solon, Ohio, USA) for approx. 3 weeks. Decalcified and dehydrated tissue samples were embedded into paraffin and 5−7 μm thick longitudinal sections were cut (Microm HM335E, Microm

International GmbH., Walldorf, Germany). Paraffin sections were placed on gelatine-coated glass slides and left dried overnight at 37 °C.

After dewaxing and rehydration sections were stained with dimethylmethylene blue (DMMB, Aldrich, Steinheim, Germany), picrosirius red (PS, Polysciences, Warrington, USA), hematoxilin-eosin (H&E) according to the manufacturer's protocol (Constantine and Mowry, 1968; Módis, 1974, 1991; Kiraly et al., 1996). After staining sections were covered with DPX (Fluka Chemie, Buchs /Switzerland).

Computer-assisted image analysis of the PA filled and non-filled donor tunnels.

During our preliminary microscopic observation we found a lot of thick trabecular bones with abnormal structure appear in the donor tunnels filled with PA however, thin osteon units of bone with normal structure are visible in the control (not operated) areas (Figure 2 and 3). To quantitatively analyse our observations computer assisted image analysis was preformed briefly described below.

Images from different samples of 6 different horses were captured by a Nikon Eclipse 800 microscope (Nikon Corporation Instruments Company, Japan) equipped with a Spot RT-slider (Diagnostic Instruments, Sterling Heights, MI, USA) CCD camera. Acquired and presented images were representative of all the samples examined. After system calibration, 500x720 μm areas of each samples were digitalized. Altogether 31 control and 31 PA filled donor tunnels were compared to each other and the quantitative analysis was performed using Image Pro 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

Mann-Whitney test was used to analyze the results statistically.

Polarized light microscopic measurement

Polarized light microscopy was used to quantify the organization changes within the collagen network of repair tissue in case of the PA filled and its surrounding. Sections stained with PS were examined by polarization microscope using 40x objective (Zetopan-pol, Reichert, Wien, Austria). 100 measurements per sample have been carried out both in case of PA filled and control tunnels.

RESULTS

Macroscopic inspection showed that the tissue built up in PA filled donor tunnels was milder compared to the neighbouring regions, which were well distinguishable.

Residuums of PA helped in orientation during microscopic examination in all the three stainings. The surfaces of the PA filled donor tunnels were recovered by fibrocartilage, the inner trabecules were thicker and showed abnormal organisation compared to trabecular meshwork of surrounding tissue (Figure 2 and 3). In some cases polinuclear giant cells were shown next to the PA particulums. Compared to the ortochromatic trabecules of the receiving area the osteon units of the PA filled donor tunnels showed purple red metachromasia on the DMMB stained sections (Figure 2).

Computer assisted image analysis

Hereby we provided clear evidences, that the engaged area of the trabecules developed in the PA filled donor tunnels was significantly wider ($p<0.05$) compared to the control trabecules' (Figure 4 and 5).

Data were analysed using Mann-Whitney statistical method. The analysis resulted in significant difference between the averages of engaged area of trabecules per ROI ($p = 5.34x10^{-6}$) (Figure 5). The engaged area of trabecules in PA-filled donor tunnels was 25% higher compared to intact spongiosa located next to the control tunnels (Figure 5).

Polarized light microscopic measurement

We analyzed the structure and the pattern of orientation of osteon units on PS stained sections where PA particulums showed homogenous dual-fraction (Figure 3). 100−100 parallel measurements were performed to explore the collagen fiber orientation and the statistical analysis

did not show significant differences between the PA filled donor tunnels and the control areas ($p = 0.5229$) (Figure 6). It refers to the fact that the spatial orientation of the collagen molecules does not differ in the trabecules of the donor cannels filled with PA and control areas.

DISCUSSION

The most important result of our study was that several new osteon units have been developed in PA filled donor tunnels, during the two years period following mosaicplasty procedure. These new osteon units showed microscopically irregular but submicroscopically intact and regular collagen structure.

It was previously showed that PA has an osteoconductive effect (Radder et al., 1996; Du et al., 2002). We aimed - first in the literature - to compare the structure of the bone tissue grow up under the influence of porous PA to the original rests.

Beside to earlier results that in human experiments PA helps the reconstruction of the articular surface (Módis et al., 2005a) the bone forming effect confirm the clinical usefulness of this biopolymer. We were looking for answers for the following questions while explaining our results: (1) how evolves bone in the PA-filling material placed into the osteochondral tunnel; (2) why does the osteon units developed here have a more massive and more irregular microscopic structure compared to the intact spongiosa in its surrounding; (3) how could it be explained that the collagen, the biggest amount of macromolecular component of the organic bone matrix which shows irregular structure by microscope, has a regular submicroscopic orientation pattern.

The answer is relatively easy to the first question. The porous PA gets in touch with the bone marrow and bone tissue of the intact surrounding. Osteoprogenitor cells can migrate from the bone marrow (maybe from the surface of the surrounding bone trabecules) to the surface of the PA particulums where they differentiate into osteoblasts which produce the bone matrix. We cannot tell the mineralization level of the newly evolved bone because we analyzed the sections of decalcinated tissue. We also do not know which degradation product of the PA can initiate chemical signalling in osteoblasts.

We suggest the speculative answer to the second question is according to that the surface of the PA is never covered by hyaline cartilage but by fibrous cartilage, which has a much lower bearing capacity. We did not observe it only in some cases of our samples but it was also reported in human biopsy samples before (Módis et al., 2005a; Módis et al., 2005b). It is well-known that the proteoglican (PG) content of the hyaline cartilage is significantly higher compared to fibrocartilages (Röhlich, 2006). These molecules, which form huge size of aggregates and have a significant hydration shell, are responsible for weight-bearing capacity of the intact articular cartilage (Helminem et al., 1987). It is possible that the increased amount of trabecular bone have to compensate the decreased weight-bearing capacity of the fibrocartilage tissue.

The most difficult is to answer the question why the orientation pattern of the collagen molecules does not change, although the bone trabecules show irregular structure compare to the surrounding. The (predominantly type I) collagen molecules, which build the collagen fibers, connect to each other with a covalent cross binding in the extracellular space (Hay, 1991; Módis, 1991; Röhlich, 2006). It is possible that the so-called 'self-assembly' creates the regular structure of collagen fiber until the enzymes, which are responsible for the covalent bindings between the collagen molecules, the different glycoproteins, which interact with collagen fibrillums, and PG molecules are present in the micro-surrounding giving place to the fibrillogenesis. We did not examine these components we registered only the final result of the process.

Experiences in further models in the field of cell-biology and biochemistry could establish the use of this biodegradable and biocompatible material.

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Competing interests

The authors have declared that no competing interests exist.

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FIGURE LEGENDS

Figure 1

Picture of the biodegradable porous polymer used to fill up the tunnels.

Figure 2

Representative light microscope pictures of picrosirius red (PS) (A and C) and dimethylmethylene blue (DMMB) (B and D) stained sections of PA-filled donor tunnels (A and B) and its surrounding (C and D) (100x magnification). Please note the difference in meta- and orthochromasia (B vs. D), thickness (A and B vs. C and D) and regularity of osteon units of the PA-filled tunnels and its surroundings.

Figure 3

Representative light (A and C) and polarized light (B and D) microscope pictures of picrosirius red stained sections of PA filled donor tunnels (A and B) and its surrounding (C and D). PA particulums showed homogeny dual-fraction under polarized light (B) furthermore lamellas of new thick trabecules evolved in PA filled donor tunnels shows irregular ordination compared to the neighbours (B vs. D).

Figure 4

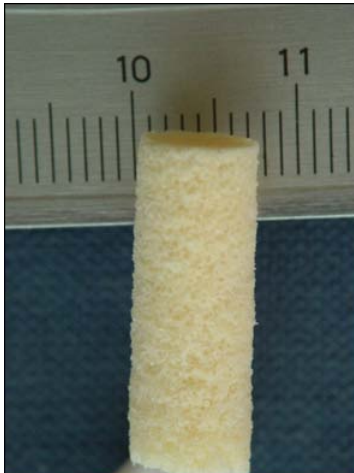
Column diagram shows the mean engaged area of trabecules per ROI (%) in each samples. 5-5 ROI per sample were digitalized both from PA filled and control areas. Engaged areas of the trabecules developed in the PA filled donor tunnels are represented by black and the control by gray columns.

Figure 5

Diagram shows the average of engaged area of trabecules per ROI (%). Enlargement of area (%) of control tunnels is represented in grey and PA-filled donor tunnels in black. The engaged area of trabecules in PA-filled donor tunnels was higher compared to control tunnels.

Figure 6

Results of quantitative polarized-light microscopy experiments from determination of collagen-fiber orientation of trabecules. Diagram shows the number of measured values belonging to different dual-fraction path length range (nm). Grey columns represent the control samples, black columns are the values of PA-filled donor tunnels. Distribution of values (nm) of PA-filled donor tunnels closely resemble the values of control-tunnels. Mann-Whitney statistical analysis resulted without a significant difference between the PA-filled and control tunnels ($p = 0.5229$). There was not observed any difference between the collagen-fiber orientation of control trabecules and the PA-filled ones.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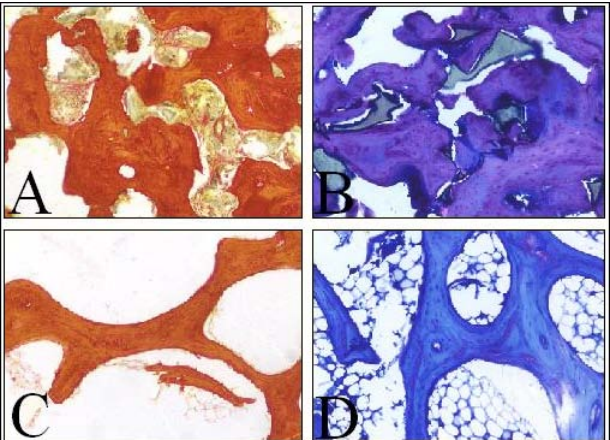
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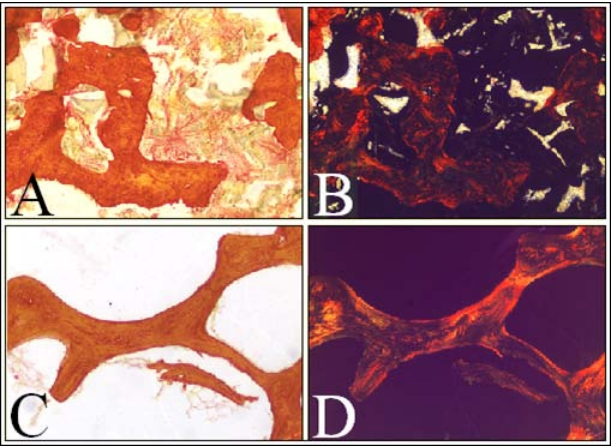
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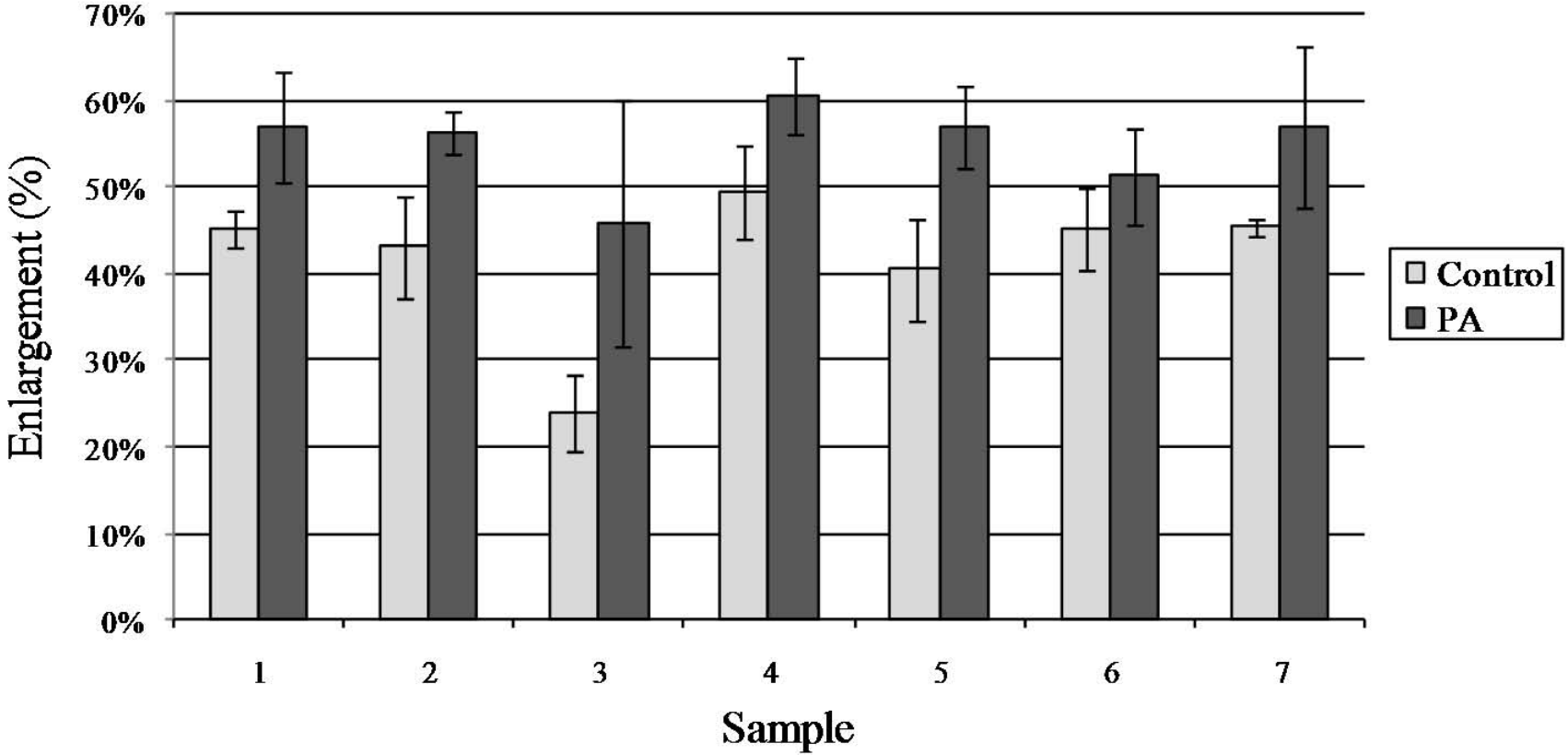
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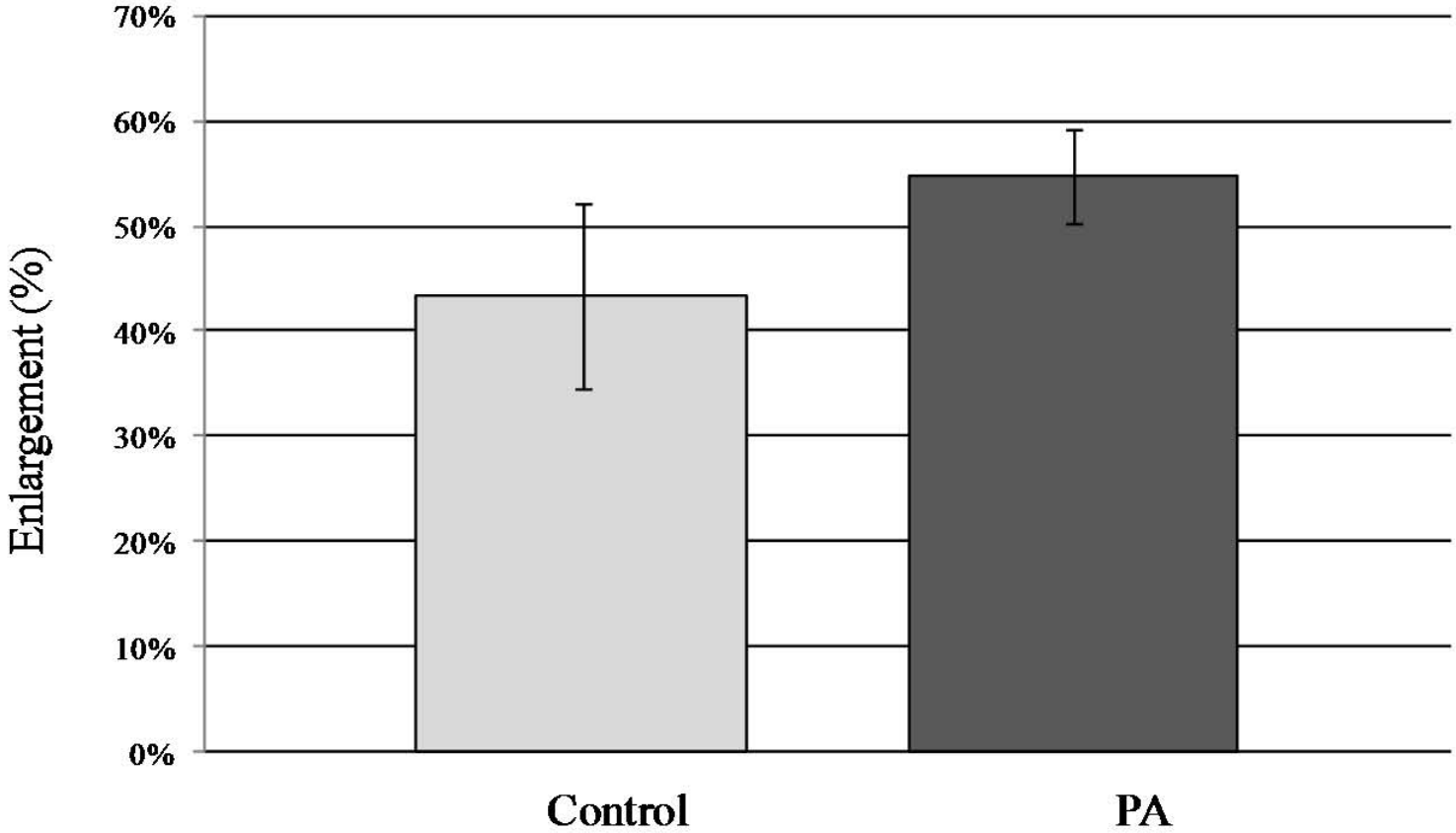
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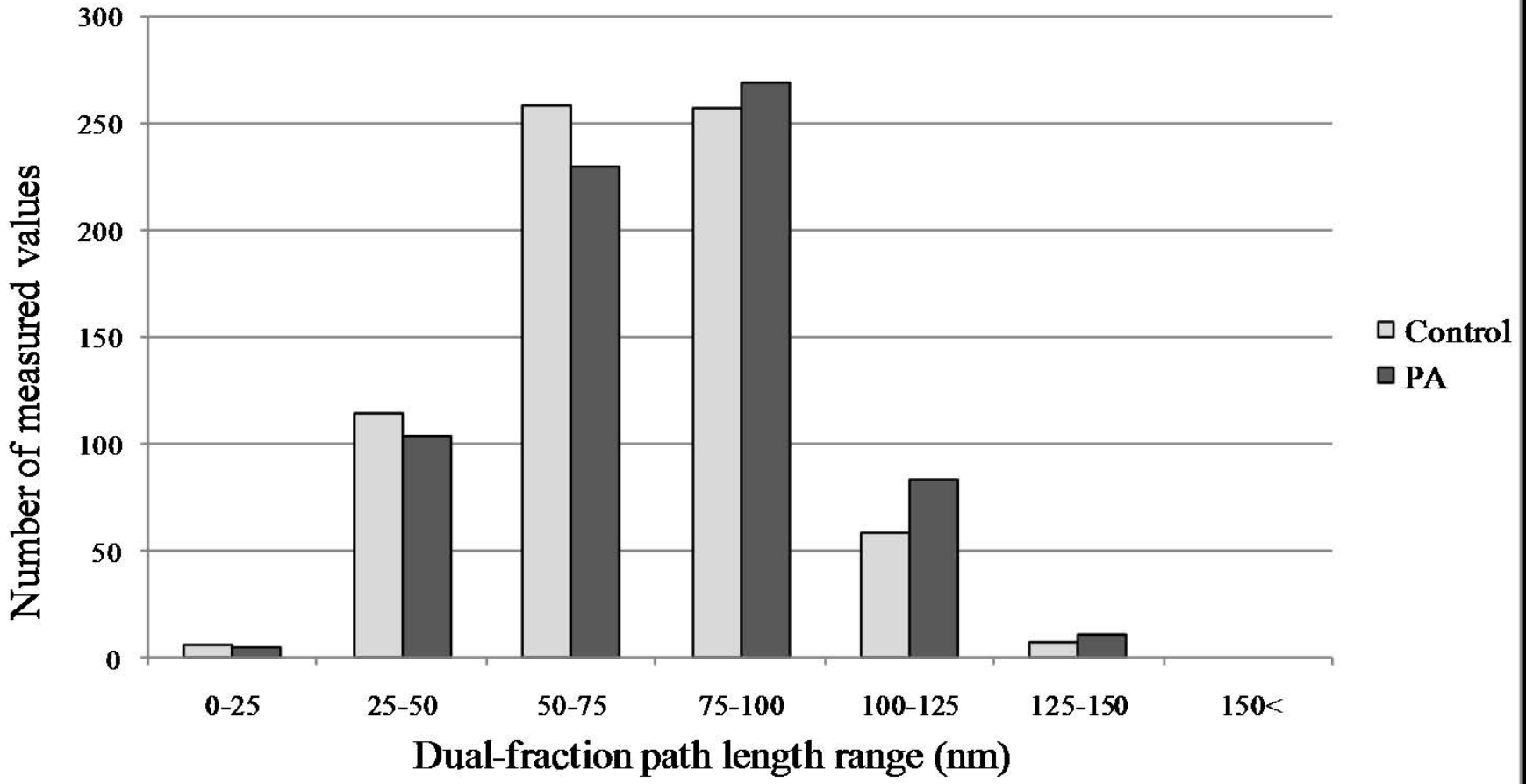
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