Histomorphometric analysis of knee synovial membrane in dogs undergoing leg lengthening by classic Ilizarov method and rapid automatic distraction

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Abstract

Purpose This pilot study aimed to test the theory that different lengthening methods affect the microscopic structure of knee joint synovium in diverse ways.

Methods This was a descriptive and analytical cross-sectional study of synovium carried out in two experimental models of canine leg lengthening using the Ilizarov fixator. Group 1 ($n=6$) used manual gradual distraction most commonly used in clinical practice at one millimetre/day divided into four equal increments, 0.25 mm at each increment. Group 2 ($n=7$) used an increased rate of automatic distraction at three millimetres/day divided in 120 increments, 0.025 mm at each increment. At the end of distraction and after fixator removal, the animals were euthanised. Control was via intact dogs. The thickness of the synovial lining layer, number of synovial cell rows, and numerical density of subsynovial microvessels were assessed in digital images for semiautomated computerised analysis of semi-thin sections stained with toluidine blue and methylene blue-basic fuchsin. Comparison of synovitis manifestation was made with grading scale. The vascular and nerve changes in the subsynovial layer were also compared.

Results Group 1 developed marked synovitis, synovium hypervascularisation, degeneration of the nerve fibres in subsynovial nerves with the tendency to regeneration. Group 2 had moderate to mild degree of synovitis with progressive degenerative changes in subsynovial vessels and nerves.

Conclusion Both methods used are unfavourable for the state of the joint synovium, but modify it in different ways.

Introduction

The Ilizarov method [1] has become widespread because it is an excellent tool in many fields of orthopaedic surgery—management of types II–IIIB open tibial shaft fractures [2, 3], crush injuries of hindfoot [4], complex tibial plateau fractures [5], tibial bone defects [6], infected non-union of the tibia [7], limb lengthening [8–11] and basic research of distractional osteogenesis [12]. But problems of limb-lengthening patients' functional rehabilitation, in particular maintenance of joint motion, remain unsolved [13, 14]. To our knowledge, few studies concerning this problem have dealt with histological changes in articular cartilage during experimental limb lengthening [15–17]. It was found that chondrocytes and extracellular matrix destruction and repair occurred both in the deep and superficial cartilage zones. Synovium plays an important role in articular cartilage changes [18]. However, there is no information about the structural response of synovium to limb lengthening.

Materials and methods

Experiments were carried out in accordance with internationally accepted Principles of Laboratory Animal Care (NIH Publication no. 85–23, revised 1985), and our institutional ethical committee approved the protocol. A total of 16 mongrel adult dogs that weighed 20–25 kg with 18–20 cm leg length were used in this study. Three animals formed the intact group and 13 dogs were operated upon. Transverse shin bone osteotomy at the proximal metadiaphysis level and osteosynthesis by the Ilizarov fixator was performed in group 1 ($n=6$). Lengthening protocol involved a five-day latent period, and then lengthening was started by manual movement of graded traction nodes at the rate of one

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millimetre/day in four increments of 0.25 mm which was performed for 28 days for a total of 28-mm lengthening (15 % increase of the initial length of shin bones). The fixator was removed after the bone healed (35 days of fixation). The animals were euthanised and the material for histology was obtained at the end of distraction (postoperative day 33) and 30 days after the fixator removal (postoperative day 98). In group 2 ($n=7$) leg lengthening with an automatic distractor was performed after mid-diaphyseal osteoclasis. The distraction protocol involved five-day latency as in group 1, but distraction rate was three millimetres/day in 120 increments (increment length 0.025 mm), for a total of 28-mm lengthening achieved in ten days, and then the fixator was removed after bone consolidation (fixation 30 days only). The animals were euthanised at the end of the distraction period (postoperative day 15) and 30 days after the fixator removal (postoperative day 75). The parts of synovium were excised from the suprapatellar zone, subjected to aldehyde-osmium fixation and embedded in Araldite. Semi-thin (0.5–1.0 μm) sections were prepared using “Nova” ultratome LKB (Sweden), stained with toluidine blue and methylene blue-basic fuchsin. Tissue sampling and processing, sectioning, staining and histomorphometric measurements were made according to standard methodology, which includes an improved large shear semi-thin sections technique [19]. Semi-thin sections allow one to ignore the Holmes effect, the large shear (4–8 mm^2 instead of standard 1 mm^2) provided sample representativeness. Every tenth slice was selected and three to four sections from every animal were analysed. The semi-thin sections and staining procedures used provided good cellular detail in the light microscope and in digital images transferred to a workstation for analysis. The preparations were investigated and digitised using the photomicroscope “Opton” (Germany) connected with the “DiaMorph” program complex (Russia, Moscow). Histomorphometry was performed with the “VT-Master-Morphology” program (VideoTest, Russia, St. Petersburg). A reference set of 20 images from each animal was selected for validation. Systematic random sampling protocols were used. One well-experienced histomorphometry

expert (biologist) inspected the classified objects indicated by coloured overlays (for synovium thickness) or dots (for all microvessels with visible lumens) on the digital images. About 100 random samples of synovium thickness for each animal were made during every measurement. Subject identity was revealed at the completion of quantification. Thickness of synovial lining layer, number of synovial cell rows, and numerical density of subsynovial microvessels were assessed. The synovitis was evaluated semiquantitatively using the expert scale [20]. The vascular and nerve changes in the subsynovial layer were also compared. Histomorphometric data were evaluated for statistical differences using the software package Attestat Program, version 9.3.1 (developed by I.P. Gaidyshev, Certificate of Rospatent official registration No. 2002611109) with unpaired *t*-test and Mann–Whitney *U*-Test, designed for small samples ($n_1, n_2 \geq 3$). A significant value of $p < 0.05$ was set a priori.

Results

At the end of distraction

In group 1 the synovium lining layer thickness was 73 % up on normal. Lining cells were mainly of the fibroblast-like type: round, fusiform, polygonal; sprout-like shapes dominated in the superficial row of cells (Fig. 1a). There were areas with absence of the lining layer or with signs of cell death. The numerical density of microvessels in the subsynovial layer exceeded the norm slightly and not significantly (Table 1). Hypertrophy of media and adventitia was revealed in the small arteries (Fig. 1b). Longitudinal orientation of smooth muscle cells (SMC) dominated the media. The SMC hydropic (vacuolar) degeneration and necrotic death were not unusual in microvessels. Endoneurial oedema was moderately marked in subsynovial nerves, endoneurial macrophages and fibroblasts content was increased (Fig. 1c). Wallerian degeneration of some

Fig. 1 Fragments of transverse semithin sections of the knee synovial membrane in group 1 dogs at the end of the distraction period. Methylene blue-basic fuchsin (a, b) and toluidine blue (c) staining. a

Thickening of the lining layer. b Media hypertrophy in subsynovial layer artery. c Nerve of the subsynovial layer with endoneurial oedema. Instrumental magnification 1250 $\times$ (a) and 500 $\times$ (b, c)

Table 1 Morphometric characteristics of synovium in intact control and experimental groups

Parameter	Intact	Group 1 (manual distraction)		Group 2 (rapid autodistraktion)	
		End of distraction	After the fixator removal	End of distraction	After the fixator removal
Thickness of the outer layer (μm) (mean $\pm$ SD)	26.08 $\pm$ 2.14	44.81* $\pm$ 10.63	28.17 $\pm$ 2.31	50.06* $\pm$ 5.89	36.45* $\pm$ 7.82
Number of synovial cell rows (range)	1–2	2–4	1–2	2–4	2–3
Numerical density of microvessels (mm^{-2}) (mean $\pm$ SD)	335.05 $\pm$ 28.88	381.26 $\pm$ 17.33	439.03* $\pm$ 37.88	369.7 $\pm$ 46.21	300.39 $\pm$ 43.99

*Significance ($p < 0.05$) between intact control and experimental group (1 or 2)

nerve fibres was noted, but some endoneurial tubes already contained regenerative axonal sprouts.

In group 2 the synovium lining layer thickness was 100 % up on normal due to cell sizes and number of cell rows increase (Fig. 2a). The representation of macrophage-like cells was increased in some parts of the lining layer, but oval-rounded and fusiform fibroblast-like cells were more numerous. Spasm or obliteration of some small-caliber arteries was revealed (Fig. 2b). The lumens of many arterioles were also closed due to spasm or protruding endotheliocytes; the increase in sizes and cellularity of perivascular spaces was noted. There were areas of neointimal hyperplasia in the arteries with open lumens. The vacuolar dystrophy of many SMC was noted in veins. The microvessels numerical density was 10.4 % up on the intact ($p > 0.05$). The crimped perineurium (Fig. 2c), subperineurial and endoneurial oedema, macrophage accumulations in subperineurial spaces, the increase in macrophage number in endoneurium, oedema of nonmyelinated fibres and myelin hernias were revealed in subsynovial nerves.

Thirty days after the fixator removal

In group 1 the synovium lining layer thickness and cell rows number were decreased and became comparable with intact (Table 1). Many of the synovial cells were destroyed; those containing only cell detritus areas were noted. Polygonal

fibroblast-like cells dominated the other parts. Numerous macrophage-like cells showed signs of increased activity; they contained a significant number of vacuoles and pinocytotic vesicles (Fig. 3a). The numerical density of microvessels in the subsynovial layer increase was 31 % up on the intact ($p < 0.05$). The number of mast cells increased, some of them exhibited the signs of partial or complete degranulation. The number of perivascular cells around microvessels increased as well. The bundles of collagen fibres in the subsynovial layer were disorganised and fragmented. Endoneurial oedema or subperineurial oedema loci were noted in subsynovial nerves, as well as the increase in macrophage number in the subperineurial space and endoneurium, signs of nerve fibre degeneration and regeneration.

In group 2 the thickness of the synovium lining layer decreased in comparison with the previous period, but it was 36.2 % thicker than normal. The lining layer was dominated by flattened and polygonal fibroblast-like cells (Fig. 3b). Fibrosis was noted in the upper subsynovial layer, as well as induration of intercellular substance, collagen fibre destruction, the increased content of mast cells, and lipomatosis. The microvessels numerical density was 18.8 % down from the previous stage; the difference from the intact value was not statistically significant. Signs of necrotic cell death, wall disintegration, lumen deformation and obliteration, and sclerosis were revealed in many microvessels. The number of

Fig. 2 Transverse semi-thin sections of the knee synovial membrane in group 2 dog at the end of the distraction period. Toluidine blue (a, c) and methylene blue-basic fuchsin (b) staining. **a** Hypertrophy of the

synovial membrane lining layer. **b** Obliteration of the subsynovial layer artery. **c** Circulatory disorders in the subsynovial layer nerve. Instrumental magnification 500x

Fig. 3 Transverse semi-thin sections of the canine knee synovial membrane 30 days after the fixator removal. **a** Group 1, toluidine blue staining. **b** Group 2, methylene blue-basic fuchsin staining. Instrumental magnification 500x

degenerated nerve fibres in the subsynovial layer nerves increased significantly as compared to the previous period.

Table 2 shows the results of synovitis grading in groups 1 and 2.

Discussion

To our knowledge, we have the first information about the histological response of joint synovium to limb lengthening including changes in its cellular content, inflammatory reactions and neurovascular disturbances. We acknowledge the limitations of our study. Further research is needed to better define the spectrum of pathological and adaptive changes in comparable experimental conditions and time points. Two experimental models of canine leg lengthening have been studied in this experiment. The manner and level of bone integrity breaking, the daily rate and rhythm of distraction in group 1 are most commonly used in clinical practice. In group 2 the increased daily rate of automatic distraction has been tested. At first glance, the descriptive and quantitative data obtained are not comparable because the groups differ in several experimental conditions. However, we took the opportunity to evaluate a wide range of destructive and reparative reactions of the synovial sheath under such different conditions. Hyperplasia of the synovium lining layer was revealed in both groups. The dynamics of synovitis in group 1 and group 2 was diametrically opposite.

Reorganisation of the synovial membrane vascular bed in group 1 reflects the hyperdynamic circulation, high activity of angiogenesis and hypervascularisation. It should be mentioned, that in group 1 the signs of nerve fibre regeneration was found at the end of distraction, 33 days postoperatively. In group 2 they were not revealed until after 75 days. But the adaptive changes in group 1 were accompanied by marked synovitis. The numerical density of microvessels in group 2 was comparable with normal; but the destructive changes in vessels, particularly the state of its arterial-arteriolar bed were symptomatic to reduction of blood supply which is the most likely cause of more marked signs of nerve degeneration. The findings in group 1 were consistent with changes in the articular cartilage in such conditions [21] which were deeper than in mid-diaphyseal leg lengthening with one millimetre/day rate in four increments [17, 22]. A similar relation was revealed in the histomorphometric analysis of lengthened muscles in these series of experiments [23]. In combination with the mentioned facts the data concerning joint synovium leads to the conclusion, that proximal (meta-diaphyseal or metaphyseal) level of leg lengthening is not optimal for functional rehabilitation. This must be taken into consideration in clinical practice. In group 2 an attempt was made to make the lengthening technique less traumatic; the level of osteoclasia was remote from the knee joint, the increment of distraction was decreased ten-fold, the period for limb with fixator in place was reduced by 23 days, not only due to more rapid distraction, but due to reduction of the period of

Table 2 Comparison of synovitis manifestation with expert scale developed by V. Krenn et al. (2006)

Criteria	Group 1 (manual distraction)		Group 2 (rapid autodistraction)	
	End of distraction	After the fixator removal	End of distraction	After the fixator removal
Thickening of the outer layer	1	0–1	1–2	1
Cellularity of the subsynovial layer	1	2	1	1
The degree of manifestation of plasmocytic-lymphocytic infiltration	0	2	1	0
Sum of points	2	4–5	4	2

fixation. Earlier formation and consolidation of the callus occurred under these conditions, but neurovascular disturbances were marked in the synovial membrane. Now we did not relate the fast automatic distraction to clinical implementation although the consolidation of bone regeneration was accelerated, and this fact is remarkable. The safest regimen for soft tissues of slow automatic distraction (one millimetre/day in 60 increments) was discovered by G.A. Ilizarov [24] and was confirmed after his death in various studies of cartilage, muscles and nerves [17, 22, 25, 26]. The optimal rate and frequency of fast automatic distraction might be developed in the future. We believe it has potential to be incorporated in the treatment process to improve the results of limb lengthening.

Conclusions

Structural variables of the knee synovium during leg lengthening depend substantially on the lengthening technique.

Conflict of interest The authors declare no conflict of interest.

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