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Proteoglycan synthesis rate as a novel method to measure blood-induced cartilage degeneration in non-haemophilic and haemophilic rats

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Abstract

Introduction: Haemophilic animal models are used to study blood-induced cartilage damage, but quantitative and sensitive outcome measures are needed.

Aim: To develop a novel quantitative method for detecting early cartilage degeneration in a haemophilic rat model of blood-induced joint damage.

Methods: The ^{35}Sulphate incorporation ($^{35}SO_4^{2-}$ assay) was applied to tibial and patellar cartilage of wild-type rats to quantify baseline proteoglycan synthesis and to evaluate the effect of 4-day blood exposure in vitro. Next, haemarthrosis was induced in 39 FVIII-deficient rats and characterized by changes in knee joint diameter and development of bone pathology (using micro-CT). Four- and 16-day posthaemarthrosis proteoglycan synthesis rate (PSR) was assessed using the $^{35}SO_4^{2-}$ assay, with the contralateral knee as control.

Results: In vitro, a decrease in PSR in tibial and patellar cartilage was demonstrated following blood exposure. In vivo, joint diameter and development of bone pathology confirmed successful induction of haemarthrosis. In the blood-exposed knee, tibial and patellar PSR was inhibited 4 and 16 days after induced haemarthrosis. Interestingly, at day 16 the proteoglycan synthesis in the contralateral knee was also inhibited to an extent correlating with that of the blood-exposed knee.

Conclusion: For the first time, early changes in cartilage matrix synthesis upon blood exposure were quantified with the $^{35}SO_4^{2-}$ assay in a haemophilic rat model, establishing this assay as a novel method to study blood-induced cartilage damage.

KEYWORDS

arthropathies, cartilage, experimental animal models, haemarthrosis, haemophilia

Astrid E. Pulles and Kåre K. Vøls contributed equally.

1 | INTRODUCTION

Joint damage upon bleeding causes significant morbidity in patients with haemophilia,[1] and adds to joint degeneration after trauma[2] and major joint surgery.[3] While the synovial inflammatory response following a single joint bleeding is considered transient,[4,5] the damaging effect on cartilage is prolonged and irreversible.[4,6-8] Even short-term blood exposure leads to impairment of cartilage matrix turnover due to chondrocyte apoptosis,[7,9] causing loss of proteoglycans that over time may lead to clinically evident joint damage.

Given the irreversible and severe consequences of blood exposure, targeted therapy to intervene in the process of blood-induced cartilage damage is crucial but lacking. A better understanding of the pathophysiological mechanisms, and in particular the earliest changes induced by haemarthrosis, may provide new insights to identify novel treatment options.[10] Investigating these processes in haemophilic patients is challenging, since the early stage of arthropathy often proceeds asymptomatically and evaluation mainly relies on indirect parameters such as imaging[11] and systemic biochemical markers.[12,13] Consequently, rodent models have proven indispensable for studying blood-induced joint damage, displaying translatable pathological changes in the tibiofemoral and patellar compartment following an induced haemarthrosis.[14,15] Cartilage damage is especially pronounced in the factor VIII deficient ($F8^{-/-}$) rat[14] in which apoptotic chondrocytes and loss of proteoglycans were identified histologically within days following an induced haemarthrosis.[9] However, histological evaluation is time consuming, less sensitive for subtle (early) changes, and results are subject to interpretation despite initiatives to harmonize semi-quantitative scores.

To improve the evaluation of early blood-induced cartilage damage in the $F8^{-/-}$ rat model, a quantitative outcome parameter able to detect the very early cellular changes is needed. Blood exposure results in chondrocyte apoptosis and induces permanent disturbances in cartilage matrix turnover, causing loss of proteoglycans essential for resilience of cartilage tissue.[23] Determining the proteoglycan synthesis rate (PSR), by incorporation of radioactive ^{35}Sulphate in cartilage ($^{35}SO_4^{2-}$ assay), is an eligible method to detect early signs of cartilage degeneration [16] and is measurable before histological alterations manifest.[17]

Although not commonly used, in rodent models of other degenerative joint diseases, the $^{35}SO_4^{2-}$ assay has been used to measure proteoglycan synthesis in cartilage from the tibia[18,19] and the patella.[20,21] Moreover, this method has previously been applied in human cartilage explants and larger animal models to evaluate blood-induced cartilage damage,[8,22] but has not been used in a haemophilic rodent model before.

The aim of this study was to develop a novel method to detect early cartilage degeneration in a haemophilic rat model of joint damage by experimentally inducing a joint bleed. Therefore, the $^{35}SO_4^{2-}$ assay was first applied to tibial and patellar cartilage of wild-type (WT) rats and the effect of in vitro blood exposure was evaluated. Secondly, an in vivo study in haemophilic rats was conducted to evaluate the effect of an induced haemarthrosis on the PSR in the short (after 4 days) and longer (after 16 days) term.

2 | MATERIALS AND METHODS

2.1 | Study design

Healthy cartilage from WT rats was used for ex vivo studies to determine baseline PSR and to measure the in vitro effect of 4-day blood exposure (Table 1). The effect of blood exposure in vivo was measured in $F8^{-/-}$ rats 4 and 16 days after induction of a single haemarthrosis. After euthanasia, the hind legs were transported to the University Medical Center Utrecht (UMCU, the Netherlands), and cartilage proteoglycan synthesis was assessed within 24 hours of euthanasia. All ex vivo and in vitro studies were performed at the UMCU. In vivo studies were conducted in $F8^{-/-}$ rats at Novo Nordisk A/S (Maaloev, Denmark). Experiments were approved by the Danish Animal Experiments Committee under the Danish Ministry of Environment and Food, as well as the Novo Nordisk Animal Welfare Body. All procedures were performed according to the Danish Animal Experimentation Act and the EU Directive 2010/63/EU. In all studies rats of both genders and aged approximately, three months were included.

2.2 | Cartilage isolation

Keeping the knee flexed at 90 degrees, the patella tendon was cut at the level of the joint space and removed including the patella. Subsequently, the lateral, medial and posterior boundaries of the joint cavity were cleaved, thereby disengaging the femur and tibia. The tibia was fixated in a bench vice to slice full-thickness cartilage fragments from the tibial plateau by use of a scalpel (surgical blade size 15; Swann Morton). A maximum of two slices were combined to represent the tibial cartilage of a single joint and a weight of more than 0.8 mg was considered sufficient for further analysis. The weight of the patellar cartilage was not measured due to the interference of the attached tissue. Instead, equally sized samples were provided by punching the central part of the patella using the cannula of a bone marrow biopsy set (T-Lok Bone Marrow Biopsy Needle, 8Gx4", Argon Medical Devices). Next, the cartilage explants from tibia and patella were separately transferred to culture medium in a 96-well round-bottomed microtiter plate. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% *heat-inactivated foetal bovine serum* (FBS; Gibco), glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin sulphate (100 µg/mL; all Paisley, UK) and ascorbic acid (85 µmol/L; Sigma).

2.3 | Determination of proteoglycan synthesis rate

Proteoglycan synthesis in cartilage explants was determined ex vivo or after in vitro culture by adding 4 µCi $Na_2{}^{35}SO_4$ (NEX-041-H carrier free; DuPont) to the explants for 4 hours during which radioactive labelled sulphate is incorporated in newly synthesized proteoglycans. After 4 hours of pulse labelling, the explants were washed twice in cold phosphate-buffered saline. The patellae were

TABLE 1 Study design

Group	Rats	n	Joint bleed induction[a]	Euthanasia DK[a]	μCT[a]	Euthanasia NL[b]	Culture blood −/+[b]	PSR[b]
Baseline	WT	6				Day 0		Day 1
In vitro culture	WT	9				Day 0	Day 1	Day 5
4-day group	$F8^{-/-}$	14	Day −4	Day 0	Day 0			Day 1
16-day group	$F8^{-/-}$	18	Day −16	Day 0	Day 0			Day 1

[a]Performed at Novo Nordisk, Denmark (DK).

[b]Performed at UMCU, the Netherlands (NL).

In vitro studies were conducted at the University Medical Center Utrecht (UMCU) to assess baseline proteoglycan synthesis in tibial and patellar cartilage of wild type (WT) rats (n=6) ex vivo 24 h after euthanasia, and to assess the effect of 4 days in vitro blood exposure on proteoglycan synthesis in tibial (n=4) and patellar (n=5) cartilage of WT rats. Subsequently, an in vivo study was performed at Novo Nordisk, Denmark. Knee haemarthrosis was induced in factor VIII deficient (F8-/-) rats and were euthanised at day 4 (4-d group, n=14) or 16 (16-day group, n=18). Then, the knees were micro-CT imaged and transported to UMCU to determine proteoglycan synthesis rate (PSR) in the tibial and patellar cartilage within 24 h of euthanasia.

decalcified in 1 mL 0.5 mol/L ethylenediaminetetraacetic acid (EDTA) overnight. Subsequently, all samples were digested for two hours at 65°C with 2% papain (Sigma) and stored at −20°C. Next, the glycosaminoglycans (GAGs) were precipitated by 0.3 mol/L hexadecylpyridinium chloride monohydrate (CPC; Sigma) in 0.2 mol/L NaCl and dissolved in 3 mol/L NaCl. The amount of radioactivity was measured by liquid scintillation analysis and normalized to the specific activity of the pulse medium, labelling time, and cartilage weight in case of the tibial explants. As a measure of cartilage matrix PSR, sulphate incorporation rate is expressed as nanomoles of sulphate incorporated per gram weight of tibial cartilage tissue (nmol/h.g) and as nanomoles of sulphate incorporation (pmol/h) per patella.

2.4 | Baseline proteoglycan synthesis in WT rats

Tibial and patellar cartilage from WT rats (3 males, 3 females; Sprague Dawley, Taconic, Lille Skensved, Denmark) not subjected to an induced haemarthrosis was obtained *postmortem* and used to determine baseline PSR.

2.5 | Proteoglycan synthesis in WT rats after 4-day blood exposure in vitro

Patellar and tibial cartilage from 9 surplus WT rats (all females, Sprague Dawley, bred at UMCU, the Netherlands) were obtained and cultured in culture medium for 4 days in a 96-well round-bottomed microtiter plate at 5% CO2 in air, 37°C, and 95% humidity. Patellar (n = 5) and tibial (n = 4) explants obtained from the left knee were cultured in the presence of 50% volume/volume (v/v) whole blood, drawn in a BD vacutainer heparin tube from a healthy WT rat, to mimic a joint bleed.[22] The samples derived from the contralateral knee were cultured in medium only. After 4 days of culturing, the explants were washed by two 20-minute incubations in culture medium to remove blood components, and the PSR was determined, as described above.

2.6 | Assessment of cartilage degeneration in a haemophilic rat model of joint damage by experimentally inducing a joint bleed

The in vivo study included 39 $F8^{-/-}$ rats (Sage: SD-$F8^{tm1sage}$; 18 males, 21 females) on a Sprague-Dawley background, bred at Novo Nordisk A/S (Maaloev, Denmark). As the study was exploratory, the number of animals used considered that up to 20% of the rats could be euthanized due to humane endpoints and that sufficient cartilage isolation could be unsuccessful in up to 20% of the knee joints. The rats were housed and monitored daily as detailed in Supporting Information. All invasive procedures were performed under inhalation anaesthesia (5% isoflurane, 0.7 L/min O_2 and 0.3 L/min N_2O for induction; 2% isoflurane, 0.7 L/min O_2 and 0.3 L/min N_2O for maintenance).

In brief, anaesthetized rats received a subcutaneous (SC) dose of buprenorphine analgesia (0.05 mg/kg, Temgesic, Indivior UK Limited) and haemarthrosis was induced by needle puncture as previously described[14,15] and detailed in Supporting Information. To confirm successful induction of haemarthrosis, joint swelling was characterized by measuring the knee diameters before induction and 24 hours after induction, as previously described.[23] At both time points, the diameter of each knee joint was measured five times with a digital calliper (Mitutoyo Corporation). The delta diameter was calculated as the difference of the mean diameter of the blood-exposed knee minus the mean diameter of the contralateral knee.

Four ('4-day group') and 16 ('16-day group') days after induction of haemarthrosis, animals were euthanized by intracardial injection of 1 mL pentobarbital (Mebumal, SAD, Amgros I/S, 50 mg/mL) while in general anaesthesia.

Knees were excised *postmortem* and subsequently imaged with micro-computed tomography (micro-CT, Quantum FX, Perkin Elmer) at field of view 20 mm, 90 kV, 160 μA with 4.5 minutes acquisition time. Micro-CT scans were blindly evaluated for pathological bone remodelling with Quantum FX 2.2 (Perkin Elmer) by reviewing 2D images in the coronal, sagittal and transverse plane. Each scan

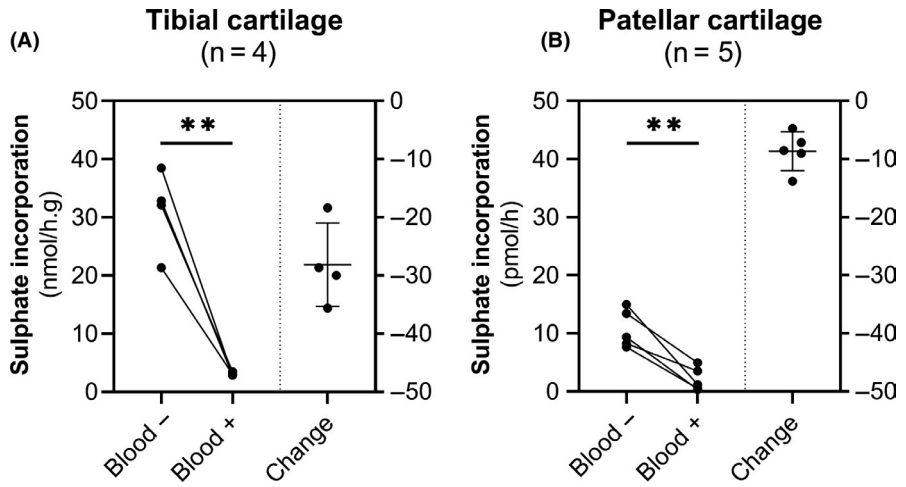

FIGURE 1 Proteoglycan synthesis after four day culture in vitro in wild type rats. Four-day blood exposure in vitro resulted in a significant decrease in sulphate incorporation rate in both tibial (A, n = 4; expressed as nmol of sulphate incorporated per hour per gram of tissue) and patellar (B, n = 5; expressed as pmol of sulphate incorporated per hour) cartilage, resulting in a negative change (blood-exposed knee minus contralateral knee, depicted as mean ± standard deviation). Paired t test for the difference in proteoglycan synthesis between cartilage exposed to blood (blood +) or culture medium only (blood -), $^{**}P \leq .01$

received a score from 0 to 7 based on the presence/absence of seven pathological bone changes: osteophytosis (femur, patella and tibia), periosteal bone remodelling (femur, patella and tibia) and/or subchondral cyst, as previously described.[24]

Following micro-CT imaging, the hind legs including the skin were stored in a 50 mL tube in a foam box at a constant temperature of approximately 4°C and transported to UMCU by airplane. Within 24 hours of euthanasia, tibial cartilage and patellar cartilage from both knees were obtained and the PSR measured ex vivo, as described above.

2.7 | Statistical analysis

Differences in PSR, joint diameter and micro-CT scores between paired samples (left and right knee of the same animal) were analysed using the paired t test or the Wilcoxon signed-rank test, when appropriate. Differences in PSR between groups were analysed using the Mann-Whitney test. Correlation between the level of PSR in the blood-exposed and contralateral knee was analysed using the Pearson correlation coefficient. Results were considered significant if $P < .05$. Graphic presentation and statistical analyses were performed using GraphPad Prism (Version 8.0.1; GraphPad Software Inc).

3 | RESULTS

3.1 | Blood exposure decreases PSR in tibial and patellar cartilage of WT rats in vitro

First, cartilage of healthy bleeding-naïve control animals (n = 12 knees) was obtained to establish baseline PSR directly after isolation. On average, a total of 1.2 mg ± 0.4 (mean ± standard deviation (SD)) tibial cartilage per joint were obtained. Tibial and patellar baseline PSR was 12.9 nmol/h.g ± 8.4 and 22.3 pmol/h ± 5.1, respectively.

Next, the effect of blood exposure on cartilage was evaluated in vitro. After 4 days of culturing in vitro, the PSR of healthy tibial cartilage (n = 4) was 31.2 nmol/h.g ± 7.1, compared to 3.1 nmol/h.g ± 0.3 upon blood exposure, corresponding to a 90% decrease (Figure 1A, $P = .004$). In patellar cartilage (n = 5), the PSR was decreased by 81% (Figure 1B, healthy (10.7 pmol/h ± 3.3) vs blood-exposed cartilage (2.1 pmol/h ± 2.0), $P = .004$).

3.2 | *Induction of haemarthrosis causes joint swelling and pathological bone remodelling in F8$^{-/-}$ rats*

In total, 32 out of 39 animals subjected to haemarthrosis completed the study (4-day group n = 14, 16-day group n = 18). Successful induction of haemarthrosis was confirmed by measuring the diameter of the knees; the day after induction, the diameter of the injured knee was significantly increased compared to the contralateral side (4-day group: median 2.78 mm vs −0.02 mm, $P < .001$; 16-day group, median 2.65 mm vs −0.02 mm, $P < .001$, Figure 2).

After euthanasia, blood-induced joint damage was confirmed by scoring the knees for presence of pathological bone remodelling on micro-CT (Figure 3A). The blood-exposed knees had a significantly higher bone pathology score than the contralateral knees in the 16-day group (median score of 5 vs 0, $P \leq .001$), but no difference in the bone pathology score was observed between the injured knee and contralateral knee in the 4-day group (median score of 0 vs 0, $P = .22$). In the 4-day group, 3 out of 14 rats developed bone pathology in the blood-exposed knee (score 1-2), whereas in the 16-day group, 13 out of 18 rats developed bone pathology in the blood-exposed knee

(score 3-7, Figure 3B). In the latter group, one contralateral knee had a score of 1. Bone changes were equally distributed between the femur, patella and tibia (data not shown).

3.3 | Haemarthrosis results in decreased tibial PSR

Equal amounts of tibial cartilage were obtained from the contralateral and blood-exposed knees (4-day group: 1.4 mg ± 0.6 vs 1.4 mg ± 0.6, 16-day group: 1.3 mg ± 0.6 vs 1.5 mg ± 0.4). Samples not meeting the minimum weight were excluded (4-day group n = 1/14, 16-day group n = 4/18).

Four days after induction of haemarthrosis, the PSR of tibial cartilage from the contralateral knee was 5.1 nmol/h.g (4.0-16.7) (median with interquartile range (IQR)), compared to 2.5 nmol/h.g (1.5-8.1) in the blood-exposed knee (Figure 4A). This corresponds to a significant decrease of 52% in PSR of the blood-exposed knee compared to the contralateral knee (P = .007) and 81% compared to bleeding-naïve control animals (P = .005). One statistical outlier was excluded from this analysis.

Sixteen days posthaemarthrosis, a low proteoglycan synthesis was not only noted in the blood-exposed knees (2.0 nmol/h.g (1.1-6.6)), but also in the contralateral knees (2.7 nmol/h.g (0.8-4.5)) (Figure 4B; P = .855). Blood exposure resulted in a decrease of 85% in PSR compared to the control group (P = .003).

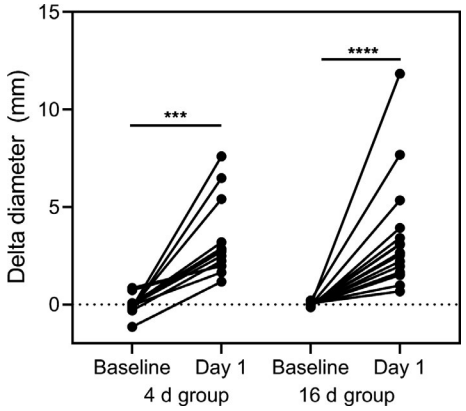

FIGURE 2 Joint swelling confirms induction of haemarthrosis in F8$^{-/-}$ rats. The diameter of the induced and contralateral knee was measured before and one day after induction of haemarthrosis. The difference between the injured and contralateral knee, the delta diameter, increased significantly on Day 1 in both the groups euthanized 4 and 16 d after injury. ***P < .001, ****P < .0001, Wilcoxon's signed-rank test

3.4 | Haemarthrosis results in decreased patellar PSR

In a subgroup of animals (4-day group n = 7, 16-day group n = 6), the patellar PSR was assessed, demonstrating similar results as seen in the tibia. Induced haemarthrosis led to a decreased PSR of 60% compared to the contralateral knee after 4 days (Figure 4C, contralateral knee 31.9 pmol/h (20.7-38.4) *vs* blood-exposed knee 13.4 pmol/h (10.4-18.4), P = .016) and a decrease of 40% compared to bleeding-naïve controls(P = .005).

Sixteen days after the induced haemarthrosis, comparable low PSR was seen in both knees (Figure 4D, contralateral knee 11.2 pmol/h (9.3-19.4) *vs* blood-exposed knee 12.2 pmol/h (10.7-18.3), P = .844), and blood exposure led to a significant decrease in PSR of 45% compared to the control group (P = .003).

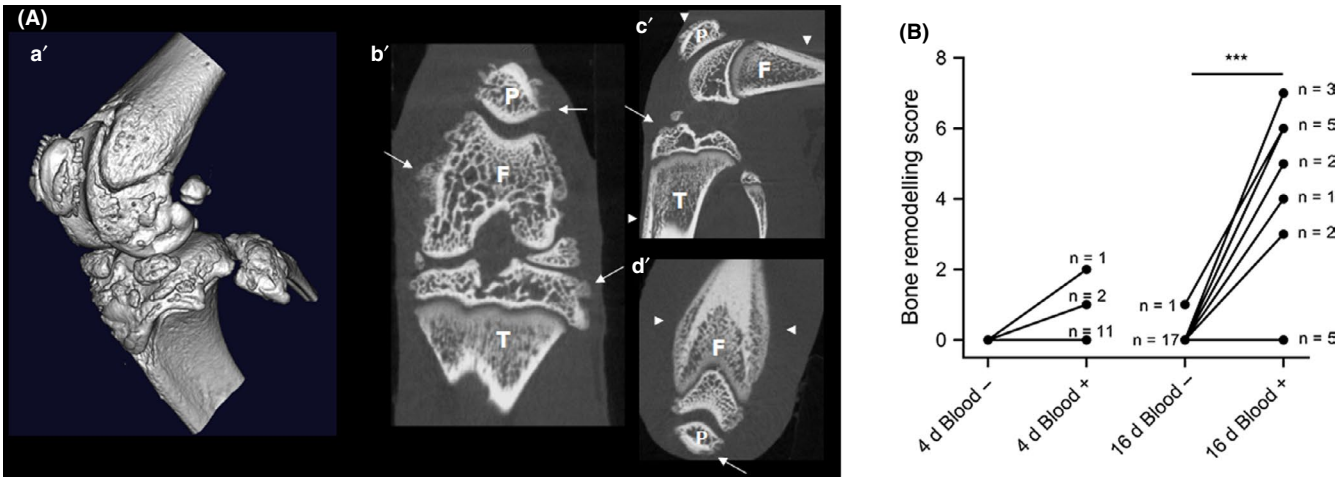

FIGURE 3 Bone pathology after induced haemarthrosis. Knee-injured F8$^{-/-}$ rats were euthanized 4 or 16 d (d) after injury, and pathological bone remodelling scored from 0 to 7. A, Injured knee of F8$^{-/-}$ rat displaying extensive bone remodelling. a': 3D volume of the injured knee. b', c', d': Coronal (b'), sagittal (c') and transverse (d') section of the injured knee showing osteophytosis (arrows) and periosteal bone remodelling (arrowheads) on femur (F), patella (P) and tibia (T). B, Bone remodelling score was significantly increased in the injured (blood +) vs contralateral (blood -) knees of F8$^{-/-}$ rats euthanized 16 d after injury. ***P < .001, Wilcoxon's signed-rank test

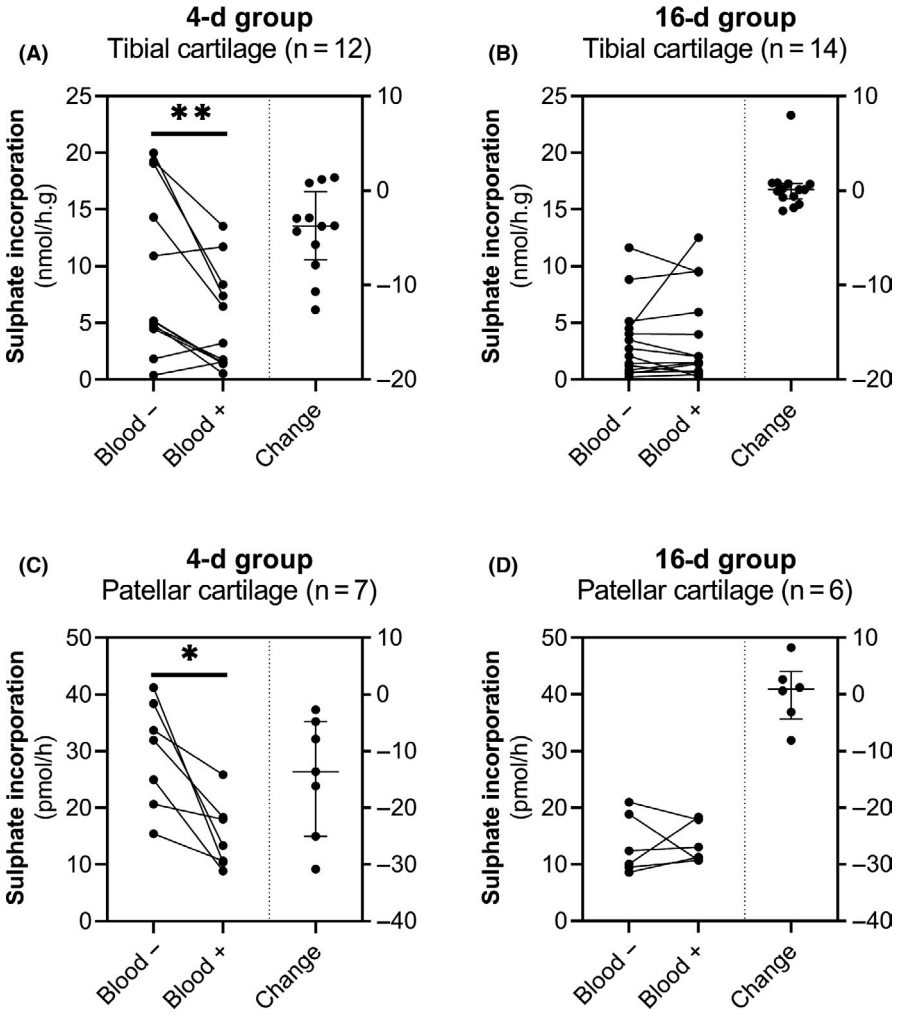

FIGURE 4 Proteoglycan synthesis rate 4 and 16 d after induced haemarthrosis in $F8^{-/-}$ rats. Sulphate incorporation was measured 4 and 16 d (d) after an induced joint bleed, in tibial (A; 4 d, C; 16 d; normalized to tissue weight) and patellar (B; 4 d, D; 16 d) cartilage. At 4 d, the proteoglycan synthesis rate was significantly decreased in the blood-exposed compared to the contralateral knee, $^{*}P \leq 0.05$, $^{**}P \leq .01$, Wilcoxon's signed-rank test. Change (blood-exposed minus contralateral knee) is depicted as median ± interquartile range

3.5 | Induced haemarthrosis affects the cartilage of the contralateral knee

Sixteen days after induced haemarthrosis, the PSR rate in the contralateral knees also appeared to be decreased. To analyse this, we compared the PSR across the 4- and 16-day group (Figure 5A and B). While there was no difference in the PSR between the injured knees of the 4- and 16-day groups (tibia $P = .491$, patella $P = .624$), the PSR in the contralateral knees was significantly lower in the 16-day group compared to the 4-day group (tibia $P = .013$, patella $P = .008$). In addition, no statistically significant differences in PSR were found between the contralateral knee at day 4 and the control group (tibia $P = .225$, patella $P = .100$), whereas the PSR in the contralateral knees in the 16-day group was significantly different from the control group (tibia $P \leq .001$, patella $P = .007$). Moreover, in the 16-day group, we found a significant correlation between the PSR in the blood-exposed *vs* the contralateral knee for the tibia (Figure 5C and D, $r = .803$, $P \leq .001$), but not the patella ($r = .259$, $P = .620$).

4 | DISCUSSION

In the present study, we demonstrate that proteoglycan synthesis can be quantified with the $^{35}SO_4^{2-}$assay in healthy tibial and patellar cartilage of the rat and that it is affected by blood exposure in vitro. Further, we demonstrate for the first time in a haemophilic animal model that induced joint bleeds lead to decreased proteoglycan synthesis, establishing the $^{35}SO_4^{2-}$ assay as a novel method for detecting early blood-induced cartilage damage.

The proteoglycan synthesis found in healthy cartilage of WT rats after the 4-day culture was clearly higher than in human and canine cartilage cultured in vitro under the same conditions.[4,6-7,22,25] This might be explained by a higher turnover of cartilage in small animals.[26] The relative inhibition of proteoglycan synthesis after 4 days 50% v/v blood exposure in vitro in tibial (90%) and patellar (81%) cartilage was comparable to data from human and canine explants (74%-99%).[4,6-7,22,25]

After confirmation of the use of the $^{35}SO_4^{2-}$ assay to study blood-induced cartilage damage in an in vitro model, feasibility of the assay was tested in an in vivo design. Whereas previous studies

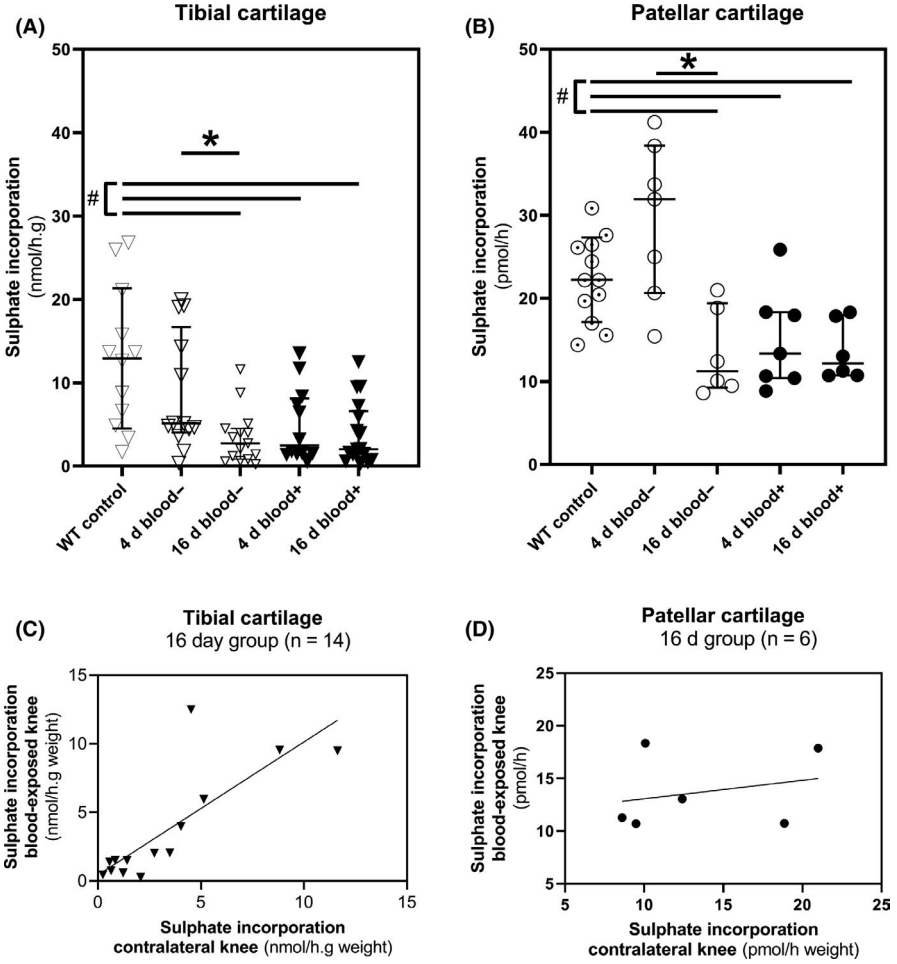

FIGURE 5 Evaluation of proteoglycan synthesis rate in the contralateral knee. A and B: Sulphate incorporation in the tibia (A) and patella (B) of bleeding naive control wild-type (WT) rats and blood-exposed (blood+) and contralateral (blood-) knees of $F8^{-/-}$ rats, 4 and 16 d after haemarthrosis induction. Sulphate incorporation in the tibia and patella of the contralateral knees was significantly decreased in rats euthanized 16 d after induced haemarthrosis compared with rats euthanized 4 d after induced haemarthrosis, $^{*}P < .05$, Mann-Whitney test. A significant difference compared to control animals was seen in all blood-exposed joints, as well as the contralateral joints of the 16-day group, $^{\#}P < .05$, Mann-Whitney test. C and D: Sulphate incorporation was significantly correlated between the injured knee and the contralateral knee regarding the tibia (C; $r = 0.803$, $P \leq .001$), but not the patella (D; $r = .259$, $P = .620$) 16 d after haemarthrosis

have measured a blood-induced decrease in proteoglycan synthesis either following in vitro blood exposure[6-7,25] or following intra-articular injection of blood in WT animals,[8,27,28] this is the first study in which a decrease in proteoglycan synthesis has been demonstrated in haemophilic animals. Upon induced haemarthrosis in haemophilic rodent models, the entire joint is rapidly filled with blood,[15] and the bleeding remains unresolved for several days.[9,29] In contrast, intra-articular injection of autologous blood is cleared quickly (<1 day) in WT animals.[30] Nonetheless, our findings of decreased proteoglycan synthesis in the blood-exposed knee 4 days after injury corroborates with reports from WT animals injected intra-articularly with autologous blood, but with a more pronounced relative decrease in proteoglycan synthesis (52% in tibia and 60% in patella vs up to 36% in literature[4,8,28,31]). This may reflect a higher degree of blood exposure in haemophilic animals.

Surprisingly, we did not find any difference in PSR between the blood-exposed knee and the contralateral knee 16 days after injury. This observation did not reflect an enhanced synthesis in the blood-exposed knee, as seen in the canine model,[8] but rather a decreased synthesis in the contralateral knee. The persistent, low proteoglycan synthesis observed in the blood-exposed knee may be due to the relatively large and untreated bleed induced in the haemophilic model, in combination with the previously discussed slower clearance of blood from the joint. Second, the decreased proteoglycan synthesis in the contralateral knee appears to be related to the degree of cartilage damage in the blood-exposed knee, a finding described in rodent models of degenerative joint disease as well.[32] This correlation was demonstrated for tibial, but not patellar cartilage. It is possible that shifted weight-bearing following an induced joint bleed[29,33] increases the mechanical load on the contralateral leg,[34] potentially damaging the cartilage.[32] Further, haemophilic rats are susceptible to spontaneous (micro)bleeds that could lead to direct cartilage damage,[35] a phenomenon which could be enhanced by increased weight-bearing in the contralateral joint. In addition, previous studies have shown that in haemophilic rodent models, an induced joint bleed elicits a local and systemic angiogenic stimulus and upregulation of pro-inflammatory cytokines.[5,29,36] Thus, it cannot be ruled out that contralateral cartilage damage is induced by systemic modulators induced by the joint bleed. Moreover, neurogenic inflammation has been suggested as a cause of contralateral damage.[37,38] Local inflammatory processes induce local neurogenic stimulation, which may cause a bilateral response of the nervous system resulting in bilateral cartilage degeneration.[39]

On histology, cartilage and bone changes in the $F8^{-/-}$ rat occur simultaneously,[9] whereas we observed a significant decrease in proteoglycan synthesis before pathological bone remodelling became evident (on day 4). Possibly, this reflects the high sensitivity of the $^{35}SO_4^{2}$ assay for detecting early cartilage damage.[17] In

contrast to the cartilage, the bone of the contralateral knee was not affected after 16 days, as evaluated by micro-CT. Pathological bone remodelling and upregulation of a signalling pathway leading to osteopenia have been shown to correlate with the amount of blood in the joint,[15,40] and thus, it is possible that spontaneous microbleeds systemic changes induced by the bleed or increased weight-bearing in the contralateral knee are not sufficient to elicit bone changes.

Our study has some limitations. The induced joint bleeds were only characterized by joint diameter, and therefore, a possible correlation between the joint bleed volume and degree of cartilage damage could not be investigated. Proteoglycan synthesis was assessed within 24 hours of euthanasia, and although in vitro experiments found no differences when measurements were done immediately or 24 hours after euthanasia (data not shown), we cannot completely rule out that time and transportation may have affected the chondrocyte activity. Also, patellar cartilage was only obtained from a subgroup of rats, and thus, conclusions are made from a relatively small number of animals (4-day group: n = 7, 16-day group, n = 6). Finally, baseline proteoglycan synthesis should ideally be assessed in $F8^{-/-}$ rats, which was not feasible due to availability of the rats. While this does not affect the conclusion that the PSR in the contralateral knees was significantly lower in the 16-day group compared to the 4-day group, the contralateral effects of haemarthrosis on PSR in this model could not be fully addressed since the baseline measurements were done in WT rats (on the same genetic background) that potentially could have a different baseline PSR. However, WT rats have the advantage that the baseline PSR was measured in the absence of potential subclinical bleeds.

5 | CONCLUSION

In conclusion, we show that the well-established $^{35}SO_4^{2-}$assay can be used to detect blood-induced cartilage damage after a single induced haemarthrosis in the $F8^{-/-}$ rat. As no current treatment specifically targets early blood-induced cartilage damage in haemophilia, this assay combined with the haemophilic rat as a pharmacological model could be used to test novel therapies. However, the use of the contralateral knee as control is questionable, as contralateral cartilage damage was seen 16 days after induced haemarthrosis.

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DISCLOSURES

K. Roepstorff is a full-time employee of LEO Pharma A/S. M. Petersen is a full-time employee of Novo Nordisk A/S. KK Vøls, K. Roepstorff and M. Petersen are shareholders of Novo Nordisk A/S. The other authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the study. KKV and KRC conducted the in vivo part of the study. AEP and KC conducted the in vitro and ex vivo part of the study. All authors contributed to the data analysis. AEP and KKV conducted the statistical analysis and drafted the manuscript. All authors revised and approved the manuscript.

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REFERENCES

1. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1-e47.
2. Shaerf D, Banerjee A. Assessment and management of posttraumatic haemarthrosis of the knee. *Br J Hosp Med*. 2008;69(8):459-460, 462–463.
3. Saksena J, Platts AD, Dowd GS. Recurrent haemarthrosis following total knee replacement. *Knee*. 2010;17(1):7-14.
4. Hooiveld M, et al. Blood-induced joint damage: longterm effects in vitro and in vivo. *J Rheumatol*. 2003;30(2):339-344.
5. Cooke E, Zhou J, Wyseure T, et al. Vascular permeability and remodelling coincide with inflammatory and reparative processes after joint bleeding in factor VIII-deficient mice. *Thromb Haemost*. 2018;118(6):1036-1047.
6. Jansen NWD, Roosendaal G, Bijlsma JWJ, DeGroot J, Lafeber FPJG. Exposure of human cartilage tissue to low concentrations of blood for a short period of time leads to prolonged cartilage damage: an in vitro study. *Arthritis Rheum*. 2007;56(1):199-207.
7. Hooiveld M, Roosendaal G, Wenting M, van den Berg M, Bijlsma J, Lafeber F. Short-term exposure of cartilage to blood results in chondrocyte apoptosis. *Am J Pathol*. 2003;162(3):943-951.
8. Roosendaal G, TeKoppele JM, Vianen ME, Van Den Berg HM, Lafeber FPJG, Bijlsma JWJ. Blood-induced joint damage: a canine in vivo study. *Arthritis Rheum*. 1999;42(5):1033-1039.
9. Christensen KR, Kjelgaard-Hansen M, Nielsen LN, et al. Rapid inflammation and early degeneration of bone and cartilage revealed in a time-course study of induced haemarthrosis in haemophilic rats. *Rheumatology (Oxford)*. 2019;58(4):588-599.
10. Pulles AE, Mastbergen SC, Schutgens REG, Lafeber FPJG, van Vulpen LFD. Pathophysiology of hemophilic arthropathy and potential targets for therapy. *Pharmacol Res*. 2017;115:192-199.
11. van Vulpen LFD, Holstein K, Martinoli C. Joint disease in haemophilia: Pathophysiology, pain and imaging. *Haemophilia*. 2018;24(Suppl 6):44-49.
12. van Vulpen LF, van Meegeren ME, Roosendaal G, et al. Biochemical markers of joint tissue damage increase shortly after a joint bleed; An explorative human and canine in vivo study. *Osteoarthritis Cartilage*. 2015;23(1):63-69.
13. Pulles AE, Mastbergen SC, Foppen W, Schutgens REG, Lafeber F, van Vulpen LFD. The combination of urinary CTX-II and serum CS-846: Promising biochemical markers to predict radiographic progression of haemophilic arthropathy-An exploratory study. *Haemophilia*. 2018;24(4):e278-e280.
14. Sorensen KR, Roepstorff K, Wiinberg B, et al. The F8(-/-) rat as a model of hemophilic arthropathy. *J Thromb Haemost*. 2016;14(6):1216-1225.
15. Vøls KK, Kjelgaard-Hansen M, Ley CD, Hansen AK, Petersen M. Bleed volume of experimental knee haemarthrosis correlates with

the subsequent degree of haemophilic arthropathy. *Haemophilia*. 2019;25(2):324-333.
16. Lafeber F, Vander Kraan PM, Van Roy J, Huber-Bruning O, Bijlsma JWJ. Articular cartilage explant culture; an appropriate in vitro system to compare osteoarthritic and normal human cartilage. *Connect Tissue Res*. 1993;29(4):287-299.
17. van Osch GJ, van der Kraan PM, van den Berg WB. Site-specific cartilage changes in murine degenerative knee joint disease induced by iodoacetate and collagenase. *J Orthop Res*. 1994;12(2):168-175.
18. Dumond H, Presle N, Pottie P, et al. Site specific changes in gene expression and cartilage metabolism during early experimental osteoarthritis. *Osteoarthritis Cartilage*. 2004;12(4):284-295.
19. van Osch GJ, van der Kraan PM, van den Berg WB. In vivo quantification of proteoglycan synthesis in articular cartilage of different topographical areas in the murine knee joint. *J Orthop Res*. 1993;11(4):492-499.
20. Bulstra SK, Kuijer R, Eerdmans P, van der Linden AJ. The effect in vitro of irrigating solutions on intact rat articular cartilage. *J Bone Joint Surg*. 1994;76(3):468-470.
21. van den Berg WB, Kruijsen MWM, van de Putte LBA. The mouse patella assay. An easy method of quantitating articular cartilage chondrocyte function in vivo and in vitro. *Rheumatol Int*. 1982;1:165-169.
22. Roosendaal G, Vianen ME, Marx JJM, Van Den Berg HM, Lafeber FPJG, Bijlsma JWJ. Blood-induced joint damage: a human in vitro study. *Arthritis Rheum*. 1999;42(5):1025-1032.
23. Elm T, Karpf DM, Øvlisen K, et al. Pharmacokinetics and pharmacodynamics of a new recombinant FVIII (N8) in haemophilia A mice. *Haemophilia*. 2012;18(1):139-145.
24. Christensen KR, Roepstorff K, Petersen M, et al. Visualization of haemophilic arthropathy in F8(-/-) rats by ultrasonography and micro-computed tomography. *Haemophilia*. 2017;23(1):152-162.
25. Roosendaal G, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Cartilage damage as a result of hemarthrosis in a human in vitro model. *J Rheumatol*. 1997;24(7):1350-1354.
26. Mastbergen SC, Lafeber FPJG. Animal models of osteoarthritis—Why choose a larger model? *US Musculoskelet Rev*. 2009;4:11-14.
27. Roosendaal G, Tekoppele JM, Vianen ME, van den Berg HM, Lafeber FP, Bijlsma JW. Articular cartilage is more susceptible to blood induced damage at young than at old age. *J Rheumatol*. 2000;27(7):1740-1744.
28. Hooiveld MJJ, Roosendaal G, Vianen ME, Van Den Berg HM, Bijlsma JWJ, Lafeber FPJG. Immature articular cartilage is more susceptible to blood-induced damage than mature articular cartilage: an in vivo animal study. *Arthritis Rheum*. 2003;48(2):396-403.
29. Bhat V, Olmer M, Joshi S, et al. Vascular remodeling underlies rebleeding in hemophilic arthropathy. *Am J Hematol*. 2015;90(11):1027-1035.
30. Sun J, Hua B, Livingston EW, et al. Abnormal joint and bone wound healing in hemophilia mice is improved by extending factor IX activity after hemarthrosis. *Blood*. 2017;129(15):2161-2171.
31. Jansen N, Roosendaal G, Wenting M, et al. Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue. *Osteoarthritis Cartilage*. 2009;17(4):433-440.
32. Meyer P, Burkhardt H, Palombo-Kinne E, et al. 123I-antileukoproteinase scintigraphy reveals microscopic cartilage alterations in the contralateral knee joint of rats with "monarticular" antigen-induced arthritis. *Arthritis Rheum*. 2000;43(2):298-310.
33. Mejia-Carvajal C, Hakobyan N, Enockson C, Valentino LA. The impact of joint bleeding and synovitis on physical ability and joint function in a murine model of haemophilic synovitis. *Haemophilia*. 2008;14(1):119-126.
34. Schött E, Berge O-G, Ängeby-Möller K, Hammarström G, Dalsgaard C-J, Brodin E. Weight bearing as an objective measure of arthritic pain in the rat. *J Pharmacol Toxicol Methods*. 1994;31(2):79-83.
35. Nielsen LN, Wiinberg B, Häger M, et al. A novel F8 -/- rat as a translational model of human hemophilia A. *J Thromb Haemost*. 2014;12(8):1274-1282.
36. Lövgren K, Christensen K, Majewski W, Østrup O, Skov S, Wiinberg BO. Acute haemarthrosis in the Haemophilia A rat generates a local and systemic proinflammatory response. *Thromb Haemost*. 2017;117(11):2092-2104.
37. von Banchet GS, Petrow PK, Bräuer R, Schaible HG. Monoarticular antigen-induced arthritis leads to pronounced bilateral upregulation of the expression of neurokinin 1 and bradykinin 2 receptors in dorsal root ganglion neurons of rats. *Arthritis Res*. 2000;2(5):424-427.
38. Bileviciute-Ljungar I, Saxne T, Spetea M. Anti-inflammatory effects of contralateral administration of the kappa-opioid agonist U-50,488H in rats with unilaterally induced adjuvant arthritis. *Rheumatology (Oxford)*. 2006;45(3):295-302.
39. Kidd BL, Cruwys SC, Garrett NE, Mapp PI, Jolliffe VA, Blake DR. Neurogenic influences on contralateral responses during experimental rat monoarthritis. *Brain Res*. 1995;688(1–2):72-76.
40. Haxaire C, Hakobyan N, Pannellini T, et al. Blood-induced bone loss in murine hemophilic arthropathy is prevented by blocking the iRhom2/ADAM17/TNF-α pathway. *Blood*. 2018;132(10):1064-1074.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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