Degenerated and healthy cartilage are equally vulnerable to blood-induced damage

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ABSTRACT

Background: Joint bleeds have a direct adverse effect on joint cartilage, leading to joint deterioration and, ultimately, to disability.

Objective: To examine the hypothesis that because degenerated cartilage has a limited repair capacity, it is more susceptible than healthy cartilage to blood-induced cartilage damage.

Methods: Healthy, degenerated (preclinical osteoarthritic) and osteoarthritic (clinically defined) human cartilage was exposed to 10% vol/vol whole blood for 2 days, followed by a recovery period of 12 days in the absence of blood. The effect of exposure to blood on cartilage was determined by measuring proteoglycan synthesis rate, release and content, as well as protease (matrix metalloproteinase (MMP)) activity.

Results: In general, exposure to blood led to a decrease in proteoglycan synthesis rate, an increase in the release of proteoglycans and in MMP activity, and therefore, ultimately, in a decrease of the proteoglycan content of the tissue. Impaired cartilage was as least as susceptible as healthy cartilage to this blood-induced damage.

Conclusion: These results demonstrate that degenerated cartilage is not more susceptible than healthy cartilage to blood-induced damage. Even though these are just in vitro findings, it remains of great importance, also, in joints already affected, to prevent joints bleeds, and when they do occur, to treat them adequately.

Articular cartilage becomes damaged when it is exposed to blood, as occurs during a joint bleed.[1 2] This damage is the consequence of the formation of hydroxyl radicals in the vicinity of the articular chondrocytes, leading to apoptosis of these chondrocytes.[3] These hydroxyl radicals form when hydrogen peroxide, synthesised by chondrocytes upon stimulation by interleukin 1, originating from activated monocytes/macrophages present in the blood, reacts with haemoglobin-derived iron from damaged and phagocytosed red blood cells. The apoptosis of the chondrocytes leads to disturbance and impairment of the cartilage matrix turnover and hence to cartilage matrix damage.[4 5]

Previously it was shown that in vitro exposure of healthy cartilage to blood leads to a severe decrease in the synthesis rate of proteoglycans, one of the main matrix components of cartilage.[4 7 9 11] Furthermore, the release of these proteoglycans from the cartilage increases upon exposure to blood. Together, this results in a decrease in proteoglycan content. Also the activity of matrix metalloproteinases (MMPs), primarily collagenases,[12 13] increases after the exposure of cartilage to blood[14] suggesting that collagen is also affected upon blood exposure. These effects are long lasting, even after a transient exposure of cartilage to a low concentration (10% vol/vol) of blood for a short period of time (2 days).[14] During a joint haemorrhage the actual exposure time and blood concentration is estimated to be far higher, >50% vol/vol for 4 days.[6]

Despite the in vitro results described above, surprisingly the current general opinion among doctors remains that a few joint bleeds are acceptable. However, cartilage already affected by a previous joint bleed, trauma or degenerative processes as seen, for example, in osteoarthritis, might even be more susceptible to blood-induced damage than healthy cartilage. The rationale for this is that osteoarthritic cartilage is characterised by a disturbed proteoglycan turnover, damaged collagen and has been associated with chondrocyte apoptosis,[9 15 16] and thus is likely to have an impaired repair capacity. This suggests that in the case of degenerated/osteoarthritic cartilage, joint bleeds may be more deleterious and specifically in these cases should be prevented or, when they occur, treated appropriately to avoid extra stimulation of the progressive degeneration with consequently severe joint damage in later years. We therefore investigated whether degenerated (preclinical osteoarthritic)[17] and osteoarthritic (clinically defined) cartilage are more susceptible to blood-induced damage than healthy cartilage.

MATERIALS AND METHODS

Cartilage culture

Three types of human cartilage were obtained: healthy (66.8 (5.5) years; male/female = 4/2), degenerated (preclinical osteoarthritic; 78.4 (3.6) years; male/female = 3/4)[17] and osteoarthritic (clinically defined; 66.5 (4.3) years; male/female = 2/4) articular cartilage. Healthy and degenerated cartilage were obtained post mortem from humeral heads within 24 hours after death of the donor. Healthy cartilage had a glossy, white, completely smooth surface and a healthy appearance. Degenerated cartilage had a macroscopic fibrillation of the surface and came from older donors without documented clinical history of joint disorders.[17] Osteoarthritic cartilage was obtained from the femoral knee condyles during joint replacement surgery. Collection of the cartilage was according to the medical ethical regulations of the University Medical Centre Utrecht.

Slices of cartilage were cut aseptically as thick as possible, excluding the underlying bone and kept in phosphate-buffered saline (pH 7.4). Within 1 h after dissection, the slices were cut into square

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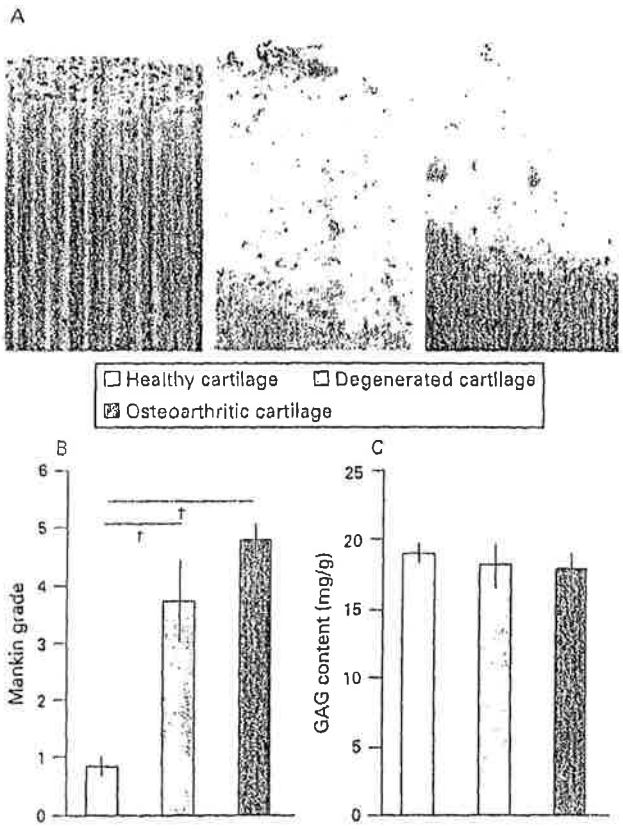

Figure 1 Characteristics of the three types of cartilage: cartilage integrity. Representative light micrographs of healthy (A; left panel), degenerated (A; middle panel) and osteoarthritic cartilage (A; right panel) and graphs depicting the average Mankin grade (B) and average proteoglycan content (C). The samples depicted in (A) were scored 0, 5, and 6, respectively, according to the modified Mankin grade.[20 21] Original magnification ×4. Number of samples: healthy cartilage (n = 6), degenerated cartilage (n = 7) and osteoarthritic cartilage (n = 6). †Significant difference ($p<0.05$) between the different types of cartilage. GAG, glycosaminoglycan.

pieces, weighed aseptically (range 5–15 mg, accuracy ±0.1 mg) and cultured individually in 96-well, round-bottomed microtitre plates in 200 µl culture medium per well according to standard procedures.[14] This culture system has been used previously and has been shown to be a reliable system for comparing human healthy, degenerated (preclinical osteoarthritic) and osteoarthritic (end-stage) cartilage tissue.[19 20 22]

For each experiment fresh blood from healthy human donors was collected into heparinised vacutainer tubes (170 IU Li-heparin/10 ml) and added in 10% vol/vol to the cartilage tissue explants immediately after it was obtained.

After an exposure of 2 days, the cartilage was washed twice in fresh culture medium for 45 minutes under culture conditions to remove all the adherent blood components. Subsequently, some of the samples were analysed. The remaining samples were cultured for an additional period of 12 days in the absence of blood. In these cultures, medium was refreshed every 4 days. These prolonged cultures in the absence of blood gave the chondrocytes the ability to recover from the blood exposure, enabling evaluation of the (ir)reversibility of the observed effects of blood exposure. After this recovery period, these cartilage samples were also analysed.

Analysis

The synthesis rate of sulphated proteoglycans was determined by a 4 h pulse labelling with $^{35}SO_4$. After precipitation of the proteoglycans from a papain digest of the cartilage tissue, radioactivity was counted by liquid scintillation analysis and normalised to the specific activity of the medium. The rate of sulphate incorporation is expressed as nanomoles of sulphate incorporated per hour per gram wet weight and per milligram DNA of cartilage tissue.[23 25]

To determine the proteoglycan content, glycosaminoglycans (GAGs) of the proteoglycans were stained, precipitated with alcian blue and determined by colorimetric assay using chondroitin sulphate as a reference.[23 25] Proteoglycan content is expressed in milligrams GAG per gram wet weight of cartilage tissue.

DNA content as a measure of the cellularity was determined fluorimetrically using Hoechst 33258 staining with calf thymus DNA as a reference.[23 25] DNA content is expressed as milligrams DNA per gram wet weight of cartilage tissue.

Proteoglycan release was determined by the loss of GAGs in the culture medium from day 3 to 6 (direct effect) and from day 11 to 14 (effect after recovery), determined as described above[23 25] and expressed as milligrams GAG per wet weight or milligrams DNA of the cartilage samples.

General MMP activity was determined in the culture supernatants using the conversion of an internally quenched fluorogenic substrate TNO211-F in the presence or absence of a general MMP inhibitor (BB94).[12 13 26] MMP activity is expressed as relative fluorescence units per second between samples with and without BB94 to correct for non-MMP protease activity, and normalised to the wet weight and DNA content of the cartilage tissue.

To determine the severity of cartilage degeneration, histological sections of the cartilage were graded for features of degeneration. Safranin-O–fast green–iron haematoxylin-stained sections from three formalin-fixed tissue samples from each donor were graded according to the modified[20] criteria of Mankin.[21]

Calculations and statistical analysis

Because of focal differences in composition and bioactivity of cartilage specifically for degenerated and osteoarthritic tissue, the results for 10 cartilage samples per parameter per donor, obtained randomly and handled individually, were averaged and taken as a representative value for the cartilage of that donor. The n value (6–7) indicates the number of experiments (that is, the number of cartilage donors). The effect of exposure of cartilage to blood according to the type of cartilage was analysed using a non-parametric test for related samples (Wilcoxon signed-rank test, two-sided), whereas comparisons between the types of cartilage were analysed using a non-parametric test for unrelated samples (Mann–Whitney U, two-sided). Differences were considered significant when $p \leqslant 0.05$.

RESULTS

Characteristics of the three types of cartilage

Cartilage integrity

Figure 1A shows representative micrographs of the healthy, degenerated and osteoarthritic cartilage. Note the presence of chondrocyte clusters, the decrease in proteoglycan staining and the surface irregularities in the degenerated and osteoarthritic cartilage. These characteristics were more prominent in the osteoarthritic cartilage than in the degenerated cartilage. The

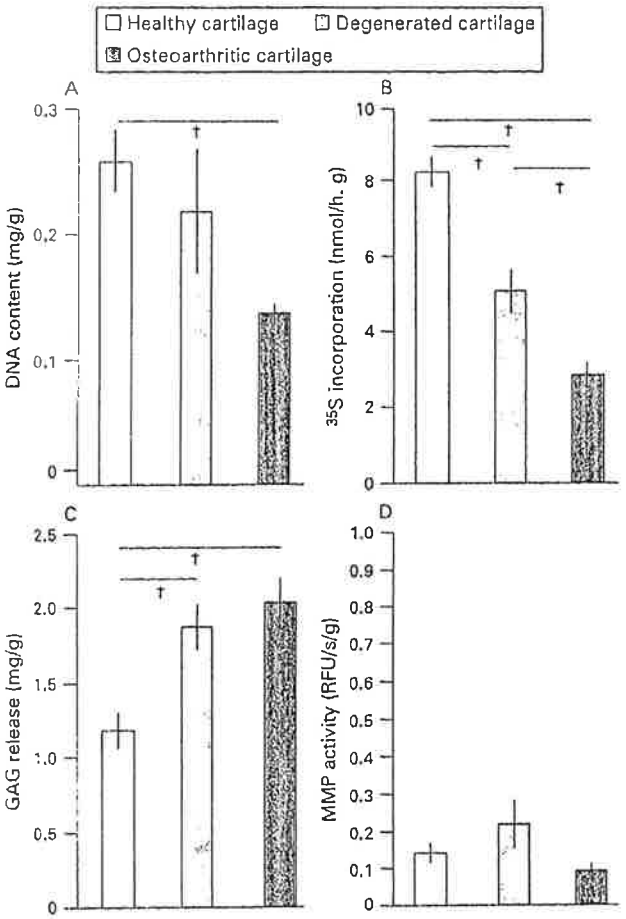

Figure 2 Characteristics of the three types of cartilage: cartilage chondrocyte activity. DNA content (A), proteoglycans synthesis rate (B), proteoglycan release (C) and matrix metalloproteinase (MMP) activity (D), normalised to the wet weight of the cartilage, are depicted. Number of samples: healthy cartilage (n = 6), degenerated cartilage (n = 7) and osteoarthritic cartilage (n = 6). †Significant difference ($p<0.05$) between the different types of cartilage. GAG, glycosaminoglycan.

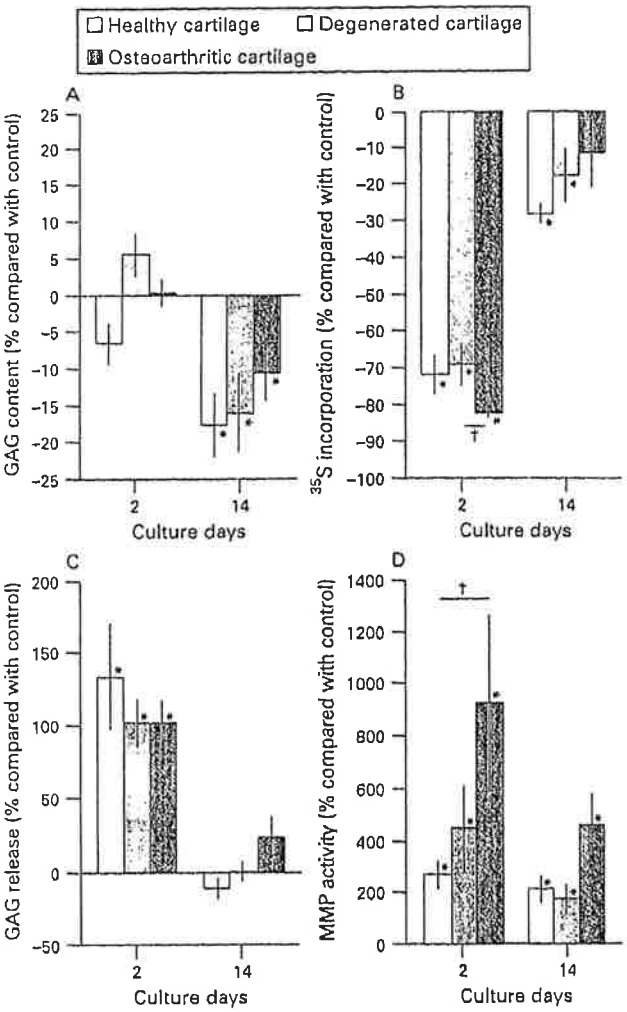

Figure 3 Effect of exposure of the three types of cartilage to blood. Human healthy (n = 6), degenerated (n = 7) and osteoarthritic (n = 6) cartilage tissue explants were exposed in vitro for 2 days to 10% vol/vol blood, followed by a 12-day recovery period. The proteoglycan content (A), synthesis rate (B), release (C) and MMP activity (D), all normalised to wet weight of the cartilage, are depicted as a percentage of their control in the absence of blood, both directly after the blood exposure at day 2 as well as after the recovery period at day 14. *Significant ($p<0.05$) change upon blood exposure; †significant ($p<0.05$) difference in the effect of blood exposure between the different types of cartilage. GAG, glycosaminoglycan; MMP, matrix metalloproteinase.

average histological grade (fig 1B) and proteoglycan content (fig 1C), all under control conditions (no addition of blood), supported the difference in degree of tissue integrity between the three types of cartilage, with degenerated cartilage giving intermediate values.

Cartilage chondrocyte activity

The variables related to chondrocyte activity showed clear differences between the three types of cartilage. The DNA content and proteoglycan synthesis rate were lower for osteoarthritic cartilage than for degenerated cartilage, and degenerated cartilage had a lower DNA content and synthesis rate than healthy cartilage (figs 2A and B, respectively). Release of proteoglycans was higher for osteoarthritic than for degenerated and healthy cartilage (fig 2C). Overall MMP activity demonstrates a different pattern; the highest activity was observed for the degenerated cartilage and this enhanced activity was lost for the osteoarthritic cartilage. Note that these differences, not statistically significant, may be irrelevant because blood could increase MMP activity multifold (see fig 3D).

Table 1 Characteristics of the three types of cartilage: cartilage chondrocyte activity normalised to cellularity (DNA content) of the cartilage

Type of cartilage	PG synthesis (nmol/h.mg)	PG release (mg/mg)	MMP activity (RFU/s/mg)
Healthy	34.2 (2.4)	4.5 (0.5)	0.6 (0.2)
Degenerated	28.7 (4.2)	10.4 (2.0)†	1.1 (0.4)
Osteoarthritic	21.9 (2.6)†	14.0 (1.4)†	1.6 (0.1)

Results are shown as mean (SD).
†$p<0.05$, significant difference compared with healthy cartilage.
MMP, matrix metalloproteinase; PG, proteoglycan; RFU, relative fluorescence units.

Table 2 Effect of exposure of the three types of cartilage to blood

Effect	Type of cartilage	DNA (%)	PG synthesis (%)	PG release (%)	MMP activity (%)
Direct effect	Healthy	6.23 (2.4)	−73.7 (5.3)*	159 (38)*	282 (72)*
	Degenerated	9.22 (7.3)	−71.2 (6.3)*	125 (28)*	489 (184)*
	Osteoarthritic	2.77 (5.7)	−82.3 (1.6)*	121 (16)*	1027 (353)* †
Effect after recovery	Healthy	−7.22 (3.0)	−21.7 (5.0)*	−1.54 (11.2)	223 (57)*
	Degenerated	−7.01 (8.1)	−8.7 (11.1)	11.0 (10.6)	221 (81)*
	Osteoarthritic	−6.72 (3.1)	−3.6 (11.2)	37.8 (19.3)*	514 (137)*

*p<0.05, significant change upon blood exposure; †p<0.05, significant difference in the effect of blood exposure of degenerated or osteoarthritic cartilage compared with healthy cartilage.
MMP, matrix metalloproteinase; PG, proteoglycan.

When the proteoglycan synthesis rate and release were normalised to cellularity (DNA content), the differentiation between the three types of cartilage remained similar (table 1). Interestingly, for MMP activity when normalised to DNA content there was a gradual increase with increasing severity of cartilage damage as to be expected.

Effect of exposure of cartilage to blood

Cartilage integrity

In fig 3 and table 2 the effects of blood on cartilage, both directly after a 2-day blood exposure of 10% vol/vol and after an additional 12-day recovery period in the absence of blood, are shown. Depicted is the change in outcome measures compared with their controls—that is, cartilage treated identically but not exposed to blood. There was no statistically significant change in the DNA content of cartilage when the cartilage had been exposed to blood, neither directly after blood exposure nor after recovery (table 2).

Proteoglycan content was not changed significantly directly after a 2-day blood exposure (fig 3A), but the adverse changes in proteoglycan turnover upon blood exposure, as depicted in figs 3B and C, resulted in a significant decrease in proteoglycan content between −10% and −20% of their controls after the recovery period. This decrease in proteoglycan content was not statistically significant different between the healthy, degenerated and osteoarthritic cartilage.

Cartilage chondrocyte activity

The proteoglycan synthesis rate was decreased directly after blood exposure with ±75% for healthy, degenerated and osteoarthritic cartilage when compared with their untreated controls (fig 3B). This decrease was slightly larger for osteoarthritic cartilage than for degenerated cartilage ($p<0.026$). After recovery, the proteoglycan synthesis rate was still significantly decreased, although by less than directly after the blood exposure. There was no significant difference in the effect of blood exposure between the three types of cartilage after recovery. Similar results were obtained when proteoglycan synthesis rate was expressed per DNA content of the cartilage (table 2).

The release of proteoglycans was increased to about twice that of control values directly after blood exposure, whereas after recovery the proteoglycan release did not differ from the untreated controls (fig 3C). The effect of blood on proteoglycan release was similar for the three types of cartilage. When proteoglycan release was expressed per DNA content of the cartilage samples, similar results were obtained (table 2) except that the release of proteoglycans from osteoarthritic cartilage was still statistically significantly enhanced after the recovery period.

Blood exposure also led to a statistically significant increase in MMP activity (fig 3D) for healthy, degenerated and osteoarthritic cartilage, both directly after exposure to blood and after recovery, although after the recovery at lower levels. The increase in MMP activity compared with their untreated controls was significant higher for osteoarthritic cartilage than for healthy cartilage ($p<0.004$). This difference was still present after recovery, although not significant anymore. When expressed per DNA content similar results were seen (table 2); the direct effect of blood on osteoarthritic cartilage being statistically significant greater than for healthy cartilage (†).

DISCUSSION

Joint bleeds, even a limited number, ultimately lead to severe joint damage.[1 3] Currently, the general opinion among doctors is that a few joint bleeds are acceptable. Although even for a first bleed under healthy conditions this might be disputed, it might well be that degenerated cartilage—for example, owing to a previous joint bleed or as a result of biomechanical influences as in osteoarthritis, is even more susceptible to blood-induced damage, because of a decreased ability to recover from such damage. However, our study could not show a clear difference in susceptibility to blood exposure between degenerated and healthy cartilage. Degenerated cartilage was at least as susceptible to blood-induced damage as healthy cartilage. The only differences seen (increased inhibition of proteoglycan synthesis and larger increase in MMP activity; figs 3B and D) were, despite statistical significance, small. Since the opposite effect was not found for any of the parameters, impaired cartilage and healthy cartilage are clearly equally vulnerable. Thus, for impaired cartilage, as seen for instance in osteoarthritis, joint bleeds should be prevented if possible and when they do occur, should be treated properly.

Owing to practical limitations, normal and degenerated cartilage were obtained from the humeral head, whereas osteoarthritic cartilage was obtained from the femoral condyles. To our knowledge no reported information is available about differences in the vulnerability to blood of cartilage from different joints or from donors of different gender. However, in all our previous experiments over the past years we have never seen differences in susceptibility of cartilage to blood between cartilage from different joints or from donors of different gender. Nevertheless, both these factors are a limitation to the experimental set-up.

Joint bleeds can occur because of a trauma, such as ligament rupture or intra-articular fracture, and do spontaneously occur in patients with the clotting disorder haemophilia. Prevention of

joint bleeds is difficult. One can avoid circumstances with a high risk of such injury, as in some sports,[27] but when joints bleeds do occur, proper treatment is needed. As we previously speculated,[14] aspiration of blood from a joint after a joint bleed, as soon as possible ideally, but at least within 48 hours, may diminish the harmful effects of a joint bleed. Studies by Ingram *et al*[28 29] and Holdsworth *et al*[30] have shown that aspiration of a joint after a joint bleed provides immediate relief of pain and an increase in range of motion. Furthermore, there is circumstantial evidence that aspiration of a joint is indicated to prevent joint damage later in life.[4 9 31 33] This study demonstrates that this is also indicated for joints that have already been harmed as in osteoarthritis. Although there is ample evidence from human in vitro studies and animal in vivo studies, a prospective controlled long-term, clinical, follow-up study, using advanced imaging techniques and analyses of serum and/or urine markers of cartilage turnover, might be indicated to demonstrate (protection from) joint damage after joint bleeds.

In cases of haemophilia, prophylactic treatment as early as possible is preferable, but expensive.[34] Therefore, sometimes it is thought that in patients with already damaged joints, there is less need for proper prophylactic treatment, because the damage has already occurred. With this study we demonstrate that blood has a devastating effect on already damaged cartilage similar to that on healthy cartilage. Therefore, it may be concluded that optimal prophylactic treatment when joint damage is already present is also important.

In this study we have used preclinical osteoarthritic degenerated cartilage and osteoarthritic cartilage as was defined and described by van Valburg *et al*.[19] The biochemical and histochemical characteristics of the three types of cartilage were similar to those described in that study. In general, the characteristics of degenerated cartilage were intermediate between those of healthy and osteoarthritic cartilage. In addition to that study, here we demonstrate the intermediate position of degenerated cartilage for proteoglycan release.

The control levels of MMP activity of the three types of cartilage are not statistically different from each other. However, there was a tendency towards a higher activity of these matrix-degrading enzymes in degenerated cartilage compared with healthy cartilage and osteoarthritic cartilage. Published reports are inconclusive in this respect. Although in the literature osteoarthritis is usually linked to an increase in MMP activity,[35 36] a decrease in the expression or activity of several MMPs has been described.[37 38] The use of different substrates and/or assays to measure the MMP activity may account for these differences. Nevertheless, exposure of cartilage to blood consistently leads to a significant increase in MMP activity, and this increase is greater for degenerated than for healthy cartilage and greater for osteoarthritic than for degenerated cartilage.

The data suggest a certain degree of reversibility of the effects of blood upon recovery. However, previous experiments have shown that the harmful effects of blood exposure are long lasting, up to at least 10 weeks, with only minor recovery.[5] The possibility cannot be excluded that the shorter exposure time with the lower concentrations of blood lead to reversibility of the observed effects. Additional studies are indicated to examine this.

The average age of the donors of the degenerative cartilage was greater than those of the donors of healthy and osteoarthritic cartilage, although the difference was not statistically significant. The possibility that age interferes with susceptibility to blood exposure cannot be excluded as has been demonstrated for dog cartilage[39 40]; the younger the animals the more susceptible to blood-induced damage. It might be that a higher vulnerability of younger cartilage has clouded a potentially higher vulnerability of degenerated cartilage, although this cannot be concluded based on the present results.

In this study we used 10% vol/vol blood exposure for 2 days. This concentration and exposure time is based on a previous study, in which we demonstrated that this combination is the minimum leading to prolonged (at least 12 days) adverse changes in cartilage.[14] Whether a higher concentration and longer exposure time would have resulted in differences in susceptibility between the three types of cartilage is not known. It might be that the decrease in proteoglycan content is greater in impaired cartilage than in healthy cartilage, because in this study a tendency towards more severe effects on the proteoglycan synthesis rate and the MMP activity in osteoarthritic cartilage were observed, which ultimately can result in a decrease of the proteoglycan content. However, further studies need to be performed to reach a conclusion about this.

Taken together, it is clear that joint bleeds have detrimental effects and impaired cartilage is at least as susceptible as healthy cartilage to blood-induced cartilage damage. Therefore, joint bleeds should be prevented when possible and because complete prevention of joint bleeds is not realistic, research must be performed to develop proper treatment of these bleeds.

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