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

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Objective. Joint bleeding, or hemarthrosis, leads in time to severe joint damage. This study was carried out to test the in vitro thresholds of exposure time and concentration that lead to irreversible joint damage, to add to the discussion on the usefulness of aspiration of the joint after a hemorrhage.

Methods. Explants of healthy human articular cartilage tissue were cultured in the presence or absence of 50% (volume/volume) blood for 1, 2, 3, or 4 days or in the presence of 0%, 5%, 10%, 20%, 30%, or 50% (v/v) blood for 4 days, followed by a 12-day period of recovery after withdrawal of blood. The effect of blood exposure on cartilage was determined by measuring the rate of proteoglycan synthesis as well as the release and content of cartilage matrix proteoglycans and the activity of matrix metalloproteinases.

Results. Exposure of cartilage to 50% (v/v) blood led to adverse changes that were largely independent of the exposure time. The adverse effects persisted after an initial exposure of up to or exceeding 2 days. Exposure of cartilage to increasing concentrations of blood for 4 days led to concentration-dependent adverse changes.

These effects persisted when the concentration equaled or exceeded 10% (v/v) blood. Moreover, after 2 days of exposure to a blood load of 10% (v/v), the adverse effects on cartilage were not reversible.

Conclusion. A 2-day exposure of cartilage in vitro to 10% (v/v) blood leads to prolonged impairment of joint cartilage. This suggests that aspiration of blood from the joint within 2 days after hemarthrosis should be considered to prevent blood-induced joint damage in the long term.

Joint bleeding, or hemarthrosis, can occur after a joint trauma such as ligament rupture or intraarticular fracture. Intraarticular bleeding also frequently occurs in the larger joints of patients with hemophilia. Moreover, in such patients, it has clearly been demonstrated that this frequent joint bleeding results in severe destruction of the joint later in life (1,2). However, limited joint bleeding or even a single incident has also been demonstrated to lead to irreversible joint damage (3–5). Blood-induced arthropathy has characteristics of both degenerative joint disease (such as osteoarthritis) and inflammation-induced joint damage (as in rheumatoid arthritis) (6). Similar to osteoarthritis and rheumatoid arthritis, blood-induced arthropathy is initiated many years before clinical manifestations become evident (7,8). Although it is a long-lasting process, it is reasonable to expect, based on these previous observations (3–5), that a minimal amount of joint bleeding is sufficient to induce severe joint damage in later years.

With regard to the mechanisms of blood-induced joint damage, our group has demonstrated that the

monocyte/macrophages within the mononuclear cell population together with the red blood cells present in the blood are responsible for the irreversible inhibition of matrix synthesis. Small amounts of interleukin-1 (IL-1), which is produced by activated monocyte/macrophages, will increase the production of hydrogen peroxide by chondrocytes. The hydrogen peroxide reacts with hemoglobin-derived iron from damaged and phagocytosed red blood cells, which results in the formation of hydroxyl radicals in the vicinity of chondrocytes. This leads to chondrocyte apoptosis and, as a consequence, to irreversible inhibition of cartilage matrix synthesis (3,4,9,10).

We have previously demonstrated that a 4-day exposure of cartilage to 50% (volume/volume) blood results in long-lasting inhibition of cartilage matrix proteoglycan synthesis and a prolonged decrease in proteoglycan content (3,11). In addition to type II collagen, proteoglycans are one of the main cartilage matrix components, both qualitatively and quantitatively (12). A 4-day period of exposure is assumed to be the natural time span of evacuation of blood from a joint (13,14), and at least 50% (v/v) blood in a synovial joint is the blood load expected to be present shortly after bleeding takes place (4). These in vitro findings were confirmed by in vivo animal studies (11,15,16). Although the effects revealed in the in vivo experiments in animals were less dramatic than has been observed in human joints, they clearly resulted in compromised joint cartilage, which is predictive of cartilage degenerative changes in the long term (11,15,16).

Although intraarticular bleeding is clearly harmful to joint cartilage, there is currently very little consensus regarding whether aspiration of blood from the joint after hemarthrosis is indicated. Studies by Ingram et al (17,18) and Holdsworth et al (19) showed an immediate relief of pain and increase in range of motion upon aspiration of the joint after bleeding. These effects were not long lasting, since after several weeks, there were no significant differences between the patients with and those without aspirated joints. It is likely that the initial differences were related to capsular pressure. Moreover, in the nonaspirated group, blood is finally evacuated from the joint via natural processes, although prior to that time, the cartilage will begin to show signs of damage; however, these destructive processes will only become clinically evident after several years.

In general clinical practice, it may be difficult to aspirate a joint immediately after the hemorrhage has occurred, because the patient may not immediately seek medical care. Moreover, physicians are reluctant to perform joint aspiration because of the potential for infection and, in the case of hemophilia, because of additional sites of bleeding. Since there is circumstantial evidence to support the view that aspiration of a joint is indicated to prevent joint damage later in life (3–6,9–11,15,16), the present study was initiated to determine the thresholds of blood exposure time and blood load that lead to long-lasting cartilage damage. A possible difference between coagulating and noncoagulating blood was taken into account, because it is not known whether blood coagulates in a joint, and if so, to what extent. The results obtained by this in vitro study might be of use in the discussion of whether aspiration of blood from the joint after a hemorrhage is indicated and whether it would be feasible in clinical practice.

MATERIALS AND METHODS

Cartilage culture. Healthy full-thickness human articular cartilage tissue was obtained postmortem, within 24 hours after death, from the humeral heads of donors who had no known history of joint disorders (mean $\pm$ SEM age at time of death 62.4 $\pm$ 2.7 years; n = 25). In previous studies it has clearly been demonstrated that this macroscopically intact cartilage is a reliable source of histologically and biochemically normal, healthy cartilage (6,20). Collection of the cartilage was carried out in accordance with the medical ethics regulations of the University Medical Center in Utrecht.

Slices of cartilage, excluding the underlying bone, were cut aseptically to the maximum possible thickness and kept in phosphate buffered saline (PBS) (pH 7.4). Within 1 hour after dissection, the slices were cut into square pieces, weighed aseptically (range 5–15 mg, accuracy within 0.1 mg), and cultured individually in 96-well round-bottom microtiter plates in 200 μl per well. Culture medium consisted of Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), ascorbic acid (85 μM), and 10% heat-inactivated pooled human male AB+ serum. Cultures were performed in a tissue incubator in an environment of 5% CO_2 in air, at 37°C.

For each experiment, fresh blood from healthy human donors was collected into vacutainer tubes (Becton Dickinson, Oxford, UK) and immediately added to the cartilage tissue explants in culture medium. In experiments in which coagulating blood was used, the blood was added before coagulation started. Because the total volume was kept at 200 μl, part of the culture medium was replaced with whole blood. Previous experiments have shown that dilution of nutrients in the culture medium or the increase in serum components that results upon adding whole blood up to a maximum of 50% have no influence on proteoglycan turnover (3). Cartilage chondrocytes are not in direct cell–cell contact with the blood cells, and the use of homologous blood instead of autologous blood is not an influential factor (results not shown).

After the cartilage tissue was exposed to blood, all of

the adherent blood components were removed by 2 wash steps, each for 45 minutes under culture conditions. Subsequently, a group of the samples was analyzed. 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 the evaluation of the reversibility or irreversibility of the observed effects of blood exposure. After this recovery period, these cartilage samples were assessed for several parameters.

Analyses of the cartilage samples directly after exposure and after 12 days of recovery included determination of the rate of proteoglycan synthesis and proteoglycan content as well as DNA content. In addition, culture medium was analyzed for proteoglycan release and general matrix metalloproteinase (MMP) activity.

Experimental design. To study the effects of exposure time, cartilage samples were cultured in the presence or absence of 50% (v/v) blood (collected in vacutainers containing 170 IU Li-heparin/10 ml) for 1, 2, 3, or 4 days. To study the effects of blood concentration, cartilage samples were cultured for 4 days in the presence of 0%, 5%, 10%, 20%, 30%, or 50% (v/v) blood (collected in vacutainers containing 170 IU Li-heparin/10 ml). To study the effects of a limited exposure time combined with low concentrations, cartilage was exposed for 2 days to 10% (v/v) blood. Both the direct effects and the effect after a recovery period were determined.

To study the effects of coagulating as compared with noncoagulating blood, cartilage samples were cultured in the presence or absence of 50% (v/v) blood for 2 days and 4 days or for 4 days in the presence of 0%, 10%, or 50% (v/v) noncoagulating blood (collected in vacutainers containing 170 IU Li-heparin/10 ml) or the same concentrations of coagulating blood (collected in vacutainers without heparin). For these assays, only the effects after the recovery period were determined. The presence of heparin itself, up to the concentrations in the final cultures, does not influence the effects of blood on cartilage (3).

Cartilage assessments. The rate of proteoglycan synthesis in the joint cartilage was determined by measuring the rate of sulfate incorporation. $Na_2{}^{35}SO_4$ (NEX-041-H, carrier free; PerkinElmer, Wellesley, MA) was added to 10-μl aliquots of 74 kBq per well, and after 4 hours of pulse labeling of the sulfated glycosaminoglycans (GAGs), the cartilage samples were washed twice with ice-cold PBS and stored at $-20°C$. Thawed samples were digested with papain (P-3125: 26.4 mg/ml in 50 mM phosphate buffer, pH 6.5, containing 2 mM N-acetylcysteine and 2 mM Na_2-EDTA; Sigma, St. Louis, MO) for 2 hours at 65°C. Papain digests were diluted to the appropriate concentrations for analysis of the proteoglycan synthesis rate and proteoglycan content as well as the DNA content.

To determine the proteoglycan synthesis rate, the GAGs in the cartilage tissue digest were precipitated with 0.3M hexadecylpyridinium chloride monohydrate (C9002-100G; Sigma). The precipitate was dissolved in 3M NaCl and the amount of radioactivity in the sample was measured, upon addition of Picofluor-40 (Packard, Meriden, CT), by liquid scintillation analysis. The rate of sulfate incorporation was normalized to the specific activity of the medium, labeling time, and wet weight of the cartilage, with results expressed as nmoles of sulfate per hour per gram wet weight of the cartilage tissue.

The proteoglycan content of the cartilage tissue digests was determined as the amount of GAGs in the cartilage tissue papain digests. GAGs were stained and precipitated with Alcian blue (A-5268; Sigma) (21–23). Staining for GAGs was measured as the change in absorbance at 620 nm, and chondroitin sulfate (C4383; Sigma-Aldrich, Swijndrecht, The Netherlands) was used as a reference. Results are expressed as mg GAG per gram wet weight of the cartilage explants.

The DNA content of the cartilage tissue digests, as a measure of cellularity, was determined in the cartilage samples using the fluorescent dye Hoechst 33258 (382061; Calbiochem, La Jolla, CA). Calf-thymus DNA (D-4764; Sigma) was used as a reference. Results are expressed as mg DNA per gram wet weight of cartilage. Regardless of the experimental conditions, there was no significant change in DNA content (cellularity) of the cartilage due to blood exposure.

Proteoglycan release was determined as the loss of GAGs in the culture medium. GAGs were stained and precipitated with Alcian blue as described above. Results are expressed as mg GAGs released per gram wet weight of the cartilage.

General MMP activity was determined in the culture supernatants using the internally quenched fluorogenic peptide substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) in the presence or absence of 12.5 mM BB94 (a general MMP inhibitor) (24–26). The MMP activity in each sample was calculated as the difference in the initial rate of substrate conversion (linear increase in fluorescence over time, expressed as relative fluorescence units [RFUs] per second) between samples with and those without BB94, normalized against the wet weight of the cartilage, with results expressed as RFUs per second per gram cartilage. The substrate was kindly provided by TNO Quality of Life (Leiden, The Netherlands).

Statistical analysis. Because of focal differences in the composition and bioactivity of the cartilage on the humeral heads, the results from 10 cartilage samples per parameter per donor, obtained randomly and each assessed separately, were expressed as the mean ± SEM and taken as representative of the cartilage of that donor. The n values (ranging 4–6) indicate the number of experiments (namely the number of cartilage donors per experiment). The data were analyzed using the nonparametric Wilcoxon's signed rank test (2-sided) for related samples, using SPSS software version 11.5 (SPSS, Chicago, IL). P values less than or equal to 0.05 were considered statistically significant.

RESULTS

Prolonged blood-induced cartilage damage according to exposure time. Proteoglycan synthesis (Figure 1a) was completely inhibited when measured directly after exposure of cartilage to 50% (v/v) blood. Even a
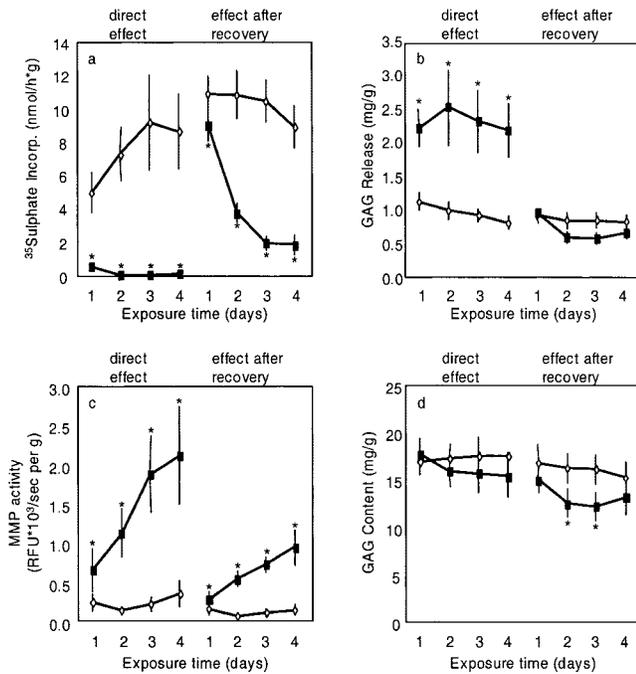
Figure 1. Exposure time–dependent effect of blood on cartilage, according to the rate of proteoglycan synthesis (**a**) measured as ^{35}S incorporation, as well as the release (**b**) and content (**d**) of proteoglycans measured as sulfated glycosaminoglycans (GAGs), and general matrix metalloproteinase (MMP) activity (**c**) measured in relative fluorescence units (RFUs). Healthy human cartilage was exposed in vitro to 50% (volume/volume) blood for 1, 2, 3, or 4 days and analyzed directly or after an additional 12-day recovery period in the absence of blood. Diamonds represent cartilage cultured in the absence of blood (controls); squares represent cartilage exposed to blood. Results are the mean ± SEM (n = 6 samples per group). ∗ = P < 0.05 versus controls.

1-day exposure was able to induce these rigorous effects, with similar effects after 4 days of exposure. Cartilage under control conditions showed a slight increase in the rate of proteoglycan synthesis immediately in the first days of culture (P not significant), which could be attributed to the culture conditions (27).

The inhibition of proteoglycan synthesis induced in cartilage by blood exposure appeared to be reversible, depending on the initial duration of exposure. After an initial 1-day exposure of cartilage to blood followed by recovery from the exposure (i.e., after withdrawal of blood), almost complete reversibility of the inhibitory effects was observed (Figure 1a), although the proteoglycan synthesis during recovery was still statistically significantly lower (−17%) than that in cartilage not exposed to blood (P < 0.05). After an initial 2-day

exposure followed by recovery, a 65% inhibition of proteoglycan synthesis (P < 0.05) was found. The initial inhibition of proteoglycan synthesis upon 3 days or 4 days of blood exposure did not exhibit a signficant recovery from exposure during the 12-day period in the absence of blood.

The release of proteoglycans was increased directly after exposure of cartilage to blood, independent of the duration of the exposure (Figure 1b). Even a 1-day exposure resulted in a doubling of proteoglycan release. Surprisingly, after the 12-day recovery period, proteoglycan release was not different from that observed in nonexposed control cultures.

The activity of the cartilage matrix–degrading MMPs increased significantly upon exposure to blood (Figure 1c). A clear dependency on exposure time was observed, since MMP activity increased with longer exposure time. After recovery, this effect was still present, although the differences were less striking.
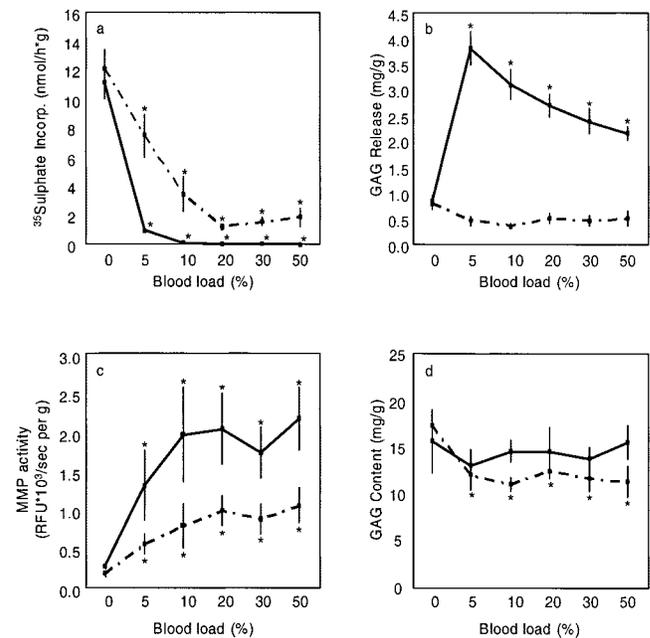
Figure 2. Concentration-dependent effect of blood on cartilage, according to the rate of proteoglycan synthesis (**a**) measured as ^{35}S incorporation, as well as the release (**b**) and content (**d**) of proteoglycans measured as sulfated GAGs, and general MMP activity (**c**) measured in RFUs. Healthy human cartilage was exposed in vitro for 4 days to 0% (control), 5%, 10%, 20%, 30%, or 50% (v/v) blood without recovery (solid lines) or followed by a 12-day recovery period in the absence of blood (broken lines). Results are the mean ± SEM (n = 5 samples per group). ∗ = P < 0.05 versus controls. See Figure 1 for definitions.
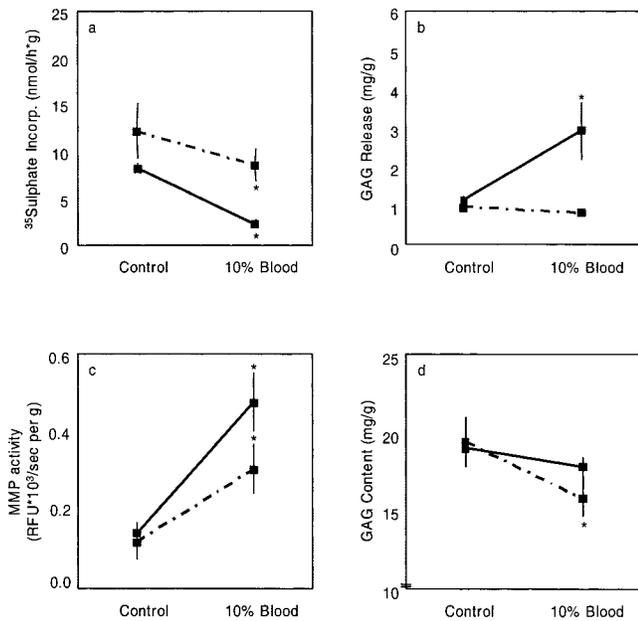
Figure 3. Effect of 2 days of exposure of cartilage to 10% (v/v) blood, according to the rate of proteoglycan synthesis (**a**) measured as ^{35}S incorporation, as well as the release (**b**) and content (**d**) of proteoglycans measured as sulfated GAGs, and general MMP activity (**c**) measured in RFUs. Healthy human cartilage was exposed in vitro for 2 days to 10% (v/v) blood without recovery (solid lines) or followed by a 12-day recovery period in the absence of blood (broken lines). Results are the mean ± SEM (n = 6 samples per group). ∗ = $P < 0.05$ versus controls. See Figure 1 for definitions.

Even after 12 days of recovery, the initial 1-day blood exposure resulted in an almost doubling of MMP activity ($P < 0.05$).

In addition, an exposure time–dependent decrease in proteoglycan content was observed (Figure 1d). This decrease continued during the recovery period and ultimately resulted in a statistically significant impairment of matrix integrity (diminished proteoglycan content) after the cartilage had been initially exposed for 2 days or 3 days to 50% (v/v) blood.

Prolonged blood-induced cartilage damage according to blood load. Exposure of cartilage for 4 days to 5% (v/v) blood led to a direct, almost complete inhibition of proteoglycan synthesis (Figure 2a). Higher concentrations of blood also yielded inhibitory effects similar to those after exposure to the 5% concentration. This decrease in proteoglycan synthesis was sustained after a recovery period, although the effects of 5% (v/v) and 10% (v/v) blood during recovery were less dramatic, with 38% and 71% inhibition of proteoglycan synthesis, respectively (both $P < 0.05$ compared with that in

nonexposed cartilage). Exposure to higher concentrations of blood effectively limited the recovery of cartilage matrix synthesis; in fact, the proteoglycan synthesis was almost completely inhibited after 12 days of recovery (Figure 2a, dotted line).

The release of proteoglycans (Figure 2b) increased directly after the blood exposure. Surprisingly, this increase showed an inverse relationship with the blood load; GAG release after exposure to 5% (v/v) blood differed statistically significantly from the release after exposure to 50% (v/v) blood ($P < 0.05$). The increase in the release of proteoglycans appeared to be completely reversible following recovery from exposure, even after an initial exposure to the highest (50% v/v) concentration of blood.

The MMP activity in the cartilage (Figure 2c) showed a blood concentration–dependent increase di-
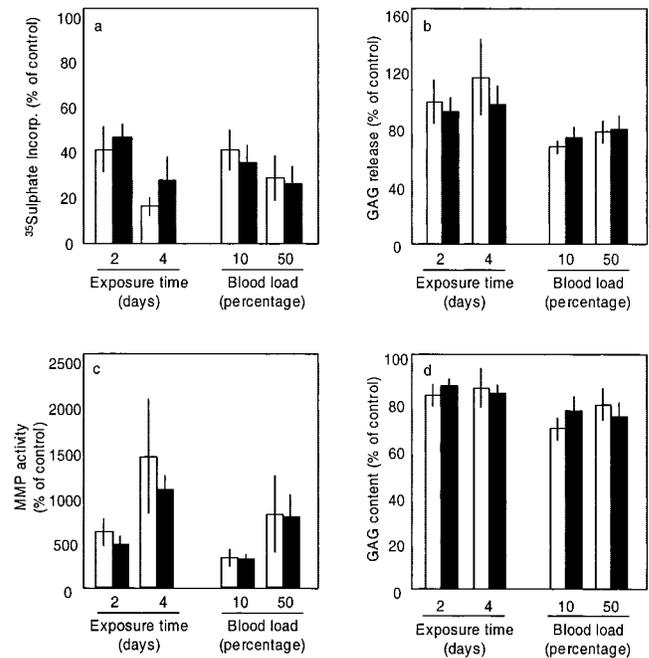
Figure 4. Effect of exposure of cartilage to either coagulating blood (solid bars) or noncoagulating blood (open bars), by exposure time and concentration, according to the rate of proteoglycan synthesis (**a**) measured as ^{35}S incorporation, as well as the release (**b**) and content (**d**) of proteoglycans measured as sulfated GAGs, and general MMP activity (**c**) measured in RFUs. Healthy human cartilage was exposed in vitro for 2 or 4 days to 10% or 50% (v/v) coagulating or noncoagulating blood, followed by a 12-day recovery period. Results are the mean ± SEM percentage of control values in the absence of blood (n = 4 samples per group). No statistically significant differences between coagulating and noncoagulating blood were observed for any of the parameters. See Figure 1 for definitions.

rectly after exposure. In addition, there was a concentration-dependent elevation of MMP activity after the recovery period, although the absolute values were lower compared with those measured directly after the exposure. Nevertheless, after exposure of cartilage to each incremental increase in concentration of blood, the MMP activity remained statistically significantly higher ($P < 0.05$) than that in control cartilage cultures.

The proteoglycan content of cartilage after the initial exposure to 5% (v/v) blood was decreased 30%, compared with that in control cartilage, after recovery from the exposure (Figure 2d). Higher blood concentrations only slightly diminished the proteoglycan content further; at 50% (v/v) blood, there was a 34% decrease in proteoglycan content after recovery, compared with that in nonexposed cartilage.

When cartilage was exposed for 2 days to 10% (v/v) blood (Figure 3) and then allowed to recover for 12 days in the absence of blood, proteoglycan synthesis was still significantly diminished (Figure 3a), MMP activity remained enhanced (Figure 3c), and proteoglycan content decreased (Figure 3d). Release of proteoglycans appeared to be only transiently increased after exposure to 10% (v/v) blood (Figure 3b) and was completely restored after 12 days of recovery.

Similar adverse effects of coagulating and non-coagulating blood on cartilage. It is not known whether blood coagulates in the joint after a hemorrhage, and if so, to what extent and how quickly the coagulation occurs. The effects of coagulating blood were therefore compared with those of noncoagulating blood. For this study, only the effects after recovery from a 2- or 4-day exposure to 50% (v/v) blood and a 4-day exposure to 10% (v/v) or 50% (v/v) blood were analyzed. In this set of experiments, the effects of noncoagulating blood were similar when compared with those of coagulating blood. As Figure 4 clearly demonstrates, the adverse effects induced by blood on any of the parameters were not significantly different for cartilage tissue cultured with noncoagulating blood as compared with that cultured with coagulating blood.

DISCUSSION

There is no consensus as to whether aspiration of blood from the joint after hemarthrosis is indicated. This study was undertaken to provide information on the thresholds of blood concentration and exposure time that lead to prolonged cartilage damage. It was demon-

strated that a 2-day exposure of cartilage to 10% (v/v) blood in vitro leads to prolonged biochemical impairment of joint cartilage, whereas a 1-day exposure to 50% (v/v) blood leads, for the most part, to only transient impairment of joint cartilage. In general, natural evacuation of blood from the joint after a hemorrhage takes more than 1 day. Based on the results from in vivo animal studies, it has been suggested that this process takes at least 4 days (13,14). Similarly, the observations from treatment of patients with hemophilia indicate that 4 days is a reasonable time span for natural evacuation of blood.

Although the present results were determined in vitro, they suggest that aspiration of blood from a joint as soon as possible but at least within 48 hours after a joint hemorrhage should be considered in clinical practice, to prevent or diminish long-lasting impairment of the cartilage tissue. Nevertheless, the blood load (exposure time and/or dose) dependency of several of the adverse effects in the joint suggests that the sooner the aspiration takes place, the better. In addition to prevention of direct cartilage damage, aspiration of blood from a joint provides immediate relief of pain and improves the range of motion (17–19). It might also be expected that aspiration decreases the risk of synovial triggering that leads to inflammatory activity (28–30). We believe that to conclusively show the benefit of joint aspiration after a hemorrhage, a prospective, controlled, long-term clinical followup study is warranted to demonstrate protection from joint damage after joint bleeding, utilizing, for example, imaging techniques or analyses of serum markers of cartilage turnover.

Translation of these in vitro results has its restrictions, and issues to be considered are numerous. For instance, in vitro, the cartilage is exposed to blood at all cutting edges instead of solely at the articular surface as occurs in vivo; this issue is currently under investigation. Furthermore, synovial fluid can possibly interfere with the blood, and neutralizing or potentiating factors might be present. Synovial tissue adds to cartilage damage by contributing to the inflammatory responses to blood exposure. Nevertheless, synovial tissue cells may phagocytize blood components and diminish the harmful effects. These issues have to be considered when translating the in vitro findings to clinical practice.

We have previously shown that exposure of cartilage to 50% (v/v) blood for 4 days leads to harmful effects on cartilage (3,4,9–11), namely the severe inhibition of proteoglycan synthesis and a direct increase in proteoglycan release and a decrease in proteoglycan

content. These effects were long-lasting, which could be attributed to the occurrence of chondrocyte apoptosis (9). The present study extends these results by showing that the effects of exposing cartilage to blood can take place within a shorter time period and after exposure to a lower blood load. Furthermore, our results indicating the reversibility of increased proteoglycan release, the exposure time– and blood load–dependent MMP activity, and the unresponsiveness of DNA content are new findings that provide more insight regarding the mechanisms of blood-induced cartilage damage.

The observed effects of blood exposure on proteoglycan turnover cannot be attributed to changes in the cellularity of the cartilage, because no significant changes in DNA content were observed. In a previous study we found that exposure of cartilage to blood results in chondrocyte apoptosis (9). In that respect, one would anticipate a decrease in DNA content in cartilage upon exposure to blood. However, articular cartilage is not vascularized and does not contain mononuclear phagocytes. Therefore, apoptotic bodies, which still contain DNA that is detectable by our assay, are not cleared from the cartilage. This explains why the DNA content is not changed significantly as a consequence of blood exposure.

The effects observed after recovery from the exposure provide an indication of the irreversibility of the effects. It could be speculated that the effects would have largely been normalized if the recovery period had been expanded. However, it has been shown previously that the harmful effects on synthesis persist for at least 10 weeks after an initial 4-day 50% (v/v) blood load (4,11). It was more feasible in the present study to conduct a followup of just 2 weeks because of the large number of experiments performed.

There was an inverse relationship between the direct proteoglycan release and the concentration of blood added. A similar inverse relationship with the exposure time was found, although this was less striking. These findings can be explained by the fact that the release of proteoglycans was measured directly after blood exposure, and not during the blood exposure itself. We assume that the amount of proteoglycans released during contact of the cartilage tissue with blood is higher when the blood load is higher, but that the amount that can be released directly after the exposure is lower with increasing concentrations of blood.

Furthermore, the release of proteoglycans completely normalized after the recovery period. As we have shown previously (4), the proinflammatory mediators IL-1β and tumor necrosis factor α (TNFα) play a role in the transient inhibition of proteoglycan synthesis measured directly after blood exposure. These cytokine-induced changes in proteoglycan synthesis were, however, not responsible for the prolonged inhibition of the rate of synthesis. It is reasonable to expect that the direct effects of exposure of cartilage to blood on proteoglycan release are induced by proinflammatory cytokines, and therefore that these effects are also transient.

In this study, we used a general assay for MMP activity in which the focus was largely on the activity of collagenases, to provide data supplementary to the proteoglycan biochemistry data. Although some of the MMP activity measured may have originated from the original blood exposure, the actual MMP measurements were performed in blood-free culture supernatants of the cartilage (shortly after the blood was withdrawn).

Our results also revealed that exposure of cartilage to blood leads to an exposure time– and blood load–dependent increase in MMP activity, both directly and, although at lower levels, after the recovery period. Active MMPs play a role by contributing to the breakdown of extracellular matrix, and release of proteoglycans is a consequence of this breakdown of cartilage matrix. It would therefore be expected that there would be a correlation between the activity of MMPs and the release of proteoglycans. However, this correlation was not evident in our experiments. The fact that the MMP assay used measures primarily collagenases and gelatinases (24–26) could explain the absence of a correlation between proteoglycan release and MMP activity. Collagen damage was not determined in this study.

Proteoglycans can also be degraded by other enzymes. The ADAMTS are a family of proteolytic enzymes that degrade extracellular matrix (31–35). Among other subtypes, ADAMTS-1, -4, and -5 have been identified as aggrecanases that are of importance in the breakdown of aggrecan, the most prominent proteoglycan in cartilage (36–39). It has also been shown that proteoglycans that were released from bovine or porcine articular cartilage after exposure to catabolic stimuli, e.g., retinoic acid, IL-1, or TNFα, were generated by the action of aggrecanases and not MMPs (31). Therefore, the increase in the release of proteoglycans as a consequence of the exposure of cartilage to blood is probably mediated by aggrecanases, whereas the increase of MMP activity leads to damage of the collagen component of cartilage. Apparently, MMP activity remains for a prolonged period of time after blood exposure, combined with the sustained inhibition of proteo-

glycan synthesis, and these changes result in persistent impairment of cartilage matrix integrity, whereas aggrecanase activity is transient, induced by transient (blood-induced) cytokines such as IL-1 and TNFα (4).

It is not known if and to what extent blood coagulates in the joint after a hemorrhage. However, we can rule out coagulation as a source of bias in the present study because we demonstrated that all of the adverse effects induced by blood were similar after exposure to noncoagulating blood as compared with coagulating blood.

Thus, the results of the present study, although determined in vitro, demonstrate that aspiration of blood from a joint after hemarthrosis, even 24 hours after the incident, should be evaluated in clinical practice. This approach has the potential to prevent cartilage damage that will become clinically visible later in life.

AUTHOR CONTRIBUTIONS

Ms Jansen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study design. Ms Jansen and Drs. Roosendaal, Bijlsma, DeGroot, and Lafeber.
Acquisition of data. Ms Jansen and Dr. Roosendaal.
Analysis and interpretation of data. Ms Jansen and Drs. Roosendaal, DeGroot, and Lafeber.
Manuscript preparation. Ms Jansen and Drs. Roosendaal, Bijlsma, De Groot, and Lafeber.
Statistical analysis. Ms Jansen and Dr. Lafeber.

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