Persistent degenerative changes in the intervertebral disc after burst fracture in an in vitro model mimicking physiological post-traumatic conditions

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Abstract

Purpose Post-traumatic disc degeneration (DD) is currently investigated with models not fully matching the clinical condition, in particular post-traumatic loading of the disc is not considered. Therefore, the aim was to establish an in vitro burst fracture model that more closely mimics the in vivo situation by including post-traumatic physiological loading and to investigate DD under these conditions.

Methods 72 rabbit spinal segments (disc/endplates + 1/3 of adjacent vertebrae) were harvested from T8/9 to L5/6 and assigned to control ($n = 36$) or trauma groups ($n = 36$). Burst fractures were induced at day 0 in the trauma group using a dropped-weight device. From day 1 to 28, all specimens were cultured at 37 °C and were dynamically loaded daily (~ 1 MPa nominal pressure, 1 Hz, 2,500 cycles). At day 1, 7, 14, and 28, 9 specimens from each group were taken for analysis: histology ($n = 2$), total disc glycosaminoglycan (GAG) content

($n = 3$) normalized to DNA, and qPCR of DD marker genes ($n = 4$) in the nucleus pulposus and the annulus fibrosus.

Results Burst fracture with post-traumatic physiological loading resulted in a 65 % loss of GAG/DNA by day 28. Histological sections confirmed the remodeling of the matrix. Catabolic (MMP-1/-3), pro-apoptotic (TNF- α , fas ligand), and pro-inflammatory (IL-1/-6, iNOS) gene transcription was substantially up-regulated in the nucleus after the trauma and did not normalize to control within 28 days. Similar results were found for the annulus on lower levels.

Conclusion An in vitro burst fracture model with physiological post-traumatic loading was established. Under these conditions, burst spinal segments undergo strong and persistent degenerative changes.

Keywords Disc degeneration · Burst fracture · Post-traumatic loading · Clinical occurrence · Trauma model · In vitro

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Introduction

Disc degeneration (DD) is strongly associated with low back pain (LBP) [1], in particular in conjunction with endplate disruption [2, 3]. Traumatic burst fractures are a main reason for endplate failure and account for up to 17 % of all major spinal fractures [4]. First evidence for a causal link of burst fracture and post-traumatic DD was provided by Kerttula et al. [5]. They reported decreased signal intensity of the nucleus pulposus (NP) on T2-weighted MRI in patients suffering from burst fractures after at least 1-year follow-up time (mean 3.7 years). Heyde et al. [6] found increased disc cell apoptosis in patients undergoing open reduction and internal fixation after thoracolumbar spine

injury. In order to support this causality and to investigate the post-traumatic degenerative process, *in vivo* and *in vitro* models have been developed. Animal trauma models can be divided into three main classes: overload models, stab incision models, and endplate perforation models [7]. All three succeed in promoting degenerative changes in the disc, but with a different emphasis on matrix remodeling, apoptosis, and inflammation. The different nature of injury may account for these variations and hence poses the question of clinical relevance, in particular as none of them mimic the clinical situation. Since a genuine *in vivo* burst fracture model is ethically challenging, Haschtmann et al. [8] established an explant model, which accounts for the dynamic nature and the high impact energy of burst fractures. Controlled fractures in rabbit IVD/EP explants were induced with a dropped weight and cultured up to 4 weeks. In a further-developed version of this model, Dudli et al. [9, 10] showed that a burst fracture of spinal segments causes strong apoptotic, catabolic, and pro-inflammatory changes in the disc and that the type of injury indeed has a substantial influence on the onset and progression of the degenerative changes. However, this model still does not represent physiological conditions, as specimens were completely unloaded after the trauma. In contrast, traumatized spinal segments are loaded in burst fracture patients, not only due to muscle tension even during bed rest but also due to daily activities and physiotherapy. Therefore, the goal of this study was to further evolve the *in vitro* burst fracture model by including physiological loading of the burst spinal segments and to investigate if post-traumatic DD also occurs under these clinically relevant conditions.

Materials and methods

Chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland) if not stated otherwise.

Full-organ intervertebral disc culture model and trauma induction

72 spinal segments (IVD/endplate with approximately 5 mm of the adjacent vertebral bodies) from T8/9 to L5/6 (9/animal) were isolated from eight New Zealand White (NZW) rabbits (female, 4–5 kg, 6 months old) as previously described [9]. Special attention was paid to the preparation of the two vertebral cross sections, since these are the contact areas with the fracture and loading devices. These surfaces were leveled, prepared parallel to each other, and perpendicular with respect to the cranial/caudal axis of the segment. Specimens were divided into a control and a trauma group and assigned to a sampling day (day 1, 7, 14, or 28), and to one of the following analysis methods: quantitative

real-time polymerase chain reaction (qPCR) ($n = 4/\text{day}/\text{group}$), glycosaminoglycan (GAG) quantification ($n = 3/\text{day}/\text{group}$), and histology ($n = 2/\text{day}/\text{group}$). Discs from the same vertebral level were assigned to each assay for all sampling points (Online Resource 1). The vertebral levels of the control groups were adjacent to the test group levels for all assays. Burst fractures were induced 6 days post-harvest (day 0) in the test group ($n = 36$), using a custom-made dropped-weight fracture device (534 g steel ball, 2 J), which ensures axial loading. The applied energy and the chosen weight of the steel ball minimize collateral damage to the specimen, while still inducing an endplate fracture. From day 1 to 28, all specimens were cultured at 37 °C, 5 % CO₂ in standard media (30 ml DMEM/F12, 5 % fetal calf serum (Labforce AG, Münsingen, Switzerland), 50 mg/ml penicillin, 100 U/ml streptomycin, 25 mg/ml L-ascorbate).

Post-traumatic dynamic loading

From day 1 to 28 after trauma, all specimens (trauma and control) were subjected to daily simulated physiological loading (2,500 cycles, 1 Hz, 0.8–1.7 MPa; Instron E10000, Instron International Switzerland). Frequencies between 0.01 and 10 Hz, pressures between 0.2 and 1.3 MPa and loading times no longer than one hour a day have beneficial, or at least not adverse, effects on disc metabolism [11]. Intradiscal pressure during walking in human is 0.53–0.65 MPa and ~1.6 MPa in sheep [12]. Quadrupeds require higher muscular activity to prevent sagittal displacement of the spine, which results in higher intradiscal loads [12]. Intradiscal pressures in this range have also been shown to occur *in vivo* during the performance of a variety of normal daily activities [13, 14]. 30–120 min of daily physiological loading has been previously shown to be ideal for inducing mRNA synthesis [15]. Since static loading is adverse in any case, discs were unloaded between loading cycles [16]. The custom-made loading device allowed for the simultaneous loading of six specimens (Online Resource 2). Each specimen was loaded with the same force (42 N). At day 1, 7, 14, and 28, 2 h after loading, 9 specimens from each group were taken for analysis.

Histology

After fixation in 4 % neutral buffered formalin, specimens were washed, dehydrated and embedded in PMMA. Samples were cut sagittally in 6 µm slices with a microtome (Leica® RM2155; Leica Instruments GmbH, Nussloch, Deutschland) and stained with Masson-Goldner. Images were taken with a Zeiss AxioCam mounted on a Zeiss 200 M microscope using 2.5 × 0.075 NA EC Plan Neo-Fluar objective and processed with Zeiss AxioVision software (Carl Zeiss AG, Feldbach, Switzerland).

Table 1 Primer sequences for quantitative real-time PCR

Gene product	Sense (5'–3')	Antisense (5'–3')	<i>T</i> (°C) ^a
GAPDH	aaggccatcaccattctcca	ggatgcgttgctgacaatct	59/62
AGG	gaggtcgtggtgaaaggtgt	gtgtggatgggtacctgac	62
COL1	ttcttggtgctcctggcattc	gcaatccgttgctcccttatg	59
COL2	gaccccatgcagtacatgcg	ccagtagtcaccgtcttcc	59
TGFb1	aagggtaccacgccaactt	ccgggttgctgctggtgtac	59
MMP-1	atacttgaaaactactacaatctg	tcttcagggttcagcatct	59
MMP-3	agccaatggaaatgaaaactcttc	ccagtggataggctgagcaaa	59
TNFa	cagcctcttctcttctctgct	ccgatcacctgaagtgc	59
FasL	acagccaacctactctctgt	cgctgacctatcatctggg	59
IL-1b	tccagacgaggcatcca	ctgccgaagctcttggg	62
IL-6	ctggtggtgctaccgttt	atggtctccaggatgctccg	62
iNOS	ccccttcaacggctgta	tctgtgacggcctgactttc	62

GAPDH glyceraldehyde-3-phosphate-dehydrogenase, *AGG* aggrecan, *COL1* collagen-1, *COL2* collagen-2, *TGFb* transforming growth factor beta 1, *MMP-1* metalloproteinase-1, *MMP-3* metalloproteinase-3, *IL-1b* interleukin-1 beta, *IL-6* interleukin-6, *TNFa* tumor necrosis factor alpha, *iNOS* inducible nitric oxide synthase, *FasL* fas ligand

^a Temperature used for annealing/extension

Quantification of whole disc glycosaminoglycan and DNA content

Specimens were digested for 24 h with papain (60° C, 125 mg/ml in 5 mM L-cystein HCl, 5 mM Na-citrate, 150 mM NaCl, 5 mM EDTA, pH 6.0). GAG was quantified using 1,9-dimethylmethylene blue binding assay [17]. From the same digestion solution, DNA was quantified with bisbenzimidol fluorescent dye (Hoechst 33258). GAG and DNA were measured for each specimen separately without pooling of tissue.

Gene transcription analysis

We selected 10 genes as marker for degenerative changes [8, 9] (Table 1). RNA isolation and purification from NP and AF as well as cDNA synthesis and qPCR were performed as described previously [18]. In the trauma group, any non-fibrous material within the nuclear space was considered as NP. Data were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and expressed as fold change compared to the control group. Gene transcription was quantified for each specimen separately without pooling of tissue.

Nitric oxide quantification

NO in culture supernatants of all samples assigned to day 28 ($n = 18/\text{group}$) was quantified indirectly by its oxidation products $\text{NO}_2^-/\text{NO}_3^-$ by Griess reaction [19].

Statistics

For statistical analysis, the software R (version 2.15.1) was used. qPCR data were evaluated using the $2^{-(\Delta\Delta C(T))}$ method. Non-parametric tests (Wilcoxon, Kruskal–Wallis with Benjamin–Hochberg correction) were performed to detect significant differences between control and trauma and between different days. In the case of a non-significant day dependency, data pooled from all days were subjected to a Wilcoxon test. Correlations were calculated using Pearson's test. GAG, DNA and NO data were evaluated by parametric tests (t test and TukeyHSD). For GAG and DNA, a two-way ANOVA on a linear model was calculated to detect trends. Significance level for all tests was $\alpha = 0.05$.

Results

Proteoglycan content and histological findings

GAG and DNA content in the control group as well as DNA in the trauma group did not change during the test period (not shown; $p = 0.97, 0.98, 0.45$). GAG in the trauma group declined steadily ($p < 0.01$) and resulted in a 64 % loss of GAG by day 28, respectively 65 % normalized to DNA (Fig. 1). Histological sections confirmed the remodeling of the matrix (Fig. 2). In the control group, the NP matrix was colorless at all days. In the trauma group, at day 1 and 7, the NP space showed a light turquoise stain, indicating an open collagen matrix. At day 14 and 28, a red

Fig. 1 DNA content, glycosaminoglycan (GAG) content, and GAG/DNA ratio of total discs. Values from trauma specimens were normalized to the respective adjacent control samples of each disc/vertebrae specimen. Trauma and control specimens were both subjected to daily simulated physiological loading. Significant differences to control groups are indicated ($n = 3$ per group and day; mean $\pm$ SD; ** $p < 0.01$, * $p < 0.05$)

stain was predominant, representing a dense unmineralized collagen I matrix.

Gene transcription in the nucleus pulposus

Endplate trauma caused an immediate strong up-regulation of catabolic (MMP-1, MMP-3), pro-apoptotic (TNF- α , fas ligand), and pro-inflammatory (IL-1, IL-6, iNOS) gene transcription (Fig. 3). Peak up-regulation was highest at day 1 (MMP-1, MMP-3, fas ligand, IL-1) or 7 (TNF- α , IL-6, iNOS). Levels at day 14 and 28 were lower than at day 1 and 7 for all catabolic, pro-apoptotic, and pro-inflammatory genes but still above control. Due to the large biological variance, up-regulations at day 14 and day 28 did not reach statistical significance ($p > 0.08$). The same pattern was found for the anabolic TGF- β , although on lower levels. Collagen-1 was on average 17.5 ± 5.8 -fold increased ($p < 0.001$) but did not reach significance at specific days. Collagen-2 shared the same pattern with collagen-1 and was significantly up-regulated at day 7. Gene transcription of aggrecan, the most predominant disc proteoglycan, was not changed ($p = 0.37$ and $p = 0.33$ for NP and AF, respectively).

Fig. 2 Photomicrographs of Masson–Goldner trichrome stained sagittal tissue sections. Anatomical level of specimens was L5/6 or L6/7, except fracture day 14 (T9/10). Original magnification $\times 2.5$.

Scale bar 1,000 μ m. Blue calcified bone, red uncalcified, dense connective tissue matrix, turquoise less dense connective tissue

Fig. 3 Gene transcription in the nucleus pulposus of fractured spinal segments compared to non-fractured control group. From left to right, genes are grouped into anabolic (AGG, COL1, COL2, TGFb), catabolic (MMP-1, -3), pro-apoptotic (TNFa, FasL), and pro-inflammatory genes (IL-1, -6, iNOS). Abbreviations are explained in

Table 1. Asterisks above lines indicate significant differences between days and asterisks above parentheses indicate significant trauma-control difference of pooled data (only in the case of non-significance at all days). $n = 4$ per group and day; mean $\pm$ SEM; ** $p < 0.01$; * $p < 0.05$

Fig. 4 Gene transcription in the annulus fibrosus of fractured spinal segments compared to non-fractured control group. From left to right, genes are grouped into anabolic (AGG, COL1, COL2, TGFb), catabolic (MMP-1, -3), pro-apoptotic (TNFa, FasL), and pro-inflammatory genes (IL-1, -6, iNOS). Abbreviations are explained in

Table 1. Asterisks above parentheses indicate significant trauma-control difference of pooled data (only in the case of non-significance at all days). $n = 4$ per group and day; mean $\pm$ SEM; ** $p < 0.01$; * $p < 0.05$

Gene transcription in the annulus fibrosus

Gene transcriptions in the AF of all catabolic, pro-apoptotic, and pro-inflammatory genes showed almost identical patterns to the NP, but were substantially lower (Fig. 4). The large biological variance impeded statistical significance. After 14–28 days, control levels were reached. TGF- β was on average 1.8 ± 0.3 -fold increased ($p < 0.05$) but did not reach significance at specific days. Collagen-1 and -2 were not significantly above control levels.

Correlation analysis of gene transcription

Pooled gene transcription data from the three classes catabolism, apoptosis, and inflammation correlated in the NP as well as in the AF (Table 2). Each class also showed a high degree of correlation between NP and AF. Anabolic gene regulation did not correlate with the other three classes, neither in the NP nor in the AF nor between NP and AF. Mutual correlations of all genes are shown in Online Resource 3.

Table 2 Correlation of gene transcription between four gene classes

	Anabolism	Catabolism	Apoptosis	Inflammation
Catabolism (NP/AF)	−0.01/−0.03			
Apoptosis (NP/AF)	0.01/−0.02	0.46***/0.43***		
Inflammation (NP/AF)	−0.04/−0.02	0.57***/0.68***	0.43***/0.36***	
NP vs. AF	0.15	0.71***	0.79***	0.78***

Top three rows: correlation between the gene classes in NP and AF. Bottom row: correlation between NP and AF for each gene class. Pearson's correlation estimate is indicated with level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The genes associated with each class are: anabolism (aggrecan, collagen-1, collagen-2, TGF- β 1), catabolism (MMP-1, -3), apoptosis (TNF- α , fas ligand), and inflammation (IL-1, -6, iNOS)

Fig. 5 NO concentration in culture supernatant. Values from trauma group were normalized to control group. Statistical differences to control groups are indicated. ($n = 18$ per group; mean $\pm$ SD; * $p < 0.05$)

Nitric oxide in culture supernatant

NO concentrations in the culture supernatant from traumatized specimens were increased at day 1 and 2 after trauma (Fig. 5). Afterwards concentrations were on control levels and not different from before trauma (day 0).

Discussion

The aim of this study was to develop an in vitro burst fracture model with physiological post-traumatic loading, in order to mimic the clinical situation and meet pathobiological conditions. Therefore, a previously established burst fracture model was extended with daily post-traumatic loading of all specimens. Under these conditions, we found that burst spinal segments exhibited strong degenerative changes. GAG content decreased by 65 % within 4 weeks. This is in agreement with strong matrix remodeling seen on histological sections. The transition in the NP from no stain, to green, to red is an indication not only for

collapsed annular fibers but also for an aberrant healing response, where anabolic and catabolic processes occur in parallel. This was corroborated by significantly enhanced anabolic (collagen-1, -2, TGF- β) and catabolic (MMP-1, MMP-3) genes in the NP. Catabolic, pro-inflammatory, and pro-apoptotic gene transcription was strongly enhanced in the NP during the first week and persisted on a lower level up to 4 weeks. These mutually correlate to a high degree in both anatomical compartments, the NP and the AF, and between NP and AF. Generally, the AF showed similar trends, although on a lower level.

The changes in inflammatory gene regulation correlated with changes of catabolic and pro-apoptotic gene transcription. This may indicate interactions between their transcription regulations. Hence, the change in one class also affects the others. It is known, for instance, that the activity of MMPs is not limited to matrix cleavage; they also modulate the inflammatory response [20]. Some MMPs, including MMP-3, were found to activate pro-IL-1 β . Conversely, IL-1 β and TNF- α , which are considered to play a pivotal role in DD [21], are potent inducers of MMP transcription [22], possibly by stimulating the NF- κ B pathway [23]. IL-1 β also increases the sensitivity of the IVD to fas ligand by increasing fas expression [24] (in this study, fas transcription was also increased on average 11.6 ± 3.2 -fold ($p < 0.01$), data not shown). Conversely, fas ligand can induce the processing and secretion of IL-1 β [25, 26]. In the presence of all these interactions, it is not surprising that degenerative discs were reported to be more sensitive to pro-inflammatory stimuli [27].

Besides inflammation, apoptosis is another hallmark of post-traumatic DD [6, 8, 9, 28]. In this study, we demonstrated that a burst fracture with post-traumatic physiological loading induces a persistent alteration in pro-apoptotic gene transcription in the NP; the death signaling molecules TNF- α and fas ligand were strongly up-regulated during the first week after trauma and persisted on a lower level afterwards. The sustained up-regulation is in agreement with a rabbit model of annular laceration [29]. However, the apoptotic effect of the few NP cells is outnumbered by a proliferative effect of the majority of disc cells. The

formation of a fibrocartilaginous tissue seen on histological sections suggest that proliferation induces a fibroblast-like cell type, which develops under the new mechanical and biochemical environment of burst fractured discs.

The up-regulation of the anabolic genes collagen-1 and -2 coincide with other trauma models [29, 30] and may be explained by the enhanced transcription of TGF- β 1, a major up-stream regulator of collagens and proteoglycans [31]. However, aggrecan gene transcription was not affected in this study. The stimulating effect of TGF β may be compensated by the inhibiting effect of the pro-inflammatory cytokines [32]. Depending on the trauma model, the stimulation [29] or the inhibition [30] is stronger. However, it is the relative amounts of collagen, proteoglycan, and protease gene transcription that determine the organ fate. As is common to all trauma models, the NP matrix is replaced by a fibrocartilaginous tissue.

TGF- β 1 has also the capability to modify iNOS translationally and post-translationally [33]. This could be the reason why NO was only increased for the first 2 days after trauma, although iNOS transcription level was increased for a longer period.

The degenerative changes not only affect the NP as reported previously [9] but also the AF. In addition, the high inter-compartment correlation of transcription regulation shows that the degenerative changes affect the whole disc simultaneously. This is consistent with the proposed “endplate-driven DD”, where the inner AF collapses into the decompressed NP under loading [34]. The few cells in the nuclear area are unable to repair the extensive damage of a matrix, which is repeatedly loaded. This causes an aberrant healing response, with increased anabolism, catabolism, and cytokines, resulting in the formation of fibrocartilaginous tissue [3]. Interestingly, we see a normalization of the response in the AF within 14 days after trauma, despite daily loading. This is in contrast to chronic inflammation in the AF caused by triple-stab injury in rat tails [35]. We speculate that stress levels in the outer annulus, in response to physiological loading, are not sufficient to provoke a chronic response in the absence of direct annular injury, despite the increased load transfer onto the annulus with nucleus degeneration.

The method of load application in this study caused higher nominal stresses in smaller specimens. Although we did not observe a significant dependency of gene expression data on the anatomical level (Online Resource 4), the trend in stress distribution opposes the physiological trend of increasing load caudally and may have contributed to the already high variance of biological samples.

The lack of an unloaded control group in the study imposes a major limitation. First, we acknowledge that one should be cautious to state categorically that the applied loading was indeed physiological. However, there is ample

literature on the beneficial, anabolic effect of mechanical loading at the levels used in this study (equivalent to ca. 0.2–1.3 MPa pressure applied to the cells, frequencies of 0.01–10 Hz, and daily loading between 30 and 120 min) [11–14]. Intradiscal pressures in this range have also been shown to occur in vivo during the performance of normal daily activities [13, 14]. As all groups were loaded, and the trauma group showed a substantial degeneration not seen in the non-trauma group, this supports that the loading level is physiological and provides a relevant base condition for evaluating the effects of injury. Second, the specific contribution of simulated physiological loading to DD cannot be assessed. Comparing loaded and unloaded spinal segments could provide important additional information on the isolated effect of the applied physiological loading on anabolism, catabolism, inflammation, and apoptosis. This should be investigated in a broader study, including trauma and control specimens, with and without post-traumatic loading. Extending the current study with unloaded control groups is not possible, since variation between animals and general experimental variation precludes pooling of data from sequentially performed experiments.

Despite these major limitations, the present study identifies several new key aspects of disc degeneration following trauma, in the presence of mechanical loading, such as nuclear disorganization and annular collapse in the loaded model, interaction of MMP activity and inflammatory response (with possible stimulation of the NF- κ B pathway) and a strong inter-compartment correlation in the immediate post-traumatic response, with the involvement of the AF, not seen in the previous unloaded model. These results are consistent with “endplate-driven degeneration” and are independent of the limitations discussed above.

The addition of post-traumatic loading after a burst fracture represents an essential step of sophistication of the trauma model. First, the presented model mimics more accurately the loading conditions in burst fracture patients and hence the degenerative process in the IVD. Second, it allows to investigate the advantages and disadvantages of different post-traumatic loading regimes in further studies. Burst fracture patients are often mobilized very early in order to maintain vertebral bone mass. However, it is conceivable that any incremental damage during the strong catabolic-inflammatory phase aggravates DD, as suggested by the triple-stab injury model [35]. Additional trauma during that time was shown to cause a persistent inflammation and enhance DD, possibly by transforming disc cells into a more active form [35]. Finally, cyclic compression of fractured spinal segments may also provide sterical access for immune cells from the vertebral bone marrow to the IVD through the formation of annular clefts. Immune cells were shown to be activated by disc cells and suggested to be involved in DD [36].

Taken together, an in vitro burst fracture model with physiological post-traumatic loading has been developed and applied to demonstrate that burst spinal segments undergo strong and persistent degenerative changes under these conditions.

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Conflict of interest None of the authors has any potential conflict of interest.

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