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PARÂMETROS GENOTÓXICOS NO DISCO INTERVERTEBRAL DEGENERADO HUMANO E SUA LIGAÇÃO À PATOGÊNESE DA DOENÇA

Dissertação apresentada à Banca de Mestrado da Universidade de Caxias do Sul para obtenção do Título acadêmico de Mestre em Ciências da Saúde.

Caxias do Sul
2019

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Orientador: Prof. Dr. Asdrubal Falavigna

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Dissertação de Mestrado submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul, como parte dos requisitos necessários para a obtenção do título de Mestre em Ciências da Saúde, Linha de Pesquisa: Engenharia e Terapia Celular

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Dedicatória

Dedico esta obra a minha amada esposa **Natasha** e aos nossos filhos **Marco Antonio e Vitor Hugo**. A vocês, meu amor incondicional !

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Sumário

Esta dissertação de Mestrado Acadêmico Stricto Sensu é apresentada no formato exigido pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul. A mesma é constituída da secção de “Introdução com referências bibliográficas”, a inclusão do artigo original submetido/publicado em periódico Qualis A na classificação da Coordenação de Aperfeiçoamento de Pessoal em Nível Superior (CAPES), e as “Considerações Finais e Perspectivas”.

1. INTRODUÇÃO

A degeneração do disco intervertebral (DIV) lombar tem sido considerada uma das principais causas de dor lombar e ciática, com redução da qualidade de vida em mais de 80% das pessoas no mundo (1). Os fatores que levam a doença degenerativa discal (DDD) são multifatoriais, relacionados ao envelhecimento e a senescência celular, gerando um elevado impacto social e econômico por afetar uma população economicamente ativa (2-7).

1.1 Lombalgia: incidência e impacto

A dor lombar e a ciática causam elevada incapacidade e morbidade, afetando mais de 500 milhões de pessoas segundo análise do *Global Burden of Disease* com um aumento da prevalência em 17,3% quando comparado aos últimos 10 anos (2, 8, 9). Estima-se que cerca de 266 milhões de casos de DDD associada à lombalgia ocorra em todo o mundo por ano, estando entre os cinco problemas mais frequentes nas emergências dos Estados Unidos, gerando um custo anual estimado de 253 bilhões de dólares (10-12). No Brasil, a dor nas costas foi considerada uma das principais causas de invalidez e auxílio-doença em 2007 (cerca de 30/100.000 contribuintes), sendo mais prevalente em homens idosos (10, 13, 14).

A fisiopatologia da DDD não está totalmente esclarecida. Faltam estudos que permitam diferenciar o processo fisiológico do envelhecimento ou senescência, do processo degenerativo patológico e doloroso (15-17). A DDD tem sido atribuída ao efeito cumulativo de fatores genéticos, envelhecimento, estilo de vida, ambiental, traumático e tabagismo (Figura1) (18-25).

1.2 Anatomia e função do disco intervertebral

O disco intervertebral (DIV) é um tecido fibrocartilaginoso composto pelo anel fibroso (AF), o núcleo pulposo (NP) e o platô cartilaginoso. O AF é definido como uma estrutura circular lamelar consistindo primariamente de fibras de colágeno tipo I que envolvem o NP. O NP possui inicialmente células notocordais substituídas gradualmente por condrócitos no processo de envelhecimento. O NP apresenta proteoglicanos e colágeno tipo II (Figura 1). O platô cartilaginoso é constituído de placas de cartilagem vascularizadas que protegem o DIV e permitem a difusão de nutrientes para o AF e o NP (5, 11, 18).

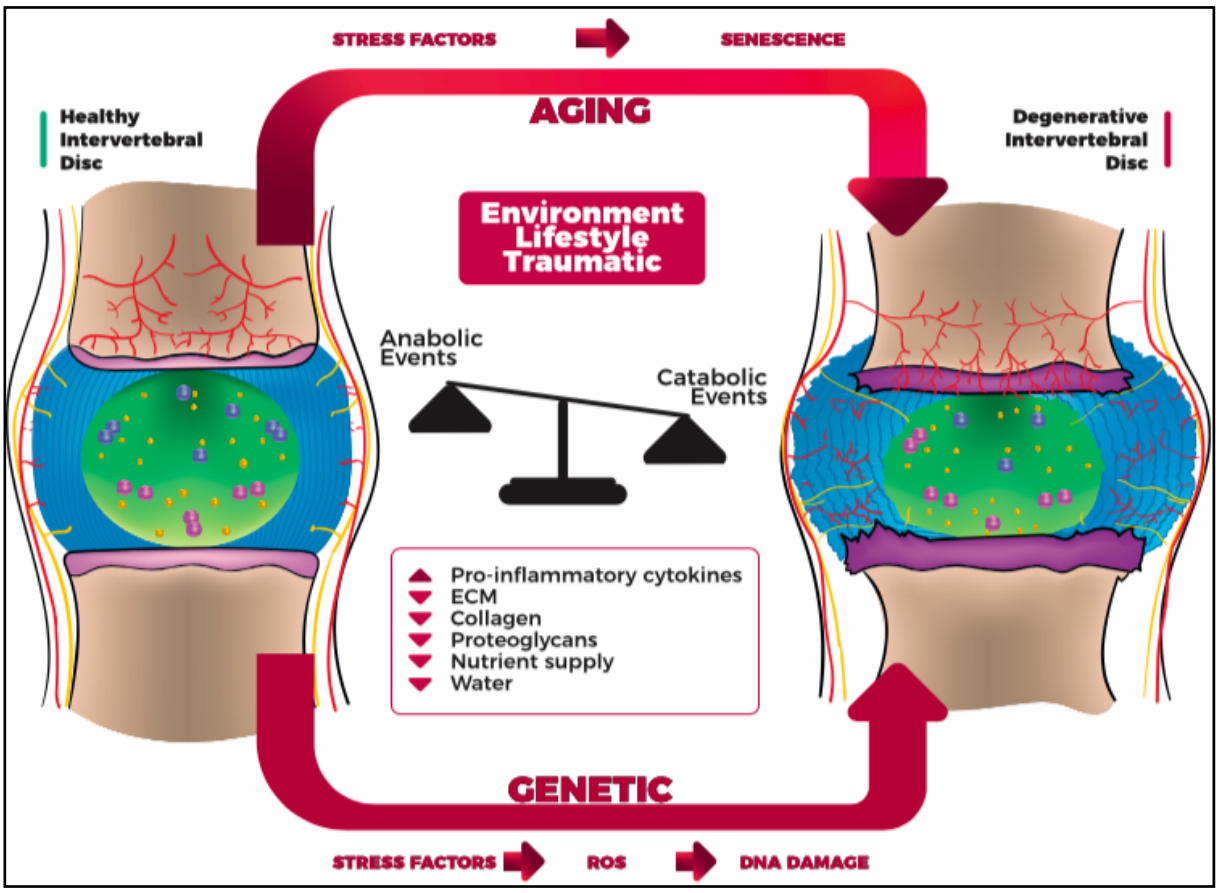

Figura 1: Desenho esquemático da estrutura do disco intervertebral saudável e degenerado e seus fatores etiológicos.

1.3 Etiologia e Fisiopatologia

Fatores genéticos e ambientais podem acelerar o processo de DDD (20). Estudos com gêmeos idênticos observaram uma taxa de hereditariedade de DDL entre 74% a 77%, ressaltando a importância do componente genético na fisiopatologia do processo degenerativo (20, 26). A DDD é decorrente de uma alteração da matriz extracelular (MEC), diminuição da produção de colágeno e proteoglicano (18). As modificações fisiopatológicas dependentes do envelhecimento começam a partir da primeira década de vida (11). O suprimento vascular diminui nas cartilagens da placa terminal e, consequentemente, há uma menor difusão de nutrientes como glicose e oxigênio para o disco, resultando em alta concentração de ácido lático e baixo pH, diminuindo a densidade de colágeno II e proteoglicanos (27, 28). A obesidade e o tabagismo são também condições independentes relacionadas a DDD (21, 24, 25). O resultado final é uma capacidade reduzida de regeneração celular e a morte celular progressiva das células do DIV (29).

1.4 Senescência Celular

A senescência celular é um processo irreversível de envelhecimento que interrompe o ciclo celular a partir do encurtamento dos telômeros, sendo esta uma via importante na fisiopatologia da DDD (11, 18, 30, 31). A senescência do DIV decorre do aumento do catabolismo celular geradas pela maior produção de enzimas de degradação e das citocinas pró-inflamatórias(30).

Evidências atuais sugerem que a senescência pode ser ativada pela erosão gradual dos telômeros, estresse oxidativo, ativação do oncogene e / ou dano ao DNA (32, 33). O dano ao DNA desencadeia uma redução progressiva dos telômeros e uma replicação incompleta do DNA pela via de sinalização p53-p21-Rb. Este processo inibe a proliferação de células discais e ativam a via de sinalização de p16-Rb, interrompendo o ciclo celular (18, 30). Mais estudos se fazem necessários para se conhecer a real influência do DNA nos fatores intrínsecos e extrínsecos da cascata degenerativa e os melhores biomarcadores de prognóstico da DDD (34).

1.5 Dano ao DNA e agentes genotóxicos

Os fatores genotóxicos podem ser classificados como intrínsecos e extrínsecos. Pode-se citar como fatores intrínsecos o estresse oxidativo com liberação de espécies reativas de oxigênio (EROs). Os principais fatores extrínsecos são a diabete mellitus, radiação e a sobrecarga mecânica (34-36). Estes danos genotóxicos impedem a replicação e transferência genética do DNA, induzindo a mutações e aberrações cromossômicas (37).

A genotoxicidade intrínseca se caracteriza pelo acúmulo de células senescentes no DIV a qual desencadeia um ambiente catabólico, chamado de fenótipo secretório associado a senescência (SASP). Este fenômeno está vinculado a danos no DNA pelo elevado potencial indutor de EROs (11, 18). Com a progressão da patologia, a difusão das substâncias diminui, provocando também a privação de oxigênio e a apoptose (38-40). Os danos ao DNA ativados pelo estresse oxidativo, promovem uma inativação de genes de reparo de DNA, incluindo Brca1, Xrcc4 e DNA ligase IV e ativação das vias p53-p21-Rb e p16-Rb (11, 30).

As EROs são fonte de importantes danos genômicos. O desequilíbrio provocado pelas EROs está envolvido no aparecimento de doenças como câncer, doenças degenerativas e envelhecimento(40). Os principais alvos das EROs são os lipídeos, proteínas, carboidratos e o DNA(7).

A apoptose é uma forma de morte celular que ocorre de forma programada, podendo ser estimulada durante o desenvolvimento, organogênese, envelhecimento, resposta inflamatória e eliminação de células após a ocorrência de dano celular por agentes genotóxicos. Dessa forma, a apoptose tem grande importância no mecanismo celular preservando a homeostase tecidual e um importante fator regulador no DDD, pois leva à redução das células DIV (6). A apoptose ocorre como resultado da geração descontrolada de enzimas de degradação MEC como metaloproteinases de matriz (MMPs) e desintegrina e metaloproteinase com trombospondina (ADAMTS) e redução de metaloproteinases da matriz inibidora de tecido (TIMPs) que são os inibidores de MMPs e ADAMTS. Além disso, foi detectada uma redução na oferta de nutrientes e fatores de crescimento, como o fator de crescimento semelhante à insulina (IGF-1), fator de crescimento de fibroblastos (FGF) e fator de crescimento derivado de plaquetas (PDGF) (6). A consequência é uma resposta inflamatória com a produção de interleucinas (IL) e fator de necrose tumoral (TNF) que ativam a produção de agentes catabólicos, determinando um processo cíclico de degradação celular que supera a capacidade de recuperação (15,30,41). A via mitocondrial da apoptose é a via mais comum desse processo. Geralmente ocorre por fatores de estresse intracelular, como danos no DNA, privação de fatores de crescimento e hipóxia. Alterações são observadas na permeabilidade da membrana mitocondrial e perda da homeostase celular, interrompendo a síntese de ATP e aumentando a formação de ROS, que promove a ativação das caspases 9 e 3(41).

Feng e cols.*(34),* estudaram a resposta das células do DIV pela tensão mecânica cíclica não fisiológica e demonstraram um aumento da senescência decorrente do efeito genotóxico de dano ao DNA nas células do NP, com formação de focos de anticorpo γ-H2A.X nos seus núcleos. A senescência prematura das células do NP começou 48 horas após o início da estimulação, sendo dependente do tempo de até 12 horas de aplicação de tensão mecânica. Este estudo sugere que quanto maior o tempo de aplicação da carga não fisiológica no disco, maior será o dano ao DNA e formação de células senescentes (34).

A prolina-glicina-prolina N-acetilada (N-Ac-PGP) é uma quimiocina derivada do colágeno e encontrada no NP do DIV, estando envolvida no processo de senescência (42). A N-Ac-PGP induz a senescência do DIV pelo aumento do catabolismo da MEC e formação de cascatas inflamatórias (35). A porcentagem elevada de células positivas para y-H2A.X sugere dano ao DNA decorrente da

produção de EROS, e o aumento da expressão das vias p53-p21-Rb e p16-Rb(35). Este estudo reforça a contribuição inflamatória na evolução da senescência.

A relação do estresse oxidativo e o dano ao DNA foi estudado utilizando o peróxido de hidrogênio (H_2O_2) por Dimozi e Zhou (43, 44), que demonstraram que a exposição por H_2O_2 correlacionou-se com aumento do dano ao DNA e a senescência prematura destas células.

A exposição ao tabaco por 7 semanas foi avaliada através de um modelo *in vivo* em ratos (45). Os ratos submetidos à inalação de tabaco apresentaram alterações degenerativas significativas e perda de componentes da matriz de proteoglicanos no DIV(45). Entretanto, esses resultados podem ser explicados apenas parcialmente pelos danos no DNA, uma vez que o efeito foi leve e não estatisticamente significativo ($p < 0,18$).

Os fatores intrínsecos e extrínsecos impactam diretamente na senescência e degeneração do DIV. Não se pode precisar a correlação entre o dano do DNA e a gravidade da senescência ou seu papel na DDD devido à heterogeneidade dos estudos e a escassez de publicações (34-36, 43-46).

O presente estudo tem como objetivo analisar a relação do grau do dano do DNA com o DDD e examinar sua resposta na apoptose, viabilidade celular, esterásica, EROs, caspase e potencial de membrana mitocondrial em discos humanos submetidos à cirurgia. Os resultados desses achados podem indicar se o dano acumulado do DNA nuclear na cartilagem do disco está associado à patogênese da DDD.

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3. ARTIGO

Genotoxicity Clues to Predict Intervertebral Disc Degeneration: A Systematic Review

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Genotoxicity Clues to Predict Intervertebral Disc Degeneration: A Systematic Review

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Running Head: Genotoxicity and Intervertebral Disc Degeneration

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Abstract

Study Design: Systematic review.

Objective: To characterize the association between DNA damage and Intervertebral disc degeneration (IDD).

Summary of Background Data: IDD is the main disorder causing low back pain and is the most promising target for intervention. Many factors can contribute to the etiology, such as genetics, environment and lifestyle, but it is not yet fully understood. DNA damage can influence this process and needs to be studied, as well as the agents that can determine these damages.

Methods: A systematic literature search of PubMed, Web of Science and Scopus was performed to identify studies related to DNA damage to the intervertebral disc.

Results: After screening 61 records, 7 articles were included according to the selection criteria. All studies showed some relation between DNA damage and IDD. However, DNA damage was always considered a secondary issue to be investigated.

Conclusions: Many factors can influence DNA damage induced by different genotoxic agents on the degenerative cascade of IVD. However, the correlation between IDD severity and DNA damage, as well as the factual role of DNA damage in disc degeneration could not be defined.

Level of Evidence: 3

Key words: DNA damage; genotoxicity; biomarker; intervertebral disc degeneration.

Introduction

Intervertebral disc degeneration (IDD) has been considered the main etiology of low back pain, and it will affect over 80% of the population worldwide at some point in their lives (1). IDD generates high disability rates and high treatment costs with medication for pain relief, physical therapy, spinal injections, and surgery (1).

Despite the high impact on the patients' lives, the etiology and pathophysiology of IDD are not fully understood (2). The degenerative disc process begins early in life and has been related to aging and cellular senescence due to change in the extracellular matrix (ECM) and decreasing collagen and proteoglycan production (2). Genetics, environmental factors, and lifestyle contribute to disc degeneration (3-7). Genetic influence on disc degeneration is up to 74% (3, 8-10). Obesity, smoking daily, and mechanical loading are independent conditions related to IDD (11, 12). A lower influence of repetitive physical loading was observed in a twins study (13). The correlation between IDD with genetics and environment was observed in the COL9A3 polymorphism and persistent obesity (14) and the whole-body vibration and IL1A-889T allele (15).

DNA damage and alterations of gene expression by extrinsic or intrinsic factors can be an important biomarker of IDD. The objective of this paper is to perform a literature review on the impact of DNA damage on IDD to determine genotoxic biomarkers as well as to indicate the best assay to evaluate DNA damage for IDD.

Methodology

The review was conducted following the methodological guidelines outlined by the Transparent Reporting of Systematic Reviews and Meta-Analyses (PRISMA) (16).

Literature Search Strategy

PubMed.gov (http://www.ncbi.nlm.nih.gov/pubmed), Web of Science (https://webofknowledge.com) and Scopus (www.scopus.com) were searched to identify articles related to DNA damage of the intervertebral disc from January 2000 to August 2018. References derived from the included studies were evaluated to find additional reference articles pertaining to the topic.

The criteria for the search in all fields were the following string: ((DNA damage OR genotoxic OR genotoxicity) AND (Intervertebral Disc OR Nucleus pulposus OR Annulus Fibrosus OR Cartilage Endplate) AND (Intervertebral Disc Disease OR Intervertebral Disc Degeneration)).

The results found in the three databases were compared, duplicate records were removed, and the rest were screened for inclusion by title and abstract review. Full-text article review was performed to ensure that all relevant papers were captured. Eligibility assessment was performed independently in a standardized manner by two reviewers (authors C.A.C. and N.F.N.). Discrepancies between reviewer assessments were discussed with an independent, blinded third reviewer (author A.F.) until a consensus was reached.

Eligibility Criteria for Study Selection

Selection criteria were stated as follows:

- the article was published in English;
- the study included analysis of DNA damage on intervertebral disc;
- the study analyzed the genotoxicity on intervertebral disc;
- the article was published in a peer-reviewed journal;

Exclusion criteria consisted of systematic reviews or meta-analyses, letters to the Editor, commentaries, papers that did not involve DNA damage and genotoxicity, or analysis of DNA damage and genotoxicity in other tissues rather than the intervertebral disc.

Data extraction and analysis

After the exclusion process, the full text of each article was reviewed. Baseline characteristics of each article were extracted from each paper: Author, year of publication, type of study, experimental model used, evaluation of DNA damage and genotoxicity, and follow up. These outcomes were compiled and organized using Microsoft Excel.

Results

All studies found in this review were published in the last five years (2013 to 2018), which shows the recent interest concerning the genotoxicity and IDD development and/or outcome. However, in most of them, the DNA damage was considered a secondary issue and not a key regulatory event to solve questions about intervertebral disc damage.

Study characteristics

The database search was performed and yielded 61 studies and a flow diagram illustrating the screening process (Figure 1- PRISMA). Among the collected studies, 20 of the articles were removed because they were duplicates. The abstracts of 41 non-duplicate articles were screened and 33 were discarded for not meeting the search criteria (6 reviews; 12 studies related to other applications and 15 did not involve the disease or DNA damage).

The remaining 8 full-text articles were included for analysis. After the full-text articles were read, one more article was excluded because it did not analyze the DNA damage, but rather DNA quantification to evaluate cellular proliferation. Finally, 7 studies were included in the present review.

The animal models and techniques used to cause and evaluate the DNA damage were different from one study to another. The majority used rat models to obtain samples for *in vitro* analysis and only one investigated the genotoxic profile in human disc cells (17). DNA damage was identified using primary antibodies, against histone γ-H2A.X in four studies (17-20), PicoGreen assay in two (21, 22) and comet assay in only one (23). Heterogeneity was also found in DNA damage-inducing agents. A mechanical agent was studied in three papers (18, 19, 22), chemical agents in five articles (17, 20-23), and both mechanical and chemical effects in only one paper (22). Mechanical agents included cyclic mechanical tension (CMT), high oxygen tension and ionizing radiation. The chemical agents employed included N-acetylated prolineglycine-proline(N-Ac-PGP), hydrogen peroxide(H_2O_2), tobacco smoke and mechlorethamine (MEC). Studies demonstrated the relationship between mechanical/chemical agents that damage the DNA of Intervertebral disc (IVD) cells and different up- or down-regulated pathways that establish genotoxic influence on IDD pathogenesis (table 1).

Discussion

IDD has been extensively studied due to the chronic condition and its high incidence, which leads to high-cost treatments and interventions that often do not provide a resolution (24). Extrinsic factors contributed to disc degeneration changes such as cigarettes and obesity (11, 12). Intrinsic factors and genetic profile also play an important role in IDD and influenced the mechanisms of cell senescence (3, 8). Despite all the investigative efforts, the real influence of the DNA damage on the degenerative cascade, the intrinsic and extrinsic factors involved and the best biomarkers to guide the disc degeneration prognosis are not fully understood. There is still a vast field of study to understand the impact of DNA damage on IDD development and prognosis.

Genetic influences

Studies on identical twins showed 74% to 77% heritability rates of IDD, highlighting the importance of the genetic component in the pathophysiology of the degenerative processes (3, 25). A high number of candidate genes and mutated alleles are responsible for the morphology and function of ECM and are related to the heritage of IDD pathophysiology (26, 27). The most common genetic variations are the polymorphisms found in genes that affect collagens I, II, III, IX, XI, and aggrecan synthesis; the release of interleukins I, VI, X, and matrix metalloproteinases enzymes II, III, IX, and vitamin D receptor (25, 26). It is impossible to precise if the alteration of IDD came from genetic or aging process.

Disc aging and senescence

The IDD has morphological and functional changes related to the aging progress (28). Different IVD structures at different times are affected during its natural process, which could become a source of pain and disability leading to the pathological condition that characterizes the IDD. Unfortunately, it is not yet possible to predict in advance which IVD will become symptomatic since there is a lack of specific biomarkers to distinguish it.

Cellular senescence consists of an irreversible cellular aging process that arrests the cell cycle of the telomere shortening (2, 29). The main hypothesis accepted for disc senescence is based on the imbalance between anabolic and catabolic mechanisms that accelerated degeneration by degrading the ECM of the disc (29, 30). The increases of matrix degradation enzymes and proinflammatory cytokines promote the degenerative process, with the catabolism outweighing the

anabolism (29) (Figure 2). The progressive reduction of telomeres triggered by a DNA damage response leads to incomplete DNA replication and activates the p53-p21-Rb signaling pathway, that promotes the replicative senescence. These reductions of proteoglycan production and collagen release create an oxidative stress environment, which increases the reactive oxygen metabolites that inhibit disc cell proliferation and activate the p16-Rb signaling pathway to induce a cell cycle arrest of disc cells in response to DNA damage (2, 29).

Genotoxic clues

Feng and coworkers (18) studied the senescent response of disc cells to mechanical stress. This experimental work demonstrated that the unphysiological cyclic mechanical tension increased premature cell senescence due to a direct genotoxic effect on DNA damage in NP rat cells by the formation of γ-H2A.X foci in the nuclei of NP cells. Premature senescence of NP cells started 48 h post-stimulation and occurred in a time-dependent manner for up to 12 hours of cyclic mechanical tension application, suggesting that the longer the application time of an unphysiological load on the disc, the greater will be the DNA damage and senescent cell formation on it will be (18). In another paper by the same research group, the authors evaluated the influence of high oxygen tension (O_2 20%) on disc cell senescence and proved that the enhancement of Reactive Oxygen Species (ROS) leads to DNA damage by upregulation of p53-p21-Rb and p16-Rb pathways to mediate premature pro-senescent effect (19).

Another intrinsic agent involved in this process is the N-acetylated proline-glycine-proline (N-Ac-PGP), a chemokine derived from collagen that was recently found in the degenerative human NP and reinforces the inflammatory contribution to the evolution of senescence (31). The relation between N-Ac-PGP and disc cell senescence was evaluated in an *in vitro* culture of rat NP cells and showed an enhancement of ECM catabolism followed by inflammatory cascades and consequent premature senescence induced by N-Ac-PGP (20). In addition, the elevated percentage of y-H2A.X-positive cells also suggested DNA damage linked to ROS production and upregulation of both p53-p21-Rb and p16-Rb pathways, without affecting telomerase activity (20).

Nasto *et al*. (21) performed a rat *in vivo* model to evaluate the exposition to an extrinsic agent of tobacco for 7 weeks (~3.5 years in humans). The rats submitted to tobacco inhalation presented significant degenerative changes and loss of

proteoglycan matrix components in the IVD. However, these findings can be explained only in part through DNA damage because the effect was mild and not significant (p<0,18). The lack of disc nutrition and vascularization common in spine degeneration needs to be further studied to prove the role of tobacco smoking-induced DNA damage (21). DNA damage in mice disc cells was also identified in chronic exposure to the cancer therapeutic agent mechlorethamine and ionizing radiation (22). These results demonstrated that DNA damage drives the loss of disc homeostasis and plays a major role in this process, starting a degenerative cascade (21; 22).

Zhou *et al.,* (23) and Dimozi *et al.,* (17) evaluated the hydrogen peroxide (H_2O_2) capability to simulate oxidative stress and induce DNA damage. The expression of chromobox homolog 8 (CBX8), an important protein that plays a role in cellular senescence and DNA repair, was correlated with increased DNA damage in rat NP cells and associated with lost type II collagen and proteoglycans amounts and cell growth inhibition (23). Interestingly, this was the only study that evaluated DNA damage by comet assay to verify DNA damage in NP cells (23). Along the same lines, the genotoxicity caused by prolonged exposure to H_2O_2 at sub-cytotoxic concentrations induced a catabolic phenotype and led to premature senescence in human intervertebral disc cells (17). Finally, the high phosphorylation of y-H2A.X and the upregulation of extracellular matrix-degrading enzymes proved the *enhancement* of DNA damage (17).

Study limitations

The limitations of this study come from the paucity of publications on DNA damage related to IDD. All studies were experimental *in vitro* cultured cells, the main source of cells was the rat NP, the methodology was very heterogeneous and different types of assay were used to establish the DNA damage, and the DNA damage was considered a secondary issue to be investigated and not a direct event that could trigger the degeneration process. Those factors explain the difficulties to correlate the intrinsic and extrinsic factors of DNA damage in disc degeneration.

It is critical to pursue further knowledge in this field of DNA damage in the IDD in human disc cells to establish the best assay and methodology. It would be useful pursuit a novel biomarker of prognosis and maximize new treatments for this pathology.

In conclusion, intrinsic and extrinsic factors directly influence DNA damage

induced by different genotoxic agents in the degenerative cascade of IVD. The factual role of DNA damage in disc degeneration need to be further studied.

Conflict of interest

The authors have declared no conflicting interest.

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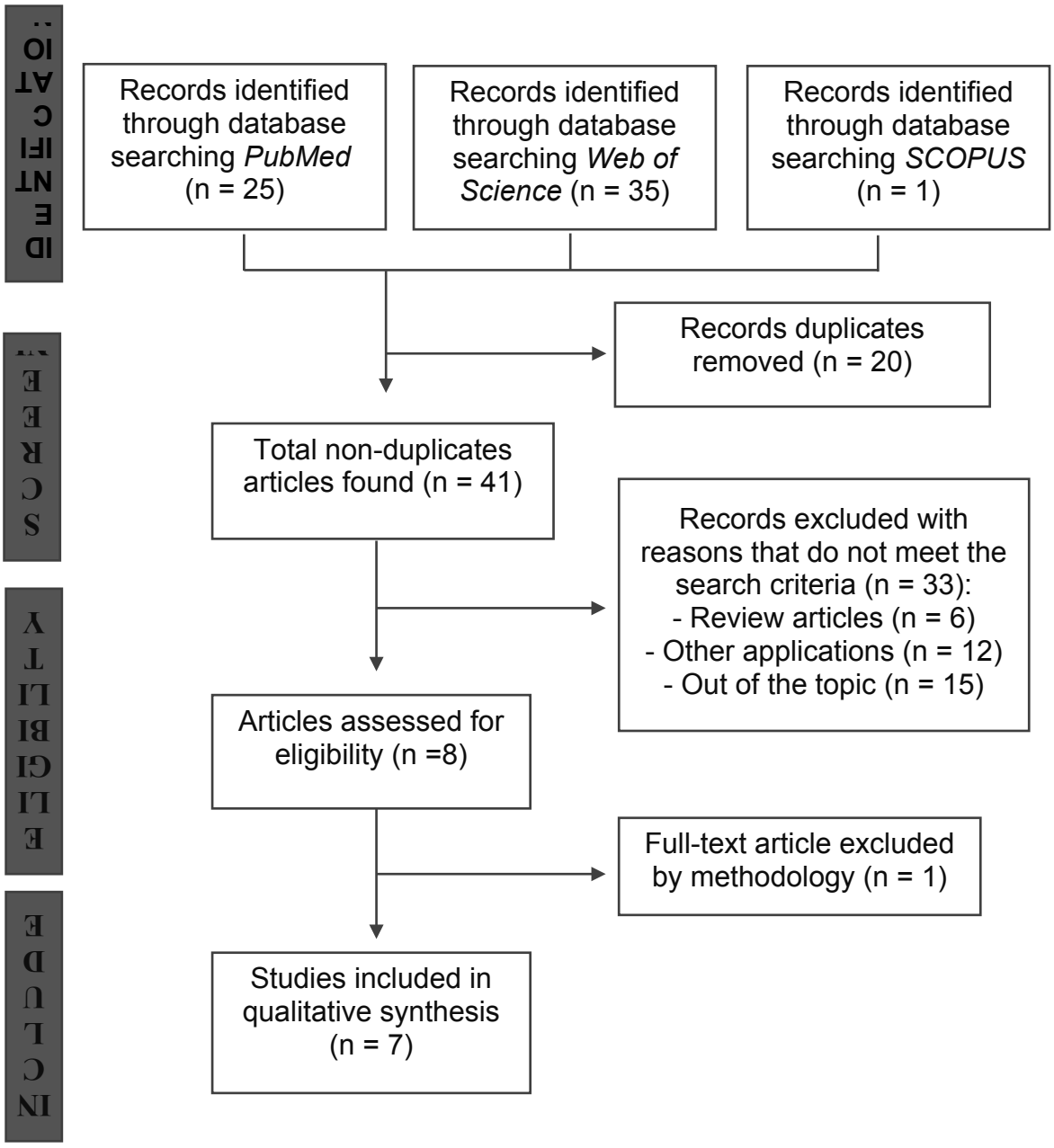

Figure 1. Systematic review flowchart, including inclusion and exclusion criteria.

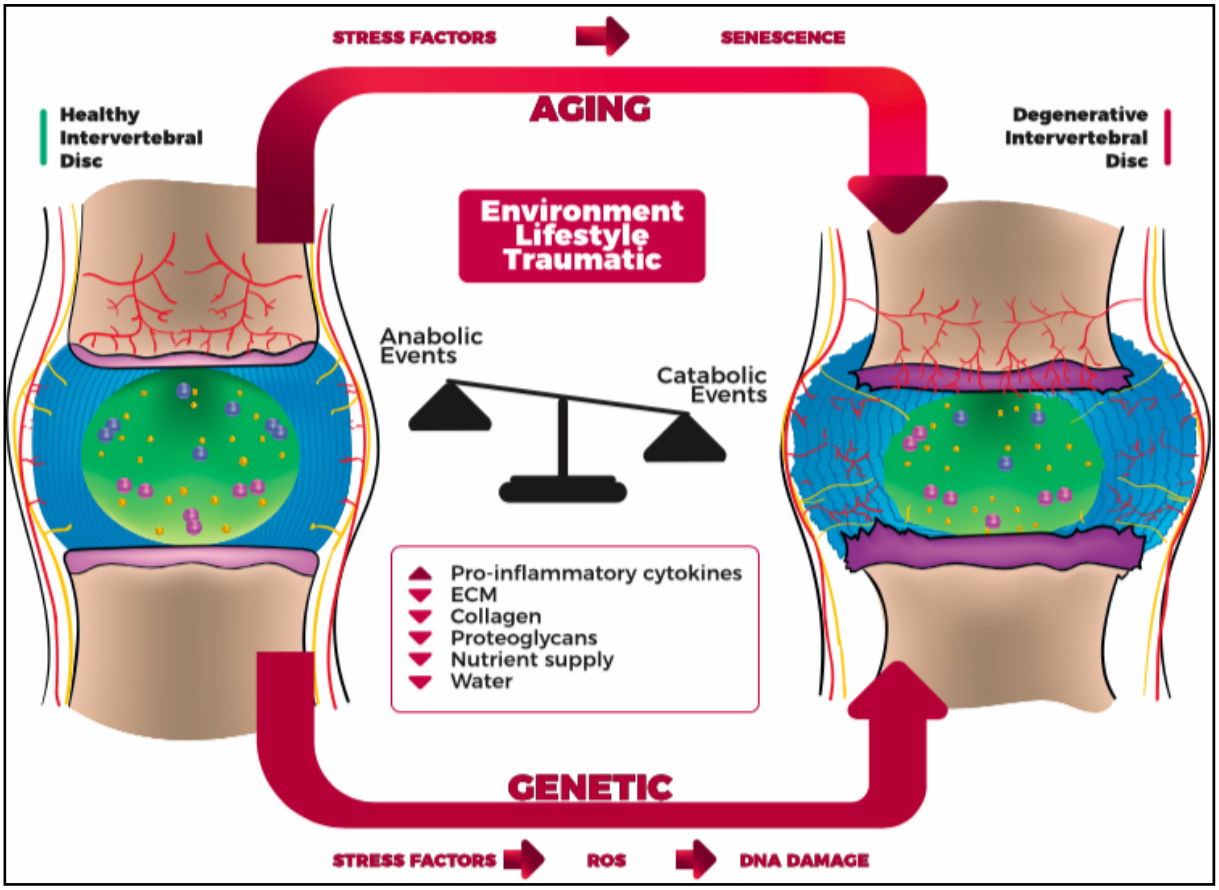

Figure 2 - Schematic drawing of the main etiological factors

Table 1- Characteristics of included studies

Author/Year	Type of DNA damage	Model	Donor tissue/ Cell types	DNA Damage Assay	DNA damage	Main pathway related to DNA damage
	Mechanical					
Feng, 2018 [18]	Cyclic mechanical tension (CMT)	*in vitro*	Rat NP cells	BrdU Incorporation and γ-H2A.X	Positive	p53-p21-retinoblastoma protein (Rb)
Feng, 2017 [19]	High oxygen tension (20% O_2)	*in vitro*	Rat NP cells	BrdU Incorporation and γ-H2A.X	Positive	p53-p21-retinoblastoma protein (Rb) P16-retinoblastoma protein (Rb)
Nasto, 2013 [22]	Ionization Radiation (IR)	*In vitro*	Rat NP and AF cells	PicoGreen assay	Positive	ROS production Direct physical effect
	Chemical					
Feng, 2017 [20]	N-acetylated prolineglycine-proline (N-Ac-PGP)	*In vitro*	Rat NP cells	BrdU Incorporation and γ-H2A.X	Positive	p53-p21-Rb and p16-Rb
Dimozi, 2015 [17]	Hydrogen peroxide (H_2O_2)	*In vitro*	Human NP cells	BrdU Incorporation Y-H2A.X	Positive	p53-p21-Rb
Nasto, 2014 [21]	Tobacco smoke	*In vitro*	Rat NP cells	PicoGreen assay	Positive	Direct effect
Nasto, 2013 [22]	Mechlorethamine (MEC)	*In vitro*	Rat NP and AF cells	PicoGreen assay	Positive	Lack of ERCC1 endonuclease
Zhou, 2013 [23]	Hydrogen peroxide (H_2O_2)	*In vitro*	Rat NP cells	**Cometa assay**	Positive	INK4A-ARF pathway

NP: nucleus pulposus; ROS: reactive oxygen species; BrdU: Bromodeoxyuridine

4. ARTIGO

Genotoxic Parameters of human degenerated intervertebral disc are linked to the pathogenesis of disc degeneration

Artigo submetido a revista Neurosurgery

Genotoxic Parameters of human degenerated intervertebral disc are linked to the pathogenesis of disc degeneration

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Conflict of interest

The authors have declared no conflicting interest.

Abstract

Study Design: Experimental study.

Objective: To evaluate the degree of damage to the DNA and the relation to the severity of the disc degeneration disease (DDD) and measure its response to this insult compared to live/dead cell parameters and reactive oxygen species activity in human disks that have undergone surgery.

Summary of Background Data: DDD is a prevalent disorder that brings great incapacity and morbidity to the world population. The pathophysiology is not fully understood and it is essential to guide the best therapy. DNA damage can influence this process but there are so far few studies to evaluate this topic and its true importance in DDD, as well as whether there is a relation between degeneration grade of degeneration and DNA damage.

Methods: An experimental study was performed with fifteen patients with grade IV or V Pfirrmann DDD classification who underwent spinal surgery for disc herniation or spondylolisthesis after failure of conservative treatment. Five patients were operated on two levels, resulting in twenty samples that were submitted to comet assay to measure DNA damage. Of these, six samples were submitted to flow cytometry and apoptosis, necrosis, cell membrane integrity, intracellular esterase activity, reactive oxygen species (ROS), caspase 3 and mitochondrial membrane potential were evaluated.

Results: All samples had DNA damage, and the average of Index damage (ID) was 78.1 (SD±65,11) and frequency damage (FD) was 49.3% (SD±26,05). There was no statistical difference between the Pfirrmann grades and the genotoxic damage. Likewise all samples of flow cytometry showed apoptosis and ROS to many different degrees.

Conclusions: DNA damage occurs in high grade degenerated human discs and contributes to activation of apoptosis pathway and ROS production that can accelerate disc degeneration.

Key words: DNA damage; genotoxicity; intervertebral disc degeneration, apoptosis, reactive oxygen species

1. Introduction

Low back pain (LBP) is a major factor in lowering quality of life (1). This condition affect more than 500 million people worldwide and the prevalence increased by 17.3% in the last 10 years (2). It is estimated that 266 million new cases of degenerative disc disease (DDD) associated with LBP occur yearly (3). The cost of treatment was estimated as $253 billion dollars annually in the United States (4). Despite the epidemiological and socioeconomic impact, the pathophysiology of DDD is not fully understood. Due to the gap in knowledge, the current treatment is based on the relief of the clinical symptoms, which includes medications, acupuncture, massage, physical therapy, weight loss, injections and surgery(5).

The aging-dependent factor initiating DDD begins in the first decade of life (4). The vascular supply decreases in the endplate cartilages and there is less diffusion of nutrients, such as glucose and oxygen, in the intervertebral disc (IVD) (6, 7). The nutrient deprivation generates an anaerobic metabolism with high levels of lactic acid concentration and lower pH. This microenvironment reduces the density and the metabolic activity of the IVD cells, mainly affecting the collagen II and proteoglycans. The constant catabolism of the IVD leads to a DDD or a functionally impaired tissue by senescence and cell apoptosis (6, 8).

Many factors contribute a different weight to the acceleration of the DDD, such as genetic, obesity, mechanical loading and lifestyle (9-16). In recent years, much has been studied about the molecular changes of IVD cells and cell senescence as the trigger for the degenerative process (17). Current evidence suggests that senescence can be activated by gradual telomere erosion, oxidative stress, oncogene activation and/or DNA damage (18, 19). The impact of DNA damage on DDD development and prognosis, the intrinsic and extrinsic factors involved, and the best biomarkers are poorly understood (17, 20-26). The present study aims to evaluate in human IVD the relationship of DNA damage with DDD severity, number of live and dead cells, and the ROS activity.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with ethical standards and approved by the Ethics Committee of the University of Caxias do Sul (CEP/UCS 2.503.156). The patients were invited by an informed consent form.

2.2. Patient Selection

The symptomatic patients with disc herniation or spondylolisthesis were evaluated and treated initially with analgesic and anti-inflammatory medication, epidural infiltration, postural care, motor physical therapy and muscle strengthening. Radiologic investigation with lumbar spine X-ray and magnetic resonance imaging (MRI) were performed if the symptoms persisted for two weeks. The surgical indication was determined by pain aggravation and progressive loss of motor function, with a clinical-radiological concordance. The final decision for surgery was made by the patient and their family after explaining the efficacy and risks of surgery, and postoperative care. The patient was invited to enter the study and signed the informed consent form if it was decided to perform surgery. The exclusion criteria were the presence of previous lumbar spine surgery or infection.

2.3. Radiological and clinical evaluation

The severity of the lumbar spine IVD was determined by magnetic resonance using the Pfirrmann scale (27). The Pfirrmann scale ranks the severity of IVD from grade I (normal) to grade V (severe degeneration) by acquisition of T2-weighted fast average spin-echo images (27).

The clinical evaluation was performed by the Oswestry lumbar functionality (ODI) questionnaire (28). The scale consists of 10 questions with six alternatives, value ranges from 0 to 5. The total score is divided by the number of questions answered multiplied by the number 5 and the result of this division is multiplied by 100. ODI is classified as minimum disability (0 - 20%), moderate disability (21-40%), severe disability (41-60%), patient who appears invalid (61-80%), and bed-restricted individuals (81-100%) (29).

2.4. Obtaining and isolating the intervertebral disc

The fragments of IVD removed during the surgical procedure were collected and washed twice in sterile vats containing physiological solution. The material was placed in sterile alkaline phosphate buffer (PBS 1X- 0.8% NaCl, 0.02% KCl, 0.02% KH2PO4, 0.088% Na2HPO4) and transported to the cell therapy laboratory.

Cell isolation was performed using the technique described by Vadalà *et al* (30) and modified by Peletti-Figueiró *et al* (31). The IVD sample of 3,454g was enzymatically dissociated [0.2mg/mL Pronase (Nuclease-free, isolated from *Streptomyces griseus*, Cat. Nº P5147-1G, Sigma-Aldrich®, Missouri, EUA) and

1mg/mL Colagenase type IA (from *Clostridium histolyticum*, Cat. Nº C0130-1G, Sigma-Aldrich®, Missouri, EUA)] in a 37°C bath, followed by successive shaking. Then the cell suspension was centrifuged and resuspended in PBS 1X (0.8% NaCl, 0.02% KCl, 0.02% KH2PO4, 0.088% Na2HPO4)(30).

2.5. Comet Assay

The comet assay was performed with some modifications from the technique described by Hartmann & Speit (32), Singh *et al.* (33) and Hartmann *et al*. (34). The isolated and resuspended cells were mixed with 0.5% low melting point agarose (Thermo Fisher Scientific Corporation, California, EUA) and placed on microscopy slides containing 1.5% agarose (Sigma-Aldrich®, Missouri, EUA). A cover slip was placed on the slides containing the material and incubated at 4° C for 10 minutes. The slides were packed in lysis solution containing 2.5M NaCl; 100mM EDTA; 10mM Tris-HCl, pH 9.5; 0.5% (v/v) Triton X-100 and 10% (v/v) DMSO ON at 4°C. Subsequently, the slides were washed, immersed in electrophoresis buffer (1mM EDTA and 300mM NaOH, pH 13) for 20 minutes at 4°C, and electrophoresed for 20 minutes at 17V and 300mA (0.9 V/cm). The material was followed by neutralization with Tris (0.4M, pH 7.5) and staining with silver nitrate for analysis under conventional light microscopy (35).

The efficiency of the technique was determined by negative and positive control. The negative control was done without the presence of cells and the positive control by the induction of fragmentation through the exposure of genotoxic agents of Bleomycin and hydrogen peroxide (1,5mg/mL of Bleomycin and 500mM of hydrogen peroxide). The hydrogen peroxide sample was verified without the addition of the compound for comparative purposes for effectiveness of the positive control.

The grade of DNA damage was measured through the size of the tail. The analysis was performed on a slide, using 100 cells per sample, that were classified in five classes, from zero, without damage, to four, maximum damage (36). The slides were checked, analyzed and determined using a conventional Leica DM2500 optical microscope (200x magnification) with image capture through the LAS V4.4 program. The damage index (ID) and the damage frequency (FD) were analyzed on the slides within the delimited area of 4.9 cm x 1.9 cm. DI is defined as the total damage suffered by the individual, while FD is defined as the number of cells that have suffered some type of damage expressed as a percentage. The ID was calculated according to the following formula: ID = [(No. of comets class 0 x 0) + (No. of comets

class 1 x 1) + (No. of comets class 2 x 2) + (No. of comets class 3 x 3) + (number of class 4 x 4 comets)]. The DI vary between the score 0 (no damage) to 400 (maximum damage) per individual. FD is calculated by the formula: FD = [(number of comet class 1) + (No. of comet class 2) + (No. of comet class 3) + (No. of comet class 4)]. FD ranging between 0 (no cell injured) to 100 (all cells injured) per subject.

2.6. Flow Cytometry

The cells isolated were conditioned in 300µl aliquots of PBS 1X (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.088% Na_2HPO_4) and adjusted to a cell concentration of approximately $1x10^6$ cells/mL for flow cytometer analysis. Intracellular esterase activity, intracellular ROS accumulation, mitochondrial membrane potential and apoptotic cells were analyzed on a FACSCalibur flow cytometer (Becton Dickison Immunocytometry Systems, San Jose, USA) equipped with an argon-ion laser emitting at 488nm. Flow cytometer data of 10,000 gated cells were acquired using CellQuest Pro software (BD Bioscience, San Jose, USA) and data were analyzed in FlowJo V.10 software (TreeStar, Inc.). Cell membrane integrity represents normal live cells.

Intracellular esterase activity was evaluated with Fungalight CFDA, AM/propidium iodide vitality kit (Invitrogen® - Thermo Fisher Scientific Corporation, Carlsbad, USA), following the manufacturer's instructions. Intracellular esterase activity represents live cells with some degree of cellular activity. To quantify intracellular ROS accumulation cells were incubated with 5µg/mL of dihydroethidium (DHE; Sigma-Aldrich®, St. Luis, Missouri, USA) for 30 min at 30°C in the dark. Cells were analyzed using FL3 (488/670) filter.

Mitochondrial membrane potential was evaluated after staining with 175nM of 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma-Aldrich®, St. Luis, Missouri, USA) for 30 min at 30°C in the dark. Cell fluorescence data were acquired using a FL1 (488/533) filter. Apoptotic cells were analyzed by the quantification of detectable phosphatidylserine exposition using an Annexin V-FITC/Propidium iodide apoptosis detection kit (Invitrogen®- Thermo Fisher Scientific Corporation, Carlsbad, USA). Staining and flow cytometer analysis were performed following the manufacturer's instructions.

The presence of active caspases was detected using CaspACE™ FITC-VAD-FMK in situ Marker (Promega Corporation, Madison, USA), that is a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK and it irreversibly binds to active

caspases. The CaspACE™ staining was performed at a final concentration of 10μM and incubated for 30 min at 30°C in the dark.

2.7. Statistical analysis

Data storage was performed in the Excel 2007 program. Statistical analysis was performed through the IBM SPSS® 22.0 for Windows program (IBM, Chicago, IL, USA). The verification of the statistical difference between the data of the Comet Assay (ID and FD) and of the grades of pathology (described by Pfirrmann) was evaluated by the non-parametric Mann-Whitney U test, taking into account significant results with $p \leq 0.05$ and $p \leq 0.01$.

The correlation of the data obtained in flow cytometry, in the clinical-radiological evaluation and in the Comet Assay was determined by Correlation, applying the Pearson coefficient. The samples with $p \leq 0.05$ and $p \leq 0.01$ considering positive and negative correlations were considered significant.

3.Results

3.1 Clinical and radiographic information

The patient's demographic, clinical, and radiographic information were described in Table 1. Fifteen patients underwent surgery for lumbar spine disc herniation or spondylolisthesis. Nine patients were male and the mean age was 53.13 (SD±12.4) the youngest age was 39 years and the oldest was 75 years. The ODI score mean was 65.9% (SD±6.2). Two-level surgery was performed in five patients, resulting in the genotoxicity analysis of the twenty samples. The most approached level was L4-L5 (n = 11), followed by L5-S1 (n = 6) and L3-L4 (n = 3). The Pfirrmann classification was grade four in twelve samples and grade five in 8 samples. There were no IVD samples with Pfirrmann grades zero, one, two, and three. Four patients with a total of six biological samples participated in the analysis by cytometry.

3.2 Comet assay

The comet assay could identify the DNA damage in all samples, demonstrating the effectiveness of the test. The average of ID was 78.15 (SD±65.1) and FD was 49.35% (SD±26.0). There was no statistical difference between the Pfirrmann grades and the genotoxic damage (ID and FD) using the non-parametric test of Mann-Whitney. Additionally, there was no statistically significant correlation using the Pearson test between the degree of degeneration and FD (table 1).

Positive genotoxicity controls induced by hydrogen peroxide (H_2O_2) and bleomycin agents were performed. Both tests had efficient genotoxicity, but greater damage was observed in the hydrogen peroxide samples, proving to be a better agent for the induction of genotoxic damage in IVD cells (fgure 1).

3.3 Flow cytometry

Of the 20 samples collected, 6 were submitted to flow cytometry (table 2). All analyzed samples showed the presence of apoptosis and cellular necrosis to a greater or lesser degree. These findings had an inverse correlation ($p \leq 0.05$) with cell membrane integrity (figure 2).

The ROS also occurred with greater or lesser intensity independent of apoptosis variation, which can be observed mainly in p14, where in the sample of L4-L5 disc had a presence of apoptosis of 61.60% and a production of ROS of 7.92%. Also found an important inverse correlation between ROS and the esterase intracellular activity ($p \leq 0.05$) (figure 3).

Similar results were observed with the presence of caspase 3. In contrast, apoptosis had a strong positive correlation ($p \leq 0.01$) with mitochondrial membrane potential (figure 3).

The results of the comet assay and flow cytometry were complemented in demonstrating the DNA damage. When the cell membrane integrity values were correlated with FD, a negative correlation ($p \leq 0.05$) was observed, meaning that when cell membrane was preserved, a lower cellular FD and a little DNA damage were expected.

Positive controls performed using bleomycin and hydrogen peroxide demonstrate a similar result to those obtained in the comet test.

4.Discussion

The pathophysiology of DDD is very complex and gaps in the literature need to be further studied. The present study confirmed that in severe grades of DDD (Grade IV and V of Pfirrmann classification) DNA damage in the nucleus pulposus cells of human IVD has been observed (table 1). In addition, it demonstrated in the same discs the presence of apoptosis, cell death and varying degrees of ROS production. Other important findings were the presence of caspase 3, changes in membrane integrity and cell esterase activity, and changes in mitochondrial membrane potential (table 2).

The DDD alterations begin early in infancy with the substitution of the producers of proteoglycan and collagen type II, that is the notochord cells, by chondrocytes-like cells (37). At the same time, the endplate cells undergo calcification, involution of the blood supply, lower permeability of the diffusion of glucose and oxygen, and reduction of catabolite removal. This leads to a loss of the balance between cellular metabolism and catabolism, activating the senescence and apoptosis pathways to regulate the DDD (4, 37).

The senescence cells presented in herniated disc have been suggested as a major trigger of disc degeneration (38). Senescence cells are characterized as an irreversible arrested cell-cycle in the G1 phase, unresponsive to mitogenic stimulation, and able to produce degradation enzymes. The harmful microenvironment decreases the functional IVD cells in discs and accelerates the DDD (39). The DNA damage is the main inducer of senescence by activating its signaling pathways (40). This study showed the DNA damage using the comet assay on human advanced IVD, regardless of the grade IV or V (Table 1). These results correlated the DNA damage with the pathophysiological process of DDD. Previous studies of disc degeneration in rats induced by extrinsic agents such as tobacco, ionizing radiation, mechlorethamine and cyclic mechanical stress, demonstrated a genotoxic effect on the DNA and proved the contribution with cellular senescence and the acceleration of DDD. The present study was performed on human degenerate IVD and its main goal is to study DNA damage by direct DNA assessment using comet assay analysis and not indirectly through cellular senescence (17, 24, 41). The accumulation of senescent cells determines a secretory phenotype associated with a senescence (SASP) microenvironment that promotes DNA damage by ROS, that is the most important source of genomic damage in humans (22). As the pathology progresses, a decrease of substance diffusion is observed and further deprivation of oxygen and apoptosis (38). The flow cytometry performed in the six samples in this study, demonstrated a higher production of ROS, a greater DNA damage, and less cellular activity. The higher production of intracellular ROS leads to lower metabolically active cells (intracellular esterase activity), with a tendency to inhibition of cell cycle proliferation and progression to apoptosis. Similarly, intrinsic agents, like ROS production, have been correlated with senescence of the nucleus pulposus cell and DDD (21, 22, 25).

Apoptosis is a form of cell death that occurs in a programmed manner, and can be stimulated during development, organogenesis, aging, inflammatory response

and elimination of cells after the occurrence of cellular damage by genotoxic agents. In this way, apoptosis has a high importance in cellular machinery preserving tissue homeostasis and an important regulating factor in the DDD, because it leads to the reduction of the IVD cells(4). The apoptosis occurs as a result of uncontrolled generation of MEC degradation enzymes like MMPs and ADAMTS and reduction of tissue inhibitor matrix metalloproteinases (TIMPs) that are the inhibitors of MMPs and ADAMTS. Moreover, a reduction of nutrient supply and growth factors, such as insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), and platelet derived growth factor (PDGF) was detected (4). The consequence is an inflammatory response with the production of IL and TNF that activates the production of catabolic agents, determining a cyclic process of cellular degradation that surpasses the recovery capacity (6, 8, 37). The mitochondrial pathway of apoptosis is the commonest route of this process. It usually occurs by intracellular stress factors such as DNA damage, growth factor deprivation, and hypoxia. Changes are observed in the permeability of the mitochondrial membrane and loss of cellular homeostasis, interrupting the synthesis of ATP and increasing the formation of ROS, that promotes the activation of caspases 9 and 3(42). This relation between apoptosis and mitochondrial membrane potential was demonstrated in our study by the flow cytometry analysis, inferring that the greater the mitochondrial membrane potential damage the greater is the cellular apoptosis.

Interestingly, in P10 and P14 samples where 2 levels were approached, both submitted to the comet assay and flow cytometry, it was observed that the level L4-L5 with Pfirrmann IV had a higher value of ID and FD, as well as of apoptosis and cell death. Instead, in the L5-S1 levels with degree of degeneration V, the value of ID and FD was lower, as well as apoptosis and cell death (Table 2). These results possibly have occurred because in the higher degrees of degeneration there is a greater isolation of adult mesenchymal stem cells (mSC) that clump together by external signaling of apoptosis in an attempt to repopulate the cells at the most damaged site. Maybe these cells have very little genotoxic damage, as seen in ID and FD (43, 44). Regarding the expression of ROS and caspase 3, the results were not shown to be equivalent. in sample P14, in grade IV of disc degeneration (L4/L5 level) there was more apoptosis and lower production of ROS and caspase 3, whereas in grade V (L5/S1 level), a lower apoptosis with greater expression of ROS and caspase 3 was obtained. This suggests that at severe levels of IVD degeneration, the DNA damage is an important factor in the pathophysiological

process of the degenerative cascade and contributes to the signaling of cellular apoptosis at higher or lower intensity but it can not be said that its expression is directly proportional to the degree of degeneration. There are certainly other intrinsic agents involved with greater pathophysiological significance, since the samples are from the same patients. Another explanation could be the different pathways (cytoplasmic and mitochondrial) of apoptosis, because although the pathways act in different ways, both activate one of the proteolytic reactions involving activation of initiator caspases (2, 9, 10) and executors (3, 6 and 7) of programmed cell death at different stages of disc degeneration (42).

The correlation of extrinsic or intrinsic agents with DDD was performed mostly by *in vitro* studies using a heterogeneous methodology. Besides that, DNA damage was considered a secondary issue to be investigated and not a direct event that could trigger the degeneration process. In the present study an evaluation of DNA in human high grade DDD was performed using two effective methods. The presence of DNA damage on the degenerated discs and the correlation with apoptosis and intrinsic inductor agents like ROS were demonstrated.

The limitations of this study were the small samples (n=6) submitted to flow cytometry and the lack of negative IVD controls, because the patients with a lower degree of disc degeneration are rarely selected for surgical treatment. It is critical to keep investigating the DNA damage in IVD cells, to better understand its relation with the degenerative cascade to maximize new modalities of treatment for this pathology.

5.Conclusion

DNA damage occurs in high grade degenerated human discs, and is an important intrinsic factor that contributes to DDD physiopathology. Likewise, DNA damage contributes to activation of the apoptosis pathway and ROS production that can accelerate the DDD and cell death.

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Table 1 - Profile of patients and Comet Assay results

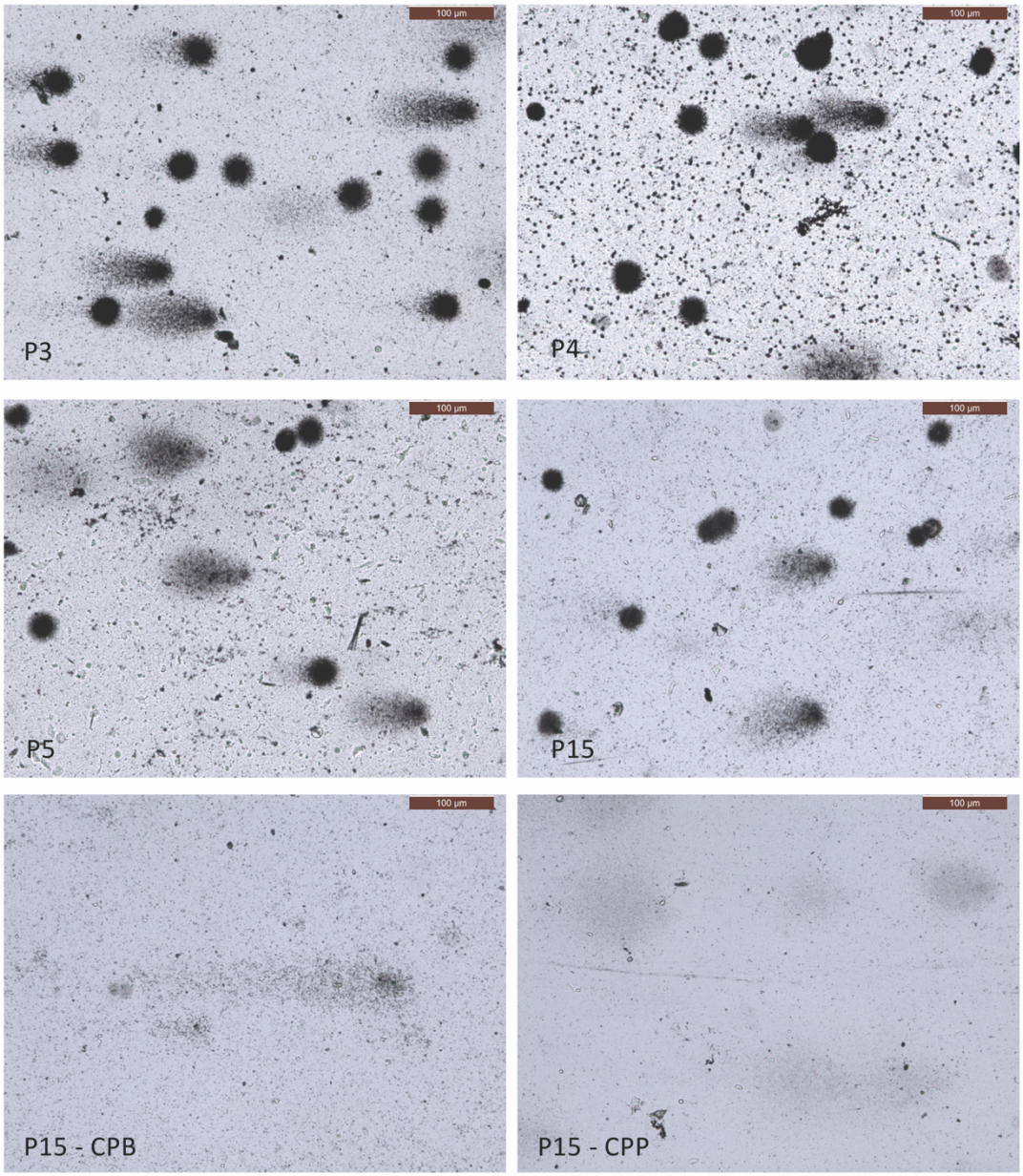
Number of patients	Age	Gender	ODI (%)	Disc level	Pfirrmann grade	Comet Assay	
						Index damage (0-400)	Frequency damage (%)
1	46	F	75	L4/L5	4	116	72
2	49	M	65	L4/L5	4	70	56
3	39	M	65	L4/L5	4	256	97
4	51	M	70	L4/L5	4	170	84
5	72	F	70	L3/L4 L5/S1	4 5	178 42	84 38
6	51	M	60	L5/S1	4	83	77
7	75	M	55	L4/L5 L5/S1	5 4	62 63	57 60
8	64	F	65	L3/L4 L4/L5	5 5	25 20	25 19
9	39	M	70	L5-S1	5	35	32
10	43	F	70	L4/L5 L5/S1	4 5	40 5	24 5
11	44	M	74	L4/L5	4	151	68
12	48	F	65	L4/L5	4	16	16
13	46	F	55	L4/L5	4	78	57
14	59	M	60	L4/L5 L5/S1	4 5	69 27	52 27
15	71	M	70	L3/L4	5	57	37

F=female M= male
ODI= Oswestry Disability Index

rmann rade	Apoptosis (%)	Necrosis (%)	Cell membrane integrity (%)	Intracellular Esterase activity (%)	ROS (%)	Caspase 3 (%)	Mitochondrial membrane potential (%)
4	41.27	34.56	23.51	62.68	46.83	22.77	35.35
5	0.78	4.87	91.47	91.33	10.94	7.51	7.97
4	37.94	39.18	17.43	64.13	31.2	51.94	29.70
4	61.60	16.65	20.40	84.10	7.93	7.52	61.10
5	10.61	20.16	51.48	46.01	39.48	47.34	12.88
5	47.3	12.9	39.7	76.4	11.89	41.09	35.57

gen

Figure 1. Comet assay samples



CPB: Bleomycin positive control
CPP: Peroxide positive control

Figure 2. (A) Incidence of apoptosis, necrosis and cell membrane integrity quantified by flow cytometry in human degenerate intervertebral disc nucleus pulposus cells with IV and V Pfirrmann grades through Annexin V-FITC and PI stained. (B) Intracellular esterase activity incidence by flow cytometry in the same cells with CFDA and PI stained.

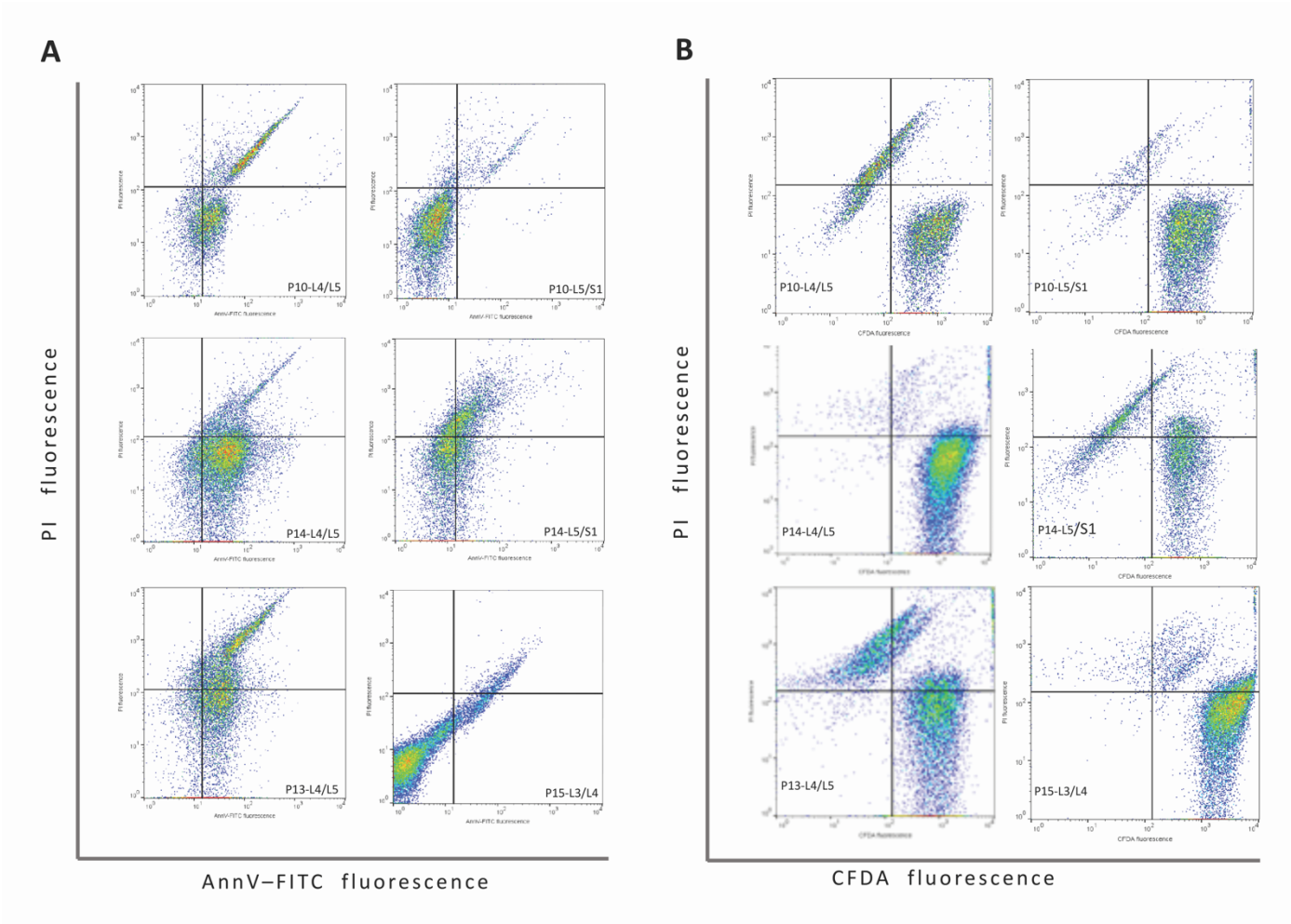
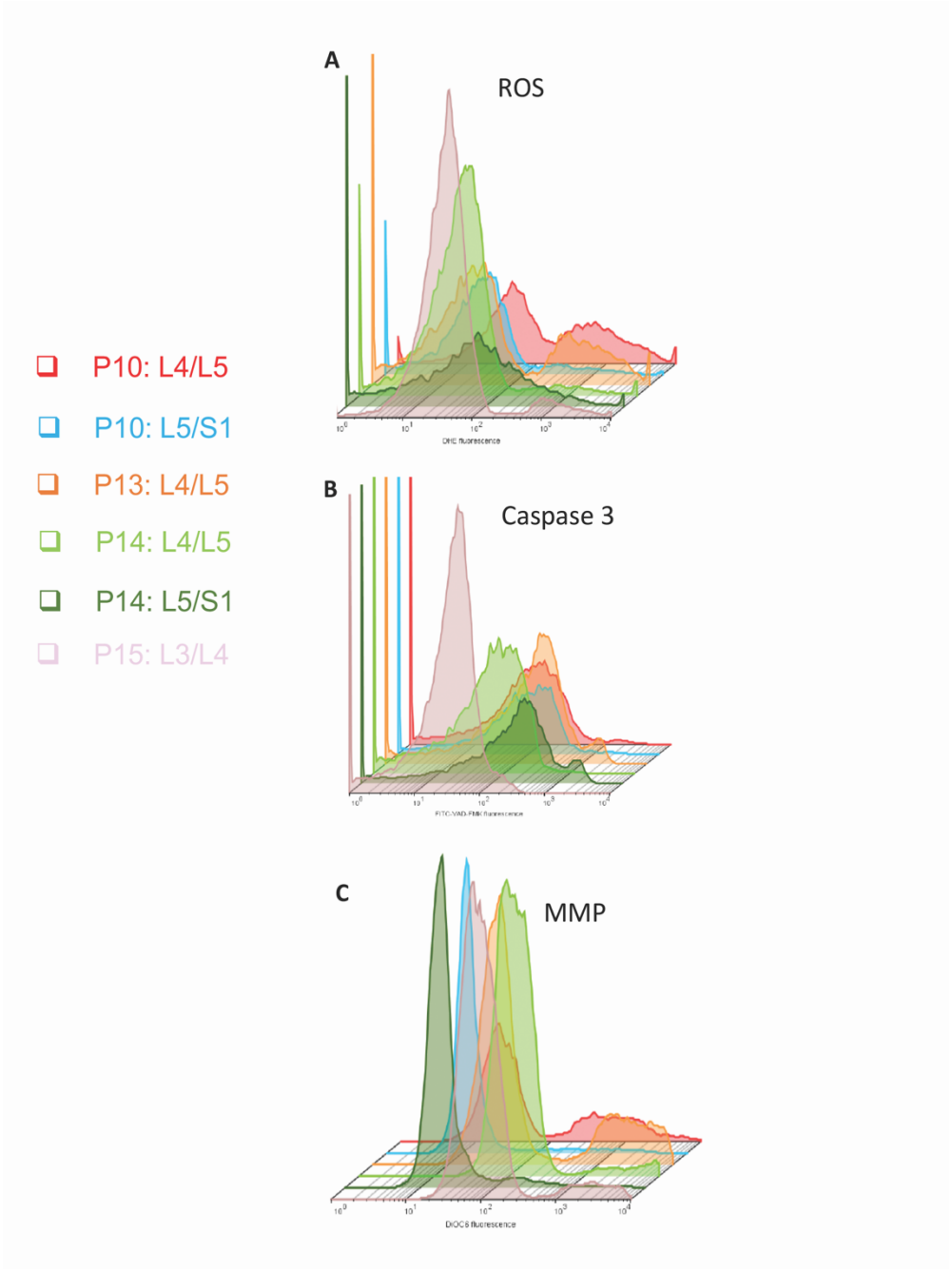


Figure 3. (A) Incidence of ROS by flow cytometry in human degenerate intervertebral disc nucleus pulposus cells with IV and V Pfirrmann grades through DHE stained. (B) Caspase 3 incidence by flow cytometry with FITC stained. (C) Mitochondrial membrane potential (MMP) quantification by flow cytometry with DiOC6 stained.



5. CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

O presente estudo demonstrou através do ensaio cometa a presença de danos no DNA das células do núcleo pulposo de discos humanos degenerados de grau IV e V de Pfirrmann. Similarmente demonstrou a presença de apoptose inicial e tardia nas células discais estudadas, bem como a indução de produção de EROs. Embora não se demonstrou uma relação estatisticamente significativa entre os danos do DNA e a gravidade de degeneração, ficou claro que em discos degenerados de grau IV e V, ocorre fragmentação de DNA e que este processo tem participação na fisiopatologia do processo degenerativo.

A realização de estudos futuros com um maior número de amostras e com discos controles de menor grau de degeneração serão necessários para estabelecer a real importância dos danos do DNA bem como os fatores que podem desencadear ou acelerar esse processo. Também poderá ser realizado no futuro a utilização de softwares existentes para a análise do ensaio cometa (Comet Assay - IV live vídeo measurement system for the comet assay - http://www.cometassay.com), onde podemos comparar os dados com a análise qualiquantitativa realizada neste estudo, além de agilizar a análise e mensurar outras características. Além disso, será interessante associarmos ao estudo genes de reparo do DNA, como Brca1, Xrc4 e DNA ligase IV por PCR Real Time juntamente com a via p53-p21-Rb por Imunohistoquímica e Western Blot.

6. ANEXOS

6.1 Anexo1

The Spine Journal

Genotoxicity Clues to Predict Intervertebral Disc Degeneration: A Systematic Review
--Manuscript Draft--

Manuscript Number:	SPINEE-D-19-00408
Article Type:	Systematic Review/Meta-analysis
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Keywords:	DNA damage; genotoxicity; biomarker; intervertebral disc degeneration
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Abstract:	Objective: To characterize the association between DNA damage and Intervertebral disc degeneration (IDD). Study Design: Systematic review. Summary of Background Data: IDD is the main disorder causing low back pain and is the most promising target for intervention. Many factors can contribute to the etiology, such as genetics, environment and lifestyle, but it is not yet fully understood. DNA damage can influence this process and needs to be studied, as well as the agents that can determine these damages. Methods: A systematic literature search of PubMed, Web of Science and Scopus was performed to identify studies related to DNA damage to the intervertebral disc.This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors have no conflicting interest. Results: After screening 61 records, 7 articles were included according to the selection criteria. All studies showed some relation between DNA damage and IDD. However, DNA damage was always considered a secondary issue to be investigated. Conclusions: Many factors can influence DNA damage induced by different genotoxic agents on the degenerative cascade of IVD. However, the correlation between IDD severity and DNA damage, as well as the factual role of DNA damage in disc degeneration could not be defined.

a. Anexo 2

Neurosurgery

Genotoxic Parameters of human degenerated intervertebral disc are linked to the pathogenesis of disc degeneration

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research-Laboratory
Section/Category:	Spine
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Manuscript Region of Origin:	BRAZIL
Abstract:	Background DDD is a prevalent disorder that brings great incapacity and morbidity to the world population. The pathophysiology is not fully understood. DNA damage can influence this process but there are so far few studies to evaluate this topic and its true importance in DDD, as well as whether there is a relation between degeneration grade of degeneration and DNA damage. Objective To evaluate the degree of damage to the DNA and the relation to the severity of the disc degeneration disease (DDD) and measure its response to this insult compared to live/dead cell parameters and reactive oxygen species activity in human disks. Methods An experimental study was performed with fifteen patients with grade IV or V Pfirrmann classification who underwent spinal surgery. Five patients were operated on two levels, resulting in twenty samples that were submitted to comet assay to measure DNA damage. Of these, six samples were submitted to flow cytometry and apoptosis, necrosis, cell membrane integrity, intracellular esterase activity, reactive oxygen species (ROS), caspase 3 and mitochondrial membrane potential were evaluated. Results All samples had DNA damage, and the average of Index damage (ID) was 78.1 (SD±65,11) and frequency damage (FD) was 49.3% (SD±26,05). There was no statistical difference between the Pfirrmann grades and the genotoxic damage. Likewise all samples of flow cytometry showed apoptosis and ROS to many different degrees. Conclusions DNA damage occurs in high grade degenerated human discs and contributes to activation of apoptosis pathway and ROS production that can accelerate disc degeneration.

b. Anexo 3

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Relação do Grau da Discopatia Degenerativa Lombar com a Genotoxicidade, Apoptose e Autofagia

Pesquisador: Asdrubal Falavigna

Área Temática:

Versão: 1

CAAE: 82871617.5.0000.5341

Instituição Proponente: Fundação Universidade de Caxias do Sul - FUCS/RS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.503.156

Apresentação do Projeto:

Trata-se de projeto intitulado Relação do Grau da Discopatia Degenerativa Lombar com a Genotoxicidade, Apoptose e Autofagia. Os pacientes sintomáticos com discopatia degenerativa lombar (DDL)serão avaliados e tratados inicialmente com medicação analgésica e antiinflamatórios, seja por via oral ou infiltração, cuidados posturais, fisioterapia motora e fortalecimento muscular. Não havendo melhora clínica em 2 semanas, os pacientes serão encaminhados para investigação radiológica através de raios-X simples de coluna e RM. A escolha do tratamento cirúrgico será feita unicamente pelo paciente após discussão, com seus familiares e esclarecidas suas dúvidas quanto ao resultado médico. Se a decisão do paciente for realizar a cirurgia, o mesmo será informado sobre o projeto de pesquisa a ser desenvolvido. Os pacientes serão plenamente informados de que o envio do disco intervertebral para análise e estudo da relação da DDL com a genotoxicidade, apoptose e autofagia. Este estudo não irá modificar o procedimento cirúrgico, uma vez que o mesmo é desprezado no lixo hospitalar. Ou seja, o planejamento, a técnica cirúrgica e suas etapas de acompanhamento não mudam com a participação ou não no projeto de pesquisa. Se o paciente estiver participando da pesquisa, partes dos fragmentos do disco retirado no processo cirúrgico para o tratamento do paciente serão armazenados em recipientes específicos contendo: I- PBS 1X (Tampão fosfato alcalino) para a avaliação de danos no DNA (Ensaio Cometa e Micronúcleos), IIFormalina 10% tamponada para análise histopatológica e imunohistoquímica e III- Nitrogênio Líquido para a verificação de fragmentação no DNA.

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Todos os recipientes utilizados na coleta serão encaminhados ao Laboratório de Terapia Celular da Universidade de Caxias do Sul para a realização das análises.
Os discos intervertebrais degenerados serão obtidos somente mediante o consentimento e assinatura do TCLE. O estudo contará com a participação de 40 pacientes. O anonimato dos pacientes serão sempre mantidos durante todo o estudo. Esta pesquisa não se trata de um estudo piloto. Foram determinados 40 pacientes, pois esta patologia apresenta 5 graus de degeneração, sendo importante para a presente proposta de pesquisa a correlação dos dados obtidos com os graus de severidade da doença. Cabe ressaltar, que para todos os ensaios avaliados neste estudo haverá representatividade amostral dos distintos graus de degeneração, permitindo a definição dos fatores prognósticos envolvidos na degeneração do disco intervertebral.O grupo controle será feito com a participação de 5 pacientes que tenham indicação cirúrgica para retirada do DIV por outras patologias, que não a degeneração do disco intervertebral. Isto permite gerar evidências comparativas adequadas e correlação estatística na definição da relação da genotoxicidade, autofagia e apoptose na DDL. Dessa forma, cabe salientar, que o disco intervertebral controle negativo da
DDL será proveniente de pacientes com escoliose em que houver a necessidade da retirada do disco intervertebral. Todos esses pacientes possuem indicação cirúrgica para a resolução do seu problema de saúde. É importante salientar que os pacientes que concordarem em doar o disco intervertebral a ser usado na pesquisa como controle negativo da DDL e participar do estudo mediante assinatura e concordância do TCLE, não serão tratados cirurgicamente com o objetivo de realizar a pesquisa. Todos os preceitos éticos já informados acima, serão seguidos também com estes pacientes. Para avaliação histológica, os discos intervertebrais coletados em formalina 10% tamponada (pH7,4) serão enviados diretamente ao Laboratório de Terapia Celular da Universidade de Caxias do Sul onde serão processados e corados com Hematoxilina e Eosina (H&E) e Tricrômico de Masson. Ainda, serão utilizados marcadores imunohistoquímicos para Caspase 3, 8 e 9 (apoptose); LC3A/B, MAP1LC3A, SQSTM1/p62 e Histona H3 (autofagia). Para análise genotóxica serão utilizados o Ensaio Cometa e Teste de Micronúcleos.

Objetivo da Pesquisa:
Objetivo Primário:
O objetivo deste estudo será avaliar através de ensaios de genotoxicidade as células do disco intervertebral e a severidade da DDL, além de,
determinar os fatores de autofagia e apoptose relacionados com a patologia e correlacionar todos os resultados obtidos com os dados clínicoradiológicos

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dos pacientes.
Objetivo Secundário:
- Verificar a histopatologia do disco intervertebral degenerado por meio de colorações histológicas.- Determinar danos no DNA do disco intervertebral
degenerado pelas técnica do Ensaio Cometa; Micronúcleos e fragmentação de DNA.- Avaliar os eventos de apoptose e autofagia no disco
intervertebral degenerado por meio da técnica de imunohistoquímica.- Mensurar a atividade das caspases 3 e 9.- Relacionar os resultados obtidos
na avaliação histopatológica, genotóxica, autofágica e apoptótica.- Correlacionar os dados clínicos com as informações radiológicas de raio-X e
Ressonância Magnética (RM).

Avaliação dos Riscos e Benefícios:
Riscos: Segundo os Itens II.22 e IV.3.b, da Resolução CNS nº 466 de 2012 toda pesquisa apresenta riscos nas dimensões física, psíquica, moral, intelectual, social, cultural ou espiritual do ser humano. Além disso, a participação na pesquisa, pode acarretar riscos ligados à manutenção do sigilo e confidencialidade durante a coleta e uso dos dados. Apesar de estes riscos existirem em qualquer pesquisa, os pesquisadores responsáveis pelo
estudo manterão o máximo de sigilo e confidencialidade possível quanto as informações do paciente. A pesquisa será realizada somente com o material do disco intervertebral descartado na cirurgia. Portanto, os riscos que podem vir a existir do processo cirúrgico estão relacionados somente ao tratamento da doença e não a pesquisa proposta.
Benefícios: Através deste estudo poderemos ter benefícios para futuros pacientes com esta patologia: (1) melhor entendimento da doença, de seus possíveis fatores etiológicos e dos mecanismos associados; (2) definição de marcadores prognósticos que possam auxiliar no tratamento da patologia e na definição de fatores degenerativos; e (3) determinação de aspectos relevantes da biologia da DDL em futuras pesquisas aplicadas que possam
contribuir com os aspectos terapêuticos da doença. Os benefícios obtidos na presente pesquisa auxiliarão futuros pacientes a terem menos sofrimento físico, psicológico e social.

Comentários e Considerações sobre a Pesquisa:
Pesquisa bem desenhada e que cumpre os preceitos éticos de proteção ao participante do estudo.

Considerações sobre os Termos de apresentação obrigatória:
Folha de Rosto assinada pelo Prof. Dagoberto Godoy. TCLE adequado. Consentimento da entidade

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Hospital Saúde assinada pelo diretor técnico.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Considerações Finais a critério do CEP:

Diante do exposto, o Comitê de Ética em Pesquisa da Universidade de Caxias do Sul, de acordo com as atribuições definidas nas Resoluções CNS 466/12 e CNS 510/16, aprova o projeto para dar início à pesquisa.

É dever do CEP acompanhar o desenvolvimento da pesquisa, por meio de relatórios parciais e final. Solicitamos que os relatórios contemplem o andamento da pesquisa, as modificações de protocolo, cancelamento, encerramento, publicações decorrentes da pesquisa e outras informações pertinentes. Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e as suas justificativas.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1051799.pdf	21/12/2017 14:24:24		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_Saude_PDF.pdf	21/12/2017 14:19:50	Asdrubal Falavigna	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Autofagia_Apoptose_IDD.docx	21/12/2017 14:19:41	Asdrubal Falavigna	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_PB_Autofagia_Apoptose_IDD.docx	21/12/2017 14:19:20	Asdrubal Falavigna	Aceito
Folha de Rosto	Folha_de_Rosto.pdf	21/12/2017 14:16:31	Asdrubal Falavigna	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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Continuação do Parecer: 2.503.156

CAXIAS DO SUL, 20 de Fevereiro de 2018

Assinado por:
Luciane Andreia Bizzi
(Coordenador)

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