Journal of Arthritis
ISSN: 2167-7921

Zwingmann et al., J Arthritis 2016, 5:2
DOI: 10.4172/2167-7921.1000195

Research Article Open access

Mitochondrial DNA Deletions are Associated with Upregulated Articular Nitrotyrosine in Human Femoral Head Cartilage

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Rec date: February 07, 2016**; Acc date:** February 24, 2016**; Pub date:** February 29, 2016

Abstract

Background: The pathogenesis of osteoarthritis (OA) has been linked with the elaboration of increased amounts of nitrotyrosine in cartilage as a stable marker of tyrosine nitration by nitric oxide. Reactive oxygen species (ROS) are known to damage mitochondrial DNA (mtDNA); this process is associated with aging of non-cartilaginous tissues. This study aimed to link intraarticular nitrotyrosine content, with mtDNA lesions and OA severity in order to gain insights into the contribution of nitrotyrosine and mitochondria to cartilage aging and the development of OA.

Methods: Femoral heads were prospectively obtained from individuals undergoing hip arthroplasty or post-mortem autopsy (22 male, 26 female, age range 16-93 years). OA severity was graded radiographically, and histologically by means of the Mankin score. Articular chondrocytes were isolated, and the 'common' mtDNA deletion quantified with a Polymerase Chain Reaction (PCR) technique. mtDNA copy numbers per chondrocyte were determined by quantitative PCR. Articular nitrotyrosine content in chondrocytes was quantified by ELISA. Multivariate associations between parameters were computed by linear regression analysis or Spearman rank correlations, as appropriate.

Results: Articular nitrotyrosine concentrations were independently correlated with both, the age of the subjects (r=0.39, p=0.01) and the presence of the common deletion (r=0.48, p=0.004), but not with the Mankin Score (p=0.84) or wild type mtDNA copy numbers. The severity of hip OA (Mankin score) however was only correlated with patient age, but not with articular nitrotyrosine, mtDNA deletions or wild type mtDNA copy numbers.

Conclusions: mtDNA lesions are correlated with articular nitrotyrosine content in hip cartilage, but a link of mitochondrial mutagenesis and NO-mediated ROS formation with the age of the subjects, or with OA severity cannot be demonstrated in this study.

Keywords: Mitochondrial DNA deletion; Nitrotyrosine

Abbreviations

mtDNA: Mitochondrial DNA, NO: Nitric oxide, OA: Osteoarthritis, PCR: Polymerase Chain Reaction, ROS: Reactive Oxygen Species, ELISA: Enzyme Linked Immunosorbent Assay

Introduction

In Osteoarthritis (OA), an age associated process leads to an aberrant cartilage structure, characterized by reduced chondrocyte numbers, loss of extracellular matrix, abnormal matrix composition and matrix calcification [1].

After age 40, the prevalence of OA in humans rises progressively. The free radical theory of aging suggests that the chronic production of endogenous Reactive Oxygen Species (ROS) and subsequent cellular damage from ROS may mediate cellular aging [2]. The pathogenesis of OA includes elaboration of increased amounts of Nitric Oxide (NO) as a consequence of up-regulation of chondrocyte-inducible NO synthetase, which in turn is induced by IL-1, TNF-α and other factors [1,2]. 3'-nitrotyrosine is a stable marker of tyrosine nitration by the peroxynitrite anion and nitrogen dioxide, which are formed as secondary products of NO metabolism in the presence of oxidants including superoxide radicals, hydrogen peroxide, and transition metal centers [3]. Increased amounts of nitrotyrosine have been detected in OA cartilage and were associated with chondrocyte death and OA severity [3-5]. It has therefore been suggested that oxidative stress may directly mediate articular aging and OA progression by contributing to chondrocyte apoptosis [6].

Mitochondria play a key role in apoptosis, and also are one of the main sources of ROS in our body [7]. The organelles contain a small

circular molecule of DNA (mtDNA), which encodes for subunits of the respiratory chain. mtDNA has been attributed a central role in aging because mtDNA lesions have been shown to accumulate over life time in several tissues of various species [8-11]. Both, point mutations and deletions have been described in mtDNA. The most frequent mtDNA deletion in aging tissue, the so called 'common deletion', consists of the absence of 4977 base pairs between positions 8468 and 13446 of mtDNA and is thought to result from slip replication between direct repeats [4]. A causal role of mtDNA mutations in mammalian aging is supported by studies demonstrating that mice with a defect in the proofreading component of polymerase-gamma, the enzyme which is responsible for the replication of mtDNA, develop a largely increased mtDNA mutation rate and premature aging [12-14]. Mitochondria are on the one hand subject to oxidative injury, but on the other hand the main cellular producers of ROS. mtDNA mutations are known to result from ROS, but at the same time also represent a perpetuator of ROS production because mtDNA-damage leads to respiratory chain dysfunction which then subsequently liberates increased numbers of ROS which then in turn either attack the respiratory chain itself or lead to further mtDNA damage [5]. ROS mediated insults on polymerase-gamma, the mtDNA replication enzyme may also cause quantitative defects in mtDNA copy number, the so called 'mtDNA depletion' [15]. ROS may therefore close a vicious circle which perpetuates mitochondrial damage and aging. Notably, such self-perpetuating damage could be initiated either by insults to mtDNA, by respiratory chain damage, by ROS, or by combinations of these factors.

This study aimed to gain insights into the mechanisms of cartilage aging and the pathogenesis of OA in human hips by quantifying the common mtDNA deletion as a marker of mitochondrial mutagenesis and investigating the associations between mtDNA deletions with the articular nitrotyrosine content as a marker of oxidative damage.

Material and Methods

Source of cartilage

This work was carried out after approval from the University of Freiburg Ethics Committee. After informed written consent, femoral heads were prospectively obtained from individuals undergoing hip arthroplasty due to OA or femoral neck fractures. Patients with necrosis of the femoral head were excluded. Femoral heads were also received from subjects undergoing autopsy in the institute of forensic medicine, provided that there were no traumatic lesions. Samples were kept buffered in HAM´s F12 with 10% FCS at 4°C until subsequent processing within 8 hours after harvesting.

Mankin score

For the scoring of OA in the femoral heads, a macroscopically affected central area of the femoral head or in the case of severe OA the more peripherally located cartilage bone border was selected. In normal looking cartilage, a central area near the ligament of the femoral head was chosen. The cartilage was dissected from the osteochondral border using an 8 mm punch device (PFM, Köln, Germany). Cartilage slices (5 µm) were cut perpendicular to the surface of the articular cartilage by standard frozen section technique. The degree of OA was scored by two independent investigators using the 14-point histological grading scale devised by Mankin et al. [6]. The Mankin grading system is based on the microscopic evaluation of decalcified sections of surgically removed femoral heads stained with Safranin O and Light Green counterstain and uses a 14 point score based on a composite of cellular changes, presence of Safranin O matrix staining and architectural changes (erosions, vessel penetration through tidemark).

Investigators were blinded to the source of the femoral heads and to all clinical information. Differences between the investigators were solved by consensus reading. The scale of the histological-histochemical grading ranged between 0 and 14 points.

Isolation of chondrocytes

Cartilage was separated from bone using a scalpel, minced to small pieces and digested with collagenase type II (Biochrom, Berlin, Germany) at a concentration of 2 g/l (320 U/l) for 12 hours at 37°C in a humidified chamber with 5% CO_2. Cells obtained from enzymatic digestion were washed in PBS and stored as cell pellets of 10^6 chondrocytes each at –80°C.

Quantification of nitrotyrosine

Chondrocytes were lysed on ice in PBS containing 1% Nonidet P40 (Roche, Germany), 2 mM EDTA and 1 µg/ml PepA. The protein concentration was measured using bicinchoninic acid (BCATM, Pierce, Rockford, USA). 50 µg of protein were used for the quantification of nitrotyrosine by means of an ELISA (HyCult biotechnology, Uden, Netherlands).

Quantification of mtDNA copy number

Total DNA was extracted from the femoral head chondrocytes using the QIAamp DNA isolation kit (Qiagen, Hilden, Germany). Copy numbers of both mtDNA and nuclear DNA (nDNA) were determined by quantitative polymerase chain reaction using the ABI 7700 sequence-detection system (Applied Biosystems, Foster City, USA) as described previously [7]. Briefly, we amplified the region between nucleotide positions 8981 and 9061 of the mtDNA of the ATP-6 gene, by use of a FAM-fluorophore-labeled probe. For the quantification of nDNA copies, we amplified the region between positions 4289 and 4342 within exon 8 of the GAPDH gene. All samples were run in triplicate. Absolute copy numbers of both mtDNA and nDNA were calculated on the basis of serial dilutions of plasmids with known copy numbers [8].

Detection and quantification of the common mtDNA-deletion

We probed for the "common" deletion by amplifying 100 ng of genomic DNA with the following extradeletional primers F8223 (TAA TTC CCC TAA AAA TCT TTG AAAT) and B13477 (AAC CTG TGA GGA AAG GTA TTC CTGC) in a PCR reaction as described previously [9,10]. By choosing a short extension cycle (30 seconds), the deleted molecule was preferentially amplified as a 324 base-pair product. The PCR product was confirmed by sequencing (MWG Biotech, Germany) to represent the common deletion. The deletion was quantified on agarose gels (Scion image) and calibrated with PCR products from templates of known ratios of wild type (from the 143B human cell line, ATCC CRL-8303) and deleted mtDNA (from 143B cybrids, homoplasmic for the "common" mtDNA-deletion).

Statistics

Groups were compared using unpaired t-tests or Wilcoxon Mann-Whitney tests, as appropriate. Correlations were computed according

to Spearman. Independent variables were tested by multiple linear regression analysis or Spearman rank correlations when they lacked normal distribution. P-values below 0.05 were considered as statistical significant. Graphics and calculations were performed using Sigma Plot (version 12.3, SPSS Inc., USA) and Stata (version 11.2 StataCorp Inc., USA).

Results

Study population

A total of 48 subjects (22 male, 26 female) was included. The median age was 64 years (range 16-93 years). A total of 48 femoral heads was analyzed. 20 femoral heads were received from patients undergoing hip-replacement for OA and 14 femoral heads were included in this study from patients with femoral neck fractures. 14 femoral heads were received from the Institute for Forensic Medicine. Causes of death were accidents (n=11), murder (n=2), and pulmonary embolism (n=1).

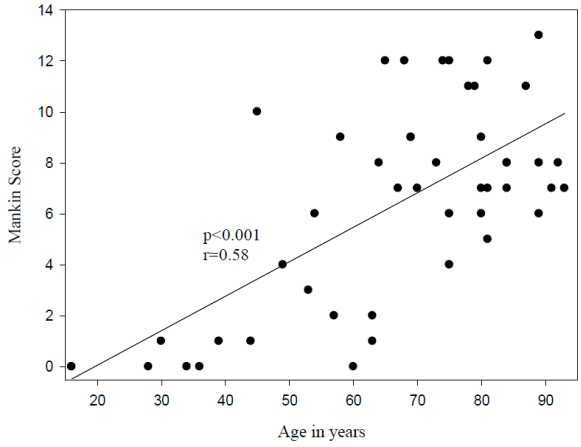

Figure 1: Osteoarthritis is correlated with age.

Influence of autolysis

We first examined whether or not the time of autolysis, or the speed of sample processing had an influence on the femoral head OA score, mtDNA or nitrotyrosine measurements. Aliquots from specimens obtained from operated patients were stored at 4°C, or room-temperature and repeatedly processed up to 72 hours after operation. In these experiments we were not able to detect an influence of autolysis on our measurements (data not shown).

Findings with advancing age

As expected, femoral heads from individuals with advanced age were more likely to have a high Mankin score, as shown by a positive correlation at univariate analysis (p<0.001, r=0.58), (Figure 1). Age was also associated with an increased articular nitrotyrosine content (p<0.004, r=0.41, Figure 2). The median mtDNA copy number among all 48 femoral heads was 108 copies /chondrocyte (interquartile range 75-146). There was however no significant correlation between the age of the patients and undeleted (wild type) mtDNA copy numbers per chondrocyte (p=0.058). Similarly, there was no correlation between the age of the study subjects and the frequency of the common mtDNA deletion (p>0.05).

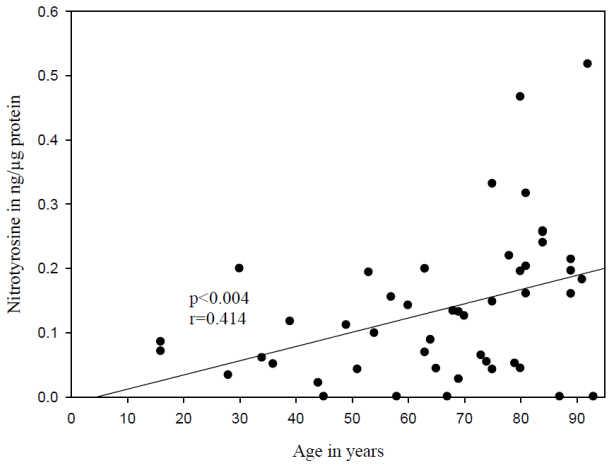

Figure 2: Nitrotyrosine content within cartilage is positively correlated with age of the patients.

At multivariate linear regression analysis, the significant association of the subjects' age with the Mankin score and the nitrotyrosine content was maintained (r=0.61, p<0.001 and r=0.39, p=0.01, respectively). In line with the univariate results, there was also no correlation between age and mtDNA mutagenesis.

Factors associated with nitrotyrosine content and mtDNA mutagenesis

As discussed above, the articular nitrotyrosine content correlated with increasing age (Figure 2). Articular nitrotyrosine content however did only borderline correlate with wild type mtDNA copy numbers in articular chondrocytes at univariate analysis (p=0.058). Importantly however, the nitrotyrosine concentrations correlated significantly with the frequency of the common mtDNA deletion (p<0.001, r=0.49, Figure 3).

These univariate findings were also replicated in the multivariate analysis (Table 1), which demonstrated that the frequency of the mtDNA deletion was positively correlated with intraarticular nitrotyrosine concentrations (p=0.004). Simultaneously, the intraarticular nitrotyrosine content was independently associated with age (r= 0.39, p=0.01), but not with the Mankin Score (p=0.84), or wild type mtDNA copy numbers.

Factors associated with increasing OA score

A significant association between the Mankin Score and the articular nitrotyrosine concentration was not found at univariate analysis (p=0.87). Also, no correlation between the Mankin Score and wild type mtDNA copy numbers was detected in chondrocytes. Similarly, the Mankin Score did not correlate with the frequency of the common deletion in mtDNA (p=0.32). There were also no differences in the frequency of the mtDNA deletion between chondrocytes derived from patients with OA (defined by a Mankin score above 5), and chondrocytes harvested from individuals with non-OA joints (defined by a Mankin score up to 5). At multivariate analysis, the Mankin score was also only associated with age (r= 0.61, p<0.001), but not with

nitrotyrosine content, the frequency of the common mtDNA deletion, or mtDNA copy numbers (Table 1).

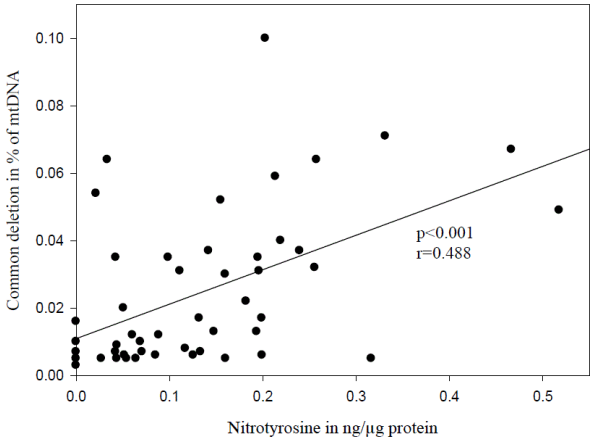

Figure 3: The frequency of the common mtDNA deletion in chondrocytes is positively correlated with nitrotyrosine content.

Discussion

Our study aimed to identify associations between articular nitrotyrosine, mtDNA mutagenesis and OA severity in human hips. We demonstrated that nitrotyrosine concentrations were associated with the age of the subjects and the presence of the common mtDNA deletion, but failed to identify a link with OA severity in terms of the Mankin Score. To our knowledge this is the first study which demonstrated a correlation between the amount of nitrotyrosine in articular chondrocytes, age, and mtDNA mutations in human cartilage.

A long-standing theory for the cause of aging [11] has implicated oxidative stress in damaged tissue proteins and nucleic acids, but up to now only some evidence is available that suggests that this process also occurs in human cartilage [4,12]. As noted above, our study however failed to identify a direct link between age and mtDNA mutagenesis in human cartilage. Another study also did not detect a correlation between mitochondrial respiratory chain function in human articular chondrocytes and age [13]. In humans, mtDNA mutations and age-related declines in mitochondrial respiratory chain functions are known to accumulate preferentially in post-mitotic tissues that are rich in oxidative metabolism such as brain, heart and skeletal muscle [14,15]. Articular chondrocytes are similar in their functionally post-mitotic nature in vivo, but manage to survive and maintain cartilage integrity in an avascular and hypoxic milieu [16].

This lack of oxidative metabolism in cartilage as an important factor of ROS production in other tissues may at least in part account for our observation that in cartilage age did not correlate with the extent of mtDNA mutagenesis in the same way as age does in other tissues [17].

Very few authors studied the role of mitochondria in OA chondrocytes. OA cartilage contains a high number of apoptotic chondrocytes; OA mitochondria were frequently described as dysfunctional [13,18], and to play an important role in apoptosis [19,20]. NO donor compounds in particular were shown to induce chondrocyte apoptosis [21,22]. Interestingly, our study did find a strong association between articular nitrotyrosine and the frequency of the common mtDNA mutation, raising the possibility of NO as a driver of mtDNA damage. An indirect mechanism of NO can however not be ruled out, because NO also acts on the respiratory chain, thereby potentially promoting the formation of superoxide, hydoxyl radicals and other oxygen dependent ROS [18].

	rho	p-value
Correlations with age		
Wild type mtDNA copy number	-0.11	0.46
Mankin score	0.61	<0.001
Nitrotyrosine content	0.39	0.01
Common mtDNA deletion	0.08	0.57
Correlations with articular nitrotyrosine content		
Wild type mtDNA copy number	0.27	0.06
Age	0.39	0.01
Mankin score	-0.03	0.84
Common mtDNA deletion	0.48	0.004
Correlations with OA severity (Mankin score)		
Wild type mtDNA copy number	-0.23	0.11
Age	0.61	<0.001
Nitrotyrosine content	-0.03	0.84
Common mtDNA deletion	-0.2	0.16

Table 1: Multivariate correlations of patient age, the frequency of the common mtDNA deletion, nitrotyrosine content and the Mankin score in human hips.

The fact that a correlation between the presence of nitrotyrosine in chondrocytes and the severity of OA was not found in our study, may point towards a role of NO-independent ROS formation in the development of OA, as previously demonstrated to contribute to the damage of articular collagen and proteoglycans [23]. Oxidative stress in OA cartilage also induces telomere instability in chondrocytes, replicative senescence and chondrocyte dysfunction [24]. The lack of association between OA severity and nitrotyrosine content in our study contrasts with the findings of others [3,25] and may also be accounted by the enormous variability of the extent and severity of the OA lesions even within single femoral heads, which makes representative cartilage sampling difficult. Some patients had areas of bare bone on the weight-bearing surface. In this situation, the only cartilage obtainable was that from sites closest to the bare area. We therefore had also investigated radiographic scores such as minimal joint space distances, but this analysis did not provide additional information (data not shown).

Our work also failed to detect any differences in the frequency of the common mtDNA deletion between joints with OA and those without OA and did not detect a correlation between mtDNA mutagenesis and the Mankin score. These findings contrast with the results from other investigators who detected higher levels of the common mtDNA deletion in patients with OA, compared to humans without OA [16]. This work however did not multivariately adjust for

large differences in the age of cartilage donors? At present, it must remain speculative, if the discrepancy between these in vivo findings may also be explained by variations in cartilage sampling. Nevertheless, in vitro work suggests that mtDNA damage can be induced by proinflammatory cytokines in chondrocytes and that OA chondrocytes are more prone to cytokine-induced mtDNA mutagenesis and apoptosis compared to chondrocytes derived from donors without OA [26].

Our data must also be interpreted with care, because associations do not equal causality. Unfortunately, it will be difficult to repeat our crossectional study in a longitudinal design because repeat cartilage sampling has ethical problems, or is performed only rarely for medical reasons. Future studies could also benefit from the simultaneous study of mitochondrial function, ROS-production and chondrocyte function.

In summary, our work identified a strong correlation between the frequency of the common mtDNA deletion in chondrocytes and the amount of intraarticular nitrotyrosine, but failed to identify a link between mitochondrial mutagenesis and patient age or OA function. Further studies are needed to give us a better understanding about the mechanisms and functional consequences of ROS production in human cartilage.

Authors Contribution

J.Z. was involved in the study design, carried out most of the laboratory studies, participated in data analysis and interpretation, and drafted the manuscript.

B.S. carried out some of the laboratory studies (quantification of mtDNA copy number, quantification of the common mtDNA-deletion), was involved in the design, acquisition of data, analysis and interpretation of the data.

M.G.P. sampled cartilage.

W.S. sampled cartilage.

N.P.S was involved in the design of the study, acquisition of data, analysis and interpretation of the data, and drafted the manuscript.

C.C.F was involved in the data analysis, data interpretation and manuscript writing.

U.A.W was involved in the design of the study, acquisition of data, data analysis, data interpretation, and manuscript writing.

All authors have given final approval of manuscript.

Conflict of Interest

None of the authors has any conflict Authors´ contribution

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All authors have given final approval of manuscript of interest with regard to the study.

Acknowledgement

The article processing charge was funded by the German Research Foundation (DFG) and the Albert Ludwigs University Freiburg in the funding programme Open Access Publishing.

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