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HYDROGEN SULFIDE BIOSYNTHESIS IS IMPAIRED IN THE OSTEOARTHRITIC JOINT

(Original article)

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ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis and it is a leading cause of disability in the elderly. Its complete etiology is not known although there are several metabolic, genetic, epigenetic and local contributing factors involved. At the moment, there is no cure for this pathology and treatment alternatives to retard or stop its progression are intensively being sought. Hydrogen sulfide (H_2S) is a small gaseous molecule and is present in sulfurous mineral waters as its active component. Data from recent clinical trials shows that balneotherapy (immersion in mineral and/or thermal waters from natural springs) in sulfurous waters can improve OA symptoms, in particular, pain and function. Yet, the underlying mechanisms are poorly known. Hydrogen sulfide is also considered, with NO and CO, an endogenous signaling gasotransmitter. It is synthesized endogenously with the help of three enzymes, cystathionine gamma-lyase (CTH), cystathionine beta-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MPST). Here, the expression of these three enzymes was demonstrated by quantitative real time polymerase chain reaction (qRT-PCR) and their protein abundance [by immunohistochemistry and Western blot (WB)] in human articular cartilage. No significant differences were found in CBS or CTH expression or abundance, but mRNA and protein levels of 3-MPST were significantly reduced in cartilage form OA donors. Also, the biosynthesis of H_2S from OA cartilage, measured with a specific microelectrode, was significantly lower than in OA-free tissue. Yet, no differences were found in H_2S concentration in serum from OA patients and OA-free donors. The current results suggest that reduced levels of the mitochondrial enzyme 3-MPST in OA cartilage might be, at least in part, responsible for a reduction in H_2S biosynthesis in this tissue and that impaired H_2S biosynthesis in the joint might be a contributing factor to OA. This could contribute to explain why exogenous supplementation of H_2S, for instance with sulfurous thermal water, has positive effects in OA patients.

112 Key words: Osteoarthritis, hydrogen sulfide; human articular cartilage; serum; mitochondria,
113 sulfurous thermal waters.

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115 **consideration for publication elsewhere.**

116

117

Abbreviations:

3-MP: 3-mercaptopyruvate

3-MPST: 3-Mercaptopyruvate sulfurtransferase

AD: Alzheimer's disease

AECOPD: Acute exacerbation of COPD

CAT: cysteine aminotransferase

CBS: Cystathionine β-synthase

cDNA: Complementary deoxyribonucleic acid

CAEIG: Galician Research Ethics Committee (*Comité Autonómico de Ética da Investigación de Galicia*)

CHD: Coronary heart disease

CO: Carbon monoxide

COPD: Chronic obstructive pulmonary disease

CTH: Cystathionine γ-lyase

CVD: Cerebrovascular disease

DMPD: N,N-dimethyl-p-phenylenediamine

H_2S: Hydrogen sulfide

IHC: Immunohistochemistry

IL: Interleukin

HPRT1: Hypoxanthine-guanine phosphoribosyltransferase

MMPs: Matrix metalloproteinases

mRNA: Messenger RNA

N: normal (non-OA), healthy control

n: Sample size

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

144 NaSH: Sodium hydrosulfide

145 NO: Nitric oxide

146 NRF2: Nuclear factor erythroid 2-related factor 2

147 OA: Osteoarthritis

148 OARSI: Osteoarthritis Research Society International

149 PAGE: Polyacrylamide gel electrophoresis

150 RA: Rheumatoid arthritis

151 RNA: ribonucleic acid

152 ROS: Reactive oxygen species

153 RT: Room temperature

154 SDS: sodium dodecyl sulphate

155 SE: Standard error of the mean

156 SOD: Superoxide dismutase

157 TAC: Total antioxidant capacity

158 TBP: Tata-box-binding protein

159 Treg: Regulatory T cells

160 VD: Vascular dementia

161 WB: Western blot

162

INTRODUCTION

Osteoarthritis (OA), the most common form of arthritis, it is a leading cause of disability in the adult population (Hunter and Bierma-Zeinstra, 2019; Chen et al., 2017). Osteoarthritis is defined by the Osteoarthritis Research Society International (OARSI) as a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness (Kraus et al., 2015). It has a high prevalence among elderly people affecting up to 40% of the population 70 years and older. The hip and knee are the joints more frequently affected, and although, in the OA joint, all tissues are implicated, the progressive destruction of hyaline articular cartilage is one of the disease's hallmarks (Chen et al., 2017). Several metabolic, genetic, epigenetic and local factors participate in OA pathogenesis and progression (Geyer and Schönfeld, 2018) but in addition, there is mounting evidence that defective mitochondrial function is also a contributing element. Mitochondrial dysfunction might result in several detrimental consequences in chondrocytes, it can aggravate cytokine-induced inflammation (Vaamonde-Garcia et al. 2012; Valcarcel-Ares et al. 2014;), modulate matrix metalloproteinases (MMPs) expression (Cillero-Pastor et al. 2013) and increase the production of reactive oxygen species (ROS) (Blanco et al. 2004). An unbalance in the anti-oxidant defense system with involvement of NRF2 (nuclear factor erythroid 2-related factor 2) might also be implicated. This factor regulates, among many other processes, the transcription and synthesis of mitochondrial anti-oxidant enzyme superoxide dismutase (SOD2), resulting in diminished anti-oxidant capacity of chondrocytes. Moreover, OA progression causes alterations in the DNA methylation profile in chondrocytes and in particular miRNAs (see for instance, Diaz-Prado et al., 2012 and Chelesci

et al., 2017)], leading to faulty regulation of the transcription and stability of genes involved in antioxidant, inflammatory, and metabolic routes. As a result, this combination of continuous ROS production and the impairement of ROS scavenging machinery leads to further mitochondrial damage in cartilage. Consequences of these include alterations in miRNA-regulated mitochondrial biogenesis as well as in redox signaling and oxidative phosphorylation (Marchev et al., 2017).

At the moment there is no cure for OA. Treatments include a combination of physical exercise, and pain and anti-inflammatory medication, but many patients end up requiring an arthroplasty. Because of this, OA has a high social and economic burden in developed countries, and strategies to both advance diagnosis and/or provide alternatives for treatment are intensively being sought.

Balneotherapy, defined as immersion in mineral and/or thermal waters from natural springs, has a long tradition as complementary non-pharmacological treatment for OA (Fioravanti et al., 2017), and recent systematic reviews have concluded that the available evidence suggests that this type of adjuvant therapy is effective and safe for the treatment of OA patients (Forestier et al., 2016; Harzy et al., 2009). Sulfurous thermal waters, i.e. those that contain hydrogen sulfide (H_2S) as their active mineral component, are amongst the more intensely investigated and several recent clinical trials have shown improvements over placebo, mostly on pain and function scales (Kovács et al. 2012, 2016). However, while progress has been made, the mechanisms underlying these effects remain poorly understood (Burguera et al. 2014; Vela-Anero et al. 2017; Fioravanti et al. 2011, Fioravanti et al., 2017).

Hydrogen sulfide is a small gaseous molecule that for a long time was known for its toxic effects and noxious odor (Beauchamp et al. 1984). However, at the end of the twentieth century it became evident that H_2S was also a physiologic gas, that it was produced endogenously in most tissues and that it could exert a variety of physiologic (or pathologic) functions (Kimura 2011; Lee Predmore et al. 2012; Rivers et al. 2012; Szabo 2007; Abe and Kimura 1996). It

215 became, next to nitric oxide (NO) and carbon monoxide (CO), the third gas to be identified as a
216 gasotransmitter (Wang 2002). Hydrogen sulfide is a highly lipophylic gas, allowing it to
217 permeate cellular membranes without a specific transporter. Thus, it can reach most tissues in
218 the organism and has a high biologic potential, since it can exert a variety of functions in many
219 biologic targets (Martelli et al. 2012). There are two endogenous routes for H_2S synthesis in
220 mammalian tissues, one enzymatic and one non-enzymatic. The non-enzymatic route, though
221 less prolific, results from the reduction of elemental sulfur to H_2S. This is achieved by means
222 of other reducing species generated through glucose oxidation, such as glutathione, lactate,
223 nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide
224 (NADH), (Martelli et al. 2012). On the other hand, the enzymatic route synthesizes H_2S with
225 the help of three enzymes, cystathionine γ-lyase (CTH, EC 4.4.1.1), cystathionine β-synthase
226 (CBS, EC 4.2.1.22) and 3-mercaptopyruvate sulfurtransferase (3-MPST, EC 2.8.1.2). L-
227 cysteine is the substrate for the production of H_2S mediated by CBS and CTH, both
228 pyridoxal-5'-phosphate-dependent enzymes. The 3-MPST-mediated synthesis of H_2S depends
229 on its interaction with cysteine aminotransferase (CAT). 3-MPST catalyzes the removal of
230 sulfur from 3-mercaptopyruvate (3-MP) forming sulfurous acid, with H_2S being as the end
231 product, while 3-MP (in addition to L-glutamate) is synthesized from the transamination
232 between L-cysteine and α-ketoglutarate, which is catalyzed by CAT. In addition, there are
233 other endogenous factors that can influence the enzymes' activities. For instance, in the brain,
234 Ca^{2+} and calmodulin regulate CBS activity, and factors that increase intracellular Ca^{2+} also
235 increase CBS-dependent H_2S synthesis (Martelli et al. 2012). In a similar way, increased NO
236 levels lead to increased endogenous H_2S. However, mediators that act over H_2S synthesis
237 might have different effects in different tissues and H_2S synthesis may vary significantly
238 depending on the location of the enzymes (Zhao et al. 2003). Also, there is evidence that, as for
239 NO, endogenous production of H_2S can be induced by exogenous stimuli (Li et al. 2005;
240 Zhang et al. 2006; Fox et al 2012).

The expression and distribution of these enzymes are tissue specific and have been reviewed elsewhere (Kimura 2011; Renga 2011; Kamoun 2004). CBS exerts its action predominantly in the brain, the nervous system and heart tissues, although it can also be found in uterus, placenta ileum, kidney, liver, and pancreatic islets (Szabo 2007). On the other hand, CTH is predominantly expressed in the vascular smooth muscle, the intestine, liver and kidney, but it is also found in ileum, uterus, brain, pancreatic islets and placenta (Kimura 2011; Martelli et al. 2012). CBS and CTH are both exclusively found in the cytosol while 3-MPST can be found in the cytoplasm as well as in mitochondria. With respect to tissue distribution, 3-MPST has been located in the heart, kidney, liver and also in the brain (Kamoun 2004; Shibuya et al. 2009).

Articular cells, specifically primary chondrocytes and chondrogenically differentiated mesenchymal progenitor cells express CTH and CBS (Fox et al. 2012). However, to the best of our knowledge there is no reference in the recent literature to 3-MPST expression or activity in joint cells or tissues.

The objectives of the present work were to verify the presence of H_2S synthesizing enzymes in cartilage, quantify H_2S biosynthesis in this tissue and compare the obtained values in OA and non-OA conditions.

MATERIALS AND METHODS

Materials

Disposable plastic was purchased from BD (BD Bioscience, Madrid, Spain). Biopsy punches were purchased from Kay Medical Europe Gmbh, Solingen, Germany. Vacuette® serum collection tubes were from Greiner Bio-One (Medline International Iberia S.L.U, Madrid, Spain). Sodium sulfide (Na_2S), sodium salicylate, ascorbic acid, NaOH, paraformaldehyde, chloroform, 2-propanol and glycogen were purchased from Sigma-Aldrich (Sigma-Aldrich Química S.A, Madrid Spain). Antibodies for cystathionine γ lyase (CTH, ab54573) and

cystathionine β synthase (CBS, Ab54883) were from Abcam® (Abcam® plc, Cambridge, UK). That of mercaptopyruvate sulfur transferase (3-MPST, sc-376168) was from Santa Cruz Biotechnology (Heidelberg, Germany). Reagents for tissue histology were from Merck, Panreac or Sigma-Aldrich except for DePex (Gurr®, VWR International Eurolab S.L. Barcelona, Spain). Reagents for immunohistochemistry were from Dako (Dako, Barcelona, Spain). Reagent for molecular biology were from Merck (absolute ethanol), Fermentas, Fisher Scientific (DNase enzyme), Life Technologies, Thermo Fisher Scientific [RNaseZap®, TRIzol®, and Superscipt® Vilo^{TM} cDNA (complementary deoxyribonucleic acid) synthesis kit], Qiagen (QIAzol lysis reagent®, QIAshredder tubes and RNeasy mini kit) and Roche Diagnostics (LightCycler® 480 Probes Master, Taqman probes and primers), all through their local commercial representatives in Spain.

Patient and tissue selection

Osteoarthritic and non-OA (normal, N) human tissue was obtained from samples included in the Sample Collection for Research on Rheumatic Disease started by Dr. Francisco Blanco García. This collection was authorised by the Galician Research Ethics Committee (*Comité Autonómico de Ética da Investigación de Galicia, CAEIG*) with registry code 2013/107 and has been inscribed in the National Registry of Biobanks (Registro Nacional de Biobancos), Collections Section code: C.0000424. Informed consent was obtained from all the donors.

Isolation of articular hyaline cartilage

Articular cartilage explants were isolated from femoral heads, condyles or tibial plateaus with the help of a scalpel, first cutting thick slices and subsequently cutting cylindrical disks with a biopsy punch (6 mm in diameter). Disks for H_2S quantification were used immediately, those for RNA (ribonucleic acid) isolation were frozen in liquid nitrogen and stored until use, and

292 those for immunohistochemistry (IHC) were fixed, dehydrated and included in paraffin (see
293 below).
294
295 ***Tissue RNA extraction***
296 The protocol used is divided in two steps:
297 1.1 Tissue disaggregation: 100 to 130 mg of cartilage were pulverized with the help of a
298 metallic mortar. This instrument had previously been cleaned with RNaseZAP® and
299 immersed in liquid nitrogen to keep it cold throughout the procedure. The cartilage powder
300 was transferred to a round bottomed Eppendorf (2 mL) and 1 mL of QIAzol® was added.
301 An UltraTurrax® homogenizer was used to further disaggregate the sample and break the
302 small cartilage pieces. After this, the tubes were subjected to circular mixing for 20 min,
303 followed by 3 min centrifugation at 14000x *g*.
304 1.2 Isolation and recovery of total RNA: in this step, a triphasic separation of ARN, ADN and
305 proteins was achieved by adding 0.2 mL of chloroform. Tubes were vigorously mixed and
306 incubated for 2-3 min at room temperature (RT), followed by centrifugation (12000x *g*,
307 15 min, 4 ºC). The aqueous phase containing the ARN was transferred to a QIAshredder
308 tube and the protocol for the RNeasy Mini kit for the isolation of RNA from animal tissue
309 was followed. Finally, RNA was recovered into 30 µL of RNase-free water and quality and
310 quantity determined as described below.
311
312 ***qRT-PCR analyses***
313 RNA concentration was quantified with a NanoDrop^{TM} spectrophotometer (Thermo Scientific,
314 Madrid, Spain) at a wavelength of 260 nm. RNA quality and purity were verified by
315 calculating the A260/A280 ratio.. An Agilent 2100 Bioanalyzer (Agilent Technologies Spain
316 S.L., Madrid, Spain) was used to verify RNA integrity. To eliminate residual genomic DNA,
317 total RNA was treated with DNase enzyme (Fermentas, Fisher Scientific, Madrid, Spain) and

then further processed to synthesize complementary (c) DNA. Specifically, cDNA was synthesized from 0.5 µg (or the total amount available if less) of total RNA with the Master Mix SuperScript® VILO™ with a total volume of 10 µl in a Thermocycler (Gene Amp PCR System 9700, Applied Biosystems, Madrid, Spain). qRT-PCR experiments were performed with Taqman probes (Universal Probe Library set, Roche) and on a LightCycler1 480 Instrument (Roche, Mannheim, Germany). Roche Assay Design Center available at www.universalprobelibrary.com was used to design primers and probe assays. Designed assays are described in Table 1. PCR reactions consisted of a pre-incubation (95ºC, 10 min), (up to) 45 cycles of amplification including incubation (95ºC 10 s), extension (60ºC, 30 s) and cooling (72ºC, 1 s) and final cooling ramp (40ºC, 20 s). Reference genes were selected from a reference gene panel using GeNorm software. Selected reference genes for cartilage were HPRT1 and TBP. Relative levels of expression were calculated using qBase+ software (www.BioGazelle.com). Data were normalized against the mean value obtained for N tissue, which was normalized to 1, and were expressed as relative expression levels.

Western Blot (WB) analyses

Protein extracts of freshly isolated chondrocytes from cartilage of healthy or osteoarthritic patients (n=3 per group) were obtained and later separated by SDS (sodium dodecyl sulphate)-PAGE (polyacrylamide gel electrophoresis) using previously described methods (Vaamonde-García et al., 2019). Membranes were incubated with the following antibodies: anti-3-MPST (1.200; Santa Cruz) overnight or anti-tubulin (1.2000; Sigma) for 1 hour. Western blots were visualized with an anti-mouse secondary antibody (1.1000; DAKO A/S, Glostrup, Denmark) and ECL chemiluminescent reagents (Merck-Millipore) in a luminescent image analyzer LAS-3000 (Fujifilm). Tubulin expression was used as loading control.

Immunohistochemistry

Cartilage biopsies were fixed in 3.7% paraformaldehyde, dehydrated in increasing concentrations of alcohol and embedded in paraffin. Paraffin blocks were cut in 4 μm sections with a microtome, deparaffinized, cleared with xylene and hydrated in a series of increasing grade alcohol. Sections were used for immunohistochemistry. Antibodies, pretreatments and dilutions were included in Table 2. Dako Real™ peroxidase blocking solution (10 min at room temperature) was used to block endogenous peroxidase activity. Then, slides were washed with phosphate buffer solution (PBS) and incubated with the primary antibody. Antigen–antibody interactions were determined with the rabbit/mouse peroxidase/DAB DAKO REAL™ EnVision™ detection kit. Sections were couterstained with H-E. Slides were dehydrated in graded alcohol, cleared in xylene and mounted in DePeX. The primary antibody was omitted in one of the sections (negative control). Slides were visualized in an Olympus Dx61 optical microscope, (Olympus España S.A.U., Barcelona, Spain). Staining intensity was quantified using ImageJ software (version 1.37a; http://imagej.nih.gov/ij/) over 12 representative fields per condition. All cells in each image were quantified, usually between 3 and 10.

Serum separation

Blood donors were either healthy volunteers without symptoms or history of OA or patients with diagnosed OA in any joint. Peripheral blood was extracted in Vacuette® tubes with separating gel. Tubes were centrifuges at 3000 rpm, 10 min and serum was collected from above the gel. Written consent was obtained from all the blood donors.

Biosynthesis of H_2S from cartilage disks

For H_2S quantification we used an ion-selective microelectrode (Lazar Research Lab. Inc., USA, model LIS-146GSCM) attached to a voltage meter (Model 6230N, Jenco Electronics, LTD, Taiwan). A calibration curve was prepared with Na_2S standards. An anti-oxidant buffer stock solution was prepared with sodium salicylate, ascorbic acid and NaOH in distilled water,

according to the electrode instructions. This stock solution was further diluted 1:3 in distilled water to prepare the working solution. For H_2S biosynthesis quantification in cartilage, 6 mm disks were placed in polystyrene tubes with 200 µL of saline and 200 µL of the anti-oxidant buffer working solution. Tubes were sealed and incubated at 37 ºC for 2 h. After this, the microelectrode was immersed in the fluid, and the voltage value was recorded and converted to H_2S concentration with the calibration curve. Biosynthesis of H_2S from cartilage was expressed as nmoles H_2S/g cartilage, mean ± SE. Values from OA and N tissues were compared.

Quantification of H_2S in human serum samples

Each serum sample (200 µL) was mixed 1:1 with the anti-oxidant buffer working solution in a polystyrene tube, sealed with parafilm M® and incubated at 37 ºC for 1 h. After this, the microelectrode was immersed in the serum and H_2S concentrations were calculated as explained above. Levels obtained in the OA sera were compared to those in the N group and were expressed as µM H_2S, mean ± standard error of the mean (SE), or median (25^{th}-75^{th} quartiles).

Statistical Analyses

Results are expressed as mean ± SE, or median (25^{th}-75^{th} quartiles) where appropriate. Statistical analyses were performed with R software (version 2.15.2) (R Core Team 2012) except for qRT-PCR experiments. Univariate analyses of variance were performed to analyze differences in H_2S biosynthesis and concentration between non-OA and OA cartilage and serum, respectively. A Pearson correlation test was done of H_2S serum values and age of the donors. qBase+ software was used to perform the statistical analysis (one-way ANOVA followed by a post hoc test) of the qRT-PCR results. In all cases, a *p* value lower than 0.05 was considered significant.

RESULTS

Tissue expression of CBS, CTH and 3-MPST in cartilage by qRT-PCR

The mRNA expression of the three enzymes relevant for H_2S synthesis was compared in OA and non-OA cartilage (n=15 and 4, respectively) (Fig. 1). No significant differences were found in the mRNA levels of CTH or CBS between the non-OA and OA conditions, although CBS expression levels were slightly lower in the latter (Fig 1 left and middle bars, respectively). Interestingly, 3-MPST mRNA expression was significantly lower in OA cartilage with respect to N cartilage, in fact, approximately 80% lower (Fig. 1, right bars).

Immunohistochemistry (IHC) of CBS, CTH and 3-MPST on OA and non-OA cartilage

Next, the abundance of the three enzymes was compared in OA and non-OA cartilage through immunohistochemistry (Fig. 2). Quantification of IHC slides is included in Table 3, and these analyses supported the results obtained by qRT-PCRs. CTH levels were similar in N (Fig. 2A) and OA (Fig. 2B) cartilage. CBS showed lower abundance in OA cartilage, although this did not reach significance (Fig. 2C: N cartilage; Fig. 2D: OA cartilage). Remarkably, significant differences were again found when comparing the abundance of 3-MPST protein in N (Fig. 4E) and OA (Fig. 4F) cartilage (*$p < 0.01$), being significantly reduced in this latter condition.

Western Blot analyses of 3-MPST on OA and non-OA cartilage

As further supporting evidence, we performed WB analyses to evaluate protein abundance of 3-MPST in normal and OA cartilage (n=3 in each case). In this case, only this enzyme was analyzed since it was the only one that showed significant differences both in gene expression and IHC. Fig. 3 includes representative images of the resulting blots, showing reduced levels of 3-MPST in OA cartilage with respect to the normal tissues and confirming the results previously shown for mRNA expression and IHC.

422

423 **H_2S biosynthesis in cartilage from OA and non-OA patients**

424 Hydrogen sulfide biosynthesis from cartilage disks, measured as described in the methods
425 section, was 0.105 ± 0.042 nmoles/g of cartilage (mean ± SE, n=13) in OA tissue and 0.433 ±
426 0.110 nmoles/g of cartilage (mean ± SE, n=5) in N tissue. Fig. 4 includes a boxplot
427 representing median, 25th and 75th quartiles and minimum and maximum values for OA and
428 healthy tissue. Medians and quartiles values were 0.056 [0.016, 0.080] and 0.457 [0.296,
429 0.593] nmoles/g of cartilage for OA and healthy cartilage, respectively. Demographic
430 information for these donors was included in Table 4. A univariate analysis of variance was
431 performed to detect significant effects, including sex and age as co-variables. Hydrogen sulfide
432 biosynthesis from OA cartilage was significantly lower than that from tissue free of OA
433 (p=0.034). Neither sex nor age had any significant influence over the results (p=0.556 and
434 0.798, respectively).

435

436 **H_2S concentration in serum of OA and non-OA patients**

437 Mean H_2S levels in serum from OA patients was 56.48 ± 7.24 μM (mean ± SE, n=38) whereas
438 H_2S levels in serum from non-OA donors was 72.18 ± 10.66 μM (mean ± SE, n=28). Fig. 5A
439 includes a boxplot representing median, 25th and 75th quartiles and minimum and maximum
440 values for OA and healthy tissue. In this case, medians and quartiles values were (45.02 [20.9-
441 79.3] μM) and (68.9 [16.4 95.1] μM) for OA and N tissue, respectively. Demographic data for
442 these donors was included in Table 5. A univariate analysis of variance was performed, and no
443 differences were found between both groups (p=0.678). The univariate analysis included sex
444 (Fig 5B) and age as co-variables, demonstrating that neither of these variables had a significant
445 influence on the results (p=0.589 and 0.783). In addition, a Pearson correlation test also
446 demonstrated that H_2S concentration and age (Fig. 5C) were not correlated (r= -0.018,
447 p=0.8925).

448

449 **DISCUSSION**

450 The objective of the present work was to confirm the presence of H_2S synthesis enzymes in
451 cartilage and to evaluate if there might be differences in the H_2S concentration or its
452 biosynthesis between healthy and OA cartilage.

453 We analyzed the gene expression and protein levels of these enzymes in this tissue, finding no
454 significant differences for the two cytoplasmic enzymes, CBS and CTH, although there seemed
455 to be a tendency for lower CBS values in OA cartilage. The only publication, as far as we
456 know, that has investigated the implication of these enzymes in OA pathology used
457 chondrocyte-like cells (chondrogenically differentiated mesenchymal progenitor cells, CH-
458 MPCs) and primary human chondrocytes. That study demonstrated that these cells express
459 both enzymes, and that CTH expression could be induced by stimulating the cells with
460 interleukin (IL)-1β, tumor necrosis factor (TNF) α, IL-6 or lipopolysaccharide (LPS) (Fox et al.
461 2012). However, in contrast with our study, which is performed directly on cartilage tissue,
462 they used cells and made no mention of 3-MPST. With respect to other tissues, LPS also
463 induced CTH expression in liver, kidney and lung in a mouse model (Li et al. 2005). These
464 results suggest that, as is the case with NO, H_2S might have a physiologic synthesis enzyme
465 (CBS) and an inducible one (CTH). Therefore, the tendency detected in cartilage for CBS
466 might imply that the expression of the enzyme responsible for the physiologic H_2S synthesis in
467 healthy cartilage is reduced with the onset of OA, thus contributing to its degenerative incline.

468 More distinctly, our results showed significant differences in 3-MPST mRNA expression and
469 protein levels in cartilage. While CBS and CTH are only found in cell's cytosol, 3-MPST is
470 responsible for producing H_2S in the mitochondria (Kamoun 2004; Yadav et al. 2013).

471 We then investigated if this reduced 3-MPST expression might result in lower H_2S levels
472 locally in the joint and/or in peripheral blood. First, we investigated the local biosynthesis of
473 H_2S in cartilage comparing OA and no-OA tissue. For H_2S quantification we used an ion-

474 selective microelectrode. The different methods to quantify H_2S concentration in biological
475 samples are somewhat controversial. Most of the studies that have evaluated H_2S in plasma or
476 serum made use of the methylene blue spectrophotometric method (Whiteman et al. 2009).
477 Other methods available are ion or gas chromatography, an amperometric sensor or HPLC
478 (Olson 2009, 2014; Ubuka 2002; Richardson et al. 2000). These might result in more accurate
479 values, but they are quite arduous and require expensive equipment. Although these methods
480 are widely used, some authors have expressed their skepticism with the reported values. In fact,
481 a study by Olson (2014) reviewed the different methods for quantification suggesting that most
482 published results have overestimated H_2S levels. Their reasoning is that reagents used to either
483 acidify or basify the samples alter their concentration, or else, their sensitivity is poor at low
484 concentrations.
485 We chose to use the specific sulfide microelectrode because of its affordability, ease of
486 handling and the possibility to measure in real time. Others have used this method to quantify
487 H_2S levels in serum/plasma or tissues related to other pathologies in animal models or human
488 subjects. For example, in their relevant paper, Yang et al. (2008) used a similar sulfide
489 selective electrode to quantify H_2S production from several tissues, including aorta, heart and
490 brain, and the H_2S concentration in the serum of wild type, $CTH^{+/-}$ and $CTH^{-/-}$ rats to
491 demonstrate the physiologic function of H_2S as a vasodilator and its role as a regulator of blood
492 pressure. Also, a sulfide sensitive electrode was used to measure plasma H_2S levels and
493 myocardial tissue H_2S concentration in a hyperhomocyteinemia rat model (Chang et al. 2008)
494 and to measure H_2S concentration in the culture medium of isolated rat aortas to investigate the
495 implication of H_2S in the aortic L-arginine/NO pathway (Geng et al. 2007).
496 Using this methodology, we found significant differences between H_2S biosynthesis in OA vs.
497 N cartilage [0.056 (0.016, 0.080) and 0.457 (0.296, 0.593)], nmoles/g of cartilage,
498 respectively). There are, that we know of, no other studies that quantified H_2S production in
499 directly in articular tissues, but differences have also been found in H_2S levels in synovial fluid

(SF) from OA patients compared to those in SF from rheumatoid arthritis (RA) patients [25.1 (18.8-34.8) vs. 62.4 (46.6-95.5)] μM, respectively; median (25^{th}-75^{th} quartiles)] (Whiteman et al. 2010b).

We subsequently determined that these differences in H_2S concentration in the joint were not seen in peripheral blood. Our values in serum, median [25^{th}-75^{th} quartiles] for OA [45.02 (20.9-79.3) μM] and N [68.9 (16.4-95.1) μM] serum, are somewhat higher than those in the work of Whiteman and coworkers (Whiteman et al., 2010b), [36.2 (17.1-66.3) μM] for OA and [37.6 (27.4-41.3) μM] for N plasma, but their study also found no significant differences between the OA and healthy groups. Of note though, their methodology differed from ours in several aspects, namely: a) they measured values in plasma rather than serum; b) their blood samples were frozen before centrifugation; c) H_2S was quantified through zinc trap spectrometry, and d) their sample size (n=4) was considerably smaller than ours (n=38).

However, our data is in contrast with those published by another group (Muniraj et al. 2014). They did find significant differences between the OA (n=16) and the healthy control group (n=30), but contrary to ours, OA values were higher. Values estimated from their figure were approximately 5-6 μM for the N group and 22-24 μM for the OA group, and they used the methylene blue assay. These OA levels are somewhat lower than those of Whiteman *et al*. but their values for the N group are considerably lower than those reported in other published data (see Table 6 for a summary of H_2S values on human serum/plasma available from the literature).

There are several other examples of pathologies in which a decline in H_2S levels or biosynthesis was found. For example, in animal models with chronic renal failure (Sen et al. 2009), hypertension (Zhao et al. 2008) or diabetes (Brancaleone et al. 2008), and in studies related to neurodegenerative diseases such as Alzheimer's (Tang et al. 2008) or Parkinson's (Hu et al. 2010), H_2S concentration in the plasma/serum of the animals or its biosynthesis in the involved tissues were reduced. More importantly, many of these results have been

reproduced in human studies. Plasma H_2S levels in hypertensive children (51.9 ± 6.0 μM; 10.5 ± 3.2 yrs) were lower than those of the control group, (65.7 ± 5.5 μM; 10.5 ± 0.7 yrs) (Chen et al. 2007). In patients with type 2 diabetes H_2S levels were significantly lower than those of age- and body mass index (BMI)-paired controls, both in lean and overweight groups (Whiteman et al. 2010a). In patients with Alzheimer's, plasma H_2S concentration was negatively correlated the severity of the disease (Liu et al. 2008). Also, a significant inverse correlation was found between serum H_2S levels and chronic obstructive pulmonary disease (COPD) stage (Chen et al. 2005).

On the other hand, there are also some cases, generally of acute inflammation, in which H_2S levels and/or its biosynthesis are elevated. For instance, in animal models of sepsis, plasma H_2S concentration in LPS-treated mice was markedly elevated (Li et al. 2005) and in rats, LPS induced a significant increase in the expression and activity of CTH and CBS (Collin et al. 2005). Also, cerulein, used to provoke pancreatitis in mice, induced H_2S synthesis in the pancreas (Tamizhselvi et al. 2007). In these cases the use of D,L-propargyl glycine (PAG) (a pharmacologic H_2S synthesis inhibitor) improved symptoms and/or reduced H_2S concentrations. Even in human patients with sepsis, H_2S concentrations were up to 4-fold higher than those of healthy controls (Li et al. 2005). However, a theory has recently been put forth that in these cases, H_2S has an anti-inflammatory role, and this represents the cells' attempt to arrest or counteract the inflammatory cascade (Whiteman, 2010a, 2011). Further research in this area is needed to confirm this theory.

Collectively, after investigating the endogenous production of H_2S in the joint, comparing subjects with OA with non-OA controls, we found that in the presence of OA, H_2S production in the joint is reduced and this seems to be associated with at the least a reduction in the mRNA and protein expression and abundance of the 3-MPST enzyme. A possible association between deficient mitochondrial H_2S biosynthesis and osteoarthritis, as suggested by our data, would be in agreement with the now established theory that mitochondrial dysfunction is a contributing

552 factor to OA pathogenesis (Cillero-Pastor et al. 2013; Blanco et al. 2004; Maneiro et al. 2003;
553 Rego-Perez et al. 2008; Blanco et al. 2011, Marchev et al., 2017). Several *in vitro* studies have
554 demonstrated that, in OA chondrocytes, the mitochondrial respiratory chain (MRC) complexes
555 II and III have lower activities than in normal chondrocytes (Maneiro et al. 2003) and that
556 mitochondrial dysfunction could enhance cytokine-induced inflammation in articular
557 fibroblast-like synoviocytes (FLS) and chondrocytes (Vaamonde-Garcia et al. 2012; Valcarcel-
558 Ares et al. 2014). Here, it cannot be elucidated with the available information if mitochondrial
559 dysfunction is a cause of this 3-MPST reduction and, consequently of reduced H_2S levels in
560 cartilage, or else it is its consequence. Also, a paper by Modis and coworkers (2013)
561 demonstrated that oxidative stress (induced by 50-500 μM H_2O_2) inhibits the synthesis of H_2S
562 mediated by 3-MPST in mitochondria isolated from murine hepatoma cells. Since OA is
563 characterized by increased production of ROS (Henrotin et al. 2003, Geyer et al., 2018) this
564 mechanism might also occur in cartilage and contribute to the lower H_2S levels found in our
565 study.
566 Our study might also contribute to explain the positive effects exerted by non-pharmacologic
567 treatments with sulfurous mineral thermal waters on OA patients (Kovács et al. 2012, 2016).
568 The mechanisms of action of sulphurous waters (or mineral waters in general) on rheumatic
569 pathologies are still being investigated. Most likely, the observed benefits result from the
570 combined effects of the hydrostatic force, the temperature of mineral water, and its chemical
571 composition. The chemical components present in the water, including H_2S, are believed to be
572 absorbed through the skin before acting at systemic level (Fioravanti et al., 2011;
573 Nasermoaddeli et al., 2005). For instance, in healthy volunteers that underwent a cycle of
574 hydropinic therapy with sulfurous drinking water, plasma levels of lipid (malondialdehyde)
575 and protein oxidation products (carbonyls and advanced oxidation protein products) were
576 decreased with respect to controls without therapy. Increased plasma total antioxidant capacity
577 (TAC) was also found after the treatment with sulfurous drinking water (Benedetti et al.,

578 2009). Similar results were seen in OA patients that took sulfur baths (Ekmekcioglu et al.,
579 2002) or the combination of sulfur-based mud baths and hydropinotherapy (Benedetti et al.,
580 2010). These patients also reported significantly lower pain scores and presented lower serum
581 TNF-α and cartilage oligomeric matrix protein concentrations (Benedetti et al., 2010).
582 Furthermore, a significant decrease in serum levels of adiponectin and resistin, known to
583 contribute to OA development and progression, was seen in patients with knee OA after a cycle
584 of mudpack plus balneotherapy (Fioravanti et al., 2015),. Also, data from several *in vitro* and
585 *in vivo* studies has shown that pharmacologic administration of H_2S reduces inflammation and
586 catabolism markers in OA joint cells, including chondrocytes (reviewed in Burguera et al.
587 2016). Further, H_2S can also reduce inflammation by acting on cells of the immune system. For
588 instance, sodium hydrosulfide (NaSH) significantly increased the release of IL-10 and
589 counterbalanced the formation of ROS on primary human monocytes *in vitro* (Prandelli et al.,
590 2013). In a different study, this compound also induced cell death and impaired proliferation in
591 CD4+, CD8+ lymphocytes and natural killer cells, reducing their cellular cytotoxic response
592 (Mirandola et al., 2007). In addition, Yang and collaborators demonstrated that H_2S deficiency
593 in mice leads to an impairment in the differentiation of regulatory T (Treg) cells (Yang et al.,
594 2017) and that injection of H_2S donor GYY4137 in an H_2S-deficient mice model restored
595 immune homeostasis and Treg cell numbers. So, this might be an additional mechanism by
596 which H2S exerts its anti-inflammatory effects.
597
598 **Conclusions**
599 Here, we have demonstrated for the first time the presence of CBS, CTH and 3-MPST in
600 hyaline cartilage and shown that gene and protein expression of 3-MPST, responsible for H_2S
601 synthesis in the mitochondria, was reduced in tissue from OA patients. We believe this is, at
602 least in part, responsible for reduced H_2S biosynthesis in OA cartilage, which was quantified
603 with a specific sulfide microelectrode. However, quantification of H_2S concentration in serum

from peripheral blood revealed no differences between OA patients and healthy controls, suggesting that this dysregulation of the H_2S synthesis route is a local phenomenon in the joint. This provides additional supporting evidence that mitochondrial dysfunction is a contributing factor to OA. Accordingly, exogenous non-pharmacologic (through sulfurous mineral waters) or pharmacologic (through synthetic releasing compounds) administration of H_2S emerges as a valid therapeutic option in OA, and further research both with preclinical models and high quality randomized controlled trials is needed in this area to demonstrate clinical efficacy and find appropriate routes of administration and doses.

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Author contributions

All authors were involved in drafting or critically reading the manuscript for important intellectual content, and all authors approved the final version. Conception and design: EF Burguera, R Meijide-Failde, FJ Blanco. Data acquisition: Á Vela-Anero, L. Gato-Calvo, C Vaamonde-García. Analysis and interpretation of data: Á Vela-Anero, EF Burguera, FJ Blanco. Obtaining of funding: R Meijide-Failde, FJ Blanco.

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Competing interest statement

Authors declare they do not have any conflict of interest.

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Table 1. List of qRT-PCR assays used

GENE	RefSeq (mRNA)	Sequence	Position	Probe n#	Amplicon (bp)
HPRT1	NM_000194.2	5'-tgatagatccattcctatgactgtaga-3' 5'-caagacattctttccagttaaagttg-3'	434-460 535-560	22	127
TBP	NM_003194.4	5'-gcccatagtgatctttgcagt-3' 5'-cgctggaactcgtctcacta-3'	104-124 223-242	67	139
CBS	NM_000071.2	5'-aggagaagtgtcctggatgc-3' 5'-taggttgtctgctccgtctg-3'	1051 – 1070 1128 - 1147	17	97
CTH	NM_001902.4	5'-gcatttcaaaaacggaatgg-3' 5'-ctcatgctgtggatgagagg-3'	948 – 967 1028 - 1047	61	100
3-MPST	NM_021126.4	5'-acatcaaggagaacctggaatc-3' 5'-gatgtggccaggttcaatg-3'	739 – 760 833 - 851	77	113

Table 2. List of antibodies used

Antibody	Isotype	Manufacturer	Pretreatment	Dilution
CBS Mouse monoclonal	Recombinant IgG2a	Abcam®	TRIS- EDTA pH 9	1:500
CTH Mouse monoclonal	Recombinant IgG1	Abcam®	TRIS- EDTA pH: 9	1:500
3-MPST Mouse monoclonal	H-11 IgG1	Santa cruz biotechnology	TRIS- EDTA pH: 9	1:500

Table 3. Intracellular enzyme positivity (mean ± SE) in healthy and OA cartilage. *p< 0.05 with respect to healthy tissue

	Healthy	OA
CBS	6.60 ± 4.03	4.38 ± 1.30
CTH	12.18 ± 2.02	12.92 ± 4.14
3-MPST	14.63 ± 4.41	*5.72 ± 1.84

Table 4. Demographic data of cartilage donors for H_2S biosynthesis quantification

	OA patients	Healthy donors
Age in years (range)	75.2 (53-96)	73.2 (67-80)
Men/Women	5/8	1/4

Table 5. Demographic data of serum donors

	OA patients	Healthy donors
Age in years (range)	68.3 (51-92)	33.2 (23-68)
Men/Women	6/27	5/23

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1015 Table 6. Summary of recent literature reporting H_2S values in human plasma/serum.
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Reference	Method	Sample	Healthy controls			Pathologic donors				Statistics
			age	n	H_2S μM	pathology	Age	n	H_2S μM	
Chen *et al*. (2007)	Sensitive sulfur electrode	Plasma	10.57±0.73	30	65.70±5.50	Hypertension	10.48±3.23	25	51.93±6.01	Mean±SD
Liu *et al*. (2008)	-	Plasma	-	23	45±7	AD VD CVD	-	31 28 20	34±7 36±5 37±7	-
Richardson *et al*. (2000)	Ion chromatography after microdistillation	Whole blood (Frozen -20ºC)	-	4	Depending on meat intake: None: 42±15 240g: 47±12 420g:28±1	-	-	-	-	Mean±SD
Hyspler *et al*. (2002)	GC-MS technique	Whole blood	-	15	35-80	-	-	-	-	-
Li *et al*. (2005)	Zinc acetate/DMPD	Plasma	57-80	5	43.8±5.1	Septic shock	70-88	5	150.5±43.7	Mean±SE
Whiteman *et al*. (2010)	Zinc trap spectrometry	Plasma	54.8±16.46 65.0±6.1	11 16	Lean: 38.9 (29.7, 45.1) Overweight: 22.0 (18.6, 26.7)					
Jiang *et al*. (2005)	Sulfide sensitive electrode	Plasma	-	17	51.74±11.94	CHD	-	40	26.10±14.27	Mean±SD
Chen *et al*. (2005)	Commercial sulfide sensitive electrode	Serum	71-80 61-70 50-60	13	35.7±1.2 34.0±0.9 36.1±1.1	COPD	65.6 ± 1.6	37	51.1± 3.0 (non smokers) 49.8 ± 3.8 (smokers)	Mean±SE
						AECOPD	76.7 ± 1.3	27	~ 35	

1017 Figure legends

1018 **Fig. 1** CTH, CBS and 3-MPST mRNA relative expression in OA vs. OA-free (normal)
1019 cartilage was analysed by qRT-PCR (n=15 and 4, respectively). Relative mRNA
1020 expression values were calculated with qBase+ software. Data were normalized against
1021 the mean value obtained for normal tissue, which was normalized to 1. One-way
1022 ANOVA and multiple comparison post-hoc tests were performed to identify significant
1023 differences. Expression of CTH in OA and non-OA cartilage was similar. There was a
1024 tendency for lower levels of CBS in OA cartilage with respect to the OA-free tissue.
1025 Expression of 3-MPST in OA cartilage was significantly lower than in OA-free
1026 cartilage (*$p < 0.01$).

1027 **Fig. 2** Levels of 3-MPST protein from OA and N cartilage were evaluated by Western
1028 blot demonstrating lower abundance in the former (n=3 for each group).

1029 **Fig. 3** Representative images of CTH (A-B), CBS (C-D) and 3-MPST (E-F)
1030 immunohistochemistry in normal (A, C, E) and OA cartilage (B, D, F). Images were
1031 taken at 40x. Scale bar represents 50 μm.

1032 **Fig. 4** Boxplot representing median, 25th and 75th quartiles and minimum and maximum
1033 values of H_2S biosynthesis in OA and healthy cartilage quantified with a selective
1034 sulfide microelectrode. A univariate analysis of variance was performed to detect
1035 significant effects, including sex and age as co-variables. Hydrogen sulfide biosynthesis
1036 from OA cartilage was significantly lower than that from healthy tissue (*p=0.034).
1037 Neither sex nor age had any significant influence over the results (p=0.556 and 0.798,
1038 respectively).

Fig. 5 A) Boxplot representing median, 25th and 75th quartiles and minimum and maximum values of H_2S concentration in OA and healthy sera quantified with a selective sulfide microelectrode. A univariate analysis of variance was performed to detect significant effects, including sex and age as co-variables. There were no significant differences in the H_2S concentration in both groups (p=0.678). The univariate analysis also demonstrated that sex (B) did not significantly influenced the results (p=0.589). In addition, a Pearson correlation test showed that H_2S concentration and age (C) were not correlated (r= -0.018, p=0.8925).

Figure 1

Fig. 1

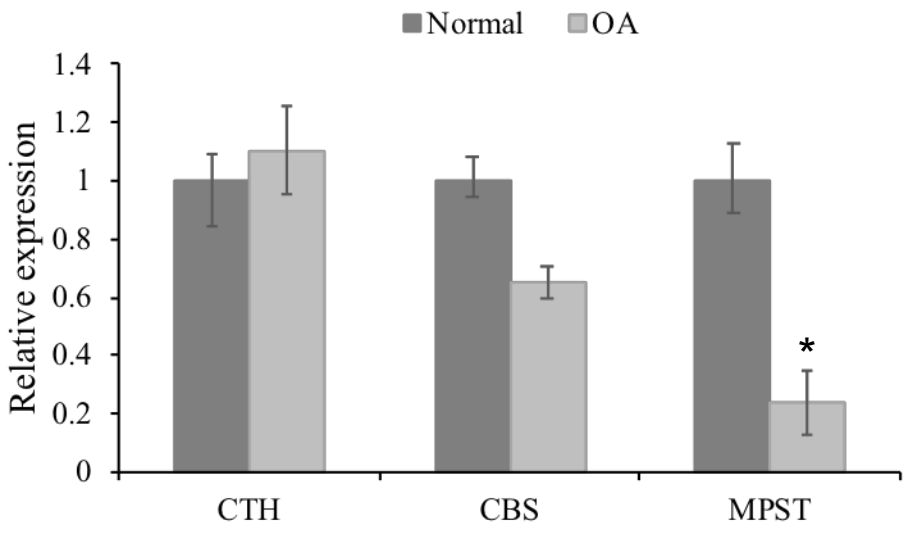


Figure 2

Fig. 2

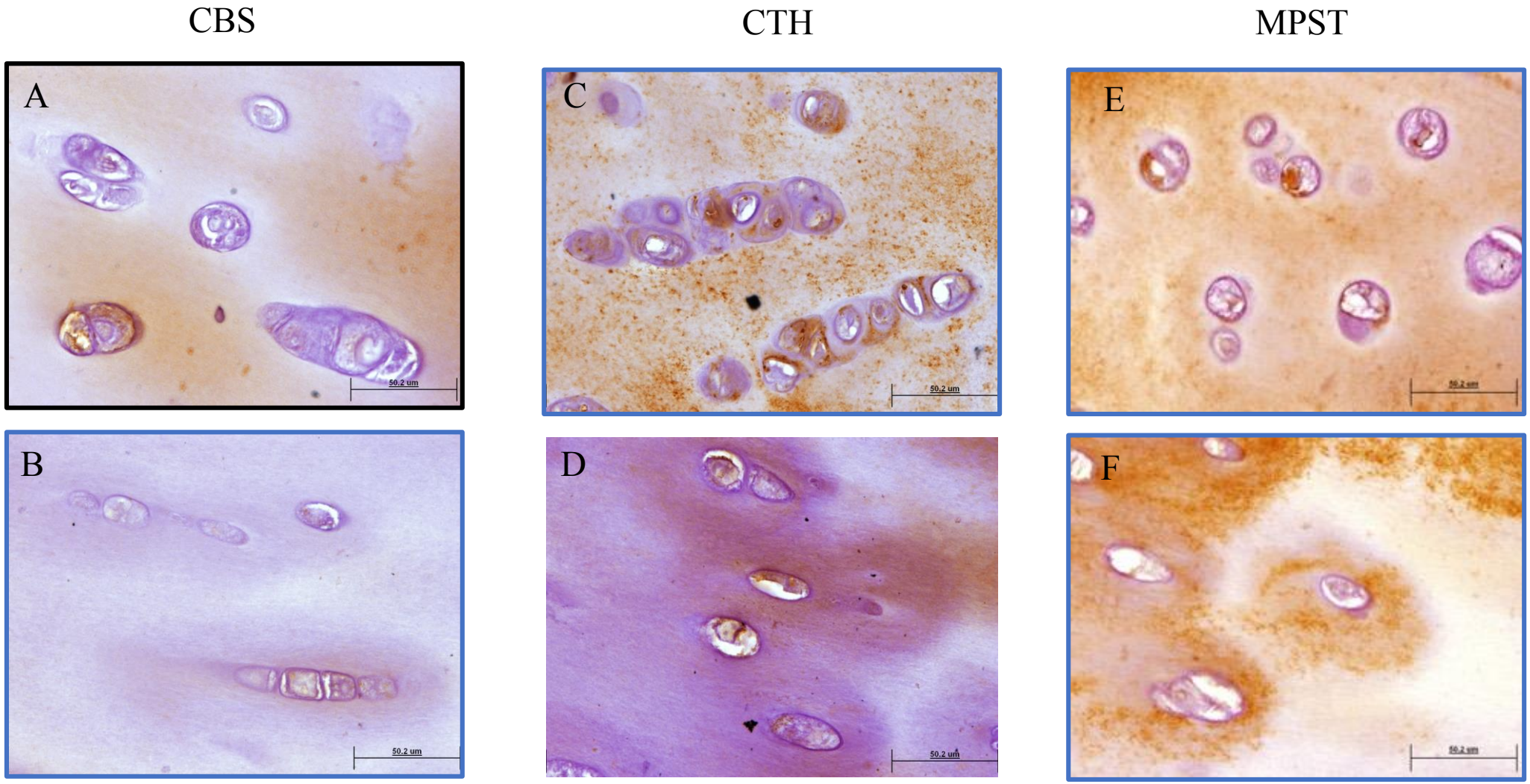
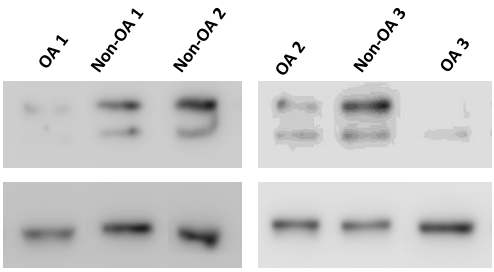


Figure 3

Fig. 3



3-MPST (37 kD) (Santa Cruz; sc-376168)

Tubulin (50 kD)

Figure 4

Fig. 4

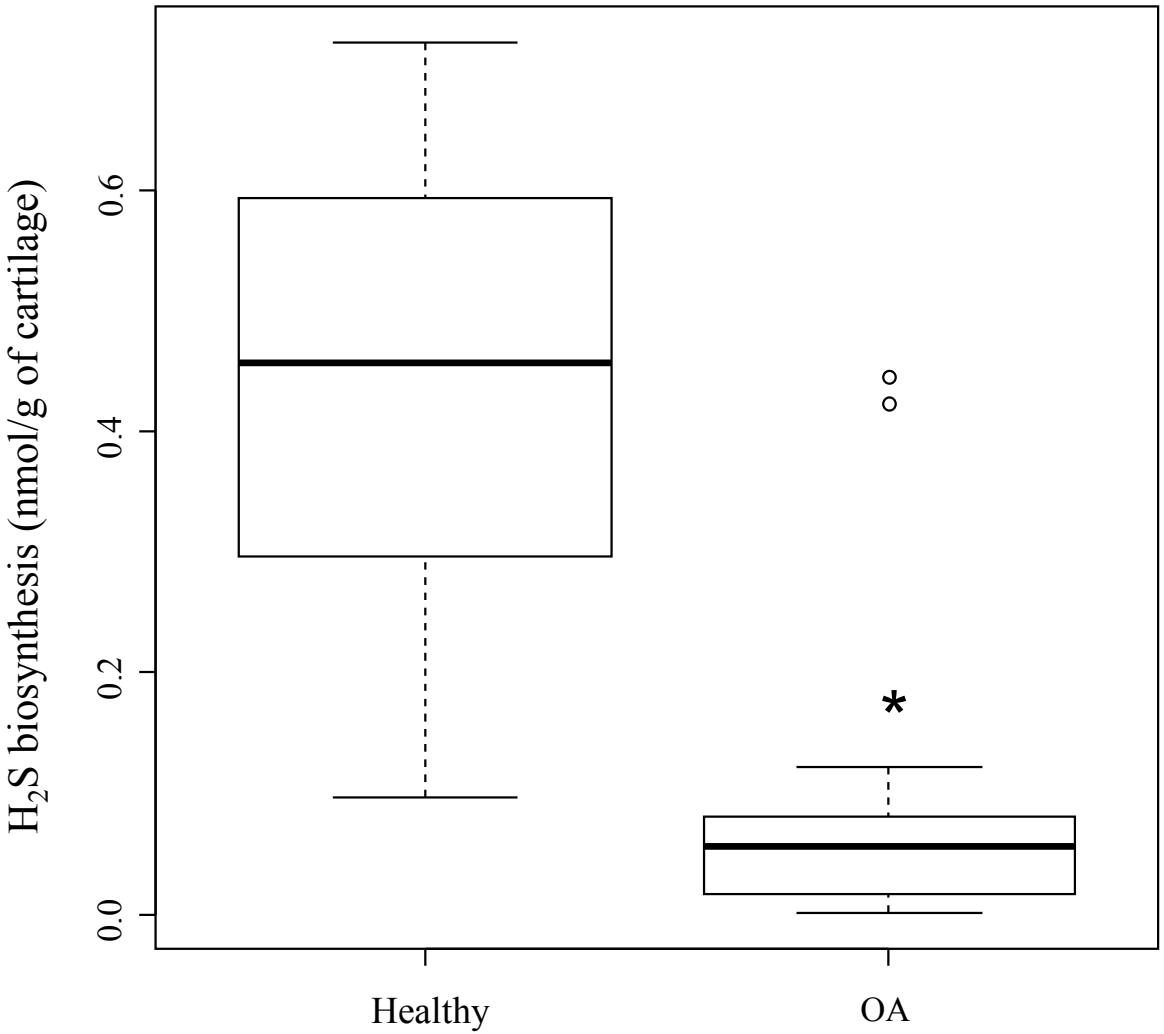


Figure 5

Fig. 5