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Comprehensive analysis of mitochondrial functions and inflammatory state of synovial membrane samples from patients suffering from arthritis

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ABSTRACT

Purpose: There is growing evidence for the role of mitochondrial dysfunction in osteoarthritis (OA) and rheumatoid arthritis (RA). However, quantitative comparison of mitochondrial derangements in these main arthritis forms in clinical settings is missing. Therefore, our aim was to characterize altered mitochondrial functions in synovial membranes of OA and RA patients.

Methods: Synovial tissue samples were taken intraoperatively and subjected to high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Austria) to analyze mitochondrial oxygen consumption. The oxidative phosphorylation (OxPhos) capacity and the relative contribution of respiratory complexes I and II were determined, together with the coupling of the mitochondrial respiratory chain (coupling control ratio, CCR). The release of cytochrome C (shuttles electrons between complexes III and IV) was also determined, in parallel with biochemical and histopathological evaluation of the samples.

Evaluation: With our protocol, we provided quantitative clinical data making RA and OA comparable with respect to mitochondrial dysfunction. Further studies are needed to elaborate future RA and OA therapies with mitochondrial targets.

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Sampling

1 Synovial fluid and tissue samples from the knee joint synovium are taken with the permission and signed consent of the patients. Synovial fluid is aspirated prior to incision, with an 18 G needle. Synovium tissue samples of 1x1 cm in size are obtained from the suprapatellar pouch, sparing the Hoffa's fat pad.

2 Synovial fluid samples are placed into Eppendorf tubes and transported on ice directly to laboratory testing. Synovium tissue samples are placed into 4% (v/v) neutral buffered formalin and phosphate buffered saline (PBS), and transported on ice directly to undergo biochemical analyses, histopathological evaluation and high-resolution respirometry respectively.

Assessment of mitochondrial functions

3 The tissue samples are homogenized in 200 µl of MiR05 respiration medium with a glass Potter-Elvehjem homogenizer.

4 Subsequently, the homogenates are weighed into the detection chambers, 50 µl in each, which are calibrated to 200 nmol/ml oxygen concentration in room air.

5 First, the steady-state basal oxygen consumption of the homogenates (respiratory flux) is measured. Glutamate (10 mM) and malate (2 mM) are used in combination to induce C I-linked respiration. Complex II-linked baseline respiration (10 mM succinate-fuelled, in the presence of C I inhibitor 0.5 µM rotenone) is determined, then oxidative phosphorylation capacity is measured by adding saturating ADP (5 mM final concentration).

6 Cytochrome C release (an indicator of inner mitochondrial membrane damage) is determined as described previously. The intactness of the inner mitochondrial membrane is assessed after adding cytochrome C (10 µM)

7 Leak respiration (LEAK) is measured in the presence of C V inhibitor oligomycin (Omy) (1 mM)

8 Thereafter, protonophore agent carbonyl cyanide p-trifluoromethoxy-phenyl-hydrazine (FCCP) (0.5 µM) is added to measure ETC coupling.

9 Finally, residual oxygen consumption (ROX) is determined by adding 1 µM rotenone (Rot) and 1 µM antimycin-A (Ama).

Biochemical analyses

10 Measuring tissue xanthine oxidoreductase (XOR) activity: Synovial membrane samples are homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg ml^{-1} soybean trypsin inhibitor, and 10 µg ml^{-1} leupeptin. The homogenate is centrifuged at 4 ^{o}C for 20 min at 24.000 g, and the supernatant is loaded into centrifugal concentrator tubes. The activity of XOR is determined in the ultrafiltered supernatant by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the

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presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue

11 Measuring tissue myeloperoxidase (MPO) activity: The MPO activity is measured in synovial tissue according to the method of Kuebler et al . Briefly, the synovial tissue is homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 M polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min. at 24.000 g. The MPO activity of the samples is measured at 450 nm (UV-1601 spectrophotometer; Shimadzu, Japan). The data is referred to the protein content.

12 Measuring nitrotyrosine levels: Free nitrotyrosine, as a marker of peroxynitrite generation, is measured by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI). The synovial fluid is centrifuged at 15,000g. The supernatants are collected and incubated overnight with antinitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse antirabbit IgG) microplates followed by development with Ellman's reagent. Nitrotyrosine content is normalized to the protein content of the homogenate and expressed in ng/mg.

Histopathological analysis

13 Intraoperatively harvested synovium biopsies are assessed with light microscopy and confocal imaging. Confocal imaging with a laser scanning endomicroscope (CLSM, FIVE1 system, Optiscan, Victoria, Australia) is started immediately after retrieving the synovial sample. The rigid confocal probe (excitation wavelength 488 nm; emission detected at 505–585 nm) is mounted on a specially designed metal frame and gently pressed onto the inner surface of the joint capsule (1 scan/image, 1024 x 512 pixels and 475 x 475 µm per image). For the *in vivo* staining, 0.01% acriflavine (Sigma-Aldrich, Budapest, Hungary) is applied topically [24]. The analysis is performed twice separately by the same investigator using a semiquantitative histology score (S0-S4) based on widening of synovial lining, neoangiogenesis, collagen fibre disorganization and fragmentation, as described previously [27]. For traditional light microscopy, the samples are fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. 5-µm thick sections are cut and then stained with haematoxylin and eosin (H&E).

Laboratory testing of synovial fluid

14 Proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and receptor activator of nuclear factor kappa-beta ligand (RANKL) levels are measured in the synovial fluid samples using commercially available ELISA kit according to the manufacturer's instruction (Sigma-Aldrich, Budapest, Hungary).

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