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The role of reactive oxygen species (ROS) in osteoarthritis patients

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Abstract
Reactive oxygen species (ROS) serve as cell signaling molecules for normal biologic processes. The generation of ROS can also provoke damage to multiple cellular organelles and processes, which can ultimately disrupt normal physiology. Reactive oxygen species (ROS) are produced mainly during oxidative phosphorylation and by activated phagocytic cells during oxidative burst. The excessive production of ROS can damage lipids, protein, membrane and nucleic acids that may contribute to the development of oxidative stress mediated diseases. In this study, healthy controls and osteoarthritis patients were evaluated for the oxidant-antioxidant status by measuring ROS production.

Keywords: Oxidative phosphorylation, antioxidant, oxidative stress, osteoarthritis

Introduction
Oxidation reaction in living cells produce free radicals, reactive oxygen species (ROS) and their derivatives. ROS are the most important class of radicals generated in living systems. They are oxygen derived radicals and contain one or more unpaired electrons in the outermost orbital shell and are called free radicals [1]. They are unstable and highly reactive. These harmful compounds can accumulate over time, causing extensive structural damage or even cell death. The cytotoxic effects of ROS can cause a variety of health problems including inflammatory disease [2]. The ROS productions are involved in the initiation of inflammatory response [3] and initiated inflammatory state that may result in chronic inflammatory disease states including arthritis [4]. Under physiological conditions, ROS are important to maintain the cell redox state and play a role in cell signalling, differentiation, proliferation, growth, apoptosis, cytoskeletal regulation and phagocytosis. If the concentration of ROS is increased beyond physiological conditions they can damage the different cellular components. Osteoarthritis is an important problem for both the individual and society [5]. Osteoarthritis (OA) is the most common form of arthritis and characterized by softening and disintegration of articular cartilage. The progressive deterioration and loss of articular cartilage leading to an irreversible impairment of joint structure are the final pathogenic events common to osteoarthritis [6]. It is the most common form of musculoskeletal joint disease. The present study was aiming to know oxidative stress condition or how free radicals and ROS play influential role in physiological and pathophysiological process in osteoarthritis patients. These free radicals can damage a wide variety of cellular constituents.

Methodology
This assay was done by two steps such as the Ficoll® density gradient separation of PBMC and ROS detection assay.

A) Ficoll® density gradient separation of PBMC
Peripheral Blood Mononuclear Cells (PBMCs) are blood cells with round nuclei, such as monocytes and lymphocytes, with the lymphocyte population consisting of T cells, B cells, and natural killer (NK) cells. PBMCs are a critical component of the immune system, playing an integral role in the body's defense mechanisms. Cellular assays using PBMC cultures form the backbone of immune monitoring studies in clinical diagnostics and therapeutic design. This protocol is used to fractionate defibrinated or anti-coagulant–treated peripheral blood (or buffy coat) using Ficoll® in a conical tube. It is important to maintain the same volumetric ratio of sample to Ficoll. First, 3ml phosphate buffered saline (PBS) was transferred to a new 15 ml tube from stock PBS solution. Then 3ml blood (equal volume) was poured to that 3ml PBS containing tube from EDTA vial. After that it was mixed (PBS + Blood) [1:1] ratio by repeated pipetting. Then 5 ml of ficoll was taken to an another new 15ml tube. Carefully, the mixing sample or diluted blood from the first tube (PBS + blood) was poured over the ficoll.

Then the total preparation was centrifuged without brake, applied at 2000rpm for 30min at 18-24°C. After centrifugation, carefully the tube was removed from the centrifuge. After that the upper plasma layer was drawn off carefully and the PBMC layer (band formation) was transferred to a eppendorf tube. This PBMC layer containing eppendorf tube was centrifuged at 1rpm for 5 or 10 min. Then the supernatant was drawn off and re-suspended the pellet was mixed in appropriate volume of PBS (500µl) in eppendorf tube.

B) ROS detection assay

That 500µl PBS containing re-suspended pellet was divided into four eppendorf tubes. This eppendorf tubes were centrifuged at 400g for 5 minutes. The supernatant was drawn off and 200µl ROS 3-plex detection reagent was mixed and incubated under normal tissue culture conditions for 2 hours with periodic shaking. After 2 hrs incubation, it was centrifuged at 400g for 5 minutes to remove the ROS 3-plex detection mixture. It was washed with 1X wash buffer, centrifuged them at 400 X g for 5 minutes and removed the supernatant. The cells were treated with an experimental test reagent. ROS inducer (Pyocyanin) was added to two test tubes and ROS inhibitor (N-acetyl-L-cysteine) was added to other test tubes. [All treatments were performed under normal tissue culture conditions]. After treatment all tubes were centrifuged at 400 X g for 5 minutes. The supernatant was drawn off and washed with 5ml 1X wash buffer. Again centrifuged them at 400 X g for 5 minutes and removed supernatant. After centrifugation washed with 100µl wash buffer and 20µl aliquot was taken on microscopic slides, covered with cover slip and were analyzed under fluorescence microscope.

Results

Study of ROS of osteoarthritis patients compared to healthy subject

In Fig 1, untreated sample or normal sample was showed only low auto-fluorescent in any channel. In Fig 2, it was observed that when ROS inducer was added to the sample, then the sample was induced with ROS inducer and cell size was increased, exhibited bright green fluorescence cytoplasmic staining in the presence of ROS inducer detection reagent. In Fig 3, it was observed that when ROS inhibitor was added to the sample, then the sample was inhibited with ROS inhibitor upon induction and cell size was decreased, exhibited bright green fluorescence cytoplasmic staining in the presence of ROS inhibitor detection reagent. In Fig 4 and 5 (pre-treated sample), it was observed that there were increased level of height and weight than normal cell sample, was increased cell size and levels of oxidative stress and was given uniform green cytoplasmic staining in the presence of the oxidative stress detection reagent.

The measurement of ROS generating cell compared to control cell is tabulated in Table 1. It was observed that the height (cm) and width (cm) was increased in control + ROS inducer cell 6.07 and 2.75 while decreased in control + ROS inhibitor cell 2.79 and 2.15 when compared to healthy or control subjects 3.97 and 3.12. In another study, it was observed that there was an increasing trend of height and weight in pre-treated sample of inducer and inhibitor 8.75 and 3.18 and 4.10 and 2.20 respectively in comparison with control 3.97 and 3.12 as an indication of oxidative stress.

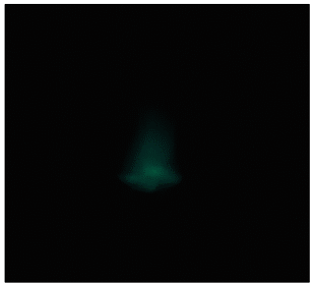

Fig 1: Normal or control cell of healthy subjects

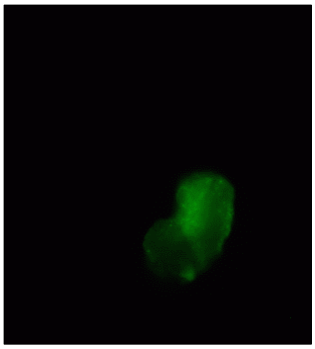

Fig 2: Control + ROS inducer (Pyocyanin). When ROS inducer was added with control sample then the control sample was induced with ROS inducer or this picture showed that cell size was increased

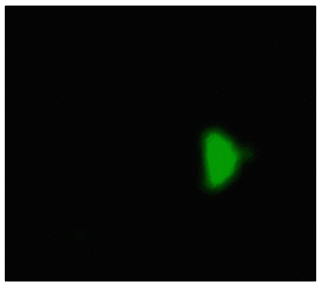

Fig 3: Control + ROS inhibitor (N-acetyl-L-cysteine). When ROS inhibitor was added with control sample then the control sample was inhibited with ROS inhibitor as well as the picture showed that cell size was decreased or cell looked like control sample

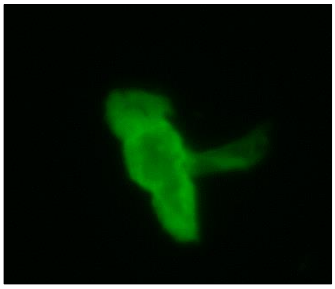

Fig 4: Sample before treatment (Pre-treated sample)

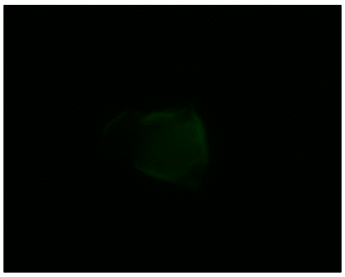

Fig 5: Sample before treatment (pre-treated sample)

Table 1: Measurement of ROS generating cell compared to control cell

Samples	Height (cm)	Width (cm)
Control cell	3.97	3.12
Control + ROS inducer	6.07	2.75
Control + ROS inhibitor	2.79	2.15
Pre-treated sample (Fig 4)	8.75	3.18
Pre-treated sample (Fig 5)	4.10	2.20

Discussion

Formation of reactive oxygen species (ROS) in body can cause oxidative damage to biological macromolecules that may contribute to the development of oxidative stress mediated osteoarthritis disease. Recent studies have suggested that human articular chondrocyte can actively produce reactive oxygen species (ROS). ROS are released during inflammation of the synovial membrane of synoviocyte. These oxidative activities play an important role in the chondrocyte catabolic process being the mediators and effectors of cartilage damage. The damaging effect of the process is initiated by a chain reaction that provides continue supply of free radicals which initiates further per-oxidation. It is reported that synovial cavity damage associates with fluctuating oxygen pressure in the joint, overproduction of free radicals and lack of oxygen-processing enzymes and free radical-scavenging molecules [7]. Free radicals are formed in both physiological and pathological conditions in mammalian tissues. The unlimited production of free radicals is an important factor in the tissue damage induced by several pathophysiologies [8]. Oxidative stress represents a state of increased levels of reactive oxygen species (ROS), also termed as 'oxidants'. This function is controlled physiologically by concentration of oxygen signal transduction and maintenance of redox homeostasis [9]. Fig 2, 4 and 5 describes a state of increased levels of reactive oxygen species, overproduction of ROS, as well as the deficiency of enzymatic and non-enzymatic antioxidant defence mechanism creates an imbalance in the equilibrium of antioxidant status which governs a wide array of diverse disorders. Fig 3 describes low level of ROS as the result of ROS inhibitor was added to the sample, then it was inhibited with ROS inhibitor and the cell size was decreased or cell looked like control sample.

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