ORIGINAL ARTICLE

Estimation of Plasma HNE (4-Hydroxy-2-Nonenal), $PGF_{2\alpha}$ (Prostaglandin $F_{2\alpha}$) and 8-OHDG (Hydroxy-Deoxy-Guanosine) Levels in patients with Rheumatoid Arthritis in Cosmopolitan Lahore

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ABSTRACT

Aim: Estimation of plasma levels of oxidative stress markers; HNE, 8-OHdG and Prostaglandin $F_{2\alpha}$in patients with rheumatoid arthritis, and highlighting the significance of these biomarkers in the pathogenesis and inflammatory destruction of effected joints in rheumatoid arthritis in the adult population in Lahore

Study Design: Case control study

Methods: Current study included seventy adult males, and among these individuals, fifty patients were diagnosed as having rheumatoid arthritis with joint complications, while rest 20 individuals served as healthy control subjects.

Data collection and analysis: Plasma HNE levels were estimated by utilizing HPLC method, and the eluted fraction contained HNE band with an intense yellow color with absorbance recorded at wavelength 370nm. For the estimation of 8-iso-$PGF_{2\alpha}$, EIA standard was added in every test tube, finally wavelength was recorded at 405-420nm. (8-OHdG) was estimated in the serum of patients with the help of commercially available ELISA kit, resultant solution was incubated and washed, and then conjugate enzyme was added, finally reading the incubation plate at 450nm

Results: In the disease group, significantly increased plasma concentrations of HNE and Prostaglandin $F_{2\alpha}$ (p=0.019) and (p=0.033) respectively were observed; (9.65±1.06 µmol/ml) and (47.56±3.15pg/ml) respectively, compared with healthy control subjects (2.06±0.05 µmol/ml) and (1.84±0.0023 pg/ml) respectively. Interestingly, 8-OHdG concentration in plasma appeared to be most significantly raised (p=0.015) in patients with RA (1.52±0.016 pg/ml), compared with healthy control subjects (0.023±0.001 pg/ml).

Conclusion: Current study highlights the significance of inflammatory and oxidative biomarkers in pathogenesis and progression of rheumatoid arthritis in adult population in Lahore. Presented study demonstrated that 8-OHdG levels were most significantly related to joint destruction in patients diagnosed with RA. Plasma levels of HNE and$PGF_{2\alpha}$ were of less significance in the pathogenesis of RA in the adult selected population

Keywords: HNE (4-hydroxy-2-nonenol), 8OHdG (8-hydroxy-deoxy-guanosine), $PGF_{2\alpha}$ (prostaglandin F2α)

INTRODUCTION

Rheumatoid Arthritis (RA) is a multisystem, autoimmune disease, characterized by progressively severe joint deformities, persistent chronic synovial inflammation, articular destruction and bony erosions, leading to varying degrees of crippling disabilities. The overall prevalence has been reported to be 0.5-1% of the total world population (Silman*et al.,* 2002). In the urban population of southern Pakistan, Karachi, the prevalence of RA was approximately 0.142%, while in northern Pakistan, the estimated prevalence was about 0.55% (Hameed *et al.,* 1995). Recently, increased attention was focused on the activity of reactive oxygen metabolites (ROS) in plasma and joint synovium as well. Commonly implicated oxygen derivatives in RA were superoxide free radical anions (O_2^-), hydroxyl free radicals (H_2O_2), lipid peroxyl radicals (LO^-), lipid alkoxyl radicals (LOO^-) and lipid peroxides (LOOH). These free radicals (O_2^-,H_2O_2,LO^-,LOO^-andLOOH) are generated in excess, in the intense inflammatory environment in the effected joints of RA patients. This excessive generation of highly reactive oxygen radicals (ROS) damaged the local joint proteins, lipids, synoviocytes DNA and components of the joint matrix. Modification and alteration of these structural and cellular proteins led to impaired biologic functions, rapid apoptosis and joint damage (Baynes *et al.,* 2000).

Extensive lipid peroxidation was caused by these highly reactive radicals, and thisheralded an era of detection of a variety of plasma bio-markers for lipid peroxidation for evaluation of disease progression.In a study conducted in Turkey, significantly high levels of malondialdehyde (MDA) and xanthine oxidase (XO), in plasma were reported in patients with RA, when compared with control subjects (Kocabaş *et al.,* 2010).In patients of rheumatoid arthritis, excessive generation of reactive free radicals accelerated the defects in the antioxidant systems. For example, an important antioxidant Catalase has shown significantly reduced activity in plasma of many patients suffering from RA (Karatas *et al.,* 2003). Authors in the past working on the role of reactive nitrogen species (RNS) had detected a significant rise in the plasma nitrite concentrations in RA patients. Similarly, biochemical analysis of the synovial contents in RA patients also revealed high levels of nitrite radicals, higher than that expected in serum. This led the authors to suggest that high concentrations of NO were generated in excess within the affected joints (McInnes *et al.,* 1996). Although many studies had focused on the role of oxidative stress in the patho-physiology of rheumatoid arthritis, it had, however, been documented that there is a significantly decreased

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concentrations of various anti-oxidants in the sera of RA patients (Hwang, 2007).These antioxidants were known to provide protection against progressive lipid peroxidation in the patients with rheumatoid arthritis. The antioxidant defense system was, therefore, significantly compromised in patients of rheumatoid arthritis. There was a marked shift in the oxidant/antioxidant balance in favor of lipid peroxidation, which further led to the tissue damage most commonly observed in this disease.

HNE (4-Hydroxy 2- Nonena l)is an oxidative marker, and its increased level is seen in enhanced lipid peroxidation chain reaction, and is, therefore, implicated in the pathogenesis of several diseases such as rheumatoid arthritis, diabetes and cancer. In RA, these HNE products enhanced the mitochondrial pathway of cellular apoptosis by increasing the release of cytochrome c from mitochondria of synovial cells, which subsequently activated the caspases, leading to enhanced apoptosis, characteristically seen in RA. HNE has been, therefore, linked in the pathology of various inflammatory disorders such as RA, diabetes, atherosclerosis and cataracts (Salvayre *et al.,*2010).Recently, a study demonstrated increased serum levels of 4-HNE in patients with RA, compared to healthy controls (Gurkan *et al.,*2016).

8-OHdG (Hydroxy-deoxy-guanosine) is another critical bio-marker of oxidative stress and DNA oxidation in patients of degenerative diseases, particularly rheumatoid arthritis. Increased synovial fluid concentration of 8-OHdG confirmed the role of gradual oxidative destruction of joints in rheumatoid arthritis.Oxidative damage to DNA by oxygen radicals resulted in the production of (8-OHdG) 8-hydroxy-2'-deoxyguanosine, which was detected in high concentration in urine of patients of RA, compared with healthy individuals. (Rall *et al.,* 2000). 8-Iso-$PGF_{2\alpha}$ (Iso-Prostaglandin $PGF_{2\alpha}$)was documented as a useful biomarker of lipid peroxidation in RA. It was derived from the membrane-bound precursor of eicosanoids, arachidonic acid, which was extensively oxidized by the free radicals in the synovial tissue and subsequently hydrolyzed by the membrane-associated phospholipases. 8-iso-$PGF_{2\alpha}$ circulated in the peripheral blood, and was then removed through urine. Increased levels of 8-IP levels in urinary samples of RA patients were observed in a previous research conducted on the patients with RA (Morrow *et al.,* 1992).In addition, it was documented that that the urinary 8-iso-$PGF_{2\alpha}$concentration had a strong association with the over-all 10-year cardiovascular risk in RA patients (Philip *et al.,* 2009).

MATERIALS AND METHODS

Study included sixty (70) adult males50 cases and 20 controls with mean age 40-70 years were included in the following study and were evaluated for different physiological and biochemical parameters. Patients under study were divided into two groups: Fifty (50) adult males, with clinical and radiographic diagnosis of rheumatoid arthritis. Twenty (20)age and sex matched healthy adult males were selected as positive controls in the present study. These selected healthy adult males had no underlying infective disease such as acquired immune deficiency syndrome (AIDS), hepatitis B (HBV) or hepatitis C (HCV) viral infection, cancer or any congenital deformity. Blood samples were equally normal with no detection of inflammatory arthritis.Patients with high erythrocyte sedimentation rate, (ESR), C-reactive proteins (CRP) and Sero-positive for rheumatoid factor (RA-factor) were included in the current study, as patients with RA. Whereas, the individuals with history of non-steroidal inflammatory drugs or antibiotic therapy in past three months were excluded from the current study. In addition, individuals with any history of arthritis, diabetes, Hypertension, Cardiovascular and liver disease were also excluded. Likewise, smokers were not included in this study. Informed consent was taken from all the patients included in this study.

Estimation of 4-hydroxynonenal (HNE): For the estimation of HNE, blood samples were drawnand mixed with 4ml citrate, once mixed, it was subsequently cooled down and was stored in a closed, sterile plastic tube. Afterwards, 3 ml of blood was centrifuged and 1 ml of plasma blood was separated it. Then added 10 µL of BHT and 0.5ml of DNPH, and stored for almost 3 hours. Afterwards it was assayed by first diluting it with the distilled water and pouring it into the Extrelut column, especially filled with almost 1.6 g of ectrelut material. Then about 40ml of dichloromethane was applied to the column, producing a yellowish color, and was then collected into a round bottom flask. Dichloromethane was then separated by applying high pressure and was immediately applied onto the adsorption chromatography and was cooled in ice. After High Pressure Liquid Chromatography (HPLC) is done, the eluted fraction contained HNE band with an intense yellow color. HNE-hydrazone was added to stop the reaction, it was allowed to evaporate on a rotary and residue was re-dissolved in 200 µL methanol and cooled in ice. Again 20µL was injected into HPLC apparatus and absorbance was recorded at the wavelength of 370nm. A peak was identified by comparing the retention time and peak height on the sample chromatograms.

Determination of prostaglandin F2A: For the estimation of $PGF_{2\alpha}$,EIA standard was added in every test tube. About 50 µl sample and 8-isoprotane AChE Tracer was added in each well. Afterwards 8-isoprotane EIA antiserum was added in the wells. Plates were then covered with the thin plastic film and were allowed to stand for 18 hours at $4^{o}C$. These wells were then emptied and rinsed with wash buffer for five times. Then about 200 µl of Ellman's reagent and 5 µl of tracer were added into it. Finally wavelength was recorded at 405-420nm (Maxey *et al*., 1992).

Determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG): 8-hydroxy-2deoxyguanosine (8-OHdG) was estimated in the serum of patients with the help of commercially available ELISA kit by Glory Science Co. Ltd. USA. For the determination, standards were prepared as mentioned in the kit protocol, followed by incubation, washing, addition of conjugate enzyme and reading the incubation plate at 450nm finally.

Statistical analysis: All the readings weregathered in the excel sheets and statistics was performed with the help of SPSS v.16 or later. Taking the (Mean±S.D) where ($p<0.05$) indicated significant results. Test applied were Independent T-test and Spearman correlation graph.

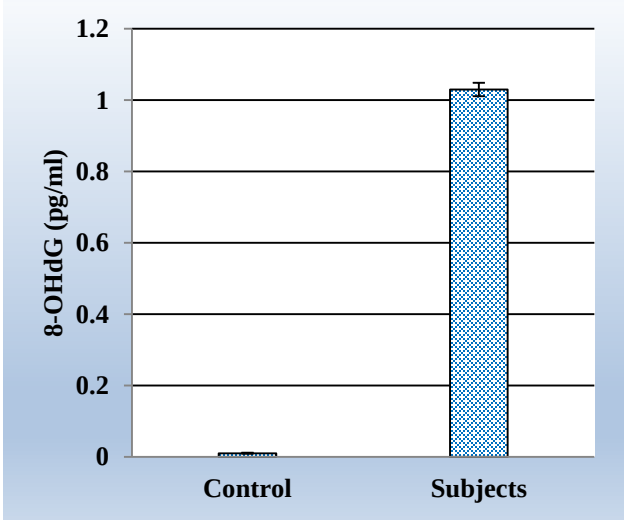
RESULTS

Plasma levels of HNE (4-Hydroxy-2-Nonenal) in rheumatoid arthritis: HNE has been considered an oxidative marker, and its increased level were seen in enhanced lipid peroxidation chain reaction, and was, therefore, implicated in the pathogenesis of several diseases such as rheumatoid arthritis, diabetes and cancer. Blood analysis from 50 diagnosed cases of rheumatoid arthritis showed significantly (p=0.019) high levels of HNE (9.65±1.06µmol/ml) compared to healthy controls (2.06±0.05 µmol/ml).

Plasma levels of prostaglandin $F_{2\alpha}$ in rheumatoid arthritis: Prostaglandin $F_{2\alpha}$ has been anothervaluable and sensitive inflammatory marker in rheumatoid arthritis. Its plasma and synovial fluid levels have been measured to evaluate joint damage. In present study, the plasma levels of 8-isoprostanes were significantly higher as compared to healthy controls. The blood samples of 50 rheumatoid patients and samples of 30 healthy controls were analyzed by radioimmunoassay (RIA) method at laboratory, and the results were compared. As described in table-1, significantly high levels (p=0.033) of Prostaglandin $F_{2\alpha}$weredetected in patients of rheumatoid arthritis (47.56±3.15 pg/ml), compared to healthy controls (1.84±0.0023 pg/ml).

Plasma levels of 8-OHdG (8-Hydroxy-Deoxy-Guanosine) in rheumatoid arthritis: 8-OHdG has been considered another critical bio-marker of oxidative stress and DNA oxidation in patients of degenerative diseases, particularly rheumatoid arthritis. Increased plasma concentration of 8-OHdG confirmed the role of gradual oxidative destruction of joints in rheumatoid arthritis. 8-OHdG measurement was performed by Colorimetric method (ELISA Kit), with sensitivity range of approximately 0.94 ng/ml - 60 ng/ml withassay time of 2 hours. Obtained samples aspirated from 50 patients of rheumatoid arthritis were analyzed for 8-OHdG and significantly increased concentration was observed (p=0.015). The levels of 8-OHdG were significantly high (1.52±0.016 pg/ml) compared with healthy controls (0.023±0.001 pg/ml).

Plasma levels of 8-OHdG



Plasma levels of Prostaglandin $F_{2\alpha}$

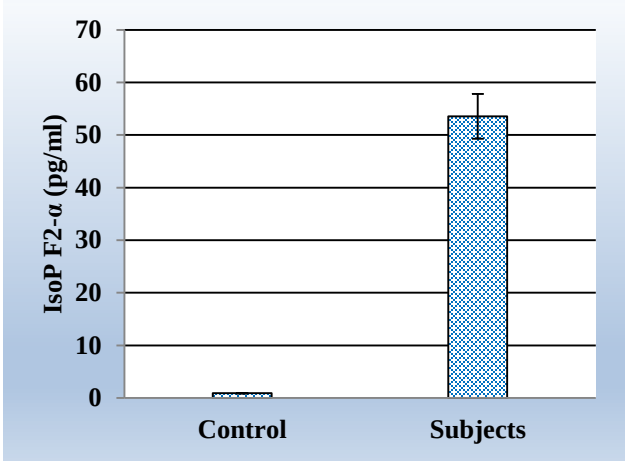


Table 1

Variables	Subject (n=288)	Control (n=100)	*P*- Value
Prostaglandin $F_{2\alpha}$	47.56±2.8	1.84±0.5	0.033
8-OHdG (pg/ml)	**1.52±0.05**	**0.23±0.02**	**0.015**
HNE (µmol/ml)	9.65±0.7	2.06±0.3	0.019

DISCUSSION

Rheumatoid arthritis (RA) is considered the most commonly occurring chronic inflammatory arthritis world-wide, estimated to affect approximately 0.5-1.0% of total world population. Unfortunately, statistics are not well documented in Pakistani population, however, prevalence is considered similar to the population world-wide. During oxidative stress, such as in RA, their concentrations of products of lipid peroxidation were significantly increased in the synovial cells, indicating enhanced lipid peroxidation.In RA, HNE production significantly enhanced the mitochondrial pathway of cellular apoptosis, by increasing the release of cytochrome c from mitochondria of synovial cells, which subsequently activated the caspases, leading to enhanced apoptosis, characteristically seen in RA. HNE has been, therefore, linked in the pathology of various inflammatory disorders such as RA, diabetes, atherosclerosis and cataracts (Salvayre *et al.,* 2010) Recently, a study demonstrated increased serum levels of 4-HNE in patients with RA, compared to healthy controls (Gurkan *et al.,*2016). In the present study on selected Pakistani population in Lahore, plasma HNE levels were found to be elevated, showing enhanced lipid peroxidation in the synovium of RA patients (p value = 0.016).

A group of scientists in 2014 documented prostaglandin $F_{2\alpha}$levels as significant marker of oxidative stress, bone erosion and disease progression in RA. They worked on 73 patients with RA, and compared the results with 62 healthy subjects. They serially measured a variety of oxidative stress markers and showed that plasma prostaglandin $F_{2\alpha}$ levels were about 9-times higher in the plasma, and approximately 3 times higher in the urine of RA patients, compared with healthy controls, thus establishing a correlation with the disease progression in RA (Luczaj *et al*., 2014). 8-OHdGis an oxidized derivative

deoxyguanosine, and considered as a primary product of DNA oxidation. It is a critical biomarker of oxidative stress, particularly measured to estimate the DNA damage in a variety of diseases, such as rheumatoid arthritis and carcinogenesis. Recently, 8-OHdG has been widely used in many arthritis studies, as a significant biomarker for endogenous oxidative DNA damage. It has been well documented that DNA damage and lipid peroxidation-derived DNA adducts were potential factors for the pathogenesis and intense inflammatory response in the joints of patients with RA (Hitchon, 2004). Previously, it has been known that the oxidative stress-related DNA damage caused breakage of DNA double and single strands, which altered the quaternary arrangement in the DNA. As a result, the DNA un-winded, and was detected in the mononuclear cells in the blood circulation of patients suffering from RA (Bhusate*et al.,* 1992). In the same lines, it was demonstrated that the baseline 8-OHdG levels were significantly greater compared to healthy controls (Laura *et al.,* 2006).

CONCLUSION

In the present study on selected Pakistani population, plasma HNE levels were found to be elevated, showing enhanced lipid peroxidation in synovium of patients with RA (p value = 0.016). Similarly, plasma levels of prostaglandin $F_{2\alpha}$ were significantly high in patients with RA, showing an enhanced, exaggerated inflammatory response in these patients (p value = 0.033). Significantly high plasma levels of 8-OHdG were observed in patients with RA, indicating oxidative DNA damage in this chronic inflammatory disease. In this study, plasma levels of 8-OHdG were most significant as compared with other biomarkers HNE andprostaglandin $F_{2\alpha}$indicating an enhanced formation of lipid peroxidation-derived DNA adducts and associated DNA damage, appearing to be crucial in the pathogenesis and intense inflammatory damage to the joints of patients with RA

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