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# Disorder Activity of Some Enzymes Plays an Important Role in Pathological Mechanism of Rheumatoid Arthritis Disease

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## Abstract:

The aim of this research to show the role of some enzymes in pathological mechanism of rheumatoid arthritis (RA) disease. Sixty patients with RA and matched number of apparently healthy volunteers were included in the study. Spectrophotometric methods were used to determine Peroxy nitrite (ONOO), Nitric oxide (NO), Nitric oxide synthase activity (NOS) cycloxygenase-2 activity (COX-2), glutathione peroxidase (GPX) activity and superoxide dismutase (SOD) activity in serum of both groups. Colorimetric assay kits were used to determine Iron. Rheumatoid factor (RF) was determined using Imuno-Latex kit. ONOO, NO levels, and NOS activity were significantly higher in the patients compared to the control group. Conversely, Iron level, SOD activity and GPX activity were significantly lower in the patients compared to the control. In patients group, RF has significant positive correlation with COX-2, NOS activity and NO level while; it has significant negative correlation with Iron level. ONOO level has significant positive correlation with NO level, NOS activity and COX-2 activity while has significant negative correlation with Iron and SOD activity. NOS activity and NO level have significant positive correlation with COX-2 activity while, they have negative correlation with Iron. Iron has significant negative correlation with COX-2 activity. In control group, COX-2 has significant positive correlation with NOS activity and ONOO level. GPX has significant positive correlation with SOD and Iron. In conclusion: disorder activity of COX-2, NOS, SOD and GPX enzymes play important role in the pathological mechanism of rheumatoid arthritis and they linked with a decreased level of serum iron

**Key words:** Rheumatoid arthritis, Cycolxygenase-2, Nitric oxide synthase, Superoxide dismutase, Glutathione peroxidase.

## Introduction:

Rheumatoid Arthritis (RA) is autoimmune disease until now considered unknown so; the cause of RA is a very active area of worldwide research [1]. RA starts with the synovial membranes inflammation that has been documented in several previous studies [2, 3]. The source of inflammation is not specific and may come from infectious agents such as viruses, bacteria, and fungi or other factors in the environment.

Inflammation leads to activate the phagocytes and other leukocytes in the synovial fluid [4]. Activated neutrophils in the joints increase the production of reactive oxygen species (ROS) [5]. The prime targets of ROS attack are the polyunsaturated fatty acids (PUFA) in the membrane's causing lipid peroxidation which may lead to disorganization of cell structure and function [6], the integrity of the plasma membrane is lost, the influx of calcium from the extracellular space and subsequently cause citrullination of extracellular proteins [7]. The presence of citrullinated proteins leads to abnormal an immune response that causes the generation of anti cyclic citrullinated peptide (anti-CCP) antibodies to develop into RA disease [7]. ROS may impair the activity of enzymatic antioxidant superoxide dismutase (SOD) [8] thus, lead to availability of superoxide union (O2•-) that reacts with nitric oxide (NO) (which synthesized from the deimination L- arginine by nitric oxide synthase (NOS)) and thus produce the highly reactive peroxy nitrite (ONOO) [9]. The conditions that help to produce high level of ONOO are the excess production of NO [2] and limited activity of SOD because this help to availability of (O2•-) which react with NO to give ONOO. The high level of ONOO may increase the activity of cycloxygenase-2 (COX-2) enzyme by oxidizing the heme iron of COX-2 enzyme. COX-2 responsible for formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. The enzyme contains two active sites: a cyclooxygenase site, where arachidonic acid is converted into the hydroperoxy endoperoxide prostaglandin G2 (PGG2) and a heme with peroxidase activity site, responsible for the reduction of PGG2 to PGH2 Thus, the enzyme acts both as a dioxygenase (incorporate O2 into a substrate) and as a peroxidase at the same time. Prostaglandins act as mediator for inflammation, sensor for pain and cause the fever [10]. All these symptoms are the distinct characteristics of RA disease. Hence, the aim of the present study was to prove this mechanism by determine the activity of the enzymes COX-2, NOS, SOD, GPX and iron level in order to find the correlation between each parameter with others and the activity of disease through the level of RF.

## Materials and Methods:

The study was conducted in 2014 (College of Science- Department of Chemistry). The samples were taken from the patients visiting Ibn-Sina hospital in Mosul City. A number of 120 volunteers were included in the study. Out of the 120 subjects, 60 apparently healthy adults were enrolled as a control group (aged 25-64 years) (group1). The remaining 60 rheumatoid arthritis patients (aged 20-70 years) (group2). Both groups were residents in Mosul City, their age, gender, and other important information were matched as shown in Table 1. Factors interfering with the results of study (Cancer, Hepatitis, Mononucleosis, Hypergammaglobulinemia etc,) excluded by history. Doctors' diagnoses were used for choosing the patients enrolled in the study. The criteria are recommended by the American Rheumatism Association [11].

About 5 mL of blood sample was obtained from each subject in both groups. The blood was left at 37 °C and then centrifuged at 3000 rpm (900 *g*) for 15 min. Serum was then separated and stored at -10 °C until analysis [12].

SOD was measured using modified riboflavin /nitro blue

tetrazolium method [13]. The principle of this method depends on the measuring the change of optical density of formazine that produced from reducing nitro blue tetrazolium by super oxide union generating in irradiating serum. According to this method the activity of SOD calculated from the following equation:

$$\text{SOD (U/L)} = \frac{\Delta A}{\varepsilon} \times 10^6$$

ΔA= absorbance of reaction solution after irradiation-absorbance of reaction solution before irradiation.
ε= the molar extinction coefficient (235 00 $M^{-1}$ $cm^{-1}$).

GPX was measured using the method of Rotruck *et al* [14]. The principle being that GPX catalyses the oxidation of glutathione (GSH) by hydrogen peroxide to give oxidized form (GSSG), the remaining GSH is then determined by Ellman's method [15]. The principle of this method depends on the reducing of Ellman's reagent [5, 5-dithio bis (2-nitrobenzoic acid)] by SH-group of glutathione to give colored product. The absorbance of this colored product measured at 412 nm, where the concentration of glutathione directly proportional with the measured absorbance. The activity of GPX calculated from the following equation:

$$\text{GPX (U/L)} = 0.23 \times \log \frac{\text{GSH1}}{\text{GSH2}}$$

GSH1= the concentration of glutathione that start from it (2 mM).
GSH2= the concentration of remaining glutathione after reaction which calculated from the following equation:

$$\text{GSH2(mM)} = \frac{\text{A}}{\varepsilon} \times 10^3$$

Where:
A= absorbance of colored product.
ε= the molar extinction coefficient (13600 $M^{-1}$ $cm^{-1}$).

ONOO was measured using modified method of Vanuffelen *et al* [16]. The principle of this method depends on the nitration of phenol by ONOO to give nitro phenol which absorbs light at 412 nm the amount of nitro phenol directly proportional with concentration of ONOO. According to this method the level of ONOO calculated from the following equation:

$$\text{ONOO (μmol/L)} = \frac{\text{A}}{\varepsilon} \times 10^6.$$

Where:
A= the absorbance of colored solution.
ε= the molar extinction coefficient (4400 $M^{-1}$ $cm^{-1}$).

COX-2 was measured by an assay for the peroxidase activity based on a colorimetric procedure [17]. This method depends on the measuring the enzyme-catalyzed oxidation of tetramethyl phenylenediamine (TMPD) by hydrogen peroxide. The blue color was measured at 610 nm. The activity of COX-2 calculated from the following equation:

$$\text{COX2 (U/L)} = \frac{\text{A2} - \text{A1}}{\varepsilon} \times 10^6$$

Where:
A2= the absorbance of reaction solution after 1 min of addition hydrogen peroxide.
A1= the absorbance of reaction solution before addition of hydrogen peroxide.
ε= the molar extinction coefficient (12000 $M^{-1}$ $cm^{-1}$).

Serum iron concentration was measured using spectrophotometeric kit method [18] from French Biolabo Company. The principle of this method depends on the dissociation of iron-transferrin bound deproteinisation by hydrochloric acid and trichloroacetic acid (TCA), the iron $Fe^{3+}$ is reduced by thioglycolic acid to iron $Fe^{2+}$. Then, ferrous iron forms with disulfonated bathophenanthrolin a colored complex where absorbance measured at 535 nm is directly proportional to the amount of iron in specimen. According to manufacturer's instructions serum iron concentration calculated by the following equation:

$$\text{Iron } (\mu g/dl) = \frac{A_{test}}{A_{standard}} \times \text{Concentration of standard } (200\ \mu g/dl)$$

NOS and NO were measured using colorimetric assay method [2]. This method depends on the determined the quantity of nitrite NO2, primary form of nitric oxide NO in biological system, using Griess reagents. In acidic solution, nitrite is converted to nitrous acid (HNO2), which diazotizes sulfanilamide. This sulfanilamide-diazonium salt is then reacted with N-(1-Naphthyl)-ethylenediamine to produce a chromophore which is measured at 540 nm. NO level calculated using standard curve prepared by taking several dilution of sodium nitrate (0-35 nm/ml) to be measured, running the experiment as described above, then plotting absorbance, measured at 540 nm, against concentration, the level of NO found from this curve. NOS activity calculated by utilize from the slope and y-intercept of the equation in the standard curve, and the sample absorbance at 540 nm. According to this method the activity of NOS is calculated from the equation of [19]:

**NOS (U/L) = {(A540 - b) / m}(VT / VS) / T**

Where:
A540 is the sample absorbance at 540 nm.
b is the y-intercept (linear regression of the standard curve).
m is the slope (linear regression of the standard curve).
VT is the final volume of the reaction in the assay.
VS is the volume of sample (ml).
T is the reaction time in minutes.
RF measured using Imuno- Latex RF kit from Wama diagnostic company where, a suspension of latex is mixed on an test card with serum containing elevated rheumatoid factor levels, a clear agglutination is seen in 2 minutes. The serum RF concentration is calculated by preparing serial dilutions of the patients' serum at 1:2, 1:4, 1:8, 1:16 using isotonic saline (NaCl 0.9%) then running the experiment as described above. The highest dilution of the serum which shows an obvious agglutination pattern is considered the titer end point. The serum RF concentration is calculated approximately by multiplying the dilution factor (i.e. 2, 4, 8 or 16) by the detection limit (8 IU/ml) to give the number of IU/ml concentration [20]. Example: If the agglutination titer appears at 1:8, the approximate serum RF concentration is: 8x8=16 IU/ml.

**Statistical Analysis**

Data were evaluated by analysis of variance (ANOVA) and t-test, adjusted for multiple comparisons. P values <0.05 were considered statistically significant. Correlations were determined by Pearson's test.

## Results:

Both groups (control and patients) were residents in Mosul City, their age, gender, BMI and other important information was matched as shown in table 1, there were no significant differences between the two groups for the physical characteristics.

**Table 1: The clinical and laboratory data of control group and RA patients.**

| Clinical and laboratory data | control | Patients | *P*-value |
|---|---|---|---|
| Number | 60 | 60 | - |
| Gender (M/F) | 30/30 | 19/41 | - |
| Age (year) | 39.75±10.22 | 43.92±13.45 | 0.058 Ns |
| Duration of RA (year) | 0 | 14.48±9.18 | - |
| BMI (Kg/m2) | 29.138±5.04 | 28.274±4.97 | 0.347 Ns |
| RF(IU/ml) | 0 | 66±49.13 | - |

Values are presented as mean ± SD, Ns: No significan

All biochemical parameters were compared between control and patients groups as shown in Table 2. ONOO level, NO level, NOS activity and COX-2 activity were significantly higher in the patients compared to the control group. Conversely, Iron level, SOD activity and GPX activity were significantly lower in the patients compared to the control.

**Table 2: The comparison of serum Biochemical parameters between control and RA patients group.**

| Parameters | Control | Patients | *P*-value |
|---|---|---|---|
| ONOO (µmol/L) | 65.06 ± 18.28 | 77.63 ± 20.53 | 0.001 |
| NO (nmol/mL) | 13.618 ± 3.607 | 16.105 ± 4.334 | 0.001 |
| Iron (µg/dl) | 145.26 ± 45.59 | 123.23 ± 50.59 | 0.014 |
| NOS (U/L) | 2.3718 ± 0.7213 | 2.8693 ± 0.8667 | 0.001 |
| COX-2 (U/L) | 4.229 ± 1.120 | 5.549 ± 1.520 | 0.001 |
| GPX (U/L) | 0.46375 ± 0.00666 | 0.447±0.011 | 0.018 |
| SOD (U/L) | 1.597 ± 0.414 | 1.413 ± 0.334 | 0.009 |

Values are presented as mean ± SD.

As shown in Table 3, in patients group, Rf has significant positive correlation with COX-2 activity, NOS activity, NO level while, it has significant negative correlation with Iron level. ONOO level has significant positive correlation with NO level, NOS activity and COX-2 activity while has significant negative correlation with Iron level and SOD activity. NOS activity and NO level have significant positive correlation with COX-2 activity while, they have negative correlation with Iron. Iron has significant negative correlation with COX-2 activity.

**Table 3: The correlations among biochemical parameters in serum of RA patients.**

| Parameters | RF | ONOO (µmol/L) | NO (nmol/mL) | Iron (µg/dl) | NOS (U/L) | COX-2 (U/L) | GPX (U/L) |
|---|---|---|---|---|---|---|---|
| ONOO (µmol/L) | r= 0.088 p= 0.503 | | | | | | |
| NO (nmol/ml) | r= 0.323 p= 0.012 | r= 0.361 p= 0.005 | | | | | |
| Iron (µg/dl) | r=-0.369 p=0.004 | r=-0.256 p= 0.048 | r= -0.514 p= 0.000 | | | | |
| NOS (U/L) | r=0.323 p=0.012 | r= 0.361 p= 0.005 | r= 1.00 p= 0.00 | r=-0.514 p=0.000 | | | |
| COX-2 (U/L) | r= 0.272 p= 0.035 | r= 0.474 p= 0.000 | r= 0.871 p= 0.00 | r=-0.537 p=0.000 | r= 0.858 p= 0.000 | | |
| GPX (U/L) | r= 0.062 p= 0.640 | r=-0.001 p= 0.994 | r= -0.008 p=0.950 | r=-0.102 p=0.440 | r= -0.008 p=0.950 | r=-0.020 p= 0.881 | |
| SOD (U/L) | r=-0.160 p= 0.223 | r= -0.571 p= 0.00 | r=-0.110 p= 0.402 | r= 0.185 p= 0.156 | r=-0.110 p= 0.402 | r=-0.175 p=0.180 | r=-0.033 p=0.804 |

r: Pearson correlation, P= p-value

As shown in table 4, in control group, COX-2 has significant positive correlation with NOS activity and ONOO level, GPX has significant positive correlation with SOD and Iron.

**Table 4: The correlations among biochemical parameters in serum of control group.**

| Parameters | ONOO (µmol/L) | NO (nmol/mL) | Iron (µg/dl) | NOS (U/ml) | COX-2 (U/L) | GPX (U/L) |
|---|---|---|---|---|---|---|
| ONOO (µmol/L) | | | | | | |
| NO (nmol/ml) | r= 0.220 p= 0.091 | | | | | |
| Iron (µg/dl) | r= -0.130 p= 0.322 | r= -0.145 p= 0.267 | | | | |
| NOS (U/L) | r= 0.220 p= 0.091 | r=1.00 p= 0.00 | r=-0.145 p= 0.267 | | | |
| COX-2 (U/L) | r= 0.504 p= 0.000 | r= 0.636 p= 0.000 | r=-0.239 p= 0.066 | r= 0.636 p= 0.000 | | |
| GPX (U/L) | r= -0.238 p= 0.067 | r= 0.096 p= 0.467 | r=0.260 p= 0.044 | r= 0.096 p= 0.467 | r= -0.199 p= 0.127 | |
| SOD (U/L) | r= -0.007 p=0.960 | r= 0.086 p=0.514 | r=0.165 p=0.209 | r= 0.086 p=0.514 | r= 0.069 p= 0.600 | r=0.290 p=0.024 |

r: Pearson correlation, p= p-value

## Discussion:

It is clear from the results of the present study that the activity of NOS, COX-2, GPX and SOD in RA patients are different from those in control and this is a proof that there is disorder in the activity of some enzymes in RA. The effect of these enzymes in the mechanism of disease seemed different, in other words, direct and indirect effect. SOD and GPX seemed from the type which has indirect effect (through their effect to level of ONOO and $H_2O_2$ molecules).

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad \ldots [21]$$

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + 2GS.. [22]$$

Similar to results that observed by a previous study [23], the current study shows that SOD activity in patients is less than in control. This reflects the high level of ONOO where, the result of the current study shows that there are significant negative correlation between SOD activity and ONOO level because the decrease in activity of SOD means the increase in level of super oxide union ($O_2^{\cdot -}$) which reacts with NO to form ONOO.

$$O_2^{\cdot -} + NO \xrightarrow{NOS} ONOO \ldots\ldots [9]$$

The current study shows that NOS is more active in RA patients compare to control and this is additional cause to increase the level of ONOO where there are significant positive correlation between NOS activity and ONOO level where, the results of the present study show that there is increase level of NO in RA patient compare to the control and this is due to increasing activity of NOS. The increase activity of NOS in RA patient might be due to the fact that NO is involved in immune, inflammatory and autoimmune basis of arthritis [24] where NO has a role in cardinal signs of inflammation [25].

GPX exhibit lower activity in RA patients the cause of this according to current study might be due to reduce the level of hydrogen peroxide (the molecule produced in SOD reaction as it shown in above equation) which consider the substrate of GPX as a result of reduced the activity of SOD this mean that the reduced activity of SOD being responsible to reduce the activity of GPX. Also the reduce activity of GPX in RA patients might be due to utilization of this enzyme for detoxification of free radicals generated in rheumatoid arthritis [1].

The current study also showed that COX-2 more active in RA patients than control and this is expected result because synovial fluid in RA patients work to stimulate COX-2 secretion [26]. The results of the study show that there significant positive correlation between COX-2 and ONOO this is due to two causes:

The first is the role of ONOO in oxidizing the heme iron of COX-2 and enabling it to transfer electron from the active site tyrosine. The resulting tyrosine radical is extracting a hydrogen atom from arachidonate, generating a radical species that react with oxygen to produce prostaglandins [27, 28].

$$\text{heme-Fe}^{III} + ONOO \rightarrow \text{heme} - \text{Fe}^{III} - ONOO \text{ complex}$$

$$\text{heme} - \text{Fe}^{III} + ONOO \rightarrow \text{heme} - \text{Fe}^{IV} = O + NO_2^- \quad .. [28]$$

$$NO_2^- + \text{Tyrosine} \rightarrow \text{Tyrosine radical} + NO_2^{\cdot}$$

The second, NOS is stimulated by the same inflammatory mediators which stimulate the expression of COX-2 where synoviocytic fibroblasts seem to be the main source of NO in rheumatoid synovium [29], thus the increase levels of NO lead to increase formation of ONOO.

The current study also showed that the level of iron is less in RA patients than in control and this is also help to increase the activity of COX-2, where the low level of iron helps ONOO molecules to reduce a large number of iron atoms which form the prosthetic group of COX-2 and activate this enzyme instead of reduce iron atoms that bound to the serum transferring [28, 30, 31], this mean that there are a compete between iron in sreum transferrin and iron in prosthetic group of COX-2 to oxidative by ONOO. This hypothesis become more obvious when you know that there are significant negative correlation between COX-2 activity and Iron level as it shown in the results of the present study. Also, the present results show that there are significant negative correlation between RF and iron level.

Finally, the current study showed that RF has significant positive correlation with COX-2 activity and NOS activity and this exhibit the direct effect of these enzymes in the mechanism of RA(through their effect to level of prostaglandins responsible for inflammation).

## Conclusion:

Disorder activity of COX-2, NOS, SOD and GPX enzymes play important role in the pathological mechanism of rheumatoid arthritis and they linked with a decreased level of serum iron

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# الاضطراب في فعالية بعض الانزيمات يلعب دور مهم في الالية المرضية لمرض التهاب المفاصل الرثوي

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**الخلاصة:**

الهدف في هذا البحث توضيح دور بعض الانزيمات في مرض التهاب المفاصل الرثوي. تضمنت الدراسة 60 مريض مصاب بالتهاب المفاصل الرثوي كمجموعة مرضى وعدد مناظر من اشخاص بالغين اصحاء كمجوعة سيطرة. استخدمت طريقة طيف الامتصاص الضوئي في تقديرمستوى البيروكسي نتريت، النايترك اوكساید، فعالية انزيمات السايكلوكس جينيز-2، النايترك اوكساید سنثيز، السوبر اوكساید دسيميوتيز و الكلوتاثايون بيروكسيديز، بينما استخدمت المحاليل الجاهزة لتقدير مستوى حديد الدم بطريقة لونية و مستوى عامل الروماتويد بطريقة مناعية. اظهرت نتيجة المقارنة للمتغيرات المقاسة بين المجموعتين ارتفاعا معنويا في مستوى البيروكسي نتريت، النايترك اوكساید وفعالية انزيمي النايترك اوكساید سنثيز والسايكلوكسي جنيز-2 عند مجموعة المرضى بالمقارنة مع مجموعة السيطرة بينما كان هناك العكس في مستوى حديد الدم وفعالية انزيمي السوبر اوكساید دسيميوتيز و الكلوتاثايون بيروكسيديز. كما اظهرت نتيجة الدراسة ان عند مجموعة المرضى عامل الروماتويد يرتبط طرديا مع مستوى النايترك اوكساید و فعالية انزيمي السايكلوكسي جينيز-2، النايترك اوكساید سنثيز بينما يرتبط عكسيا مع مستوى حديد الدم كما ان النايترك اوكساید والنايترك اوكساید سنثيز يرتبطان طرديا مع السايكلوكي جينيز-2 وعكسيا مع حديد الدم الذي يرتبط عكسيا ايضا مع انزيم السايكلوكسي جينيز-2.عند مجموعة الاصحاء اظهرت نتيجة الدراسة ان انزيم السايكلوكسي جينيز-2 يرتبط طرديا مع النايترك اوكساید سنثيز ومستوى البيروكسي نتريت كما يرتبط انزيم الكلوتاثايون بيروكسيديز بعلاقة طردية مع انزيم السوبر اوكساید دسيميوتيز وحديد الدم. يستنتج من الدراسة الحالية ان الاضطراب في فعالية انزيمات السايكلوكسي جينيز-2، النايترك اوكساید سنثيز، السوبر اوكساید دسيميوتيز وانزيم الكلوتاثايون بيرروكسيديز يلعب دور مهم في الالية المرضية لمرض التهاب المفاصل الرثوي ويرتبط بانخفاض مستوى حديد الدم.

**الكلمات المفتاحية**: التهاب المفاصل الرثوي، السايكلوكسي جينيز-2، النايترك اوكساید سنثيز، السوبراوكساید دسميوتيز، الكلوتاثايون بيروكسيديز.