Indian Journal of Clinical Biochemistry, 2003, 18 (2) 216-222

EFFECT OF TYPE II COLLAGEN TREATMENT ON THE ANTIOXIDANT STATUS IN IMMUNE TISSUES OF ADJUVANT INDUCED ARTHRITIC RATS

Nalini Ganesan*, Hariprasad Chegu, A.N.Chandrasekaran*****

**Associate Professor, Dept.of Biochemistry, SRMC & RI (DU), Porur, Chennai-600 116.*
*** Principal, Professor & HOD, Dept.of Biochemistry, SRMC & RI (DU), Porur, Chennai-600 116.*
****Retd. Professor of Rheumatology, Govt.General Hospital, Chennai Medical College, Chennai -600 003.*

ABSTRACT

Adjuvant induced arthritis (AIA) is a model widely used to study Rheumatoid arthritis (RA). In the present study, lipid peroxides level in spleen and thymus of AIA rats was observed to be significantly high compared to normal rats. A significant decrease in ascorbic acid (ASA), reduced glutathione (GSH), superoxide dismutase activity (SOD) was also observed in spleen and thymus of AIA rats compared to normal rats. There was also a steady increase in the circulating immune complex level (CIC) throughout the experimental period in serum of AIA rats. In the present investigation, it was decided to study the effect of pre and post treatment with TYPE II collagen on the antioxidant status and the circulating immune complex level in AIA rats. The results from the present work indicates that the pretreatment with TYPE II collagen was effective in bringing significant changes on all the parameters studied in AIA rats. The post treatment with TYPE II collagen was effective in bringing significant changes on the CIC immune complex level and GSH content in the thymus tissue of AIA rats. The present work suggests that the pre treatment with TYPE II collagen was more effective in suppressing the disease than the post treatment.

KEY WORDS

Adjuvant arthritis, antioxidants, type II collagen, tolerance.

INTRODUCTION

Adjuvant arthritis induced by intradermal injection of Complete Freund's Adjuvant (CFA), is a chronic, generalized reaction and is an accepted experimental model of rheumatoid arthritis (1). It has been suggested that the pathogenesis of AIA, involves the formation of immune complex which allow the dissemination of mycobacterial antigen throughout the body and ultimately gets deposited at various sites and initiates joint lesion (2).

Free radicals are highly reactive and unstable entities capable of damaging cellular components, and they contribute to the pathogenesis of a number of diseases (3). There is accumulating evidence that RA has characteristics of a free radical -produced disease (4).

Author for Correspondence

Nalini Ganesan
**Associate Professor, Dept.of Biochemistry, SRMC & RI (DU), Porur, Chennai-600 116.*

In normal cellular life, the damage by the free radical is kept to a minimum by the existence of defense mechanism. In our previous study on RA patients we have observed a decrease in the antioxidant status (5).

Collagen exists in genetically distinct types in different vertebrate tissues. So far, 19 distinct types have been isolated and most of them have been characterized in considerable detail with respect to aminoacid sequence and chromosomal localization (6). The unique immunogenic as well as arthritogenic properties of Type II collagen results from a helical conformation of its structurally distinct α-chains (7).

Oral tolerance is a safe and simple treatment strategy, which has been used to suppress experimental autoimmune encephalomyelitis, AIA and diabetes (8). This new strategy has also been used for treating diseases like multiple sclerosis, RA and uveitis. The primary mechanism by which orally administered antigen induces tolerance are via the generation of active inhibitory cytokines or clonal anergy and/or clonal deletion (9). In our

previous study, we have reported their effect on pre and post treatment with TYPE II collagen on the physiological parameters like body weight changes, paw swelling and lysosomal enzymes in immune tissues of AIA rats (10).

In RA, virtually every tissue is affected by the inflammatory process (11). Studies have revealed abnormal lymphoid function in the peripheral blood of patients with rheumatoid arthritis (12). Therefore, the primary objective of the present investigation was to study the effectiveness of pre and post treatment with TYPE II collagen on the level of serum CIC and antioxidant status in immune tissues of AIA rats.

MATERIALS AND METHODS

Animals- Female Wistar rats weighing about 75 g were obtained from King Institute of Preventive Medicine, Guindy, Chennai. The animals were acclimatized for a week to the animal house of the department. All the animals were subjected to a daily photoperiod of 12 h light and 12 h darkness and were maintained on a commercial standard pellet diet and tap water *ad libitum* .

Chemicals - Complete Freund's Adjuvant (10 mg/ ml), Type II collagen from bovine tracheal source, 1,1', 3, 3' tetra ethoxy propane, pyrogallol and TRIS were purchased from Sigma Chemicals, St.Louis, USA. Reduced glutathione, 5, 5' dithiobis 2-nitrobenzoic acid were bought from BDH England. All other chemicals of analytical grade were obtained from Loba Chemie, Bombay, Sisco Research Laboratories.

Induction of Arthritis - Adjuvant induced arthritis was induced in animals by intradermal injection into the footpad of the hind paw with 0.1 ml CFA containing 10 mg/ ml mycobacterium.

Evaluation of Arthritis - The development of arthritis were assessed by measuring the hind paw swelling of the injected and the uninjected paw with a vernier calipers as described in [13].

Treatment - Type II collagen was dissolved in potassium buffer at pH 7.6 to obtain 100 μg in 0.5 ml and the rats were fed with collagen by gastric gavage.

Experimental Design - The experimental group consisted of 30 rats divided equally into five groups.

Group I - This group formed the normal control group.

Group II - This group of animals were given a single intradermal injection of 0.1 ml CFA into the foot pad on day 0.

Group III - This group of animals were pretreated with Type II collagen (100 μg per feeding) by gastric gavage on days -7, -5 and -2, followed by intradermal injection of 0.1 ml CFA into the foot pad on day 0. This group formed the pretreated group.

Group IV - This group of matching severity of the disease, received the same dosage of collagen on 15, 17 and 19 day after the immunization with 0.1 ml of CFA. This group formed the post treated group.

Group V - This group of animals were given the same dosage of Type II collagen to normal rats. This formed another control group.

All the animals were examined carefully throughout the experimental period which was of six weeks duration. At the end of each week, the animals of each group were bled and it was used for the estimation of CIC. On the 42 nd day, the end of the experimental period, the animals were sacrificed by cervical dislocation. The tissues, spleen and thymus were rapidly removed and kept in ice-cold saline. The tissues were weighed immediately after blotting well and used for the preparation of tissue homogenate.

Preparation of Tissue Homogenate- A portion of the tissue of known weight was minced to small pieces and then homogenised in the cold room with 0.01 M Tris -HCl buffer, pH 7.4 using a high speed Potter-Elevehjem teflon homogeniser to obtain 10 % homogenate. Aliquots of this homogenate was used for the estimation of lipid peroxides, ascorbic acid, superoxide dismutase, reduced glutathione and protein. All the estimations were completed within 12-24 hrs of sacrifice.

Estimation of lipid peroxides - The tissue lipid peroxides were determined by the method of Ohkawa *et al* (14). The lipid peroxides are expressed as nmole/mg protein.

Estimation of ASA - The ASA in the tissue was estimated by the method of Omaye *et al* (15). The level of ASA is expressed as mg of ASA/ mg tissue.

SOD activity - The SOD activity in the tissues were determined by auto-oxidation of pyrogallol as described by Marklund and Marklund (16) and the enzyme activity was expressed in terms of units/ mg protein. One unit of enzyme activity corresponds

to amount of enzyme that inhibits the reaction by 50 %.

Estimation of GSH - The GSH content is estimated by the method of Moron *et al* (17) and expressed as mg of GSH / mg tissue.

Protein content - The protein content in the homogenate was determined by the method of Lowry *et al.* (18).

Estimation of CIC in sera - The CIC in the serum was measured by the method of Seth and Srinivas (19). It is expressed as polyethylene glycol index.

Statistical evaluation - The results obtained in this study were expressed as Mean ± S.D. The CIC levels were analysed by using student's "t" test. The " p" value was arrived at to assess the statistical significance of the changes observed. The results obtained for other parameters were analysed by the application of one-way analysis of variance (ANOVA). The multiple comparison to elicit the significant difference between various groups were performed by means of a Tukey's HSD test. Values of $p < 0.05$ was considered statistically significant.

RESULTS

The results of lipid peroxides and antioxidants in spleen and thymus tissues are given in Table 1 and Table 2 respectively. The analysis of the results by Tukey's multiple comparison test between various groups ($p<0.05$) are as follows:

The lipid peroxides in the spleen , thymus of group II animals were significantly higher than group I and group V. The lipid peroxide content in group III was significantly lower than group II. No significant change was observed between the group IV and group II. No significant change was observed between group I and group V animals.

The ASA content in the spleen and thymus of group II animals were significantly lower than the group I and group V animals. The ASA content in the immune tissues of group III animals were significantly higher than group II. No significant change was observed between group IV and group II animals. No significant change was noticed between group I and group V.

The SOD activity in the immune tissues of group II animals were significantly lower than the group I and group V. The enzyme activity in spleen and thymus of group III was significantly higher than group II. No significant change in SOD activity was observed in the immune tissues of group IV compared to group II animals. No significant change in SOD activity in immune tissues was seen between group I and group V animals.

The GSH content in the immune tissues of group II animals was observed to be significantly lesser than the group I animals. The GSH content of the pretreated group was significantly higher than group I. In the case of thymus tissue group IV animals also showed significant change when compared to thymus tissue of group II animals. No significant change was observed in GSH values in immune tissues of group I and group V animals.

The results of sera CIC of each group at the end of each week for a period of six weeks is represented in Figure No. I rise in sera CIC was observed in group I and group V animals. In the case of group II animals the level of sera CIC rose after the second week reaching a maximum after 4 weeks . Pretreatment and post treatment with Type II collagen significantly reduced the sera CIC throughout the experimental period.

DISCUSSION

The results of our present study indicates that the decrease in the antioxidant status is also one of the factors contributing to the pathogenesis of the disease. The observed significant increase in LP level in the immune tissues of group II animals suggests that the tissues are subjected to increased oxidative stress. Yoshikawa *et al* (20) have reported elevated levels of thiobarbituric acid -reactive substances in sera and synovia of AIA rats. In the group III animals the decreased level of LP suggests that Type II collagen was effective in controlling the extent of lipid peroxidation. No significant changes observed in group IV animals reveal that Type II collagen treatment given after the onset of the disease was not effective in inhibiting the oxidative damage.

Ascorbic acid has been shown to be protective against oxidative stress by being involved in recycling vitamin E (21). A decreased level of ASA in the immune tissues of group II animals show a decrease in the antioxidant status. Eldin *et al* (22) have shown that administration of ascorbic acid in a daily oral dose of 50 mg/kg body weight in AIA rats was effective in modulating some biochemical changes. In the pretreated group, the ASA content

was significantly higher than the group II showing the immunoprotective role of Type II collagen on the development of adjuvant induced arthritis. In the post treated group, Type II collagen was unable to bring about a change in ASA acid level in the immune tissues.

Reduced glutathione plays an important role in the protection of cells and tissue structures (23). Its role includes detoxication of xenobiotics, free radicals, peroxides and regulation of immune function. In the present study, a decrease in cellular antioxidant observed in group II animals may be one of the causes for the development of adjuvant arthritis. Low concentrations of glutathione has been implicated in Rheumatoid arthritis (24). An observed increase in tissue GSH content in the pretreated group shows that Type II collagen tends to prevent the tissue depletion of glutathione. The same reason can be attributed to the change in glutathione content in the thymus of group IV animals.

Superoxide dismutase provides the first line of defense against free radical damage. An attempt to alter the SOD activities in arthritic patients appears to have a short lived anti-inflammatory effect (25). In the present study, a decreased activity of SOD was observed in the immune tissues of group II animals. The treatment of AIA with SOD was found to inhibit the level of thiobarbituric acid reactive substances in the sera and synovial fluid of rats (20). In the case of the pretreated group, the SOD activity was significantly increased compared to group II animals. No significant change was seen in the post treated group. Evidence exists that SOD were found to inhibit the primary phase of the adjuvant arthritis but had no effect on the secondary phase of adjuvant arthritis (26). This shows that when Type II collagen is given after the onset of the disease, it has no role on the activity of superoxide dismutase.

Previous reports (27) shows the existence of immune complex in the synovial cavity. These complexes are thought to perpetuate local inflammation possibly through the release of lysosomal enzymes (28). In the present study, the level of CIC in the serum of AIA rats increased after the second week and reached a peak during the fourth week of immunization with CFA suggesting the setting in and activation of the inflammatory process. This trend was suppressed in group III animals suggesting that Type II collagen tends to act as a potent suppressing agent. A mild suppression in the level of CIC was observed in post treated group suggesting an anti-inflammatory effect.

There was no significant change in any of the parameters studied between group I and group V animals. The studies by Thompson (29) reveal that administration of Type II collagen to normal rats did not produce any clinical disease, nor did it induce a detectable serum anti-CII antibody response.

Welles and Battisto (30) have reported that immunization of rats with Type II collagen, prior to the induction of arthritis diminished their arthritogenic response. Zhang *et al* (31) observed a significant suppression of the disease as evident from the delay in the development of the disease. Cremer *et al* (32) failed to observe a significant suppression in the incidence or the severity of adjuvant arthritis after the treatment with Type II collagen.

The results of the present study put together, reveals that during the setting in and activation of the inflammatory process, there is an increase in lipid peroxidation in the tissues as a result of decreased tissue antioxidants and that Type II collagen acts more effectively when given before immunisation with Complete Freund's adjuvant..

Clinical trials by Trentham *et al* (32), Sieper *et al* (34) and Barnett *et al* (35) on rheumatoid arthritis patients using different sources and dosage of Type II collagen have observed a marginal beneficial effect with respect to number of swollen and tender joints. As these studies have shown a trend towards clinical improvement, future studies with this treatment strategy can be helpful in biochemical monitoring of disease activity.

Table 1
Levels of lipid peroxides and antioxidants in spleen

	Group I	Group II	Group III	Group IV	Group V	F ratio df (4,25)
LP(nmole/mg protein)	2.37 ± 0.8	4.5 ± 0.45	3.42 ± 0.38	3.92 ± 0.35	2.63 ± 0.39	18.64 (p<0.001)
ASA (mg/mg tissue)	0.46 ± 0.05	0.29 ± 0.04	0.39 ± 0.01	0.34 ± 0.02	0.45 ± 0.01	26.74 (p<0.001)
SOD (U/mg protein)	0.94 ± 0.17	0.59 ± 0.06	0.82 ± 0.05	0.71 ± 0.03	1.06 ± 0.11	21.35 (p<0.001)
GSH(mg/mg tissue)	2.88 ± 0.29	1.76 ± 0.29	2.28 ± 0.26	2.03 ± 0.12	2.85 0.33	19.73 (p<0.001)

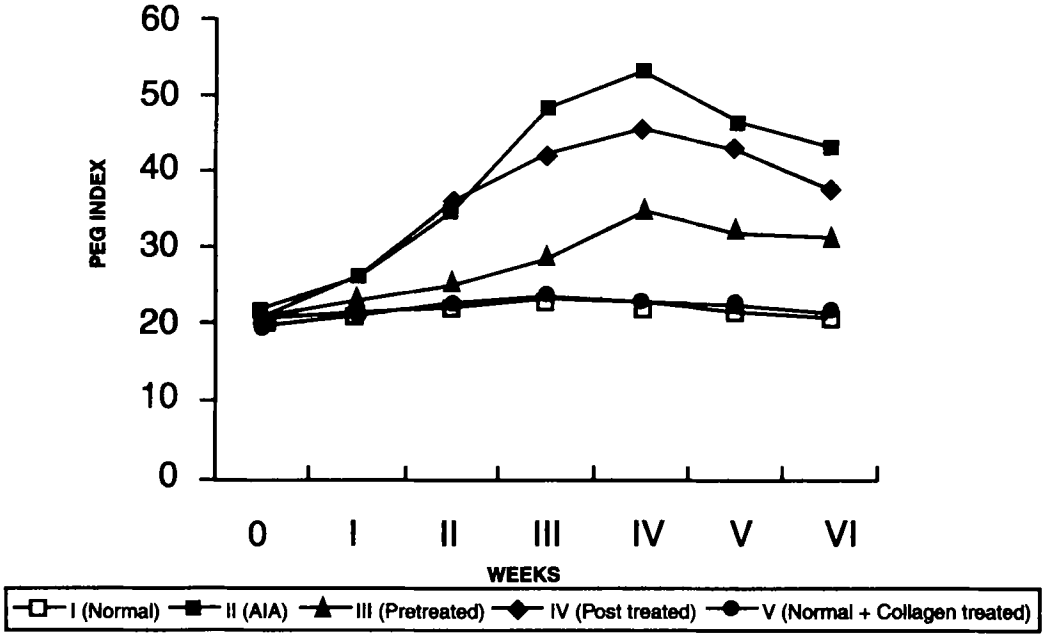
The values represent the Mean ± S.D. for six rats.
LP - lipid peroxides, ASA - ascorbic acid , SOD - superoxide dismutase , GSH - Reduced glutathione.

Table 2
Levels of lipid peroxides and antioxidants in thymus

	Group I	Group II	Group III	Group IV	Group V	F ratio df (4,25)
LP(nmole/mg protein)	3.33 ± 0.68	5.69 ± 0.6	4.68 ± 0.31	5.09 ± 0.44	3.73 ± 0.28	23.69 (p<0.001)
ASA (mg/mg tissue)	0.59 ± 0.12	0.35 ± 0.07	0.49 ± 0.02	0.44 ± 0.04	0.53 ± 0.04	11.42 (p<0.001)
SOD (U/mg protein)	3.41 ± 0.41	2.52 ± 0.35	3.17 ± 0.19	2.94 ± 0.14	3.47 ± 0.31	9.59 (p<0.001)
GSH(mg/mg tissue)	3.98 ± 0.31	2.38 ± 0.28	3.44 ± 0.35	2.87 ± 0.13	3.90 ± 0.23	37.73 (p<0.001)

The values represent the Mean ± S.D. for six rats.
LP - lipid peroxides, ASA - ascorbic acid , SOD - superoxide dismutase , GSH - Reduced glutathione.

Figure 1 Circulating immune complex in serum



REFERENCES

1. Pearson, C.M. (1964) Experimental models in rheumatoid disease. Arth. Rheum. 7, 80-86.
2. Mackenzie, A.R., Pick, C.R., Sibley, P.R. and White, B.P. (1978) Suppression of rat adjuvant by cyclophosphamide pretreatment : evidence for an antibody mediated component in the pathogenesis of the disease. Clin. Exp. Immunol. 32 , 86-89.
3. Southorn, P. and Powis, G. (1988) Free radicals in medicine I - Chemical nature and biologic reactions . Mayo Clin. Proc. 63, 381-391.
4. Greenwald, R.A. and Moy,W.W. (1980) Effect of oxygen derived free radicals on hyaluronic acid. Arth. Rheum. 23, 455-463.
5. Nalini, G., Chandrasekaran, A.N., Hariprasad, C., Gowri, C. and Poonguzhali, K. (1997) The antioxidant status in Rheumatoid arthritis. J. Clin. Biochem. Nutr. 23, 69-75.
6. Prockop, D.J. and Kivirikko, K.L. (1995) Collagens : Molecular Biology, Diseases and Potentials for therapy. Annu. Rev. Biochem. 64, 403 - 434.
7. Timpl, R., Furthmayr, H., Hahn, E., Becker, U. and Stoltz, M. (1973) Immunochemistry of Collagen . Model properties of a natural protein antigen. Behring Inst. Mitt. 53, 6- 70.
8. Weiner, H.L., Friedman, A., Miller, A., Khoury, S.J., Al -Sabbagh, A., Santos, L., *Nussenblatt, R.B., Trentham, D.E. and Hafler, D.A.* (1994) Oral tolerance: immunological mechanism and treatment of animal and human organ specific autoimmune diseases by oral admimistration of autoantigens. Ann. Rev. Immunol. 12 , 809 - 837.
9. Weiner, H.L. and Mayer, L.F. (1996) Oral tolerance : mechanism and applications. *Ann. N. Y. Acad. Sci.* xiii : 778 - 785.
10. Nalini, G., Chandrasekaran, A.N., Hariprasad, C., Joseph, C.T., Gowri, C. and Poonguzhali, K. (1998) Effect of Type II Collagen treatment in Adjuvant induced arthritis. APLAR J. Rheumatology 1, 212 - 221.
11. Brooks, P.M. (1993) Clinical management of rheumatoid arthritis. Lancet 341, 286-290.
12. Silverman, H.A., Johnson, J.S., Vaughan, J.H. and McGlamory, J.C. (1976) Altered lymphocyte reactivity in rheumatoid arthritis. Arthritis Rheum. 19, 509-515.
13. Williams, A.S., Camilleri, J.P. and Williams, B.D. (1994) Suppression of adjuvant induced by liposomally conjugated methotrexate in the rat. *Br. J. Rheum.* 33, 530 -533.
14. Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid raection. Anal. Biochem. 95, 351-358.
15. Omaye, S. T., Turnbull, J.D. and Sauberlich, H.E. (1979) Selected methods for the determination of Ascorbic acid in animal cell, tissues and fluids. Meth. Enzymol . 62 , 1-11.
16. Marklund, S. and Marklund, G. (1974) Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469- 474.
17. Moron, M.S., Despierre, J.W. and Mannervik, B. (1979) Levels of glutathione, glutathione reductase and glutathione - s- transferase activities in rat lung and liver. Biochim. Biophys. Acta 582 , 67-78.
18. Lowry, O. H., Rosebrough, N. J., Farr, A.L. and Randall, R. I. (1951) Protein determination using Folin -Ciocalteau reagent. J. Biol. Chem. 193 , 438 - 448 .
19. Seth, P. and Srinivas, R.V. (1981) Circulating immune complex in cervical cancer : simple method for detection and characterisation . Ind. J. Med. Res. 73, 926 -929.
20. Yoshikawa, T., Tanaka, H. and Kondo, M. (1985) The increase of lipid peroxidation in rat adjuvant arthritis and its inhibtion by superoxide dismutase. Biochem. Med. 33, 320-326.
21. Kagan, V.E., Serbinov, E,A., Forte, T., Scita, G. and Packer, L. (1992) Recyling of vitamin E in human low density lipoproteins. J. Lipid Res. 33, 385-397.
22. Eldin, A.A., Hamdy, M.A., Shaheen, A.A.,

Motawi, T.K. and Abd-el-Gawad, H.M. (1992) Effect of Vitamin C administration in modulating some biochemical changes in arthritic rats. Pharmcol. Res. 26, 357-366.

23. Meister, A. (1983) Selective modifications of glutathione metabolism: Science 220, 472-477.

24. Uhlig, S. and Wendel, A. (1992) The consequences of glutathione variations. Life Sci. 51 , 1083-1094.

25. Martelli, E.A. and Orgotein (1980) A review. Med. Actual. 16, 97-101.

26. Hirschelmann and Bekemeier, H. (1981) Effects of catalase, peroxidase, superoxide dismutase and 10 scavengers of radicals in carregeenin edema and adjuvant arthritis of rats. Experientia 37, 1313-1314.

28. Zvaifler, N. J. (1965) A speculation on the pathogenesis of joint inflammation in rheumatoid arthritis. Arthr. Rheum. 8 , 289-292.

29. Thompson, H. S.G. and Staines, N. A. (1986) Gastric administration of Type II collagen delays the onset and severity of collagen induced arthritis in rats. Clin. Exp. Immunol. 64, 581- 586.

30. Welles, W.L. and Battisto, J.R. (1981) Suppression of adjuvant arthritis by antibodies specific for collagen Type II Collagen. Immunol. Commun. 10 , 673 - 685.

31. Zhang, Z.J., Christopher, S.Y., Lee, Lider, O. and Weiner, H.L. (1990) Suppression of adjuvant arthritis in Lewis rats by oral administration of Type II Collagen. J. Immunol. 145, 2489 - 2493.

32. Cremer, M,A., Hernandez, A,D., Townes, A.S., Stuart, J.M. and Kang, A.H. (1983) Collagen induced arthritis in rats : antigen specific suppression of arthritis and immunity by intrvenously injected native Type II Collagen. J. Immunol .131, 2995 - 3000.

33. Trentham, D.E., Dynesius-Trentham, R.A., Orav, E.J., Combitchi, D., Lorenzo, C. and Sewell, K.L. (1993) Effects of oral administration of Type II Collagen on rheumatoid arthritis. Science 261, 1727 - 1730.

34. Sieper, J., Kary, S., Sorensen, H., Alten, R., Eggens, U. and Huge, W. (1996) Oral Type II Collagen treatment in early rheumatoid arthritis.: a double-blind , placebo-controlled, randomized trial. Arthritis Rheum. 39 , 41 - 51.

35. Barnett, M.L., Kremer, J,M., St.Clair, E.W., Clegg, D.O., Furst, D. and Weisman, M. (1997) Treatment of rheumatoid arthritis with oral Type II Collagen : results of a multicenter, double - blind, placebo controlled trial. Arthritis Rheum. 40, 290-297.