

Effect of diclofenac alone or in combination with alpha-tocopherol on the oxidative activity of polymorphonuclear leukocytes in healthy and osteoarthritic individuals

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ABSTRACT

Objectives: To investigate the effects of diclofenac alone or when combined with alpha-tocopherol on the oxidative activity of polymorphonuclear leukocytes (PMNs) in healthy and osteoarthritic (OA) patients.

Methods: The study was carried out at the College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia, over the period 1999 to 2000. Twelve healthy controls and 12 osteoarthritic patients were recruited to the study. Twelve healthy controls and osteoarthritic patients were given diclofenac 50 mg thrice daily orally, initially for 5 days then alpha-tocopherol at 200 mg thrice daily orally, was added for another 5 days. Blood samples were drawn before the start of the study (pre-treatment) and at 5 days following treatment with diclofenac alone and 10 days following treatment with diclofenac and alpha-tocopherol. Chemiluminescence (CL) response was measured for whole blood and isolated polymorphonuclear leukocytes (PMNs) on all samples.

Results: Diclofenac enhanced CL response of whole blood and of PMNs of healthy controls when stimulated with phorbol myristate acetate (PMA) and opsonized zymosan (OPZ). Co-treatment with alpha-tocopherol resulted in no appreciable change in the CL response of whole blood when stimulated with PMA or OPZ but a further significant enhancement of CL response of isolated PMNs when these cells were stimulated by either PMA or OPZ. In osteoarthritic patients, diclofenac alone and when combined with alpha-tocopherol showed no significant change in CL response of whole blood. The CL response of PMNs from OA patients was decreased by diclofenac alone. However, this inhibitory effect was not observed when alpha-tocopherol was used together with diclofenac.

Conclusion: The effect of diclofenac alone or in combination with alpha-tocopherol did not produce a consistent effect on the CL response of whole blood or isolated PMNs of healthy or osteoarthritic patients.

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Polymorphonuclear leukocytes (PMNs) phagocytic activity is of major significance in the defense against noxious stimuli.¹ Their production of oxygen free radicals is important in

destroying the offending antigen. However, these free radicals are believed to be of significance in promoting and perpetuating the inflammatory process.² Such inflammation is of relevance in

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arthritis and its symptoms. The effect of non-steroidal anti-inflammatory drugs (NSAIDs) on the function of PMN is protean, including interference with adhesion, aggregation and the oxidative process.³⁻⁵ Variable results of the effect of NSAIDs on the oxidative process of PMN was reported.⁶⁻⁸ In vitro studies on isolated PMN showed diclofenac to inhibit such oxidative processes, while its effect on whole blood was mixed but mostly enhancing.⁶⁻⁸ Alpha-tocopherol is known to have oxygen free radical scavenging properties.⁹ It is the principal lipid-soluble chain-breaking antioxidant in tissues and plasma.^{10,11} It had been shown that 3 months treatment with oral alpha-tocopherol acetate lead to a definite improvement in red cell survival, hemoglobin concentration and reticulocyte count in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency.¹² Clinical trials of vitamin E use in patients with established coronary artery disease showed that the vitamin slowed the progression of coronary disease when demonstrated by quantitative angiography as compared to controls after 2 years of supplementation.¹³ Two randomized controlled clinical trials have shown that alpha-tocopherol supplements either reduced progression of carotid lesions or caused regression of the free-radical-induced lesions.^{14,15} Diclofenac, a NSAID, is commonly used in the treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis.¹⁶⁻¹⁹ It reduces inflammation, swelling and arthritic pain by inhibiting the production of prostaglandins.^{20,21} The drug also affects PMNs function in vitro, thereby reducing chemotaxis, superoxide toxic radical generation and neutral protease production.²² Therefore, in this study, we investigated the effect of diclofenac alone or in combination with alpha-tocopherol on the oxidative process of whole blood and isolated PMNs of healthy and osteoarthritic patients.

Methods. The study was carried out at the College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA), over the period 1999 - 2000. Twelve healthy volunteers and 12 osteoarthritic patients were recruited to the study. Twelve healthy volunteers were given diclofenac 50 mg thrice daily for 5 days orally and then alpha-tocopherol 200 mg 3 times daily by the oral route was added for another 5 days. Blood was extracted before the drugs were given (pre-treatment), on the fifth day while on diclofenac, and on the tenth day while on both drugs. The 12 osteoarthritic patients were recruited from the rheumatology outpatient clinics. A 7-day period in which other non-steroidal drugs were stopped and patients were given paracetamol for pain control preceded the start of the study. The osteoarthritic

patients were given diclofenac and alpha-tocopherol in a manner similar to the above protocol. Both healthy volunteers and osteoarthritic patients signed an informed consent before they were enrolled in the study. This was carried out to conform to the guidelines of the recommendations of the ethical committee at the College of Medicine Research Center (CMRC), King Saud University, Riyadh, KSA.

Luminol, phorbol myristate acetate (PMA), phosphate buffered saline (PBS) and zymosan were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dimethyl-sulfoxide (DMSO) was obtained from Fluka-AG, Chemiche (Fabrik, Buchs, Switzerland). Diclofenac were generously donated by Ciba-Geigy Pharmaceutical Company (Basel, Switzerland) while alpha-tocopherol was donated by Hoffman La-Roche Pharmaceutical Company (Basel, Switzerland). We dissolved PMA in DMSO to give a stock solution of 2 mg/ml. This stock solution was stored at -20°C until used. The stock solution was further diluted to 20 µg/ml with phosphate-buffered saline (PBS) prior to use. Luminol (LKB-Wallac 1243-216) was dissolved in DMSO to give a concentration of 1.77 mg/ml. This stock solution was further diluted in PBS to 17.7 µg/ml prior to use. Particulate zymosan was washed twice and resuspended in PBS and the concentration was adjusted to 12.5 mg/ml. Opsonization was performed by incubating 100 µl of autologous human serum from healthy individuals and 900 µl of zymosan suspension at 37°C for 30 minute, washed with PBS and re-suspended in PBS to give a final concentration of 1.25 mg/ml.

Separation of polymorphonuclear neutrophilic leukocytes. Polymorphonuclear leukocytes were separated by using Nycodenz (Nyegaard and Co., Torshov, Norway). Fresh blood was collected by venepuncture from apparently healthy subjects and osteoarthritic patients in sterile containers with heparin (10 IU/ml, Fischer Scientific Co., NJ). We incubated 10 ml of heparinized blood mixed with one ml of 6% (weight in volume [w/v] of NaCl in water) in a 15 ml tube for 30 minute at room temperature. Then the leukocyte-rich plasma was layered over 3 ml Nycodenz solution in a 12 ml tube and was centrifuged at 1800 rev/minute for 15 minute in a Heraeus centrifuge (Model GmbH, Osterode). We added 10 ml of PBS (Electronucleonics Inc., Columbia, MD) to the bottom PMN-rich portion and centrifuged at 475 x g for 10 minute. The supernatant was discarded and the sediment was suspended with 5 ml of NH₄Cl (0.9%) and left to stand in ice for 10 minute to lyse the erythrocytes. The cells were then centrifuged as above and re-suspended in 1 ml of PBS medium.

The PMNs were then counted and viability was determined by the trypan blue exclusion test.

Chemiluminescence measurement. This was carried out according to previously reported methods.²³⁻²⁶ The effects of diclofenac alone or in combination with alpha-tocopherol on the luminol-dependent CL were determined by adding various concentrations of these drugs at 37°C to whole blood and isolated PMNs. The cells were then stimulated by PMA or OPZ and measurement of CL response begun immediately (incubation time was zero).

Effect of drugs on the viability of polymorphonuclear leukocytes. The effect of diclofenac and alpha-tocopherol on PMN's viability was tested at 60 minute following incubation at 37°C. The percentage of viable cells was estimated by the trypan blue (0.2% w/v) exclusion test. The viability of the cells was not significantly affected by diclofenac or alpha-tocopherol.

Controls. (i) Diclofenac and alpha-tocopherol in the concentrations used, did not induce a CL response of unstimulated PMNs in the presence of luminol. The background CL activity of unstimulated PMNs was always less than 1.0 millivolt. (ii) The inhibitory effect of diclofenac and alpha-tocopherol on the CL response of isolated human PMNs was readily reversible when PMNs were washed with PBS.

Statistical analysis. The results of CL were computed when appropriate by an LKB computer program which gives the maximum peak response. Results were expressed as the mean \pm SEM and were analyzed for significance between the different groups using the 2-tailed student's t-test. Results were considered significant when $p < 0.05$.

Results. Diclofenac given as a treatment to healthy volunteers in a dose of 50 mg thrice daily for 5 days orally resulted in enhancement of inflammation as measured by the CL response of whole blood and isolated PMNs compared to the pre-treatment results (Table 1). The enhancement was significant in case of whole blood but was not significant when isolated PMNs were used. Co-treatment with alpha-tocopherol resulted in reduction of the CL response (which was not statistically significant) in the whole blood samples but a further enhancement in the isolated PMNs samples. This enhancement was significant compared to the pre-treatment value ($p < 0.05$). Table 2 shows the results of treatment with diclofenac alone or in combination with alpha-tocopherol on whole blood and PMN of healthy volunteers stimulated with OPZ showing a similar trend to that seen when whole blood and PMN were stimulated with PMA (Table 1), except

that the enhancement of the CL response in whole blood was not statistically significant for diclofenac alone. Whole blood from osteoarthritic patients stimulated with PMA showed no significant difference between pre-treatment samples and after treatment with Diclofenac alone or when diclofenac was added together with alpha-tocopherol, while CL response of isolated PMN for same patients showed a significant decrease when treated with diclofenac alone. This response became insignificant when alpha-tocopherol was added together with diclofenac (Table 3). Stimulation with OPZ instead of PMA gave no significant differences between the groups (Table 4).

Discussion. The present results show that, in healthy volunteers, treatment with diclofenac alone, enhanced the CL response of whole blood and of isolated PMNs while the combination of diclofenac with alpha-tocopherol treatment of healthy individuals lead to a small decrease in the CL response of whole blood but a further increase in CL response of the isolated PMNs. In osteoarthritis patients, the combination of diclofenac and alpha-tocopherol lead to insignificant enhancement of the CL response of whole blood and PMNs. This means that unlike results of in-vitro studies, addition of diclofenac to isolated PMNs extracted from treated patients and analyzing their CL response ex-vivo showed no such inhibition by diclofenac. On the contrary, the CL response was enhanced. Pre-treating with alpha-tocopherol in addition to diclofenac lead to a small decrease in CL response of whole blood of healthy individuals. There is evidence that activated PMNs generate reactive products such as superoxide and liberate lysosomal enzymes which contribute to tissue damage.²⁷⁻³¹ It had been shown that diclofenac produces an inhibitory effect on the CL response of isolated human PMNs in-vitro.³² In the present study, diclofenac produced inhibition of the CL of isolated human PMNs of healthy volunteers or osteoarthritic patients except in case of whole blood experiments. This discrepancy in the effects of diclofenac may be explained by the fact that blood contains proteins and enzymes that may interfere with the antioxidant activity of diclofenac. The inhibitory effect is in agreement with previously reported data that showed a reduction of superoxide radical generation and neutral protease production by diclofenac in-vitro.³³ Alpha-tocopherol (vitamin E) is a naturally-occurring antioxidant in biological systems, having an apparent specificity as a lipid antioxidant. Alpha-tocopherol had been reported to protect animal tissues against oxidative damage both in-vitro and in-vivo.³⁴ Surprisingly, in the

Treatment	Chemiluminescence response (mV)			
	Whole blood	p value	PMNs	p value
Pre-treatment	10.66 ± 2.67	-	538.18 ± 18.19	-
Diclofenac	15.87 ± 3.31	0.013*	715.44 ± 228.14	0.087
Diclofenac + alpha-tocopherol	8.94 ± 2.17	0.24	822.42 ± 191.32	0.0047*

mV - millivolt, PMNs - polymorphonuclear leukocytes
 All values are expressed as mean ± SEM of 6 experiments.
p<0.05 taken as significant (in comparison with the pre-treatment group).
 *Significant at *p*<0.05

Table 1 - Effect of diclofenac alone or in combination with alpha-tocopherol on the chemiluminescence response of whole blood and polymorphonuclear leukocytes isolated from healthy volunteers stimulated with phorbol myristate acetate.

Treatment	Chemiluminescence response (mV)			
	Whole blood	p value	PMNs	p value
Pre-treatment	21.68 ± 5.74	-	1239.95 ± 91.72	-
Diclofenac	26.25 ± 6.98	0.2437	1836.00 ± 273.00	0.0005*
Diclofenac + alpha -tocopherol	15.84 ± 4.07	0.069	2456.67 ± 282.93	<0.0001*

mV - millivolt, PMN - polymorphonuclear leukocytes
 All values are expressed as mean ± SEM of 6 experiments. *p* value <0.05 taken as significant.
 *Significant at *p*<0.05 as compared to the pre-treatment group.

Table 2 - Effect of Diclofenac alone or in combination with alpha-tocopherol on the chemiluminescence response of whole blood and polymorphonuclear leukocytes isolated from healthy volunteers stimulated with opsonized zymosan.

Treatment	Chemiluminescence response (mV)			
	Whole blood	p value	PMNs	p value
Pre-treatment	12.14 ± 2.64	-	1125.70 ± 123.62	-
Diclofenac	12.99 ± 2.98	0.6124	893.67 ± 99.17	0.0049*
Diclofenac + alpha-tocopherol	13.60 ± 3.33	0.419	986.49 ± 140.30	0.09

mV - millivolt, PMN - polymorphonuclear leukocytes.
 All values are expressed as mean ± SEM of 6 experiments.
 Diclofenac dose 50 mg given 3 times daily. *Significant at *p*<0.05
 (in comparison to pre-treatment group)

Table 3 - Effect of Diclofenac alone or in combination with alpha-tocopherol on the chemiluminescence response of whole blood and polymorphonuclear leukocytes isolated from osteoarthritic patients stimulated with phorbol myristate acetate.

Treatment	Chemiluminescence response (mV)			
	Whole blood	p value	PMNs	p value
Pre-treatment	24.21 ± 4.15	-	2497.67 ± 123.62	-
Diclofenac	26.75 ± 3.73	0.2909	2256.64 ± 297.08	0.096
Diclofenac + alpha-tocopherol	27.04 ± 4.23	0.26	2714.68 ± 323.33	0.155

mV - millivolt, PMN - polymorphonuclear leukocytes. All values are expressed as mean ± SEM of 6 experiments. Diclofenac dose 50 mg 3 times daily, *p* value in comparison to the pre-treatment group.

Table 4 - Effect of Diclofenac alone or in combination with alpha-tocopherol on the chemiluminescence response of whole blood and polymorphonuclear leukocytes isolated from osteoarthritic patients stimulated with opsonized zymosan.

present study, alpha-tocopherol when given together with diclofenac failed to potentiate the inhibitory effects of the latter on the generated oxygen-derived free radicals liberated when whole blood were stimulated by either PMA or OPZ. On the other hand, when isolated PMNs were used in the assay medium, alpha-tocopherol potentiated the inhibitory actions of diclofenac when these PMNs were drawn from healthy volunteers or osteoarthritic patients. These findings corroborate previously published data where alpha-tocopherol potentiated the effects of ascorbic acid, a known oxygen-derivative free-radical scavenger.³⁵⁻³⁹

In conclusion, alpha-tocopherol failed to potentiate the inhibitory actions of diclofenac on the CL response of whole blood. However, it synergized with diclofenac when isolated PMNs were used.

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