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

*To the Editor*

I read the above paper by Al-Arfaj et al with great interest, and I would like to make a few comments concerning the paper.[1] The study examined the effect of diclofenac and alpha-tocopherol on polymorphonuclear leukocytes (PMNs) function and generated interesting results. However, I believe the authors could have elaborated more on the objectives of the study and expanded the discussion section to interpret their results in relation to the previously published data.

The authors treated patients and controls with diclofenac alone or in combination with alpha-tocopherol. The same agents were then added to isolated PMN, or to whole blood, prior to stimulation with zymosan, or phorbol myristate acetate (PMA), and measurements of chemiluminescence (CL). The rationale behind giving these agents to patients and controls for long periods was not clearly stated.

Polymorphonuclear leukocytes have a rather short half life in vivo and consequently, the circulatory pool is continuously replaced with new cells from the bone marrow.[2] Therefore, treating patients and controls with diclofenac and alpha-tocopherol for a day or longer would, in my opinion, make little difference to PMN function. This is also supported by the author's statement that washing PMN with phosphate buffered saline, prior to stimulation, reverses the effect of diclofenac on these cells.

A major draw back with this study is due to the use of CL as a measure of PMN oxidative activity. The use of CL alone has always made interpretation of results very difficult. The difficulty arises as the generated CL is due to the product, not only of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, but also of the myeloperoxidase (MPO) enzyme, released from the primary granules.[3,4] Consequently, enhanced CL response can occur as a result of increased oxidant production ($O_2^-$), increased primary granule degranulation or, of both. We have previously shown that some agents can enhance CL response by the selective enhancement of PMN degranulation of MPO, without having any effect on the activity of the NADPH oxidase.[5] Therefore, decrease or increase in CL response can not be attributed solely to increase or decrease in the oxidative activity of PMN. For these reasons, it would have been desirable to have measured PMN oxidative activity more directly ($O_2^-$ and $H_2O_2$) in the above study. Regarding the design of the experiments, I wonder whether one can compare the results obtained in days, 0 (pre-treatment) 5 and 10 and attribute any changes in PMN CL response to diclofenac and alpha-tocopherol. In my opinion, the patient and the control groups could have been divided each into 3 sub-groups; one acting as a control (pre-test), a second sub-group receiving diclofenac and a third sub-group receiving diclofenac and alpha-tocopherol. Such a design would have made direct comparison of results easier. However, an in vitro study with purified PMN from patients and control individuals could have generated enough data to conclude the full effects of these 2 agents on PMN oxidative activity. With regards to the results section, it appears that only 6 out of the 12 subjects were eventually used (n=6 experiments). Looking at the actual data, apparently the inter-experimental variability in PMN CL response (as evident by the rather large SEM) was quite wide, and with the small number of experiments, the results produced may not be that significant, particularly these generated in response to PMA stimulation. Indeed, one would not expect diclofenac and alpha-tocopherol to have any significant effect on PMA induced PMN CL response.

By passing the cell membrane receptors, PMA binds irreversibly to the protein kinase C (PKC) enzyme and induce maximum activation. Consequently, PMN response can not be further enhanced by any other agents.[6,7] Moreover, as PMN CL response induced by stimulation with PMA is generated entirely intracellularly, one would not expect alpha-tocopherol to have any effect on such results.[3]

In contrast to PMA, diclofenac appears to have increased the zymosan induced PMN CL response, although the SEM is still very large. However, like PMA, most of the CL produced by PMN in response to zymosan is intracellular;[3,4] alpha-tocopherol would not be expected to have any major effect on the CL response. Therefore, whether the additional increase in the zymosan induced PMN CL response was due to alpha-tocopherol, is difficult to say with the large SEM and the very few experiments that were carried out.

In my opinion, it is generally very difficult to interpret the results of the above study and draw any firm conclusions due to the large inter-experimental variability (as evident by the very large SEM and the SD), and the few experiments conducted. In most of the results, it would appear that the higher values of the control (pre-test) and lower values of the test would seem to overlap, making the significance and the interpretation of the results difficult.

The variability in PMN response has always been a problem and is due to many factors including the methods used to separate the cells.[8] Priming of PMN occurs during cell separation, and the extent of priming is dependent on the type of method used. We have previously developed a method for PMN separation that results in minimal cell priming and consequently, low inter-experimental variability in PMN responses.[5]

Regarding the discussion, diclofenac appeared to enhance the zymosan induced CL response in isolated PMN from the control, but not from the osteoarthritic individuals. However, these interesting results were not discussed by the authors. It is possible that in the control group, diclofenac enhanced PMN response by inhibiting prostaglandin production through the inhibition of the cyclooxygenase enzyme or by enhancing leukotrienes production or both as a result of redirecting arachidonic acid to the lipoxygenase pathway. Enhancement and inhibition of PMN function by prostaglandins and leukotrienes, has been well documented.[9,10] In contrast to the control group, PMN from the patient-group appeared to be already primed in vivo and diclofenac had no further enhancing effect on PMN CL response.

Osteoarthritis is generally regarded as a degenerative disease. However, low inflammatory reactions occur with the production of both cytokines and growth factors.[11] Therefore, one would expect PMN from these patients to be all already primed in vivo by these cytokines and growth factors and therefore, further priming in vitro would not be expected to have any further effect on PMN CL response.

Finally, the enhancing effect of diclofenac on PMN was not reconciled with previous studies where diclofenac was reported to inhibit PMN oxidative activity. It is possible that these apparently contradictory effects of diclofenac could be due to the use of different agonists and methods to stimulate and assess PMN function. For example, diclofenac would be expected to inhibit PMN oxidative function if weak agonists, such as N-formyl-methionyl-leucyl-phenylalanine (FMLP), were used to stimulate PMN. Similarly, some of the oxidant produced in response to FMLP appears extracellular, inclusion of alpha-tocopherol and diclofenac would lead to scavenging of these extracellular oxidants leading to reduction of PMN oxidative activity.

I believe the study by Al-Arfaj et al produced very interesting results, and indeed any work that generates a discussion is regarded as interesting. The work could be extended to examine the full effects of diclofenac and alpha-tocopherol on the various PMN functions, using more physiological stimuli, beside zymosan and gently prepared cells.

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## *Reply from the Author*

We read with great interest the comments made by Dr. Khalil Aziz in reference to our above mentioned paper and in response we forward the following points:

1. The control that had been described in the method's section in which diclofenac alone or combined with alpha-tocopherol was meant to test in vitro whether these drugs would have a "quenching effect" per se. Results of these control experiments were not discussed in the results section. This has to be differentiated from the in vivo experiments where samples of blood were withdrawn from normal individuals or osteoarthritic patients who had received treatments with diclofenac alone or with alpha-tocopherol.

2. The rationale for giving diclofenac or diclofenac and alpha-tocopherol for 8 days is simple. Diclofenac is usually given for a period of at least one week to osteoarthritic patients in order to see a favorable response. Therefore, it is only logical to design the experiments in such a way to faithfully mimic the real clinical situation.

3. The aim of the study was to investigate whether the addition of alpha-tocopherol to the anti-inflammatory drug, diclofenac, would potentiate the effects of the latter on the release of oxygen-derived free radicals from PMNs in vivo. We were not addressing the effects of alpha-tocopherol or diclofenac when given singly or in combination on the liberation of any one specific species of the free radicals.

4. The rationale of our study was not directed to a comparison between the effects of alpha-tocopherol and diclofenac on the response of PMNs at various intervals, following stimulation by soluble or particulate stimuli, but to investigate whether alpha-tocopherol has any potentiating effects on the PMNs response to diclofenac. This would eventually lead to doses of diclofenac being reduced. Subsequently, the side effects of diclofenac on the patient would be minimized.

5. The numbers shown in the legends to tables denote the number of experiments performed but

not the number of healthy volunteers or patients with osteoarthritis who were recruited to the study. The number of controls or patients was 12 in each group. It is noteworthy that in in vivo studies, variation in responses among individuals is quite likely.

6. Dr. Aziz made an excellent observation regarding the enhancement of the CL response which was induced by zymosan in the control experiments. The explanation afforded by Dr. Aziz in terms of the diversion of the prostaglandin pathway to the lipoxygenase arm with the subsequent release of leukotrienes is quite plausible. It is well accepted and fits nicely with what was observed.

7. Additionally, we do agree with Dr. Aziz that diclofenac might have primed the PMNs following its sub-chronic administration in vivo.

8. Finally, we found the comments made by Dr. Aziz to our present publication quite useful. We do agree that our work could be extended to further examine the full effects of diclofenac and alpha-tocopherol on the various functions of PMNs.

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