In vitro effect of different non-steroidal anti-inflammatory drugs on human polymorphonuclear leukocyte activity measured by luminol-dependent chemiluminescence of the whole blood

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ABSTRACT

Objectives: To define the well-known variability in the effects of non-steroidal anti-inflammatory drugs and to search for predictors of such variability using an in vitro model.

Methods: Polymorphonuclear leukocyte activity was measured by luminol-dependent chemiluminescence of the whole blood using barium sulphate as a stimulator. Blood was taken from 40 apparently healthy volunteers (22 males and 18 females; their age ranged from 20-50 years). Drugs (indomethacin 10ug/ml, aspirin 300ug/ml, ibuprofen 25ug/ml or diclofenac 8ug/ml) were added into the blood of each individual in vitro. The chemiluminescence was measured in a photon counting system.

Results: There was a marked inter and intra individual variation in the chemiluminescence response to the 4 non-steroidal anti-inflammatory drugs, added in vitro. The variation exhibited a continuous pattern. No statistically significant correlation was found between the in vitro effect of one non-steroidal anti-inflammatory drug and the other 3 drugs, nor between the effect of each drug and factors like age, sex, weight, height, packed cell volume, hemoglobin percentage and white blood cell count. Subjects with hemoglobin-AS type (number = 9) responded mainly by enhancement to indomethacin and diclofenac. When the number of subjects rather than the average net effect was compared according to blood groups, those with blood group A showed chemiluminescence responses towards enhancement with indomethacin and diclofenac and blood group O with aspirin. A consistent pattern of enhancement and inhibition was evident; enhancements and inhibitions by any 2 drugs involve a seemingly constant proportion of subjects.

Conclusion: Luminol-dependent chemiluminescence responses of polymorphonuclear leukocyte activity could be a good in vitro model to study the variability in response to non-steroidal anti-inflammatory drugs. Characteristics of each individual are not able to predict the pattern of variability. Abnormal hemoglobin and the type of blood group seem to be an interesting area for research.

Keywords: Non-steroidal anti-inflammatory drugs, indomethacin, aspirin, ibuprofen, diclofenac, chemiluminescence, polymorphonuclear leukocyte activity.

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It is well known that the response to non-steroidal anti-inflammatory drugs (NSAIDs) shows considerable inter individual variability.[1] About 40% of patients who do not respond to one NSAIDs may well respond to another.[2] Therefore, it is often necessary to try several drugs before finding one to suit a particular patient. Marked variability has also been observed within the same individual.[3] Wide variation exists not only in the response to NSAIDs but also in individual susceptibility to their adverse effect.[4] An explanation for this variability could be related to differences between agents with respect to their pharmacodynamic actions or pharmacokinetic parameters, or a combination of both.[5] Vesell and Penno[6] illustrated the multiplicity of either well established or suspected host factors that may contribute to variation of drug response in the individual. Rational therapy requires that inter individual variation caused by these factors be considered in arriving at the right choice for each patient (individualization of therapy).[6] Studying this variability, and factors contributing to it may, therefore, help in predicting patients who respond better, or those who develop adverse effects more than others. Possible mechanisms for NSAIDs variation in response include: distinct isoforms of cyclooxygenase enzyme (COX-1 and COX-2),[7,8] NSAIDs enantiomers and stereoisomerism,[9,10] variation in pharmacokinetic parameters,[11] and variations in the mechanism of action.[12] Non-steroidal anti-inflammatory drugs are found to affect various functions of leucocytes e.g. chemotactic activity, adhesivity, as well as, phagocytic (oxidative) activity. The results of previous studies on oxidative activity are variable. For example, a review of literature on the effect of indomethacin on polymorphonuclear leukocyte (PMN) oxidative activity revealed that 10 studies showed inhibition,[12-21] 3 showed enhancement[12,15,16] and 4 showed no effect.[13,15,16,22] Most of the above-cited studies used blood from a small number of subjects. The present study is intended to further characterize the phenomenon of variability in the effect of 4 NSAIDs and to search for potential predictors of such phenomenon. This is to be carried out by using an in vitro model of PMN phagocytic activity measured by luminol-dependent chemiluminescence (CL) of the whole blood taken from a good number of apparently healthy volunteers.

Methods. ***Preparation of blood samples.*** Forty volunteers (22 males, 18 females) apparently healthy by history and clinical examination with ages ranging between 20 and 50 years, taking no drugs for the last one week and no NSAIDs for the last 2 weeks, were recruited for the study. Four millilitres of venous blood from each volunteer was mixed with sodium citrate as an anticoagulant and used for various analyses. All tests were performed within 3-4 hours after withdrawal of blood. Luminol (Sigma Chemical Co.) in a concentration of 1.13 millimole was used and prepared by dissolving 0.02g of luminol in 2 ml of 0.2M NaOH, diluted to 100 ml with deionised water, and stored at 4°C till used.

CL medium and inducer. A medium of the following composition was used: in millimoles, 165 NaCl, 15 Tris-HCl, 2.25 CaC12, and 25 BaSO4. Barium sulphate is in suspension form.

Drugs. Indomethacin (Sigma Chemicals Co.), Aspirin (Medex, UK), Ibuprofen (a gift from SDI, Iraq), and Diclofenac (Al-Hikma Drug Company, Jordan). Drug powders were dissolved in absolute ethanol as a stock solution. A known amount from this stock solution was diluted with phosphate buffer saline (PBS). When 20 ul from the latter diluted solution was added to 0.2 ml of blood, the final concentrations of the 4 drugs in the blood samples were: 10 ug/ml for indomethacin, 300 ug/ml for aspirin, 25 ug/ml for ibuprofen and 8 ug/ml for diclofenac. The drug solutions were added into the blood samples in covered test tubes and kept at 37°C in a water bath for half an hour before CL was measured.

CL measurement. The reaction mixture was prepared according to the method of Vladimirov et al[23] and consisted of: 1 ml of CL inducer, 100ul of luminol solution, and 125 ml of 0.2M NaOH. To this mixture, 0.01 ml of the whole blood was added and gently agitated to mix well in the measuring cuvette of the photon counting system. The cuvette was, then, placed immediately in an LKB (Wallac) luminometer and the peak CL appeared after 2 to 3 minutes and was recorded on a chart recorder. Control samples contained the drug solvents only (ethanol and PBS) in a similar dilution manner to the drugs.

Other investigations. Hemoglobin (Hb) percentage, Hb type (by cellulose acetate electrophoresis), packed cell volume (PCV), blood groups and leukocyte count, were made according to standard methods.[24] Statistical analyses were made using, when appropriate, analysis of variance to test the differences between means among multiple groups and, Chi square and analysis of proportions for categorical data. Results were considered significant when P value was less than 0.05.

Results. ***Variation in response.*** There is considerable inter and intra individual variation in the in vitro response to the 4 NSAIDs (Figure 1 and Table 1). The pattern of in vitro response varied markedly for each subject. One NSAID may cause enhancement, while the other caused inhibition (Figure 1). The proportion of subjects showing inhibition or enhancement of CL activity by addition of each of these drugs seems to be relatively constant. This amounts to one third inhibition and two thirds enhancement for each drug (Table 2).

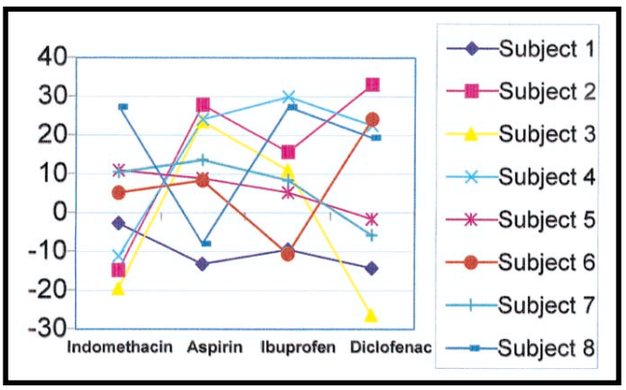

Figure 1 - Chemiluminescence response of polymorphonuclear leukocyte activity (presented as percent difference from control) of 8 subjects showing marked inter and intra individual variation to non-steroidal anti-inflammatory drugs.

Among the 27 subjects showing enhancement by indomethacin, those with enhancement induced by aspirin, diclofenac or ibuprofen were similar in number but they are not the same subjects (17 subjects for aspirin and diclofenac and 16 subjects for ibuprofen). There was no significant correlation between the in vitro effect of indomethacin and the other 3 drugs. When the 4 NSAIDs were compared according to their ability to cause significant enhancement or inhibition (arbitrarily taken to be more than 10%), diclofenac was found to cause >10% enhancement in a greater number of subjects.

Relationship with various subject characteristics. When the effect of NSAIDs on PMN activity was studied according to various parameters, no significant correlation was found with age, sex, weight, height, Hb%, PCV, or WBC count. Similarly, no significant correlation was detected between these parameters and the enhancement or inhibition caused by each drug.

Table 2 - In vitro effect of indomethacin, aspirin, ibuprofen and diclofenac on human polymorphonuclear leukocyte activity measured by luminol-dependent chemiluminescence.

Drugs (conc. used) no. = 40	% change with respect to control			
	Inhibition		Enhancement	
	No.	Mean ± SEM	No.	Mean ± SEM
Indomethacin (10ug/ml)	13	-9.4 ± 2.5	27	9.6 ± 1.7
Aspirin (300 ug/ml)	13	-7.0 ± 1.5	27	9.6 ± 1.4
Ibuprofen (25 ug/ml)	13	-9.4 ± 1.9	27	11.3 ± 1.7
Diclofenac (8 ug/ml)	12	-10.7 ± 3.1	28	13.4 ± 2.5

conc. - concentration; no. = number; SEM - standard error of the mean

Table 1 - Frequency distribution of in vitro effect of indomethacin (10 ug/ml) on polymorphonuclear leukocyte activity from 40 volunteers.

Effect of indomethacin: % difference from control	Frequency (number of subjects)
-25%	1
-20%	0
-15%	2
-10%	4
-5%	0
0%	6
+5%	8
+10%	10
+15%	3
+20%	2
+25%	2
+30%	2

(-) mean inhibition and (+) mean enhancement compared to control. The percentages represented intervals of 5% each.

Net effect of each drug versus various parameters. When the average net effect of each drug (enhancements minus inhibitions divided by the number of subjects), was compared to age, sex, Hb type and blood groups, the results are as follow: a. Age. The average net effect is towards higher enhancement in those more than 25 years of age (n=32) for aspirin, ibuprofen and diclofenac but not with indomethacin. However, these enhancements are not statistically significant. b. Sex. There was no statistically significant difference in the average net effects between males and females. c. Hb type. Only indomethacin and diclofenac resulted in more and statistically significant enhancement in Hb-AS compared to subjects with Hb-A, although the significance with diclofenac is marginal (Figure 2). d. Blood groups. No statistically significant correlation was found between the type of blood group and the average enhancement or inhibition by the 4 drugs. However, when the number of subjects showing

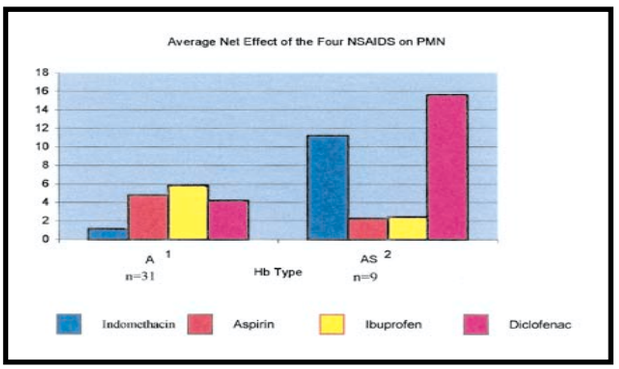

Figure 2 - Average net effect of the 4 non steroidal anti-inflammatory drugs on polymorphonuclear leukocyte activity measured by luminol-dependent chemiluminescence, presented according to hemoglobin type. (Data expressed as a percent change from control).

Table 3 - Relationship of blood groups to the in vitro effect of non-steroidal anti-inflammatory drugs on human polymorphonuclear leukocyte activity.

Blood groups	Indomethacin		Aspirin		Ibuprofen		Diclofenac	
	Enhancement n = 27	Inhibition n = 13	Enhancement n = 27	Inhibition n = 13	Enhancement n = 27	Inhibition n = 13	Enhancement n = 28	Inhibition n = 12
A+ n = 9	9	0	5	4	5	4	7	2
B+ n = 13	6	7	6	7	10	3	10	3
AB+ n = 6	5	1	5	1	4	2	4	2
O+ n = 12	7	5	11	1	8	4	7	5

enhancement or inhibition is considered according to their type of blood group, indomethacin showed a significant difference towards enhancement in blood group A and AB; aspirin in groups O and AB; ibuprofen in groups B and O and diclofenac in blood groups A and B. Comparison between the 4 NSAIDs showed that drugs causing enhancement of CL activity in a significant number of subjects, are aspirin in blood group O and indomethacin and diclofenac in blood group A (Table 3).

Correlation between the effect of each NSAID and other 3 drugs. No significant correlation was found between the effect of each drug and the other 3 drugs on PMNs activity. The number of subjects showing similar effect is relatively constant, though they are not the same subjects. The number showing enhancement by indomethacin and enhancement by each of aspirin, ibuprofen or diclofenac is 16 to 17 subjects, and those showing inhibition by indomethacin and each of the 3 drugs is also constant though much lower in number (n = 4 to 5). If the effect is opposite; inhibition by indomethacin and enhancement by others or vice versa, the number is 7 to 8, and remarkably similar for the 3 drugs. The number of subjects showing enhancement by both indomethacin and aspirin at the same time was 16 to 17, while those showing enhancement by indomethacin, aspirin and ibuprofen was 11, and by all the 4 drugs was only 7. These results were obtained when all levels of change from control were considered. Similar findings were observed when levels below 5% of change from control were considered within the biological variation of this system, and were excluded.

Discussion. The present study proved that studying PMN activity by luminol-dependent CL is a useful tool to investigate variations in the in vitro effects of NSAIDs. In view of well-known differences in response to these drugs, studies of small size utilizing small numbers of subjects may give conflicting and biased results. For example, Dale and Penfield[16] and Berradia et al[12] used only 6 and 10 volunteers. Similarly, workers Gay et al[15] and Dale and Penfield[16] reported that the in vitro effect of NSAIDs on PMN activity is dependent on the drug concentration and type of stimulant used. In the present study, a single concentration and a single stimulant produced a considerable inter individual as well as intra individual variation. In addition, these 2 types of variations do not seem to be related to each other i.e. one cannot predict variation between individuals from the pattern of response of each individual to various NSAIDs. What is remarkable in our finding is that, there is almost a constant proportion of the study population showing one type of response. Around one third of the group under investigation showed inhibition of PMN activity by each and any of the 4 NSAIDs and two thirds enhancement (overall enhancement to inhibition ratio is 2.3, increased to only 2.7 if below 5% response is excluded. This is almost uniform with each drug, although the subjects showing enhancement or inhibition are not the same for all drugs used, i.e. the pattern of response is peculiar for each drug and does not correlate with response to other 3 drugs. Another important finding is that the proportion of any 2 drugs showing a similar response is also fairly constant. For example, enhancement by indomethacin and any of the other 3 drugs (aspirin, ibuprofen or diclofenac) occurred in 16 to 17 subjects, although they are not the same subjects with any 2 drugs. This consistency could point to a genetically determined variation that is common to all 4 NSAIDs but peculiar to each one of them. In an attempt to relate variability in response to various subject characteristics to enable one to predict the effect; parameters such as age, sex, weight, height, hemoglobin concentration, PCV and WBC count, failed to correlate with the effect of any of the 4 drugs. Parameters such as age and sex were also found to account for only 15% of the variability in plasma concentration of piroxicam and naproxen in patients with osteoarthritis.[25] However, statistically significant correlation was found between the effect of NSAIDs and the type of hemoglobin and blood groups. Indomethacin, and to a lesser extent diclofenac, caused a significant enhancement in subjects with hemoglobin type AS compared to normal hemoglobin subjects.

If the enhancement of PMN activity and the production of oxyradicals are anticipated to correlate

with the toxicity of NSAIDs,[26] indomethacin and diclofenac may, then, better be avoided in subjects with sickle cell disease in favour of aspirin and ibuprofen. This preliminary postulation needs to be tested and clinically verified to see whether an increase in oxyradicals is harmful or beneficial in such patients. A significant correlation between the response to NSAIDs and the type of blood group was found when the number of subjects was taken. However, when the magnitude of average enhancement or inhibition is considered, no statistically significant results were found. In general and as shown in Table 3, when the effect of the 4 drugs were compared together according to the type of blood groups of the subjects, the clearest results are with indomethacin and diclofenac in subjects with blood group A and aspirin in blood group O. The relationship between drug toxicity and blood groups, has been previously recognized. For example with oral contraceptive pills, the incidence of thrombosis is more likely to occur in patients with blood groups A, B & AB than with O blood group.[2]

The major source of reactive oxygen species (ROS) in the whole blood is PMN and several articles had studied PMN activity by using the whole blood.[23,27,28] Whole blood is advocated to simulate the in vitro situation regarding plasma protein and interaction between various blood cells. Lymphocytes, for instance, in in vitro culture respond differently in the presence or absence of monocytes.[29] The CL response of PMN activity, therefore, appears to be a good model to study various aspects of drug effects, side effects and interactions. For example, calcium-channel blockers were shown to increase the inhibitory action of indomethacin and diclofenac.[20] The in vivo effect of these drugs on PMN activity from normal subjects and patients, for example with rheumatoid arthritis and osteoarthritis, is an important extension of this work. The importance of reactive oxygen species in the pathogenesis of arthritis is increasingly recognized. Piroxicam given to patients with rheumatoid arthritis and osteoarthritis was found to inhibit superoxide secretion in isolated granulocytes by about 25%.[30] The wide use of NSAIDs by patients with sickle cell disease and other types of hemoglobinopathies deserves careful evaluation of these drugs on PMN activity from these patients. This is needed in view of the differential effect of various NSAIDs on blood from subjects with sickle cell trait shown in this study.

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References

1. BeDell LS, editor-in-chief. Physicians Gen Rx, the complete drug reference. Missouri (USA): Mosby; 1996. p. 143, 1120, 1139, 1711.
2. British National formulary (BNF). A joint publication of the British Medical Association and the Royal Pharmaceutical Society of Great Britain: London (UK); 1998. No. 36.
3. Walker JS, Nguyen TV, Day RO. Clinical response to NSAIDs in urate-crystal induced inflammation: a simultaneous study of intersubject and intrasubject variability. Br J Clin Pharmacol 1994; 38: 341-347.
4. Scheiman, MD. NSAIDs, Gastrointestinal injury, and cytoprotection. Gastroenterol Clin North Am 1996; 25: 279-298.
5. Cashman J, McAnulty G. NSAIDs in perisurgical pain management. Mechanisms of action and rationale for optimum use. Drugs 1995; 49: 51-70.
6. Vesell ES, Penno MB. Assessment methods to identify sources of interindividual pharmacokinetic variations. Clin Pharmacokinet 1983; 8: 378-409.
7. Pairet M, Engelhardt G. Distinct isoforms (COX-1 and COX-2) of cycloxygenase: possible physiological and therapeutic implications. Fundamen Clin Pharmacol 1996; 10: 1-15.
8. Dubois RA, Eberhart CE, Williams CS. Introduction to eicosanoids and the Gastroenteric tract. Gastroenterol Clin North Am 1996; 25: 267-277.
9. Dubois N, Muller N, Lapicque F, Gillet P, Netter P, Royer RJ. Stereoselective protein binding of NSAIDs: Pharmacological implications. Therapie 1993; 48: 325-329.
10. Rudy AC, Bradley JD, Ryan SI, Kalasinski LA, Xiaotao Q, Hall SD. Variability in the dispostion of Ibuprofen enantiomers in osteoarthritic patients. Ther Drug Monit 1992; 14: 464-470.
11. Hundal Q. Studies on the possible relationship between levels of piroxicam and naproxen in biological fluids from patients with osteoarthritis as well as reactive arthritis and clinical events [doctoral thesis]. Oslo (Norway): The National Hospital; 1994.
12. Berradia N, Marchand-Arvier M, Humbert JC, Vigneron C. Effects of indomethacin and diclofanac on some functions of polymorphonuclear neutrophils. J Pharm Pharmacol 1988; 40: 806-808.
13. Smolen JE, Weissmann G. Effects of Indomethacin, 5, 8, 11, 14. Eicosatetraynoic acid (Etya) and P-Bromophenacylbromide (BPB) on Lysosomal enzyme release and superoxide anion generation by human polymorphonuclear leukocytes. Biochem Pharmacol 1980; 29: 533-538.
14. Bodaness RS, Chan PC. Reaction of indomethacin with singlet molecular oxygen. Biochem Pharmacol 1980; 29: 1337-1340.
15. Gay JC, Lukens JN, English DK. Differential inhibition of Neutrophil superoxide generation by NSAIDs. Inflammation 1984; 8: 209-222.
16. Dale MM, Penfield A. Superoxide generation by either 1-oleoyl-2-acetylglycerol or A23187 in human neutrophils is enhanced by indomethacin. FEBS Letts 1985; 185: 213-217.
17. Rao BA, Sisodia P, Janardhan A, Sattur BP. Inhibition of superoxide anion production from activated phagocytes by anti-inflammatory drugs. Indian J Exp Biol 1986; 24: 644-646.
18. Friman C, Johnston C, Chew C, Davis P. Effect of diclofenac sodium, tolfenamic acid and indomethacin on the production of superoxide induced by N-formyl-Methionyl Leucyl-Phenylalanine in normal human PMNs. Scand J Rheumatol 1986; 15: 41-46.

19. Neal TM, Vissers MCM, Winterbourn CC. Inhibition by nonsteroidal antiinflammatory drugs of superoxide production and Granule enzyme release by polymorphonuclear leukocytes stimulated with immune complexes or formyl-methionyl-leucyl-phenylalanine. Biochem Pharmacol 1987; 36: 2511-2517.
20. Mustafa AA, Ali-Balia SR, Al-Tawaijri AS, Al-Dalaan A. Calcium channel blockers enhance the inhibitory action of non-steroidal anti-inflammatory drugs on the luminol dependent chemiluminescence: differential effects of Tenoxicam. Saudi Med J 1993; 14: 340-346.
21. Minakami K, Watanabe Y, Miyahara M, Kobaachi H, Kurashige T, Utsumi K. Effects of indomethacin and aspirin on the TNF-alpha-induced priming and protein tyrosyl phosphorylation of human neutrophils. Physiol Chem Phys Med NMR 1993; 25: 55-67.
22. Arthur L, Sagone, Jr. Effect of anti-inflammatory agents on the hydroxyl radical (OH) producing of zymosan stimulated human granulocytes (PMNs). Oxygen and Oxy-Radicals in Chem Biol 1981; 719-724.
23. Vladimirov YUA, Sherstnev MP, Piryazev AP. Chemiluminescence of the leukocytes of whole blood stimulated by barium sulphate crystals. Biophysics 1989; 34: 1136-1140.
24. Daci JV, Lewis SM. Practical Hematology. 6th ed. Edinburgh: Churchill Livingstone; 1984.
25. Rugstad HE, Hundal O, Holme I, Herland OBV, Husby G, Giercksky KE. Piroxicam and naproxen plasma concentration in patients with osteoarthritis: relation to age, sex, efficacy and adverse events. Clin Rheumatol 1986; 5: 389-398.
26. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 1991; 91 (Suppl 3c): 145-225.
27. Sakumoto M, Matsumoto T, Mochida O, Kubo S, Mizunoe Y, Kumazawa J. Chemiluminescence response of whole blood in patients undergoing urological operations. Int Urol Nephrol 1997; 29: 473-478.
28. Brown GE, Reiff J, Allen RC, Silver GM, Fink MP. Maintenance and down-regulation of primed neutrophil chemiluminescence activity in human whole blood. J Leukoc Biol 1997; 62: 837-844.
29. Jawad AM, Rogers HJ. The effects of flurbiprofen and indomethacin on the mitogenic response of human peripheral mononuclear cells. Immunopharmacology 1984; 7: 59-67.
30. Biemond P, Swaak AJG, Penders JMA, Eindoff CMB, Koster JF. Superoxide production by polymorphonuclear leucocytes in rheumatoid arithritis and osteoarthritis: in vivo inhibition by the antirheumatic drug piroxicam due to interference with the activation of the NADPH-Oxidase. Ann Rheum Dis 1986; 45: 249-255.