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# DECREASE OF OSTEOARTHRITIC SYNOVIAL FLUID VISCOSITY BY MEANS OF U.V. ILLUMINATION: A METHOD TO EVALUATE THE FREE RADICAL SCAVENGING ACTION OF DRUGS

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**Abstract**—Ultra-violet illumination of osteoarthritic synovial fluid induces a viscosity fall due to the production of free radicals. The authors demonstrate by means of an indirect method (i.e. ferricytochrome C reduction) and by the protection of viscosity mediated by superoxide dismutase, mannitol and catalase, that at least three oxygen products, i.e. superoxide anion, hydroxyl radical and hydrogen peroxide, are generated by u.v. illumination. Ultra-violet illumination of synovial fluid, because of its ease, could be a helpful method for the screening of the free radical scavenging effect of drugs.

In recent years, much experimental research has given due emphasis to the role played by certain free radicals in the inflammatory process [1–6].

Their pro-inflammatory effect has been seen in rats [7, 8]. Likewise, Oyanagui [4], Kuehl [9] and Puig-Parellada [10] have called the attention of several pharmacologists to the possible protective effect exerted by some non-steroidal anti-inflammatory drugs (NSAIDs*) against free radicals. In studying the scavenging action of drugs, Greenwald [11] utilized the protection of viscosity of a hyaluronic acid solution, whereas Puig-Parellada and Planas used the protection of viscosity of bovine synovial fluid.

In this paper we tried to prove that the protection of the viscosity fall of human synovial fluid could be employed as a preliminary screening assay in studying the scavenger effectiveness of drugs against some toxic products of oxygen. The viscosity decrease was induced by a free radical generating system, i.e. u.v. illumination.

## MATERIALS AND METHODS

We have employed human synovial fluid samples drawn from the knees of ten patients with osteoarthritis; quartz mercuty vapor lamp (HPK-lamp 125 W; 3500–4000 Å) was purchased from Philips, Milan, Italy; viscometer and picnometer from Carlo Erba, Milan, Italy; ferricytochrome C type III from Sigma Chemical Co., St. Louis, U.S.A; L-epinephrine from Sigma Chemical Co., St. Louis, U.S.A.; superoxide dismutase (SOD) from Istituto Farmacologico Serono, Rome, Italy; mannitol from Laboratori Baxter, Trieste, Italy; catalase from OTI Farmaceutici, Parma, Italy; acetylsalicylic acid and indomethacin from Chiesi Farmaceutici, Parma, Italy; bovine serum albumin from Sigma Chemical Co., St. Louis, U.S.A.

### *Preparation of the synovial fluid samples*

Each sample was drawn in the morning, in aseptic conditions and afterwards centrifuged at 3000 rpm for 30 min in order to obtain fluids without cells. We utilized the clear supernatant for the preparation of slides, stained with May–Grünwald–Giemsa. The colouring of the slides confirmed the absence of haematic and synovial cells.

### *Viscosity index*

The relative viscosity was determined on the same fluids using a 'down-flowing' method following previous studies by Hasselbacher [12]. The viscosity index was calculated as follows:

$$\eta_{sf} = \eta_c \cdot \frac{t_{sf}}{t_c} \cdot \frac{\delta_{sf}}{\delta_c}$$

(where $\eta$ = viscosity; $\delta$ = density; $t$ = down-flowing time; sf = synovial fluid; c = control sample $H_2O$). Each phase was performed at 21°.

### *Ultra-violet illumination of synovial samples*

Following McCord and Fridovich's [12] experiments on photolysed water, we demonstrated that u.v. illumination of synovial fluid induces a viscosity fall whose maximum level, ranging from 35 to 55% of the basal value, occurs after 4 hr [13, 14].

Indirect methods were employed to verify the production of oxygen free radicals and hydrogen peroxide, by means of u.v. illumination. As a proof of the production of superoxide anion ($O_2^-$), we took into consideration the reduction of ferricytochrome

* Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; $O_2$, superoxide anion; $OH^-$ hydroxyl radical; $H_2O_2$, hydrogen peroxide; SOD, superoxide dismutase.

C, which was added to illuminated synovial fluid [15]. Previously, we excluded any reduction of ferricytochrome C directly illuminated by u.v. The reaction mixtures (illuminated synovial fluid plus cytochrome C, 75 μM) were placed in 1-ml quartz cuvettes and the reduction of ferricytochrome C was recorded spectrophotometrically at 550 nm (Perkin–Elmer 576 ST).

The involvement of various toxic oxygen-derived free radicals as mediators of the synovial fluid viscosity fall, was observed by experiments done with scavengers.

For scavenger studies [6, 16–20] various reagents, i.e. superoxide dismutase (SOD: 1.12; 11.2; 112 μg/ml), catalase (1.12; 11.2; 112 μg/ml) and mannitol ($17 \times 10^{-3}$ M) were added to the synovial fluid prior to initiation of u.v. illumination. Besides these agents, we tested the effects of albumin at 2.5 and 25 mg/ml, in order to exclude aspecific effects.

The scavenging action of drugs derived from the viscosity index obtained after 4 hr of u.v. illumination of the synovial fluid sample plus the drug, vs the value of the same sample illuminated without the drug, as outlined below:

$$\text{protection } \% = 100 - \left(\frac{\eta_{sf} + \text{drug}}{\eta_{sf}} \times 100\right).$$

*Epinephrine autoxidation*

This assay was performed in order to confirm the results obtained with illuminated synovial fluid. Following Misra and Fridovich's method [21] we employed as a source of $O_2^-$ the epinephrine autoxidation assay, for the evaluation of the specific scavenging action of drugs. The epinephrine was used at a concentration of $5.4 \times 10^{-4}$ M in sodium carbonate, 0.05 M, pH 10.2. The adrenochrome production was recorded spectrophotometrically at 480 nm (extinction coefficient $\epsilon^{480} = 4020 \times M^{-1}$ $cm^{-1}$). The spontaneous adrenochrome production with and without albumin or drugs, was used to assess the $O_2^-$ scavenging effect.

Each experiment was performed in triplicate and the results represent the mean of the obtained values.

We studied by means of illuminated synovial fluid and epinephrine autoxidation assays the actions of acetylsalicylic acid and indomethacin.

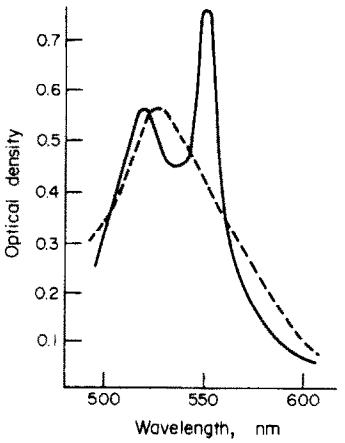


Fig. 1. Behaviour of cytochrome C in illuminated and non-illuminated synovial fluid. Synovial fluid plus ferricytochrome C, 75 μM, vs non-irradiated synovial fluid - - -; the spectrum is characteristic of the oxidized form of cytochrome C. Irradiated synovial fluid plus ferricytochrome C, 75 μM, vs irradiated synovial fluid ——; the spectrum is characteristic of the reduced form of cytochrome C. In some experiments SOD, at various concentrations (1.12, 11.2, 112 μg/ml) was added to the mixture (synovial fluid + ferricytochrome C) prior to illumination. After the illumination the spectrum of cytochrome C was recorded. An inhibition of the reduction of cytochrome C has been observed only at higher concentrations (11.2 and 112 μg/ml). The curves were obtained with a Perkin–Elmer 576-ST spectrophotometer.

## RESULTS

The reduction of ferricytochrome C to ferrocytochrome C proves that u.v. illumination of human synovial fluid really produces the superoxide anion ($O_2^-$) (Fig. 1). The superoxide dismutase protection of the synovial fluid viscosity fall confirms the above findings. We found a powerful protection at concentrations of 11.2 and of 112 μg/ml (Table 1). At the same concentrations we did not observe the reduction of cytochrome C.

A sure protection was found even with mannitol and with catalase (Table 1). No effect was seen with albumin. On the basis of these results we support the hypothesis that u.v. illumination of human synovial fluid induces the simultaneous production of

Table 1. Effect of free radical scavengers on the synovial fluid viscosity fall induced by u.v. illumination

| Drugs | Concentration | % Reduction of viscosity index after 4 hr of u.v. illumination | % Protection exerted by drugs |
|---|---|---|---|
| Synovial fluid | (basal value) | 44.5 | — |
| + Superoxide dismutase | 1.12 μg/ml | 38 | 15 |
| | 11.2 μg/ml | 3.5 | 92 |
| | 112.0 μg/ml | 1.5 | 96 |
| + Catalase | 1.12 μg/ml | 43.5 | 2.2 |
| | 11.2 μg/ml | 5.6 | 87 |
| | 112.0 μg/ml | 1.6 | 96 |
| + Mannitol | $17 \times 10^{-3}$ M | 10.8 | 75 |

Table 2. Effect of non-steroidal anti-inflammatory drugs on the synovial fluid viscosity fall induced by 4 hr of u.v. illumination

| Drugs | Concentration | % Reduction of viscosity index after 4 hr of u.v. illumination | % Protection exerted by drugs |
|---|---|---|---|
| Synovial fluid | (Basal value) | 41.6 | — |
| + Acetylsalicylic acid | $3 \times 10^{-3}$ M | 30.7 | 26.3 |
| | $3 \times 10^{-4}$ M | 37.7 | 9.3 |
| + Indomethacin | $1 \times 10^{3}$ M | 40.2 | 3.3 |
| | $1 \times 10^{-4}$ M | 33.9 | 18.5 |
| | $1 \times 10^{5}$ M | 36.6 | 12.0 |

$O_2^-$, $OH^\cdot$ (hydroxyl radical) and $H_2O_2$ (hydrogen peroxide) as suggested in photolysed water by McCord and Fridovich:

u.v. $\rightsquigarrow H_2O \rightarrow H_2O^+ + e^-$ (electron) (first step);

$H_2O^+ \rightarrow H^+ + OH^\cdot$ (second step);

$OH^\cdot + OH^\cdot \rightarrow H_2O_2$ (third step);

$OH^\cdot + H_2O_2 \rightarrow H_2O + HO_2^\cdot$ (fourth step);

$HO_2^\cdot \rightarrow H^+ + O_2^-$ (fifth step).

By this method, which offers the possibility of screening the scavenging effect of many substances against some of the most important toxic products of oxygen, acetylsalicylic acid and indomethacin show a protection ranging between 3 and 26% (Table 2). Such low values were obtained with many other NSAIDs.*

With the epinephrine autoxidation assay we observed that superoxide dismutase exerts a protection of 59% at the concentration of 0.112 μg/ml and of 90% or more at concentrations of 1.12 and 11.2 μg/ml, respectively. The protection of albumin at concentrations of 25 mg/ml and of 2.5 mg/ml was 3.33% and 0, respectively. A protection always less than 20% was seen for mannitol, catalase, acetylsalicylic acid and indomethacin (Table 3).

* U. Ambanelli, G. F. Ferraccioli, P. Fietta and A. Spisni, Screening of the free radical scavenging action of some non steroidal anti-inflammatory drugs (in preparation).

## DISCUSSION

Using human synovial fluid as substrate of u.v. illumination, we demonstrate the simultaneous production of some radicals ($O_2^-$, $OH^\cdot$) and of $H_2O_2$. In this assay, specific scavengers such as superoxide dismutase, mannitol and catalase exert a protective power on the viscosity of human synovial fluid. Therefore, we believe in the usefulness of our method of detecting the free radical scavenging effect of drugs. In fact, in a preliminary screening, other agents, usually utilized as basic treatment in rheumatoid arthritis, such as tiopronin and D-penicillamine [22, 23], presented a marked effectiveness. However, both acetylsalicylic acid and indomethacin showed no free radical scavenging action. The results obtained with a specific assay for superoxide, confirmed that even on $O_2^-$ the NSAIDs employed were scarcely effective. Comparing our data with those obtained by Puig-Parellada and Planas, who used another cell-free assay, we found similar results for acetylsalicylic acid but not for indomethacin. Nevertheless, we cannot exclude that NSAIDs might act on another biological product of oxygen, i.e. the singlet oxygen, on which indomethacin seems to act specifically [24].

NSAIDs might act even on the superoxide synthetase (NADPH-oxidase) of the cell membrane as hypothesized by Oyanagui. Further studies using both cell preparation and cell-free methods are needed to clarify these important points.

Table 3. Effect of some drugs on the inhibition of the epinephrine–adrenochrome autoxidation

| Drugs | Concentration | Adrenochrome nMoles/min | % Inhibition of epi autoxidation |
|---|---|---|---|
| L-Epinephrine | $5.4 \times 10^{-4}$ M | 22.9 | — |
| Superoxide dismutase | 0.112 μg/ml | 9.4 | 59 |
| | 1.12 μg/ml | 2.3 | 90 |
| | 11.2 μg/ml | 1.4 | 94 |
| Catalase | 0.112 μg/ml | 22.6 | 1 |
| | 1.12 μg/ml | 22.7 | 1 |
| | 11.2 μg/ml | 18.8 | 18 |
| Mannitol | $17 \times 10^{-3}$ M | 19.7 | 14 |
| Acetylsalicylic acid | $3 \times 10^{-4}$ M | 22.9 | 0 |
| | $3 \times 10^{-3}$ M | 22.9 | 0 |
| Indomethacin | $1 \times 10^{-5}$ M | 22.9 | 0 |
| | $1 \times 10^{-4}$ M | 20 | 10 |
| | $1 \times 10^{-3}$ M | 22.9 | 0 |

Many other experiments in this direction are in progress in our laboratory to understand better the molecular mechanism of the most used antirheumatic agents.

However, following Greenwald supposition that even in human chronic arthropathies free radicals might act as mediators of the articular cartilage damage [25], we believe that the therapeutic approach with scavengers such as SOD in rheumatoid arthritis [26] and mannitol or SOD in osteoarthritis is really justified [27, 28].

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