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Studies on the biological effects of ozone: 9. Effects of ozone on human platelets

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During the course of ozonated autohaemotherapy (O_3-AHT) using heparin as an anticoagulant, it was occasionally observed that a few clots were retained in the filter during blood reinfusion. This observation prompted an investigation on the effect of ozone (O_3) on human platelets. We have now shown, both by biochemical and morphological criteria, that heparin in the presence of O_3 can promote platelet aggregation. In contrast, after Ca^{2+} chelation with citrate, platelet aggregation is much reduced. The potential role of the transient formation of hydrogen peroxide (H_2O_2) in the presence of Ca^{2+} with the possible expression of adhesion molecules is briefly discussed.

Key words: glutathione, hydrogen peroxide, oxidation, ozone, platelets

Introduction

Ozonated autohaemotherapy (O_3-AHT) is a fairly well known complementary medical approach classified by the National Institutes of Health (Bethesda, MD, USA) among oxidant therapies. Since 1954[1] it has been used in countless sessions,[2] mostly in Europe, and it is carried out by briefly exposing a known volume of human blood to a gas mixture composed of about 96% medical oxygen (O_2) and 4% ozone (O_3), followed by the reinfusion of ozonated blood into the donor. The procedure normally involves autologous blood anticoagulated with citrate-phosphate-dextrose (CPD) and does not cause any side effects.[3] Following our observation[4] that O_3 can act as a mild inducer of cytokine production, we have clarified that this effect is more pronounced in blood treated with heparin rather than CPD,[5] most likely because the former anticoagulant preserves the physiological Ca^{2+} level (about 1 mM) in the plasma. In fact, addition of Ca^{2+} up to 5 mM enhances the induction but also causes a slight increase in haemolysis. On the basis of the empirical observation[2] that O_3-AHT is beneficial in chronic viral hepatitis, particularly in immunosuppressed patients, it appeared interesting to correlate the enhancement of cytokine production to the O_3 concentration. However, when the latter reaches 70–80 µg/ml per g of blood, in spite of having added up to 30 IU heparin per ml of blood, some samples show an unusual tendency to form small clots. This observation compelled us to begin an investigation on the effect of O_3 on platelets, which are cell components, probably even more sensitive than erythrocytes to oxidative stress for the relatively low repertoire in antioxidant enzymes.[6–8] The biochemical and morphological study presented here shows that O_3 can exert marked aggregation, and indicates a clear limitation for the simultaneous use of heparin and O_3.

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Materials and methods

Ozone generation and measurement

O_3 was generated from medical-grade O_2 using electrical corona arc discharge in the last generation O_3 generator (Model Ozonosan PM100K, Hansler GmbH, Iffezheim,

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Germany), which allows the gas flow rate and O_3 concentration to be controlled in real time by photometric determination at 253.7 nm, as recommended by the Standardisation Committee of the International O_3 Association.

Reagents

Anticoagulants were either heparin (calcium salt, 30 IU/ml blood) normally used for therapeutic purposes (Calciparina, Italfarmaco) or ACD (citric acid, Na citrate, glucose) (Haemonetics, Braintree, USA). Adenosine diphosphate (ADP) was from Sigma Chemical Co. (St. Louis, MO, USA), and for studying aggregation a 0.5 mM solution was prepared.

Preparation of platelet-rich plasma (PRP) samples

Both ACD and heparinised platelet-rich plasma (PRP) were prepared from the same blood samples (60 ml) drawn, after informed consent, from five fasting (12 h) non-smoker volunteers between the ages of 23 and 27 years, who were considered to be healthy and had not ingested platelet-active medication for at least 2 weeks.

Nine parts blood were anticoagulated with either one part ACD or with one part of saline containing heparin so that its final concentration was 30 IU/ml. Blood was centrifuged at 200 $\times$ g for 20 min and platelets were measured with a Coulter counter. An average platelet count of 3×10^3/ml plasma was used. A further centrifugation of PRP at 6000 $\times$ g for 15 s gave a platelet-containing pellet and a supernatant platelet-free plasma used for biochemical determinations.

O_2 and O_3 delivery to biological samples

A predetermined volume of the O_2/O_3 gas mixture at three O_3 concentrations (20, 40 and 80 µg/ml per ml of PRP) was collected with a teflonated disposable syringe and immediately introduced into a second syringe containing an equivalent volume of PRP via a 'y' connector. Final gas pressure remained at normal atmospheric pressure. In order to obtain reproducible results, it needs to be emphasised that O_3 is a very reactive gas so that extremely rapid and precise handling is required. The PRP samples were gently but continuously mixed with the gas for up to 30 s, and afterwards they were dispensed into test tubes for various analyses. Control samples were either untreated or mixed with an equal volume of O_2. It is worth mentioning that O_2 represents at least 95% of the O_2/O_3 mixture.

Platelet aggregation

Platelet aggregation was monitored by the change in light transmission at 650 nm in an Apact dual-channel Labor aggregometer, according to Born.[9]

Biochemical determinations

(1) Determination of thiobarbituric acid-reactive substances (TBARS): in order to evaluate the relevance of lipid peroxidation, TBARS were assessed according to Pompella *et al.*[10]
(2) Total antioxidant status (TAS) in plasma samples was assessed according to Rice-Evans and Miller.[11]
(3) Protein thiol groups (PTG) were measured in plasma according to Hu[12], using procedure 1 with 5,5′-dithio-bis(2-nitrobenzoic acid) DTNB dissolved in absolute methanol.
(4) Total reduced glutathione (GSH) content of platelets was promptly determined on a platelet-containing pellet after acid precipitation of proteins with 5% (w/v) TCA according to the method described by Tietze.[13]

Electron microscopy evaluation

Scanning electron microscopy (SEM) Platelets were concentrated onto polycarbonate filters (Unipore, 0.2 µm) and, after careful washing with saline solution, were fixed with Karnovsky fixative (paraformaldehyde 4%, glutaraldehyde 2.5% in 0.1 M cacodylate buffer) for 3 h at 4°C, washed with the same buffer, postfixed in OsO4 1% in 1% veronal acetate buffer for 2 h at 4°C, dehydrated in graded alcohols and then, after immersion in tetramethyl silane, dried at 30°C. Afterwards samples were coated with gold and examined with a Philips 505 SEM at 30 kV.

Transmission electron microscopy (TEM) PRP samples were fixed with Karnovsky fixative (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer) using a volume ratio of 3:1 and a gentle mixing; after about 30 s the gelified samples were cut into small cubes, further fixed with Karnovsky solution for 3 h at 4°C, then washed with cacodylate buffer, postfixed in 1% OsO_4 in veronal acetate buffer for 2 h at 4°C, dehydrated in graded alcohols and embedded in araldite using propylene oxide as an intermediate diluent. Ultra-thin sections were cut from the sample using an LKB NOVA ultramicrotome and stained with uranyl acetate and lead citrate before examination using a Philips CM10 electron microscope at 80 kV.

Statistical analysis

Whenever possible, results obtained from five donors were expressed as means±SD. Statistical evaluation of the experimental data was performed with Student's *t*-test for paired samples with ≤ 0.05 as the minimal level of significance ($P < 0.05^{+}$ and $P < 0.01^{++}$).

Results

Representative tracings of platelet aggregation due to either PRP in heparin or in ACD are reported in Figure 1.

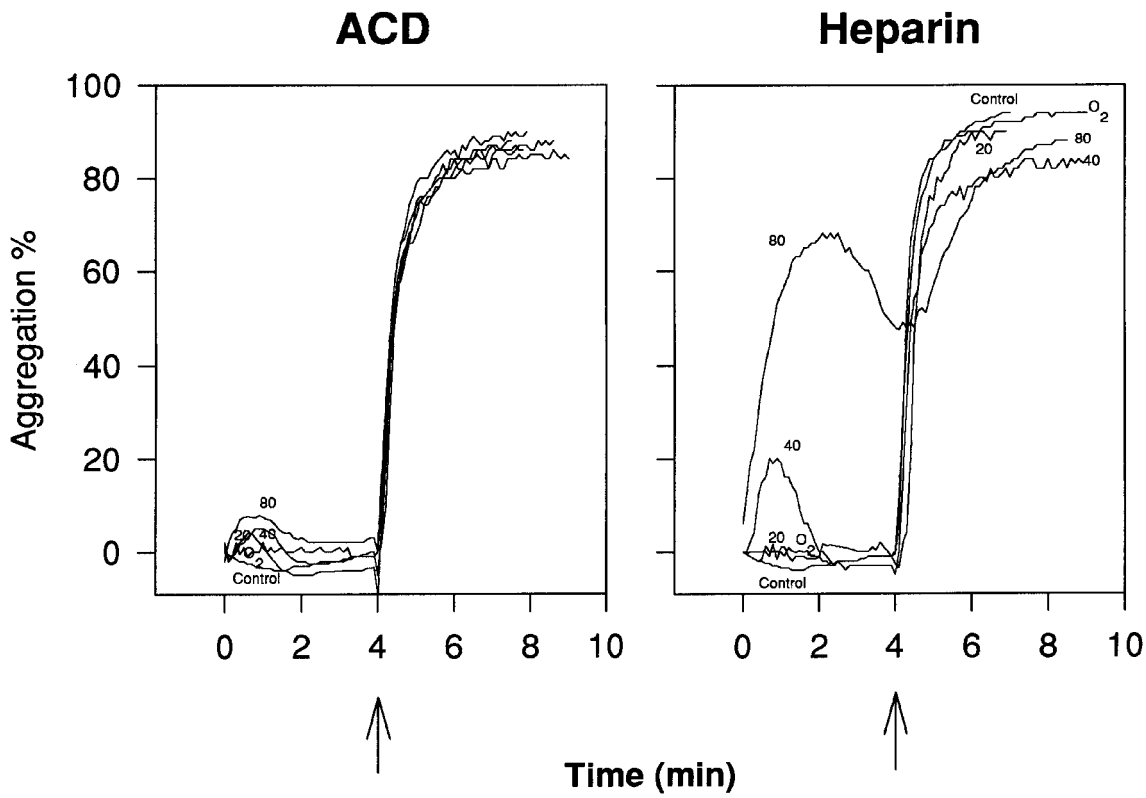

Figure 1. Representative tracings of platelet aggregation induced by progressively increasing O_3 concentrations (20, 40 and 80 μg/ml per ml PRP). O_3 causes immediate and dose-dependent aggregation only in heparinized PRP (right panel). Aggregation profiles of PRP in ACD are reported in the left panel. After 4 min, ADP induces full aggregation (arrow).

Control and O_2-treated samples do not show spontaneous aggregation. However, when heparinised PRP is pre-treated for 30 s with three concentrations of O_3 (20, 40 and 80 μg/ml per ml PRP) Figure 1 (right panel) shows a striking dose-effect relationship: a mean of either 20±6 or 68±14% spontaneous aggregation occurs immediately due to an O_3 concentration of 40 or 80 μg/ml. Full aggregation occurs after 4 min upon addition of ADP. The induction of immediate aggregation due to 80 μg/ml O_3 is quite reproducible and it is very slight in ACD treated PRP (Figure 1).

The increase of TBARS as a marker of peroxidation due to progressively increasing O_3 concentrations, is shown in Figure 2. O_3 at 20 μg/ml per ml of PRP induces a significant ($P < 0.05$) increase over control and O_2-treated samples. Neither heparin nor ACD interfere with TBARS formation. Evaluation of TAS in both heparinised and ACD plasma samples showed a typical slight decrease in approximate relation to the oxidant concentration (Figure 3, top panel). As usual, PTG showed a far more pronounced decrease consistent with the idea that protein-SH groups, in comparison to uric acid and ascorbic acid, are preferentially oxidised by ROS (Figure 3, bottom panel).

With the aim of clarifying the reason for platelet aggregation induced by O_3 in the presence of the physiological Ca^{2+} level (~ 1 mM), we have examined the GSH content and the morphology of platelets in both anticoagulants. It is interesting to note that there are no significant changes in the GSH content of platelets even after exposure to 80 μg/ml O_3 (Figure 4).

SEM examination shows that there is a marked formation of platelet aggregates in heparinised PRP exposed to 40 and 80 μg/ml O_3 per ml of plasma (Figure

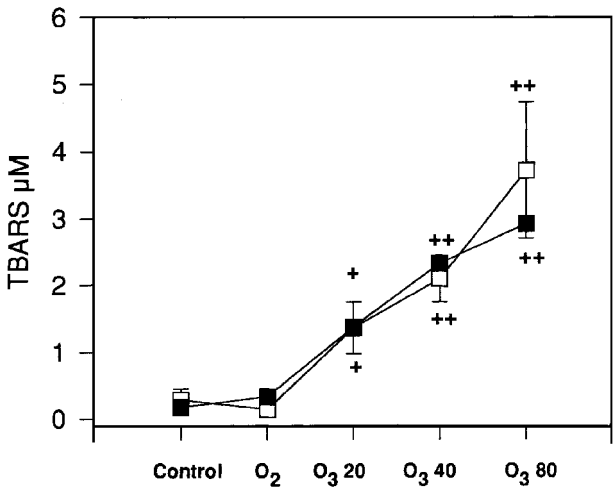

Figure 2. Plasma levels of TBARS in PRP samples (□, ACD, ■, heparin) treated with either O_2 or increasing concentrations of O_3 (μg/ml per ml of PRP).

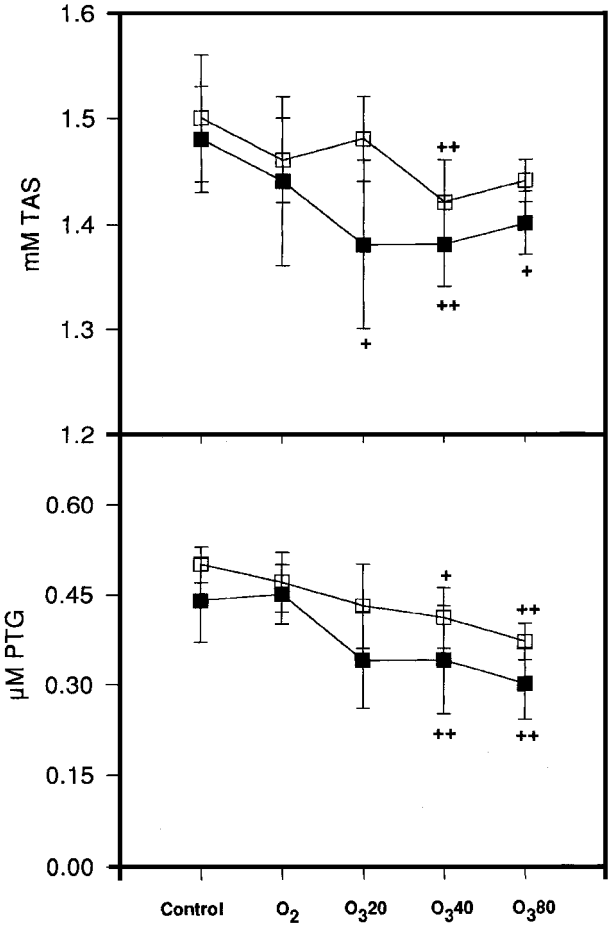

Figure 3. Effect of 30 s exposure of either O_2 or O_3 (20, 40 and 80 µg/ml per ml of PRP) on total antioxidant status (TAS) and protein thiol groups (PTG). (□) ACD; (■) heparin-treated PRPs).

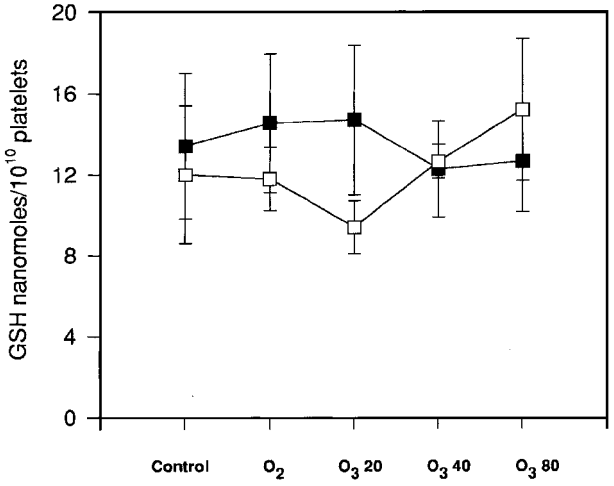

Figure 4. GSH content in control, oxygenated and ozonated platelets obtained from PRPs anticoagulated with either ACD (□) or heparin (■).

5). Similar aggregates are clearly visible after TEM (Figure 6), while ACD-treated samples show normal morphology.

Discussion

There is no doubt that ozonation of both ACD- and heparin-treated PRP induces an almost linear increase of TBARS that have been used as an index of lipid peroxidation and an ozone marker. It must be emphasised that the range of O_3 concentration used (20–80 µg/ml per ml of PRP) is within the therapeutic window used in medical practice. Concentrations either below 20 or higher than 80 µg/ml appear to be either ineffective (probably corresponding to a placebo effect) or toxic, respectively, as haemolysis begins to increase significantly only above 100 µg/ml. This result is largely due to the powerful and articulate antioxidant system present in plasma and blood cells, constituted by hydrophilic, lipophilic scavengers and antioxidant enzymes.[14–16] Indeed Figure 3 clearly shows that both plasma PTP and, to a lesser extent, TAS decrease progressively during ozonation and 'sacrificial' molecules serve to neutralise the process of oxidation due to the cascade of reactive oxygen species (ROS) generated by ozone decomposition. Moreover, it is remarkable that the platelet content of GSH is not significantly modified even after exposing PRP to an O_3 concentration of 80 µg/ml. This result was not unexpected because it is known[17] that platelets can rapidly restore their GSH content after treatment with oxidants. In comparison, using similar experimental conditions, a GSH decrease of 15–20% has been measured in erythrocytes, although these cells also can recover their normal GSH content 30–50 min after O_3 exposure.[18]

The phenomenon of platelet aggregation is very reproducible but, because it is very rapid, it could be missed if the exposure to O_3 lasts longer than 30 s. From Figure 1 it can be noted that it was partially reversible and did not abrogate ADP-induced aggregation. Besides being observed macroscopically, Figures 5 and 6 illustrate, both by SEM and TEM, how platelets are clumped together. At this stage we do not know which are the biochemical and molecular events leading to aggregation but we can hypothesise that Ca^{2+} has a critical role as, after its chelation with citrate, aggregation does not occur. For the future, it would be interesting to perform experiments in the presence of hirudin which, unlike heparin, does not appear to cause any potentiation of platelet responses. It is unlikely that a small amount of ADP released from erythrocytes contributes to the 'spontaneous' aggregation because only PRP, practically erythrocyte free, was exposed to ozone.

Another important factor is the transient presence of H_2O_2 as one of the ROS generated during O_3 decomposition.[19] Pryor[20] has postulated that H_2O_2 and aldehydes are the terminal products after O_3 reacts with unsaturated fatty acids. In a previous paper[19] we have shown that up to 28 µM of H_2O_2 are formed immediately after ozonation of plasma exposed at an O_3 concentration of 80 µg/ml. H_2O_2 has an average half-life of 2.5 min, and disappears owing to traces of catalase and peroxidases activity. However, H_2O_2 lifetime is long enough to

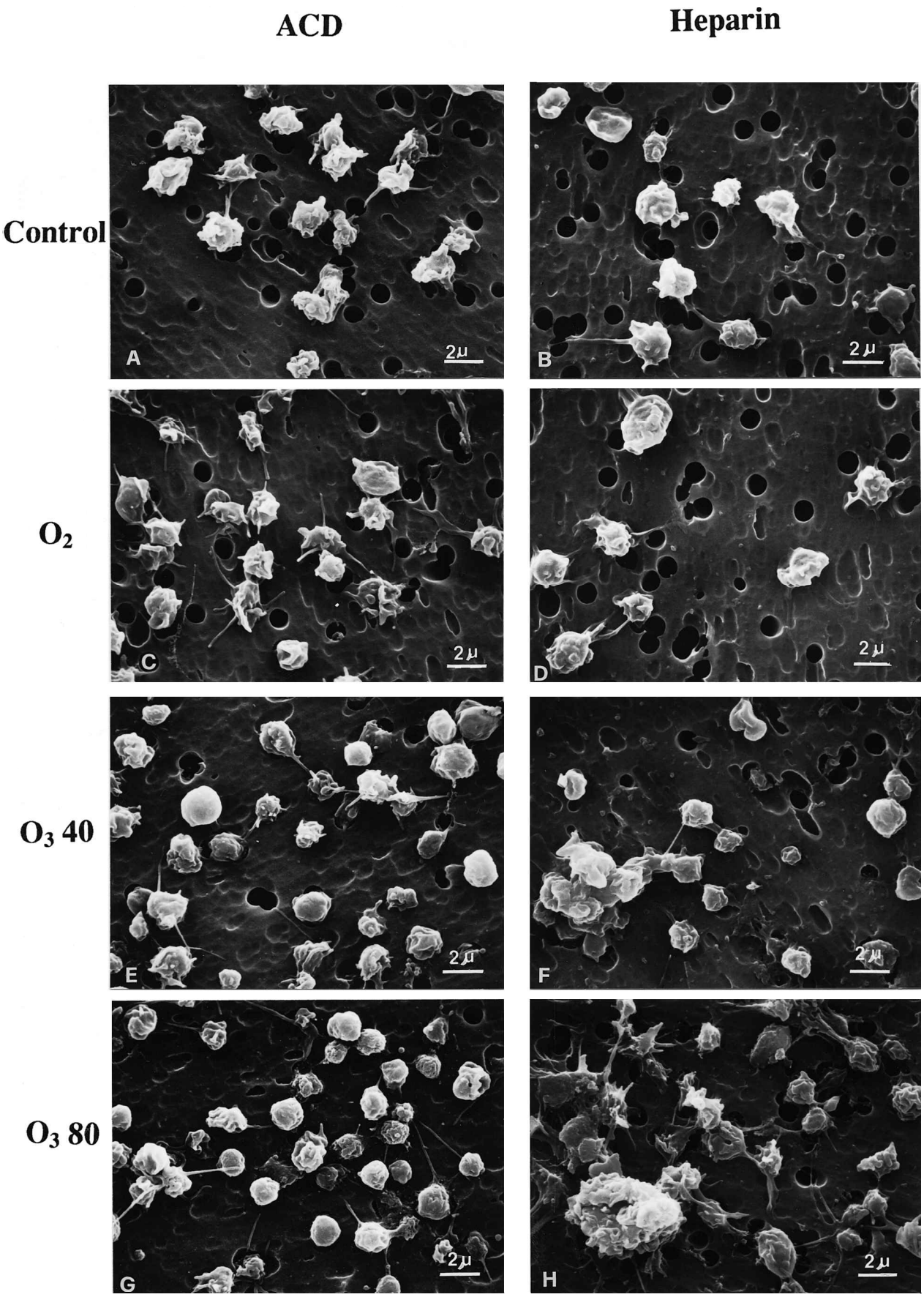

Figure 5. Scanning electron microscopic examination of human PRPs either in ACD (left panel) or in heparin (right panel). Platelets exposed to O_3 concentrations of 40 and 80 μg/ml per ml PRP form aggregates visible on the right-hand side.

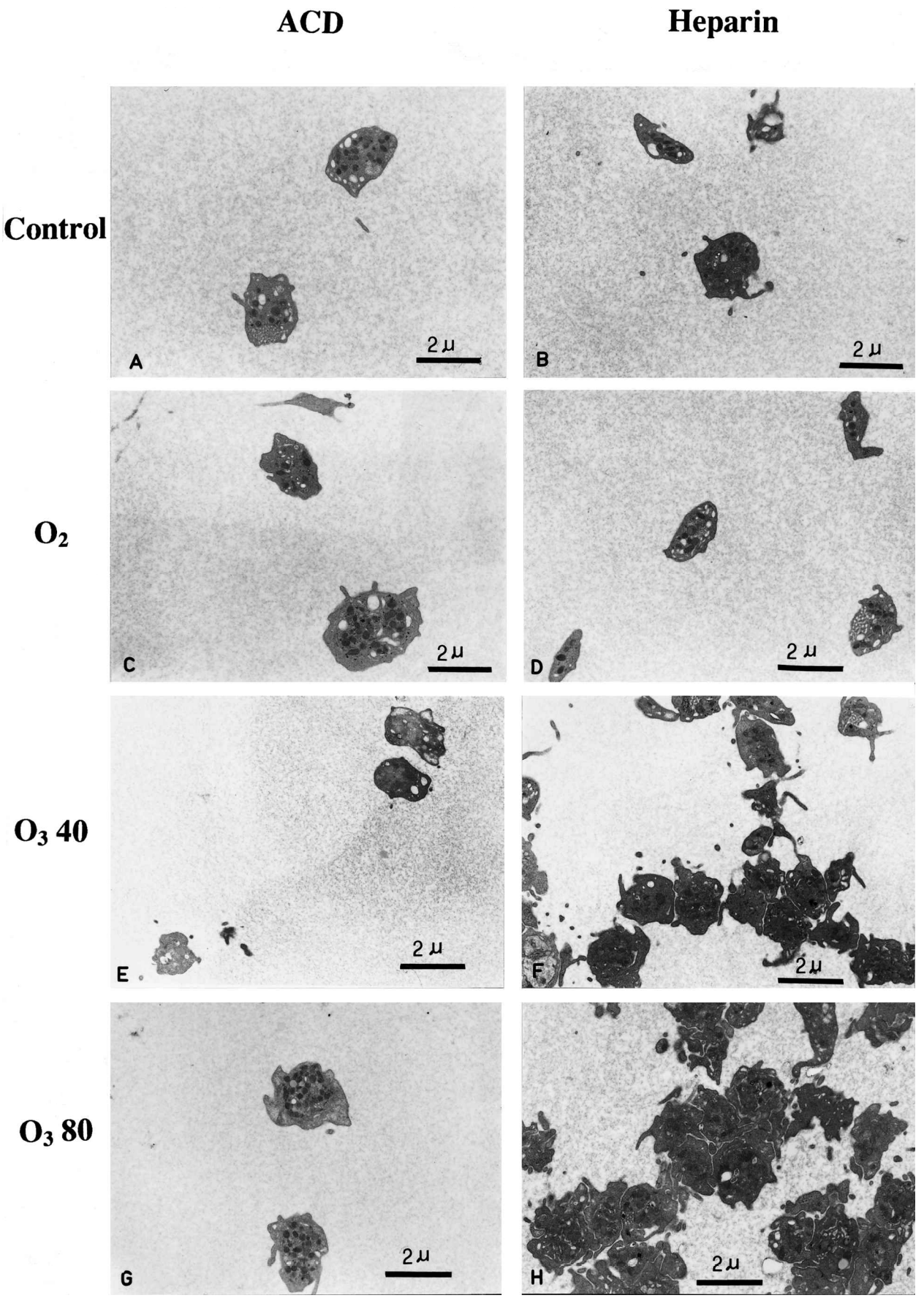

Figure 6. Transmission electron microscopic examination of human PRPs either in ACD (left panel) or in heparin (right panel). Platelets exposed to O_3 concentrations of 40 and 80 μg/ml per ml PRP form aggregates visible on the right-hand side.

trigger aggregation of platelets, which has been clearly shown to occur even in the presence of nanomolar concentrations of H_2O_2.[21–23] It remains unclear whether O_3, besides generating H_2O_2 in plasma, is also able to induce the endogenous production of H_2O_2 from platelets.[24] It remains also undetermined whether exposure of platelets to O_3 causes a change in the expression of cell-surface molecules or enhances the release of products from granules. We have just started an investigation aimed to assay several factors in the plasma, because this aspect may have important practical implications. O_3-AHT with blood anticoagulated with ACD has been widely used for treating hind limb ischaemia with apparently beneficial results and avoiding amputation in several patients.[25]

Before this study it was not known whether heparin could give better results than ACD but, on the basis of the present results, we have to be cautious because heparin in the presence of O_3 favours platelet activation, and this may lead to thromboembolism, although no apparent side effect has been ever noted *in vivo* during treatment. On the other hand, it will be instructive to examine whether platelet aggregation *ex vivo* enhances the release of factors, such as transforming growth factor β[26,27] or platelet-derived growth factor,[28] that may be responsible for accelerating the healing of torpid ulcers observed[25] in hind limb ischaemia patients after a course of O_3-AHT.

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