Ozone and procaine increase secretion of platelet-derived factors in platelet-rich plasma

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ABSTRACT

Platelet-rich plasma (PRP) is gaining more and more attention in regenerative medicine as an innovative and efficient therapeutic approach. The regenerative properties of PRP rely on the numerous bioactive molecules released by the platelets: growth factors are involved in proliferation and differentiation of endothelial cells and fibroblasts, angiogenesis and extracellular matrix formation, while cytokines are mainly involved in immune cell recruitment and inflammation modulation. Attempts are ongoing to improve the therapeutic potential of PRP by combining it with agents able to promote regenerative processes. Two interesting candidates are ozone, administered at low doses as gaseous oxygen-ozone mixtures, and procaine. In the present study, we investigated the effects induced on platelets by the *in vitro* treatment of PRP with ozone or procaine, or both. We combined transmission electron microscopy to obtain information on platelet modifications and bioanalytical assays to quantify the secreted factors. The results demonstrate that, although platelets were already activated by the procedure to prepare PRP, both ozone and procaine induced differential morpho-functional modifications in platelets resulting in an increased release of factors. In detail, ozone induced an increase in surface protrusions and open canalicular system dilation suggestive of a marked α-granule release, while procaine caused a decrease in surface protrusions and open canalicular system dilation but a remarkable increase in microvesicle release suggestive of high secretory activity. Consistently, nine of the thirteen platelet-derived factors analysed in the PRP serum significantly increased after treatment with ozone and/or procaine. Therefore, ozone and procaine proved to have a remarkable stimulating potential without causing any damage to platelets, probably because they act through physiological, although different, secretory pathways.

Key words: platelet activation; platelet growth factors; cytokines; ozone therapy; transmission electron microscopy.

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Introduction

The advent of regenerative medicine has transformed the landscape of healthcare, offering innovative strategies to harness the body's inherent healing mechanisms for tissue repair and functional restoration.

Among the myriad of therapeutic approaches, platelet-rich plasma (PRP) has emerged as front-runner for its high regenerative properties, attributed to the platelet reservoir of hundreds of bioactive molecules[1,2] involved not only in haemostasis but also in inflammation modulation, angiogenesis, and cellular proliferation and differentiation.[3-5]

PRP has beneficial effects in diverse clinical applications, including orthopaedics, sports medicine, dermatology, and pain management. Positive outcomes have been obtained in conditions such as tennis elbow[6] and knee osteoarthritis.[7] In dermatology, PRP is utilized for skin rejuvenation, scar revision, and hair restoration.[8,9] Furthermore, PRP has demonstrated efficacy in wound healing of chronic skin lesions such as diabetic foot ulcers and venous leg ulcers.[10,11] In dental and oral surgery, PRP has been used to enhance postoperative healing, promote bone regeneration, and improve the outcomes of implant dentistry and periodontal procedures.[12-14]

Attempts are ongoing to improve the therapeutic potential of PRP by combining it with agents able to promote inflammation modulation and regenerative processes.

An interesting candidate is ozone (O_3), which is used at low doses as gaseous O_2-O_3 mixtures for complementary therapeutic purposes in various medical disciplines including orthopaedics, dermatology, and dentistry (recent reviews in[15-19]). The therapeutic efficacy of low-dose O_3 would rely on the induction of an oxidative "eustress"[20] that stimulates the cell antioxidant and cytoprotective pathways through activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) without inducing injury or inflammation.[21-24] Moreover, low O_3 concentrations have been demonstrated to promote angiogenesis, wound healing and tissue preservation from injury,[25-30] all key events in regenerative medicine. Remarkably, injections of PRP in association with O_3 proved to give better results than PRP alone in treating osteoarthritis[31,32] or tendon-to-bone healing[33] in both humans and animals.

Procaine (Pr), a local anaesthetic agent, has attracted attention for its potential role in tissue regeneration beyond its analgesic properties. In fact, at low concentrations Pr demonstrated antioxidant and cytoprotective properties in various disease states such as inflammation, sepsis, intoxication, atherogenesis and neurodegeneration, resulting in tissue repair and, ultimately, in "anti-ageing" effects.[34] In addition, Pr is the preferred local anaesthetic in neural therapy (a complementary treatment designed to resolve chronic pain) thanks to its ability to influence cytokine metabolism, such as Interleukin 6 (IL-6) and Tumour Necrosis Factor α (TNF-α), and promote anti-inflammatory response.[35-37] These properties have led to the exploration of Pr therapeutic potential in comparison with PRP[38] and the combination of the two treatments to enhance the regenerative processes is currently under discussion among clinicians.

In this context, in the present study we provide evidence concerning the effects of the *in vitro* treatment of PRP with O_3 or Pr or both O_3 and Pr. The study was carried out with PRP obtained from healthy subjects using a combined approach that included a comparative analysis of the ultrastructural features of platelets, providing information on their structural integrity and functional modifications, as well as a quantitative evaluation of growth factors and cytokines secreted, which play an important role in the therapeutic efficacy of PRP.

Materials and Methods

PRP samples were prepared from blood drawn from three healthy non-smoker volunteers (one male and two females aged 31-70 years, *i.e.*, the authors MAL, OA and MM), who did not take any drug for at least two weeks and did not undergo ozone therapy for at least three months. This study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013, and all participants signed an informed consent prior to blood sampling and investigation. From each subject, 36 mL of blood were collected in a syringe containing 4 mL of 3.8% sodium citrate (Fidia Spa, San Mauro Torinese, TO, Italy) as anticoagulant. It is known that sodium citrate is unable to activate platelets[39] and significantly reduces platelet aggregation in blood ozonation in comparison to heparin.[40] PRP was obtained by using the cell concentration unit Duografter II with its specific Push-Out tubes and syringes (Fidia Spa), according to the manufacturer's double spin method. Briefly, the blood was distributed in the tubes and centrifuged at 180 rpm for 8 min. The supernatant (plasma) and the upper cell pellet corresponding to the platelet-rich fraction and buffy coat were aspirated and put in a second tube that was centrifuged at 200 rpm for 10 min. After that, the platelet pellet and the lower half of plasma were collected as PRP. For each experiment 3 mL PRP samples were put in 20 mL polypropylene (O_3-resistant) syringes and treated with: i) a gaseous mixture of O_2-O_3 (16 µg O_3/mL O_2) in the proportion of 1:1 (*i.e.*, 3 mL PRP + 3mL gas) or ii) 3 mL of 2% Pr hydrochloride (Fidia Spa) (final Pr concentration 1%) or iii) 3 mL of 2% Pr hydrochloride plus 16 µg O_3/mL O_2 in the proportion of 1:1. For sake of simplicity, these experimental conditions hereinafter will be referred as O_3-treated, Pr-treated and Pr+O_3-treated samples, respectively.

O_2-O_3 gas mixtures were generated from medical-grade O_2 by an OZO2 FUTURA apparatus (Alnitec Srl. Cremosano, CR, Italy). The concentration of O_3 was chosen as the most commonly used in the treatment of many orthopaedic and dermatologic diseases, while 1% Pr is the standard concentration for this anaesthetic. Control samples were treated with air. The treatments took 20 min, and then each sample was processed for both transmission electron microscopy and bioanalytical assays.

Ultrastructural analysis of platelets

Morphological analyses were carried out at transmission electron microscopy, in order to analyse the effects of the exposure to O_3, Pr and Pr+O_3 on the fine features of platelets. To avoid possible morphological alterations due to pre-fixation cell handling, platelets were fixed in their plasma by adding an equal amount of fixative solution made of 5% glutaraldehyde and 4% paraformaldehyde in PBS. In detail, after treatment, the pellets were fixed for 2 h at 4°C; then, the liquid was removed and the samples were washed with PBS, post-fixed with 1% OsO_4 and 1.5% $K_4Fe(CN)_6$ for 1 h at 4°C, dehydrated in acetone and embedded in Epon resin.

Semithin sections (2 µm in thickness) were stained with 1% aqueous toluidine blue and observed in an Olympus BX51 microscope (Olympus Italia Srl, Segrate, MI, Italy) equipped with a QICAM Fast 1394 Digital Camera (QImaging, Surrey, BC, Canada) for image acquisition.

Ultrathin sections (70-90 nm in thickness) were stained with Reynolds' lead citrate and observed in a Philips Morgagni transmission electron microscope (FEI Company Italia Srl, Milan, Italy) operating at 80kV and equipped with a Megaview III camera (FEI Company Italia Srl) for digital image acquisition.

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Growth factor and cytokine secretion

Aliquots (200 µL) of the plasmatic fraction of PRP collected 20 min after gas and/or Pr exposure were frozen in liquid nitrogen and stored at -80°C until analysis. The amount of 13 platelet-derived factors was evaluated. The analysed growth factors were: Epidermal Growth Factor (EGF), Fibroblast Growth Factor 2 (FGF-2, also known as bFGF or FGF-β), Platelet-Derived Growth Factor (PDGF-AB), Vascular Endothelial Growth Factor (VEGF). The cytokines were: Interleukin 1 Receptor antagonist (IL-1Ra), IL-1β, IL-2, IL-6, IL-10, IL-13, Interferon α (INF-α), IFN-γ and TNF-α. Quantitation of the factors was conducted with a Luminex™ FLEXMAP 3D™ instrument (Bio-Rad Laboratories, Segrate, MI, Italy) coupled to the software Bio-Plex 6.2. Briefly, 180 µL aliquots of plasma were put in a 96-well plate. Superparamagnetic microspheres conjugated with fluorophores and antibodies against the target factors were added to the assay wells, then the plate was loaded into the Luminex system for reading and signal quantitation. Samples were run in duplicate.

Values of concentrations (expressed as pg/mL) were transformed into percentages based on the relative control values in order to make comparable the results obtained in the three subjects.

Statistical analysis

For each analysed factor, the Kolmogorov-Smirnov test was performed in order to verify the hypothesis of identical distributions in subjects for the same factor. Since this test confirmed equal distributions, the data for each factor were pooled according to the experimental condition (*i.e.*, control, O_3-treated, Pr-treated and Pr+O_3-treated samples) and the mean ± standard error (SE) were calculated. Statistical comparison was performed by the one-way analysis of variance (ANOVA) test followed by Dunn's test for pairwise comparisons (Bonferroni corrected p-values). Statistical significance was set at $p \leq 0.05$.

Results

Ultrastructural features of platelets

At light microscopy, all PRP samples appeared to be mostly composed of closely packed platelets, while leukocytes occurred in low amounts (Figure 1). At transmission electron microscopy, the morphological features of platelets from the three subjects were similar in each experimental condition. No damaged or abnormal platelets were found in any sample.

In control samples platelets showed irregular shape due to many elongated protrusions (Figure 2). They contained numerous α-granules, characterised by a homogeneous, moderately electron dense content, and a few dense (δ) granules, characterised by a dense core surrounded by a clear space.[41,42] The open canalicular system, a surface-connected complex tubular network derived from plasma membrane invagination,[43] was well developed and often dilated; some platelets showed tubular dilations containing finely granular material suggestive of secretory activity *via* exocytosis. Mitochondria and endoplasmic reticulum were well preserved, glycogen granules were abundant. Microvesicles were rarely found among platelets, suggesting a scarce secretory activity *via* vesicle release.

Platelets of O_3-treated samples resembled the control ones but they were richer in elongated protrusions and in tubular dilations containing finely granular material (Figure 3), while microvesicles were rarely observed, thus suggesting a high secretory activity through exocytosis rather than vesicle release.

Platelets of Pr-treated samples showed a more regular shape in comparison to control, with a few short protrusions (Figure 4). The α-granules were numerous and dense granules were frequently observed. The open canalicular system was well developed and composed of small tubules often grouped in clusters whereas dilations were occasionally found, suggesting a low exocytic activity. Mitochondria and endoplasmic reticulum were well preserved, and glycogen was abundant. Many microvesicles protruding from the platelet surface were observed; moreover, microvesicle clusters were frequently found enclosed in vacuoles inside platelets, protruding from their surface and free in the extracellular environment, thus suggesting a marked secretory activity through vesicle release. Platelets of Pr+O_3-treated samples resembled the Pr-treated ones, but they showed more irregular shapes due to some elongated protrusions (Figure 5). The open canalicular system frequently showed dilations with finely granular material suggestive of high exocytic activity, and only rare clusters of small tubules were observed. Similarly to Pr-treated samples, numerous microvesicles and microvesicle clusters occurred, suggesting a high secretory activity through vesicle release.

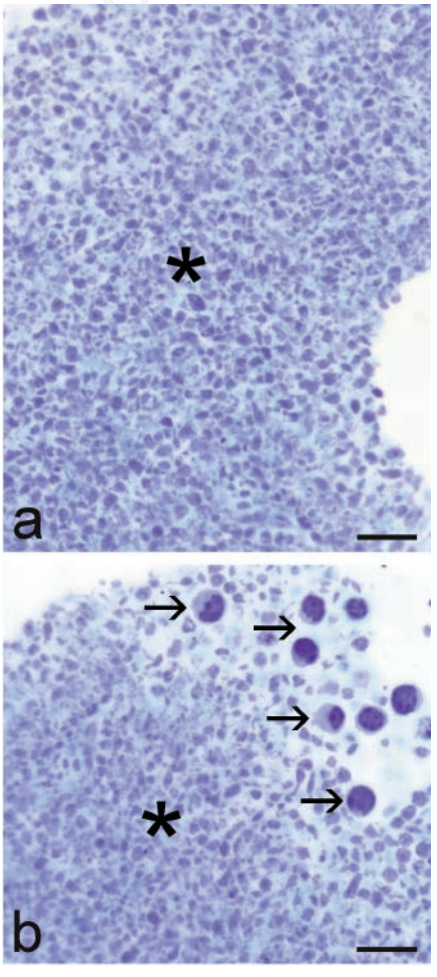

Figure 1. Light microscopy images of PRP pellets. **a**) The large majority of the sample is made of platelets (asterisks). **b**) Some leukocytes (arrows) sometimes occur at the border of the platelet pellet. Scale bars: 20 µm.

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Growth factor and cytokine secretion

Significant differences were found in the amount of most platelet-derived factors considered in the PRP plasma from samples treated with O_3 and/or Pr in comparison to control (Figures 6 and 7). As for growth factors, EGF and FGF-2 showed a significant increase after all treatments, with values significantly higher in Pr+O_3-treated samples than in the O_3- or Pr-treated ones (Figure 6 a,b). VEGF increased significantly after O_3 treatment, whereas Pr and Pr+O_3 did not induce any effect (Figure 6c). PDGF-AB did not undergo any change after all treatments (Figure 6d). Among cytokines, IL-1β, IL-6, IL-10 and TNF-α (Figure 7 a-d) showed a significant increase after treatment with both Pr and Pr+O_3, whereas they did not change after O_3 exposure. IL-2 and INF-α significantly increased after all treatments, with values significantly higher in Pr- and Pr+O_3-treated samples than in the O_3-treated ones (Figure 7 e,f). Finally, the values of IFN-γ, IL1-Ra and IL-13 were below the detection limit in all samples.

Discussion

Our morphological and bioanalytical study of the effect of O_3 and Pr on PRP revealed significant changes in platelet structure as well as plasmatic concentrations of platelet-derived factors.

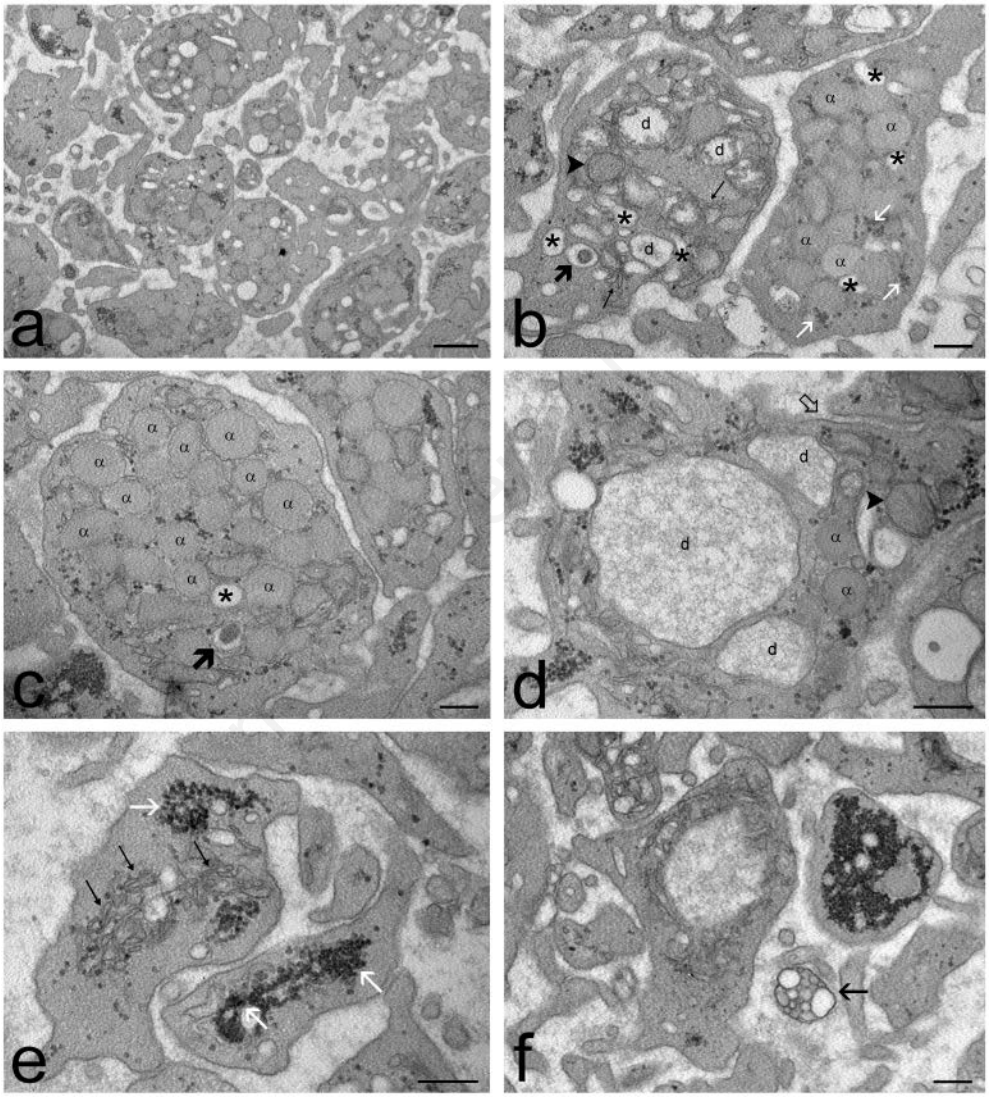

Figure 2. Transmission electron micrographs of control PRP samples. **a**) General view of the sample: the platelets show features typical of activation such as elongated surface protrusions and enlarged open canalicular system. **b**) Platelets containing α-granules (α), dense granules (thick arrow), mitochondria (arrowhead), endoplasmic reticulum (thin arrows), glycogen granules (white arrows), and a developed open canalicular system (asterisks) with evident dilations (d). **c,d**) Two platelets showing different activation stages: in **c**) α-granules (α) are numerous and the open canalicular system is not dilated (asterisks), a dense granule is indicated by a thick arrow; in **d**) canalicular dilations (d) are very evident and some α-granules (α) occur in close proximity. Note the tubule belonging to the open canalicular system that opens to the platelet surface (open arrow). A mitochondrion is indicated by an arrowhead. **e**) High magnification detail showing the well-developed endoplasmic reticulum (thin arrows) and glycogen clusters (white arrows). **f**) A rare microvesicle cluster (arrow) occurs in the extracellular space. Scale bars: a) 500 nm; b-f) 200 nm.

Platelets of control samples showed the typical morphological signs of activation compared to resting platelets, such as many elongated protrusions, and well-developed and dilated open canalicular system.[42,44,45] Some platelets showed remarkable canalicular dilations containing a finely granular material corresponding to the content of α-granules released in the surface-connected open canalicular system.[46] Occasionally, single microvesicles and clusters of microvesicles were observed, according to the heterogeneous types of membrane vesicles known to be released by platelets.[45,47,48] Taken together, these observations demonstrate a remarkable secretory activity carried out mainly by α-granules excretion rather than microvesicle release. The activated status of control platelets is consistent with the double spin procedure used to prepare PRP, aimed at yielding highly concentrated platelet-derived factors by stimulating platelet activation. Notably, this procedure did not induce any structural damage, as highlighted by transmission electron microscopy.

O_3 treatment did not change the general morphology of platelets but made their shape even more irregular with respect to control and increased the number of large canalicular dilations with α-granule content. On the other hand, microvesicle release remained scarce. This suggests that O_3 stimulates platelet secretory activity mostly *via* α-granule exocytosis in the open canalicular system but not *via* microvesicle release. Low O_3 concentrations

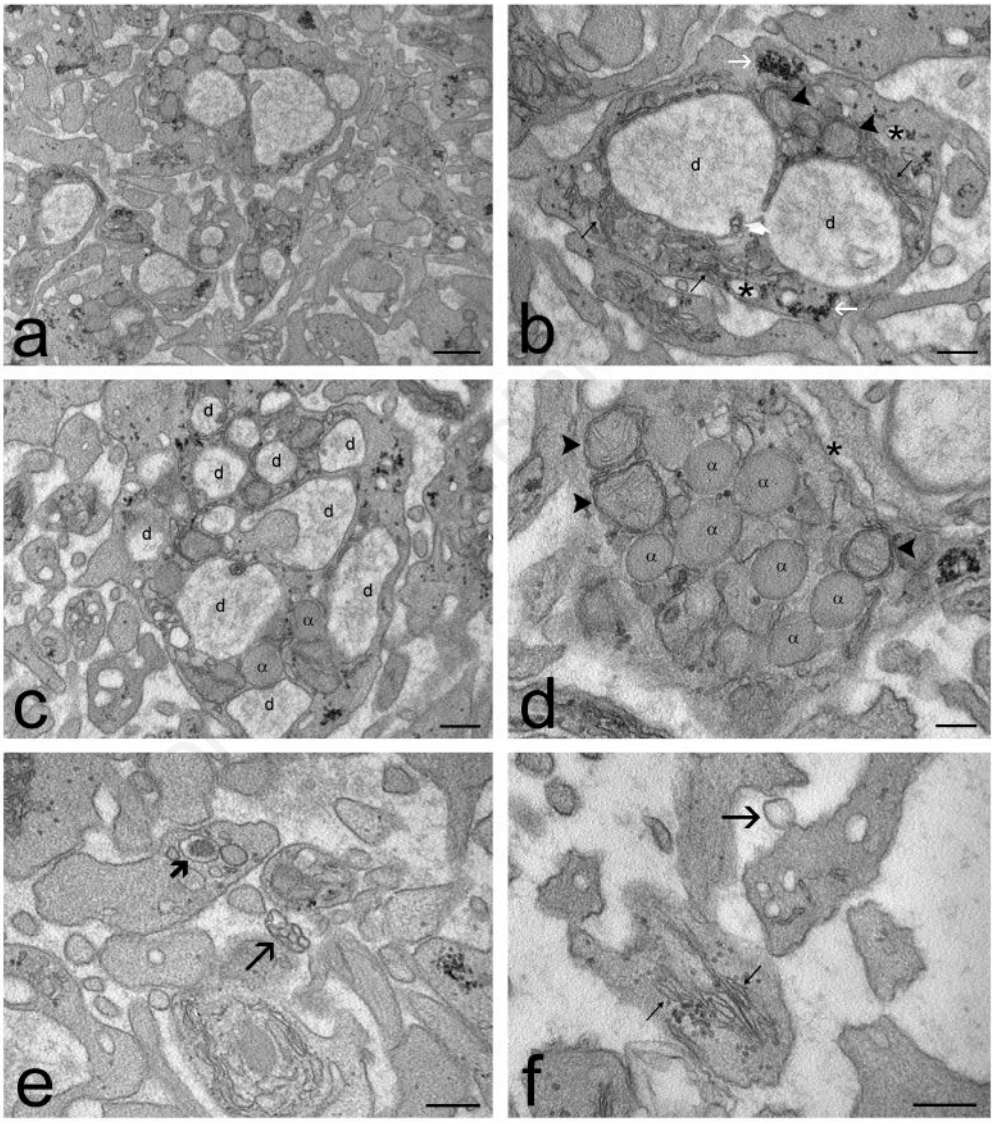

Figure 3. Transmission electron micrographs of O_3-treated PRP samples. **a**) General view of the sample: the platelets show features typical of activation, with many elongated surface protrusions and enlarged open canalicular system. **b**) A platelet showing two large, intercommunicating (white arrowhead) canalicular dilations (d), tubules (asterisks) belonging to the open canalicular system, mitochondria (arrowheads), endoplasmic reticulum (thin arrows) and glycogen clusters (white arrows). **c**) Most platelets show many canalicular dilations (d) and a few α-granules (α). **d**) Some platelets appear less activated, containing many α-granules (α) and moderately enlarged open canalicular system (asterisk); mitochondria are indicated by arrowheads. **e**) A rare microvesicle cluster (arrow) occurs in the extracellular space. A dense granule is indicated by the thick arrow. **f**) A microvesicle (arrow) budding from the platelet surface. Note the well-developed endoplasmic reticulum (thin arrows). Scale bars: a) 500 nm; b-f) 200 nm.

(such as 16 µg/mL) are known to induce mild oxidative stress unable to cause cell damage but able to influence several -although not fully understood- cell functions (reviews[24,49]). Interestingly, mild ozonation can affect the molecular organization of cytoskeleton,[50,51] which plays a key role in cell protrusion formation, organelle trafficking and endo-exocytosis, as well as it occurs in platelets.[52-56] Accordingly, it may be hypothesized that, under our experimental conditions, O_3 affects cytoskeletal proteins promoting the formation of new protrusions at the platelet surface as well as α-granule exocytosis, consistently with the higher concentration of some platelet-derived factors secreted in the plasma. The optimal preservation of cytoplasmic organelles demonstrates that this O_3-driven stimulus did not induce any structural damage to platelets.

The treatment with Pr markedly changed the general aspect of platelets, reducing surface protrusions and canalicular dilations. Pr is a well-known inhibitor of platelets activation by reducing calcium mobilization from intraplatelet storage pools and Ca^{2+} efflux across membranes.[57-59] This is consistent with the small amount of elongated surface protrusions that represent a marker for platelet activation,[42,44,45] observed in our Pr-treated samples. Pr also reduces α-granule exocytosis;[60] consistently, in our Pr-treated platelets the reduction in dilation of the open canalicular system and the occurrence of many small tubules often grouped in clusters

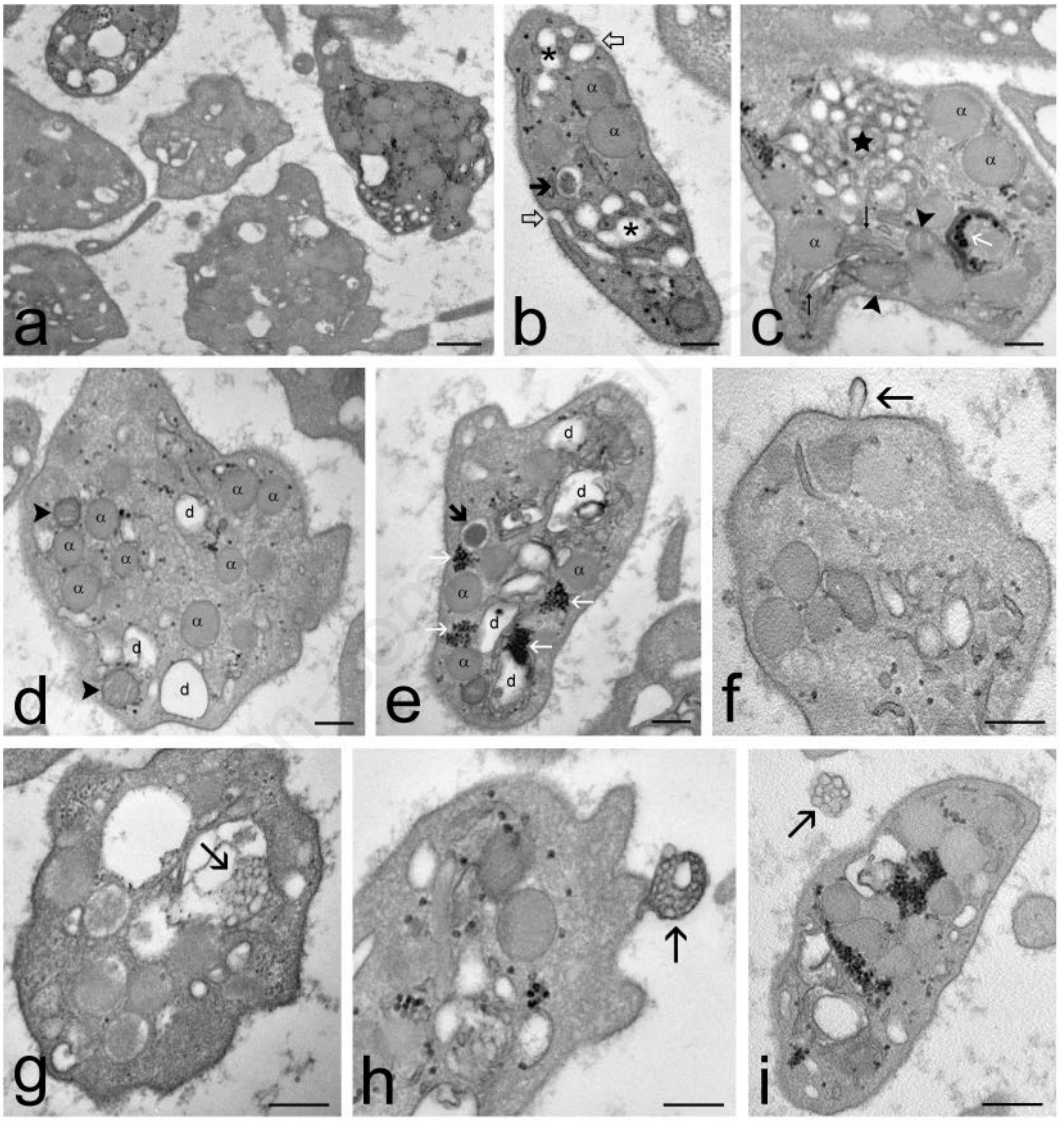

Figure 4. Transmission electron micrographs of Pr-treated PRP samples. **a**) General view of the sample: the platelets appear as activated but the surface protrusions are few and short, and the open canalicular system is moderately enlarged. **b**) A platelet containing α-granules (α) and dense granules (thick arrow). Tubules of the canalicular system (asterisks) open to the platelet surface (open arrowheads). **c**) The open canalicular system often forms clusters of small tubules (star). The platelet contains also α-granules (α), mitochondria (arrowheads), endoplasmic reticulum (thin arrows) and glycogen clusters (white arrow). **d,e**) Some platelets show moderate canalicular dilations (d). α-granules (α); dense granules (thick arrow); mitochondria (arrowheads); glycogen clusters (white arrows). **f**) A microvesicle (arrow) budding from the platelet surface. **g-i**) Microvesicle clusters (arrows) occurring **g**) inside a canalicular dilation, **h**) budding from the platelet surface and **i**) free in the extracellular space. Scale bars: a) 500 nm; b-i) 200 nm.

clearly indicate a decreased exocytic activity. However, the overall secretory activity of Pr-treated platelets was not reduced, as demonstrated by both microscopy and bioanalytical data. In fact, ultrastructural observations showed that platelets greatly increased microvesicle release, both as single units but especially as clusters, and most of the analysed platelet-derived factors occurred in higher amount in Pr-treated PRP than in control samples. It is known that Pr changes cell membrane fluidity inducing blebbing[61] and this could facilitate microvesicle release. Notably, despite the evident morphological modifications induced by Pr, no platelet damage was observed, demonstrating the safety of this treatment.

The combination of Pr and O_3 treatments resulted in platelets similar to Pr-treated samples but showing more elongated surface protrusions and more canalicular dilations containing finely granular material; in addition, the clusters of small tubules disappeared. These features are an index of increased α-granules secretion[46] and are probably due to the above-discussed effects of O_3. Notably, Pr+O_3-treated platelets maintained also a high secretory activity *via* microvesicles, as demonstrated by ultrastructural observations. This is probably the reason for the high plasmatic concentration of most of the secreted factors analysed.

Taken together, ultrastructural observations demonstrated that O_3 and Pr differentially affect platelets structural features, but both of them exert a stimulating effect on the release pathways.

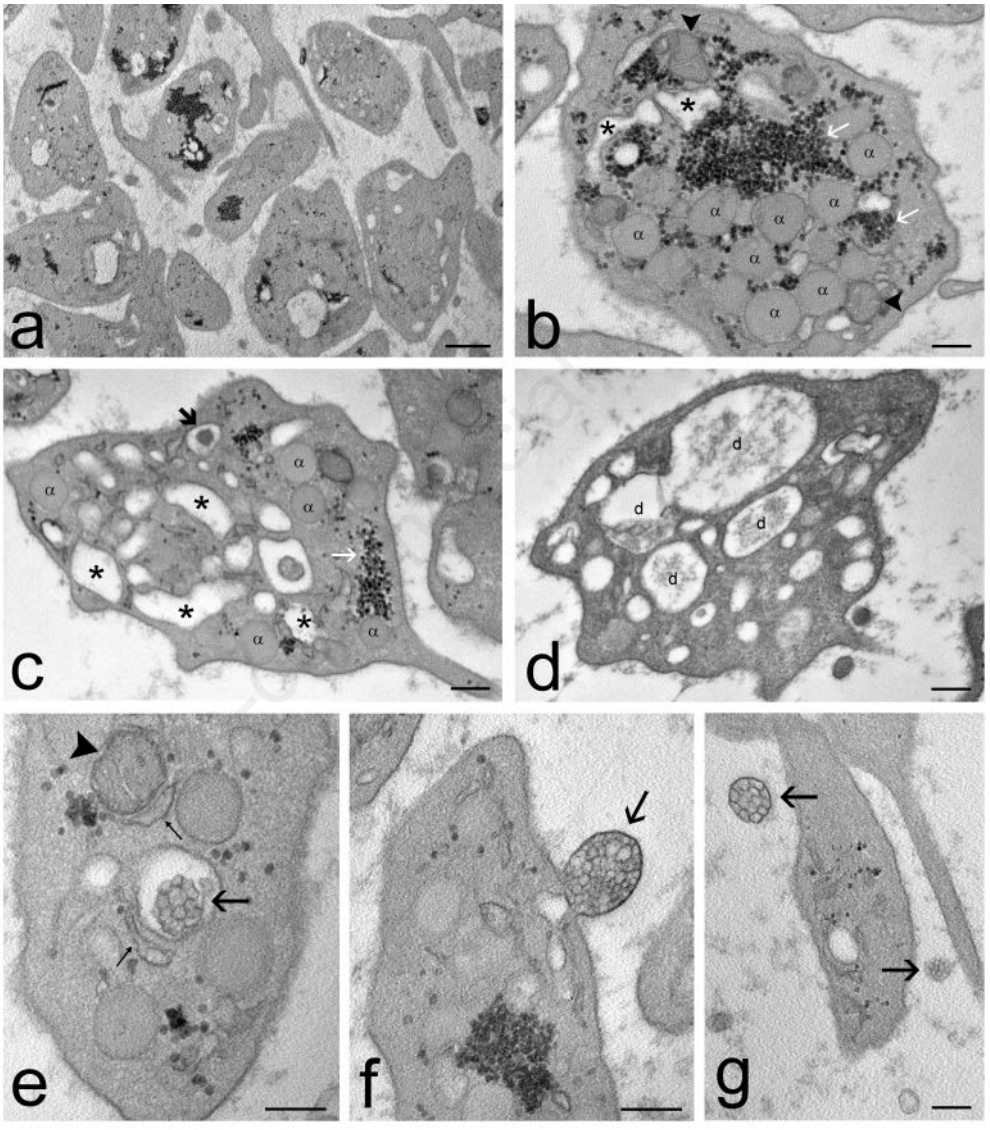

Figure 5. Transmission electron micrographs of Pr+O_3-treated PRP samples. **a**) General view of the sample: the platelets show few and short surface protrusions and many dilations of the open canalicular system. **b,c**) Some platelets contain many α-granules (α), dense granules (thick arrow) and moderately dilated open canalicular system (asterisks). Mitochondria (arrowheads); endoplasmic reticulum (thin arrows); glycogen clusters (white arrows). **d**) Some platelets show many evident dilations (d) of the open canalicular system. **e-g**) Microvesicle clusters (arrows) **e**) occur inside canalicular dilations, **f**) bud from the platelet surface, and **g**) are distributed in the extracellular space. A mitochondrion is indicated by an arrowhead; endoplasmic reticulum cisternae are indicated by thin arrows. Scale bars: a) 500 nm; b-g) 200 nm.

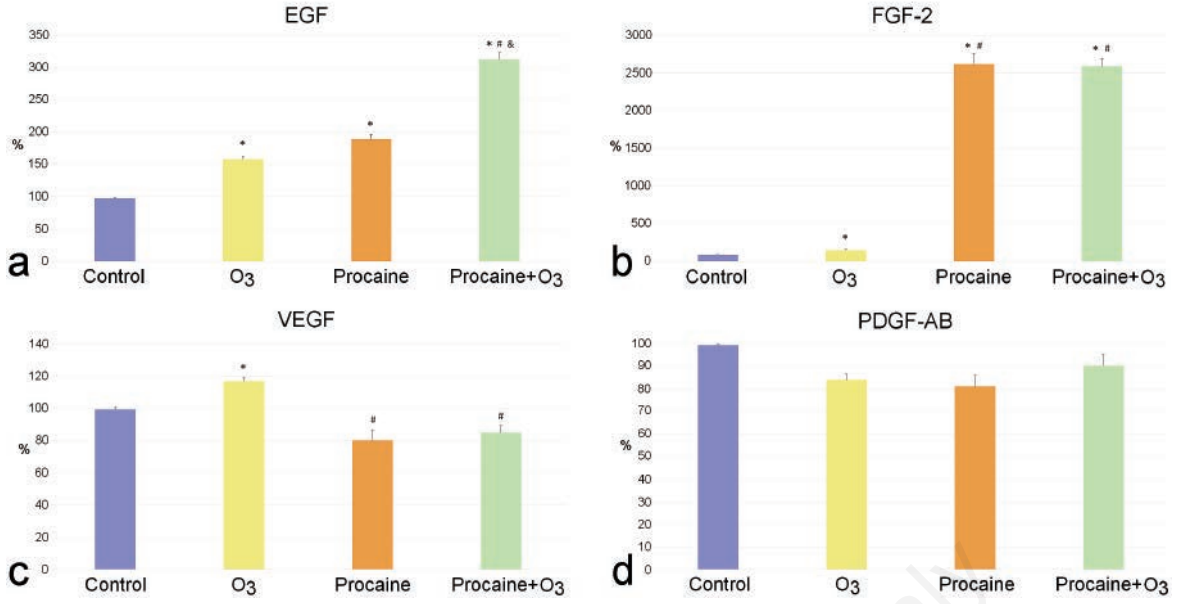

Figure 6. Mean value ± SE of platelet-derived growth factors detected in the PRP plasma of control, O_3-treated, Pr-treated and Pr+O_3-treated PRP samples after 20 min from treatment (three experiments in duplicate). Control values were set at 100%; *significant difference with control; #significant difference with O_3-treated samples; &significant difference with Pr-treated samples.

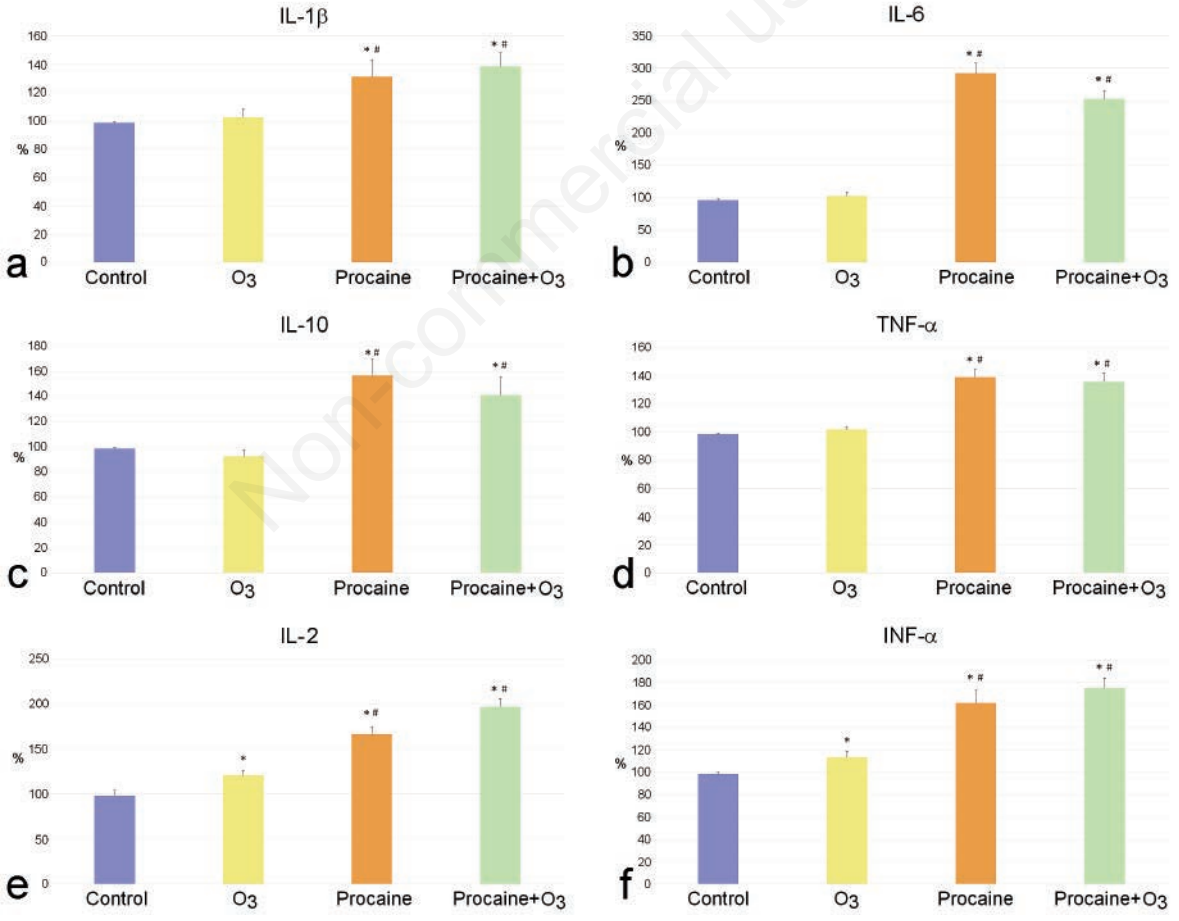

Figure 7. Mean value ± SE of platelet-derived cytokines detected in the PRP plasma of control, O_3-treated, Pr-treated and Pr+O_3-treated PRP samples after 20 min from treatment (three experiments in duplicate). Control values were set at 100%; *significant difference with control; #significant difference with O_3-treated samples.

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Consistently, O_3 and Pr treatments applied to PRP increased factor release in the plasma.

The thirteen factors analysed in our study are known to be present in PRP,[3,62,63] although some inconsistencies are present in the literature due to different experimental conditions (*e.g.*, method to obtain PRP, sample volumes, anticoagulant factors, platelet concentration). These platelet-derived factors play key roles in many physiological and regenerative processes: growth factors (EGF, FGF-2, PDGF, VEGF) are involved in growth, proliferation and differentiation of endothelial cells and fibroblasts, vessel wall permeability and angiogenesis, and extracellular matrix formation, while cytokines (IL1-Ra, IL-1β, IL-2, IL-6, IL-10, IL-13, INFα, IFN-γ, TNF-α) are mainly involved in immune cell recruitment and inflammation modulation (recent review in[5]). Therefore, these platelet-derived factors are of primary importance for the therapeutic potential of PRP since they are essential for wound healing, bone regeneration and cartilage repair.[5]

In our study, most of the analysed factors were found in all plasma samples, with the exception of IL1-Ra, IL-13 and IFN-γ, which were below the detection limit. Nine of the ten detected factors showed significant modifications in their plasmatic concentration following at least one treatment.

Pr- and Pr+O_3 induced a significant increase of IL-1β, IL-6, IL-10 and TNF-α release in comparison to control, whereas O_3 did not influence the secretion of these factors. IL-1β, IL-6 and TNF-α are all released *via* both α-granules and microvesicles[64,65] and, although scarce information is available about IL-10 in PRP, its presence has been reported in platelets secretion[62,63] suggesting similar secretory mechanisms. It may be hypothesized that the increased microvesicle release induced by Pr is responsible for the increased secretion of these factors. On the other hand, O_3 alone proved to be unable to induce an incremental effect on the secretion of these factors. It cannot be excluded that the O_3-driven stimulus would require a time longer than 20 min, as demonstrated for other platelet-derived factors in a previous *in vitro* study on ozonated human platelets.[66]

IL-2, INF-α and FGF-2 showed a significant increase in all treated samples in comparison to control, with values of Pr- and Pr+O_3-treated samples significantly higher than O_3-treated ones. The three factors are known to be secreted by platelets *via* both α-granules and microvesicles[5,41] and the above-discussed ability of O_3 and Pr to stimulate both release pathways is probably the reason for the observed plasmatic increment of these factors. Concerning in particular IL-2, it has been demonstrated that low O_3 concentrations stimulate IL-2 release by lymphocytes,[67] which occur in PRP although in low amounts, and IL-2 stimulates platelet secretory activity:[68] this could contribute to the increased amount of IL-2 found in O_3-treated samples. The strikingly augmented microvesicle release induced by Pr could explain the higher increase of IL-2, INF-α and FGF-2 in both Pr- and Pr+O_3-treated samples.

EGF showed a similar increase in both O_3- and Pr-treated samples in comparison to control, suggesting that the release of both α-granules and microvesicles play a similar role in the secretion of this growth factor.[5,41] Consistently, when the O_3 and Pr are combined, they act synergically thus significantly improving the amount of EGF in comparison to samples treated by O_3 or Pr only.

VEGF showed a significant increase only in O_3-treated samples in comparison to control. This growth factor has been found in both α-granules and microvesicles[5,41,46] but probably the increase in α-granules release induced by O_3 is more efficient than microvesicle release in incrementing the plasmatic content of this factor.

The plasmatic value of PDGF-AB was not affected by any treatment, suggesting that platelet activation due to PRP preparation promoted its strong release and no further implementation could be obtained by O_3 or Pr. A previous study reported that *in vitro* ozonisation of human platelets increased PDGF-AB release;[66] however, this discrepancy may be explained by the different experimental conditions applied, with special reference to the higher O_3 concentration used (40-80 μg O_3/mL).

In conclusion, the results of this study demonstrate that *in vitro* treatment of PRP with 16 μg O_3/mL O_2 or 1% Pr or both is able to induce differential morpho-functional modifications in platelets that always result in an increased release of factors. It is worth noting that the treatments were performed on PRP *i.e.*, a concentrate of platelets already activated through the preparation protocol; therefore, the modifications observed in the treated samples represent additional effects occurring during the 20-min exposure to O_3 and/or Pr. Hence, O_3 and Pr proved to have a remarkable stimulating potential without causing any damage to platelets, probably because they act through physiological -although different- secretory pathways. It is worth noting that the absence of cell damage is of primary importance to avoid inflammatory reactions in patients. We are aware that this work has been conducted on a limited number of subjects and must be considered as a pilot study, but the interesting results obtained make O_3 and Pr promising candidates for further research aimed at improving the therapeutic potential of PRP.

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