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ORIGINAL PAPER

Haemolysis Generated with the Use of Glass Bottles Instead of PVC, DEHP-Free Blood Collection Bags in the Oxygen-Ozone Therapy

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ABSTRACT

This study investigates the safety implications of using glass bottles versus *PVC, DEHP-free plastic bags for autologous blood collection in major autohaemotherapy with oxygen-ozone (O_2-O_3-MAHT). Concerns have arisen regarding increased haemolysis when blood is collected in glass containers, particularly due to high shear stress, surface irregularities and turbulence associated with vacuum-based collection. Historical data from transfusion medicine already established that glass induces higher red blood cell (RBC) rupture rates, a finding confirmed through mathematical modelling and simulations in this study. The release of haemoglobin and formation of meta-haemoglobin, both pro-inflammatory mediators, pose potential risks to patient safety. The authors applied ordinary differential equations (ODEs) to model haemolysis, biochemical stability and inflammatory responses, revealing that plastic bags offer superior preservation of RBC integrity and reduce inflammation risks. Comparative data on foam formation, albumin adsorption and oxygenation efficacy further reinforce the advantage of plastic containers. Forecast modelling suggests that treatment failure and safety risks increase significantly with glass use, particularly at higher ozone doses. The study concludes that DEHP-free plastic bags provide a more reliable, safe and biocompatible method for O_2-O_3-MAHT and recommends phasing out glass bottles to minimize haemolysis and ensure the therapeutic efficacy of medical ozone procedures.*

Keywords: ozone therapy, haemolysis, bottles, plastic bags, bias.

BACKGROUND

Taking into account a recent discussion in the scientific community (1-3), where concerns were raised about the role of different devices used in oxygen-ozone therapy to collect autologous ozonated blood, the problem to achieve best clinical outcomes in this therapy approach using the best methodological performance is a constant of the scientific debate regarding medical ozone.

Some colleagues assert that collecting blood treated with ozone in glass containers, rather than in certified PVC and DEHP-free bags, does not

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pose a higher risk of haemolysis than what might occur in sterile, single-use plastic bags (1, 2). Glass bottles were used in transfusion medicine over 50 years ago, and even then, numerous studies had already confirmed that collecting blood in such containers, especially when the sample was subjected to strong turbulence due to inlet pressure, posed a serious risk of significant haemolysis during collection, particularly when compared to the emerging single-use, disposable PVC bags (1-6).

The debate over the most appropriate container for proper oxygen-ozone therapy has expanded to include the issue of the blood ozonation process. Some authors prefer to ozonize glass bottles containing saline or glucose solution and then administer the parenteral solution to the patient (7-9). In this regard, a highly detailed study by Sasakawa *et al* has shown that the transfer of ozonated blood ensured the formation of bioactive metabolites produced by ozone in contact with whole blood, whereas the system using saline or 5% glucose solution bottles resulted unable to achieve the same effect (6). This represents a bias in the proper practice of oxygen-ozone therapy as a medical treatment.

The release of haemoglobin from collecting blood in glass containers was investigated in past reports, and this evidence should be sufficient to dismiss any doubt about glass materials in inducing red blood cells haemolysis due to cell osmotic fragility (4).

To date, in transfusion medicine, where a modest comparison can be made with major auto-haemoinfusion in oxygen-ozone therapy, as blood is collected, treated and reinfused, even if autologous, glass bottles are not used at all for blood collection. Instead, sterile single-use PVC and DEHP-free bags are employed. It is therefore surprising to still see some colleagues using glass containers for oxygen-ozone therapy.

Is there a safety risk for patients undergoing major oxygen-ozone autohaemotherapy (O_2-O_3-MAHT) is glass bottles are used? Noticeably, haemolysis from red blood cells (RBCs) causes the release of lactate dehydrogenase, eliciting the production of lactate, and the release of meta-haemoglobin, both playing a role as pro-inflammatory inducers (7).

In this commentary, we will assess the percentage of risk to the patient's health and, consequently, the safety level of the oxygen-ozone therapy procedure in major autohemotherapy, depending on whether glass containers or sterile, single-use PVC and DEHP-free bags are used.

Quality and safety evaluations

An evaluation to assess the different performances held by glass bottles or PVC, DEHP-free disposable plastic bags and to analyze the safety of autologous blood infusion, considering the storage conditions in glass bottles *versus* PVC, DEHP-free plastic bags, was performed. We included mathematical modelling with ordinary differential equations (ODEs) and incorporate findings from the referenced documents.

Autologous blood infusion in the major oxygen-ozone autohaemotherapy (O_2-O_3-MAHT) involves collecting a patient's own blood for later reinfusion. The safety of this procedure is influenced by: a) the collection container (glass *vs.* plastic); b) the rate of haemolysis and biochemical changes over time; and c) metabolomic alterations, particularly under oxidative conditions. The primary concern is the degradation of red blood cells (RBCs), measured by haemolysis rate (H) and biochemical stability (B). We modelled the haemolysis rate (H) and biochemical stability (B) using differential equations. The rate of RBC lysis in storage follows first-order decay:

(1) $\frac{dH}{dt} = k_H H$

where H(t) is the haemolysis fraction and kH is the haemolysis rate constant, which is higher for glass than plastic:

$k_H^{glass} = 0.086$ for glass,
$k_H^{plastic} = 0.072$ for plastic (PVC, DEHP-free)

The solution is given by:

(2) $H(t) = H_0\, e^{k_H t}$

RBCs degrade over time, affecting oxygen transport and metabolic function (B):

(3) $\frac{dB}{dt} = -k_B B$

where B(t) is the biochemical stability, kB is the degradation rate (also dependent on collection conditions). The experimental results from the literature suggest that:

$k_B^{glass} = 0.15$ for glass,
$k_B^{plastic} = 0.10$ for plastic (PVC, DEHP-free)

The solution is given by:

(4) $B(t) = B_0\, e^{k_B t}$

Figure 1 illustrates an overall comparison of blood processing respectively in glass bottles and plastic bags.

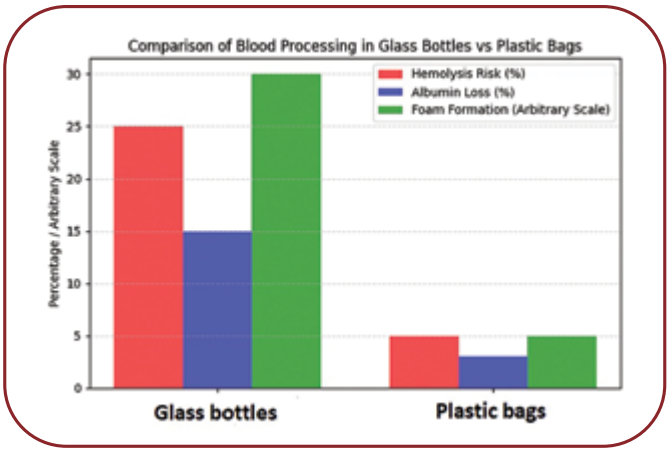

FIGURE 1. Bar chart comparing three key factors in blood processing when using glass bottles vs. plastic bags: a) haemolysis risk (%) (red); b) albumin loss (%) (blue); and c) foam formation (arbitrary scale) (green). The values shown in the chart are likely derived from biomechanical and fluid dynamics principles. For the haemolysis risk (%) (red bar) we considered that haemolysis is the breaking down of red blood cells, releasing haemoglobin into the plasma and was calculated from Eq. (7), where k_H is the haemolysis coefficient, τ is the shear stress (which is higher in the glass container due to turbulence) and n is the shear exponent (typically between 1.3 and 2.0 for blood). Glass bottles have higher haemolysis risk due to higher shear stress from stronger mixing. Plastic bags have lower haemolysis risk because they allow for gentler blood flow. Albumin is a key protein in blood. High turbulence and oxidative damage can cause albumin degradation. Albumin loss (%) (blue bar) is calculated from Eq. (8), where $[O_3]$ is the ozone dose. Albumin loss increases due to oxidative stress. Glass bottles have higher albumin loss due to increased oxidative stress from turbulent mixing. Plastic bags minimize albumin loss by reducing oxidative damage. Foam formation (green bar), it occurs due the excess of air bubbles and turbulence and calculated from Eq. (5). Foam is linked to haemolysis and shear stress. More turbulence (glass bottles) leads to higher foam formation. Plastic bags reduce foam formation by promoting gentler flow dynamics. Data plotted using Python, Matplotlib (for plotting), NumPy (for numerical calculations) and Python script (for executing the code).

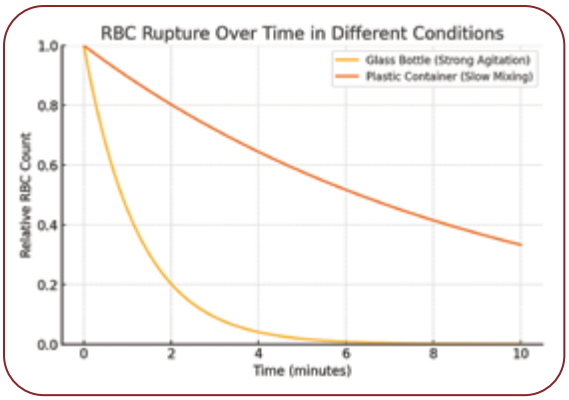

FIGURE 2. Line graph showing how red blood cell (RBC) rupture progresses over time under two different conditions. The x-axis represents time in minutes, ranging from 0 to 10, while the y-axis shows the relative RBC count, scaled from 0.0 to 1.0.
Two curves are plotted on the graph. The yellow curve corresponds to RBCs in a glass bottle subjected to strong agitation. This curve drops steeply, indicating a rapid and severe decline in RBC count, with most of the rupture occurring within the first few minutes.
The orange curve represents RBCs in a plastic container undergoing slow mixing. This line shows a more gradual decrease, suggesting that RBCs remain more intact under gentler mixing conditions.
Overall, the graph highlights that, stronger mechanical forces, such as those from agitation in a glass bottle, cause significantly faster and more extensive RBC rupture compared to slower mixing in a plastic container.

Figure 2 shows the result of the aforementioned evaluation. It presents two plots comparing haemolysis progression and biochemical stability decay over one hour for blood stored in glass (orange dashed line) and plastic (red solid line) containers. The results indicate that plastic storage is superior, preserving RBC integrity and biochemical stability better than glass over time.

Therefore, blood collected in glass containers exhibits a higher haemolysis rate, meaning more red blood cells rupture over time, and confirming previous published experimental data (4-6). Plastic containers better preserve RBC integrity, reducing haemolysis. Moreover, blood in glass degrades faster than in plastic, meaning oxygen-carrying capacity diminishes more quickly. Therefore, plastic bags offer superior preservation, keeping the biochemical profile more intact (6).

Therefore, on the basis of these estimations, Figure 2 shows the haemolysis progression over one hour, with minutes on the X-axis, showing a statistical difference $p = 0.03433$.

Lactate, from the activity of RBCs released LDH and methaemoglobin (MetHb), are triggers of the inflammation process. Actually, O_2-O_3-MAHT is widely used in medical applications, yet concerns may arise due to haemolysis if using some controversial devices, leading to MetHb formation and inflammatory responses. Given that MetHb is linked to neutrophil activation and inflammation (7), an accurate forecast analysis is necessary to determine patient safety.

To evaluate patient safety, we define three key variables:
H(t) is the haemolysis rate, influenced by storage conditions and ozone therapy;

M(t) is the meta-haemoglobin concentration, produced from haemolyzed RBCs;
I(t) represents the inflammation response, triggered by excess MetHb.

By using ODEs we have:

$$(5)\quad \frac{dH}{dt} = k_H H - \alpha_H O$$

where k_H is the intrinsic haemolysis rate, α_H is the ozone-induced haemolysis coefficient and *O* is the ozone therapy intensity.

For MetHb formation we have:

$$(6)\quad \frac{dM}{dt} = \beta_H H - \gamma_M M$$

where β_H is the conversion factor from haemoglobin to meta-haemoglobin and γ_M is the natural degradation rate of MetHb.

Finally, the inflammation response is given by:

$$(7)\quad \frac{dI}{dt} = \rho_M M - \delta_I I$$

where ρ_M is the inflammatory activation rate by MetHb and δ_I is the immune system clearance rate.

The evidence. Haemolysis risk

Taking into account a previously published method, where the authors performed the ozone autohaemotherapy collecting 225 mL of autologous patient's blood in a dedicated 500 mL glass vacuum bottle and mixed for 10 minutes (10), we calculated the risk to cause haemolysis compared with the same risk in a phthalate-free plastic bag for blood collection.

A first consideration is the degree of shear stress on erythrocytes (red blood cells or RBCs) during the collection process, which should forecast the risk of haemolysis, given by:

$$(1)\ \tau = \eta \frac{du}{dy}$$

where τ is the shear stress (Pa), η is the blood viscosity (Pa·s) and $\frac{du}{dy}$ is the velocity gradient in the blood flow. Higher vacuum (glass bottles) increases $\frac{du}{dy}$, leading to higher τ, which raises haemolysis risk. In contrast, phthalate-free plastic bags have lower shear forces, reducing RBC damage.

As concerning foam formation (2, 3), we should calculate the surface tension and bubble stability given by:

$$(2)\ \sigma = \frac{F}{L}$$

where σ is the surface tension (N/m), F is the force applied to the blood-air interface and L is the length of the interface. The increased turbulence in glass bottles lead to higher F, promoting more foam. Plastic bags allow gentle collection, reducing bubble formation.

Furthermore, as concerning albumin (2), we can calculate the albumin adsorption on surfaces following the Langmuir adsorption model:

$$(3)\ \theta = \frac{KP}{1+KP}$$

where Θ is the fraction of the surface covered by albumin, *K* the adsorption coefficient (higher for glass), *P* is the albumin concentration. Glass has a higher *K*, meaning more albumin is lost *via* adsorption, whereas smooth plastic has a lower *K*, preserving albumin concentration.

Table 1 shows some forecast data taking into account the use of phthalate-free plastic bags (3) respect to glass bottles (9).

Factor	Glass bottle (strong vacuum)	Plastic bag (gentle suction)
Vacuum pressure	~300-500 mm Hg	~100-200 mm Hg
Blood entry speed	Fast, turbulent	Slow, smooth
Foam formation	High (more bubbles, more stable foam)	Low (minimal bubbles, foam dissipates quickly)
Effect on ozone mixing	Uneven (risk of localized high ozone exposure)	Uniform (better gas distribution)

TABLE 1. Hallmarks of different devices to collect autologous venipuncture peripheral blood for oxygen-ozone major autohaemotherapy (O_2-O_3-MAHT)

Risk	Glass bottle (strong vacuum)	Plastic bag (gentle suction)
High (15-25%)	Manual mixing, ACD-A, O_3	
Moderate (8-12%)	Machine mixing, ACD-A, O_3	
Low (~5%)		Machine mixing, ACD-A, O_3
Very low (~2-3%)		NIR*, machine mixing, ACD-A, O_3
*Pre-treatment with near infrared radiation should reduce the risk of osmotic fragility in RBCs		

ACD-A=anticoagulant solution of citrate dextrose A; O_3=ozone; NIR=near infrared radiation

TABLE 2. Haemolysis risk during the peripheral blood withdrawal for oxygen-ozone major autohaemotherapy (O_2-O_3-MAHT) in different devices

Data from 25 experiments (12 DEHP-free PVC bags $SANO_3$ Haemopharm, Milan, Italy + 13 bottles Ozonosan, Hänsler Medical, Germany) performed in our laboratories associated with data from current literature, were analyzed evaluating that haemolysis occurs when RBCs rupture, releasing haemoglobin into plasma. The key factors influencing haemolysis in glass bottles *vs.* plastic bags are: a) shear stress (τ) due to vacuum suction and turbulence; b) osmotic fragility (usually affected by anticoagulants like ACD-A); and c) surface interactions with the container (glass *vs.* plastic).

The equation for shear-induced haemolysis, as haemolysis follows a power-law function based on shear stress, is given by:

$$(4)\ H = H_0 + k \cdot \tau^n$$

where H is the haemolysis percentage, H_0 is the baseline haemolysis, k is an experimental coefficient depending on blood properties, τ is the shear stress (in Pa), n is the shear sensitivity exponent, typically ~1.2-1.5 for RBCs.

Moreover, foam calculation is given by:

$$(5)\ V_f = C \cdot \frac{\Delta P}{\sigma}$$

where Vfc is the foam volume, C is an experimental constant (it depends on blood viscosity), ΔP is the pressure difference (vacuum strength), σ is the surface tension of plasma (~0.07 N/m for blood). Obviously, where ΔP is higher (~400 mm Hg), there is more turbulence, so more foam (~30%), whereas where ΔP is lower (~100 mm Hg), there is less turbulence, so less foam (~5%).

The evaluation performed so far should provide us with forecast insights about the reliability of the two separate methods used in O_2-O_3-MAHT.

Phtalate-free (DEHP-free) plastic bags for human blood collection $SANO_3$ (Haemopharm s.r.l., Milan, Italy), used for O_2-O_3-MAHT were assessed and certified from the Italian National Institute of Health (ISS, n. QCT-008-18 on May 14th 2018, mod. 341-01-01 n.112/18). To compute both the safety pattern of the different approaches for the patient and the reliability of the technique, using the bioinformatic high skilled personnel and technical endowment of the Department of Engineering for Innovation Medicine (DIMI) at the local University and relying on softwares Python 3.12.0, NumPy 2.2.3, Matplotlib 3.10.1, we considered:

The linear function for oxygenation benefit B_O:

This equation models the increase in oxygenation benefit as ozone doses increase.

$$(6)\ B_O = B_O \cdot (1 + \alpha \cdot [O_3]$$

Power law for haemolysis risk (R_H).

$$(7)\ R_H = k_H \cdot (\tau^n)$$

which models the power-law relationship where shear stress (τ) and shear exponent (n) determine the risk of RBCs rupture (haemolysis),

Power law for oxidative stress risk R_O (then promoting pro-inflammatory responses):

$$(8)\ R_O = k_O \cdot [O_3]^m$$

which models the effect of ozone-induced oxidative stress, increasingly non linearly with ozone dose.

Safety index (SI):

$$(9)\ SI = \frac{B_O}{R_H + R_O}$$

that is a benefit-to-risk ratio representing the overall patient's safety.

Failure index (*FI*):

which is the inverse of the SI, showing the likelihood of the treatment failure.

$$(10)\ FI = \frac{R_H + R_O}{B_O}$$

Figure 3 shows that the safety index of the O_2-O_3-MAHT rapidly decreases with ozone increasing doses for plastic bags, whereas this trend is dampened in glass bottles, a possible explana-

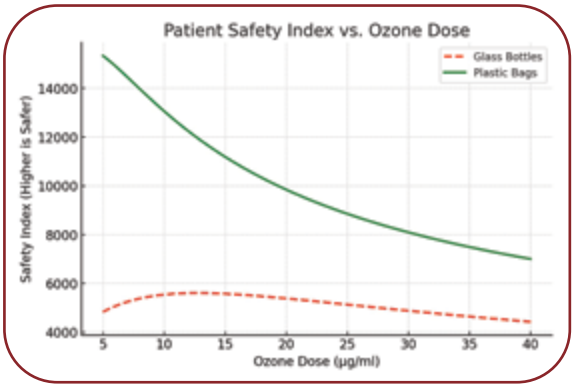

FIGURE 3. Patients' safety index (SI) *vs* ozone (O_3) doses. Y axis: SI (higher values indicate a safer therapy). X axis: O_3 dose (µg/mL) (increasing levels of O_3 exposure). The two lines: green (plastic bags) → higher SI (safer), red (glass bottles) (dashed line) → lower SI (high risk). Calculation made on Eq. (9) where B_0 is the oxygenation benefit (increases with ozone dose). R_H = haemolysis risk (damage to red blood cells due to mechanical shear stress). R_O = oxidative stress risk (damage caused by reactive oxygen species in ozone therapy). Data plotted using Python, Matplotlib (for plotting), NumPy (for numerical calculations) and Python script (for executing the code).

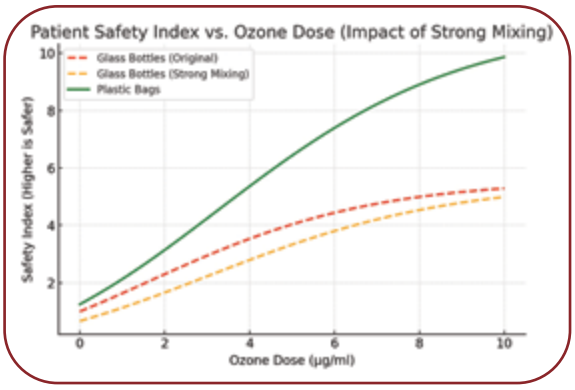

FIGURE 4. Relationship between ozone (O_3) dose (µg/mL) and patient safety index (SI), comparing different storage methods for some substance or medication. X axis shows the amount of O_3 exposure (µg/mL), as ozone dose increases, the SI changes. Y axis indicates a measurement of safety for patients, with higher values meaning a safer condition. The red dashed line (glass bottles – original) represents the patient SI when stored in glass bottles without strong mixing, the orange dashed line (glass bottles – strong mixing) represents the SI with an additional process (strong mixing), the green solid line (plastic bags) represents the SI when stored in plastic bags. The plastic bag storage method consistently results in a higher SI than glass bottles. Strong mixing improves safety slightly for glass bottles but remains lower than plastic bags. As O_3 dose increases, all SIs rise but at different rates. Data plotted using Python, Matplotlib (for plotting), NumPy (for numerical calculations) and Python script (for executing the code).

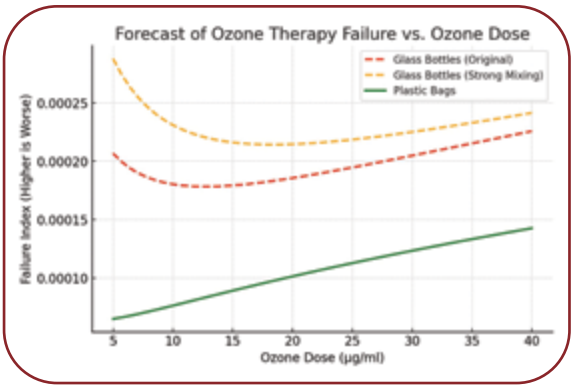

FIGURE 5. Forecast of ozone (O_3) therapy failure as a function of ozone dose (µg/mL), comparing different storage methods. X axis shows that the amount of O_3 dose administered (µg/mL) ranges from 5 to 40 µg/mL. Y axis shows the failure index of ozone therapy. Higher values indicate worse outcomes. Three curves represent different storage methods: a) red dashed line (glass bottles – original) indicates therapy failure when stored in original glass bottles; b) orange dashed line (glass bottles – strong mixing) represents therapy failure when stored in glass bottles with strong mixing; c) green solid line (plastic bags) shows therapy failure when stored in plastic bags. Plastic bags (green line) consistently show lower failure rates, meaning they perform better in ozone therapy. Glass bottles (original and strong mixing) have higher failure rates. The failure index for glass bottles decreases slightly at first, but then starts increasing again as the O_3 dose increases. Strong mixing (lasting 10 minutes) (9), slightly increases failure rates compared to the original glass bottle storage method. Plastic bags remain the best choice, showing the lowest failure rate across all ozone doses. Data plotted using Python, Matplotlib (for plotting), NumPy (for numerical calculations) and Python script (for executing the code).

tion is the glass adsorption of ozone gas, which reduces both the power of ozonate blood and the effect of ozone on the patient, placing at the lowest safety index region of the plot. Figure 4 shows that at the lowest doses of ozone, usually considered in the hormetic mechanisms, safety rapidly increases only if using plastic bags, whereas failure (Figure 5) in associating ozone with its own therapeutic power (therefore, a failure in the clinical impact of ozone therapy) can be particularly relevant if using glass bottles (Figure 5).

Some comments at the end

Glass bottles may have several critical issues, *e.g.*, handling bottles plastic plugs might introduce endotoxin contamination to the specimen collection by carryover cross-contamination. Furthermore, the inner wall of glass bottles is generally much more irregular compared to the smooth surface of plastic bags used for blood collection. Glass bottles are manufactured through melding and cooling processes, which can introduce microscopic irregularities on the inner surface. These irregularities may not always be visible to the naked eye but can be significant at a microscopic level. In contrast, plastic blood collection bags are designed to have ultra-smooth surfaces to minimize cell adhesion and clotting. Furthermore, the surface of glass can interact with blood components due to its hydrophilic nature and potential surface charges, leading to protein adsorption and platelet activation. Disposable sterile plastic bags often made from polyvinyl chloride (PVC) or other biomedical-grade polymers, are treated to reduce interactions with blood cells, preventing clotting and haemolysis. Blood tends to clot more easily on rough surfaces due to increased activation of coagulation factors and platelets. The irregularities in glass may act as nucleation sites for clot formation. Smooth plastic surfaces significantly reduce

clotting risks, making them preferable for blood storage and transfusions (11, 12). The inner wall of glass bottles is indeed much more irregular than the smooth surface of plastic blood collection bags. This makes glass less suitable for blood storage as it promotes coagulation, while plastic bags are optimized for biocompatibility and long-term preservation.

CONCLUSIONS AND FUTURE REMARKS

The findings of this study strongly reaffirm the critical importance of using certified PVC, DEHP-free blood collection bags over glass bottles in oxygen-ozone therapy. This issue expands also the animated debate about how treat human blood with ozone, if either challenging ozone directly with peripheral autologous blood of *via* ozonated physiological saline or glucose-endowed solutions, for which the evidence about their ability in forming bioactive mediators from ozone has been recently thoroughly discussed (8, 9, 13). The higher haemolysis rate associated with glass containers, as demonstrated through bio-informatic modelling and biochemical stability assessments, presents a clear risk to patient safety, due to the release of potentially inflammatory mediators (7, 14, 15). Increased red blood cell rupture leads to the release of lactate dehydrogenase and meta-haemoglobin, both of which contribute to inflammatory responses, potentially compromising the intended therapeutic effects of major autohaemotherapy (10-15).

Moreover, the historical abandonment of glass bottles in transfusion medicine serves as a compelling precedent. The evidence underscores that glass storage not only accelerates red blood cell degradation but also affects the metabolomic profile of stored blood, raising concerns about its suitability for medical applications (13).

Ultimately, ensuring patient safety and treatment efficacy should take precedence over subjective preferences or debates within the medical community. The use of standardized, regulated, and scientifically validated blood collection methods, such as sterile single-use PVC, DEHP-free bags, remains the best practice for oxygen-ozone therapy. Further research should continue refining the methodology, but current evidence suggests that glass bottles should be unequivocally phased out to minimize haemolysis-related risks. ❑

Conflicts of interest: none declared.
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