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AMELIORATION OF SYMPTOMS AND OXIDATIVE STRESS IN HOSPITALIZED CONVALESCENT POST SARS-COV-2 PATIENTS TREATED WITH RECTAL OZONETHERAPY AND NUTRITIONAL SUPPLEMENTATION

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ABSTRACT

Context: Inflammatory responses induced by SARS CoV-2 and also metabolic effects of drugs used could increase generation of endogenous reactive oxygen species which persist after viral recovering contributing to detrimental outcomes. Recommendations for therapeutic strategies are suggested in order to reestablish balance between oxidative damage and antioxidant system. *Aims:* Determining the effect of BIOPLA and rectal insufflation ozone therapy (RIO_3T) intervention in Cubans SARS-CoV-2 convalescent patients. *Materials and methods:* Forty patients received 1-BIOPLA capsule (400 mg) daily or 2-BIOPLA capsule plus RIO_3T daily during one month. The assessment of patients considers anthropometry, symptoms, chemical, haematological, and five plasma redox indexes. Twenty presumable healthy subjects were studied and considered as control group. For evaluating the effect of interventions all variables measured in final session were compared with the baseline values. Adverse reaction was checked during follow-up. Different statistical analyses were done. *Results:* Relatively to the control group, both groups of convalescent patients had significant differences in some redox, chemical and haematological indexes at baseline ($p<0,05$). Effect evaluation showed beneficial improves in both treated groups resulting in 85% of patients for BIOPLA plus RIO3T and 37% for BIOPLA ($p<0,05$). Considering all redox indexes values of groups at initial and final extraction, multivariate statistical analysis clearly separated groups, obtaining two canonical functions ($p< 0,05$). Symptoms were totally reduced. No side effects were observed. Conclusions: These results corroborate that substantial oxidative stress and symptoms persists in COVID 19 post condition ameliorating with RIO3T and BIOPLA interventions. Redox indexes diagnosis is worthwhile to conduct a more comprehensive study and manage of conditions.

KEYWORDS: Oxidative stress, SARS-CoV-2, ozonetherapy, supplementation, convalescence.

INTRODUCTION

There is lack of a specific treatment for COVID-19, a recent outbreak discovered in Wuhan, China; resulting in a highly contagious disease that spread throughout China and other countries and caused global concern and emergency. According to the World Health Organization report, 250 countries with almost 61 million of persons are affected worldwide since December 2019 to November 2020.[1-3]

Patients with COVID-19 (who were infected by SARS-CoV-2) are reported to present symptoms varying from fever, dry cough, myalgia, fatigue, and diarrhea, etc. in relation to the patients' age and morbidities associated. Recent published research suggests that SARS-CoV-2 produced marked inflammatory and immune responses

that may activate a "cytokine storm", and apoptosis of epithelial cells and endothelial cells; subsequently, vascular leakage, abnormal T cell and macrophages responses ensue and induce oxidative stress (OS) status.[4-6]

Many host cellular factors have been identified as being involved in the viral infection and the various steps of the life cycle or viral products interfere with the homeostasis of cellular metabolism, thus triggering a stress pressure to the host cell. In general, cellular metabolism steadily produces reactive oxygen species (ROS), as a byproduct of the normal aerobic metabolism, by a variety of enzymes in mitochondria, endoplasmic reticulum (ER), and peroxisome compartments, and simultaneously the oxide molecules are removed to keep the balance.[7-10] Also immunological cells produce ROS as effector response to pathogens attributable to its antimicrobial properties.[11]

OS is defined as an imbalance between the generation of oxidants and the potential of antioxidant defenses of cells, as well as the ability of damage repair mechanisms to favor an excess of oxidants. This unbalanced state can cause tissue damage or lead to the production of toxic species to all cell components.[12,13] OS may lead to disturbance in the normal oxidation-reduction state of a cell resulting in adaptation-resolving oxidant tone (eustress) or damage of the cellular components (distress).[13,14]

Related literature indicates that OS is involved in several human diseases, such as inflammation, diabetes, arteriosclerosis, autoimmune disorders, skin diseases, hypertension, cancer, eye diseases, infectious diseases, among others.[15-18] Over the past decades, the OS theory has strongly stimulated interest in the role of antioxidant defense mechanisms in removing ROS, i.e. antioxidant enzymes and non-enzymatic antioxidants.[19,20] Concerns have been raised about the benefits of redox modulation linked with adaptation of cells to OS and the ability to enhance the cell antioxidant system.[21] Therefore, the maintenance and restoration of a favorable intracellular and extracellular redox environment are vitally important for the host to maintain signaling, energetic process and combat against virus infection. [22] According to previous analyses redox biomarkers are necessary to evaluate in order to determine status but also the effect of interventions on the oxidant/antioxidant systems and its relation to clinical outcome. [23-25]

COVID-19 is seen as a disease that primarily affects the lungs, but considering OS involves in pathophysiology and its influence with inflammation and drug toxicity, it can damage many other organs as well. This organ damage may increase the risk of long-term health problems.[26-28]

Recent reports suggest that 87% of COVID-19 patients, although being negative for the viral nucleic acid test, still present a high level of inflammation and persistent symptoms for at least two months after onset of disease, despite resolution of viral infection.[29-31] A significant number of patients who have tested positive for SARS-CoV-2 virus remain unwell beyond three weeks after negative test, and also for months. Some people's recovery is prolonged with most common symptoms as fatigue, dyspnea, joint pain, and chest pain.[32] These events caused in part by persistent viraemia in other fluids and organs than nasopharyngeal, due to weak or absent antibody response, relapse or reinfection, inflammatory and other immune reactions, deconditioning, and mental factors such as post-traumatic stress may all contribute and it´s related to to oxidative tone.[29,31] Long term respiratory, musculoskeletal, and neuropsychiatric sequelae have been described for other coronaviruses (SARS and MERS), and these have pathophysiological parallels with post-acute COVID-19. Post-acute COVID-19 ("long covid") seems to be a multisystem disease, sometimes occurring after a relatively mild acute illness, requiring strategies and intervention in order to restore biological conditions.[33]

Several researches have reported that in a highly OS, meanly produced by chronic activation of immune system, the progress of COVID-19 infection can be stimulated in the host.[9,10] Redox imbalance could mediate modulation and inflict activation of transcriptional signal, exerting specific cellular dynamic including apoptosis and tissues damage which in turn might conduce to dysfunction related or not to the pathology.[13,15,16]

That's situations suggest a potential amplifying mechanism involved in CoV-induced OS. That why the attenuation of the OS by targeting several key steps in the pathophysiology process could bring about improved outcomes.[34] Initials attention appoint to nutrition as primary source to incorporate micronutrients with antioxidant qualities. Nevertheless there are some limitation to supply the necessary amount of antioxidants to counteract oxidation on diseases process, also there are a potential stress associated to repurposing drugs recommended to use as antivirals, it is required indeed a nutritional supplementation and other bioxidative therapies in order to obtain beneficial modulation of OS.[35-38]

Considering previous aspects, a multidisciplinary team supervised by Cuban Health Ministry Committee proposed an assisted convalescent protocol for post COVID-19 conditions using BIOPLA nutritional supplementation and rectal ozonetherapy based on its redox modulation properties demonstrated previously in different preclinical[39-41] and clinical conditions.[42,43]

BIOPLA is a dietary complement based on a concentrate protein - mineral elaborated from human placental after born registered as PN R10324/20 in Cuban regulatory

agency. It has been used for nutritional support to aged individual of any sex or sport practitioner since 2010 in Cuba. Diverse researches showed not adverse reactions report after use for six months or one year. BIOPLA is not virucidal but it has indirect anti-viral actions because it's anti-inflammation, anti-oxidation actions and immune enhancing properties.

Also, treating the patients with ozone/oxygen therapy (O2/O3) since initial phases of the disease is believed to be favorable and it is recommended and researched by different authors. [44-46]

Oxygen-ozone has a high solubility in plasma and induces formation of two-second messengers: peroxide (H_2O_2), ozonides and alchenals in different biological fluids.[47-48] These are the mainly reactive which interacts with the membrane proteins and receptors of the immunocompetent cells entering the cells and they interact with signal transduction proteins on the nucleus and at mitochondrial level.[47] The key action mechanism of O2/O3 therapy is the interaction of second messenger on proteasome and inflammation cascade, to regulate inflammatory process, by stimulating the nuclear factor Nrf2 and by inhibiting nuclear factor NFkB.[41,49-51]

The O2/O3 therapy can restore the right immune response by stimulating signal transduction molecules via Nrf2 and thus stimulating the nuclear transduction via specific microRNAs restoring the normal antioxidant and immunostimulating reaction. Ozone can be administered by different routes, by the systemic (major autohemotherapy, saturated saline solution, rectal and vaginal insufflation) or non-systemic technique (minor autohemotherapy and bag ozone therapy). Clinical studies in different diseases contributed with evidences of effectively actions and minor adverse reaction (adequate risk-benefit balance).[52-56] There are currently diverse clinical trials that are being carried out in China, Italy, Iran and Spain and seek to assess the effectiveness of major autohemotherapy or saline solutions on SARS-CoV-2.[57-59] There is no experimental study that is assessing the administration of systemic ozone by RIO3 technique in post COVID-19 condition. RIO3 counted with beneficial evidences of effectiveness with high safety in different clinical conditions. The present document mainly proposes to determine the effect of BIOPLA® and BIOPLA® plus RIO3 intervention in Cubans SARS-CoV-2 recovered patients.

MATERIALS AND METHODS

The complementary application of RIO3 and BIOPLA® supplementation was done in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines of the International Conference on Harmonization. The exploratory study protocol was approved by the Scientific Committee of hospital (by fast tract) on May 17, 2020 and ethics committee of University of Informatics Science Hospital in Havana Cuba. This trial has also been approved and registered at Cuban Ministry of Health by Cuban Center of clinical trials (code number RPCEC 00000342).

The application of RIO3 plus BIOPLA® was done following the criteria of a pilot, open label, phase IV clinical trial, between June 1th to July 7th 2020, aiming to first treat 20 hospitalized patients with SARS-CoV-2 recovered status with persistent symptoms, other 20 patients, also with persistent symptoms received only BIOPLA® and as a second aim, to assess the safety and efficacy of RIO3 and BIOPLA®. These 40 patients were hospitalized at Ernesto Guevara Hospital, belongs Informatics Science University of Havana. The hospital has all source documents archived in medical recording system. Clinical analyses and laboratory examinations are also available locally. The subjects of the study consist of individuals who recovering SARS-CoV-2 infection previously confirmed by PCR. In the absence of agreed definitions, for the purposes of this article we define post-acute COVID-19 convalescent condition to individuals with symptoms extending beyond three weeks since negative PCR test. All patients were treated during acute COVID-19 with Kaletra (Lopinavir/Ritonacir), Chloroquine and Interferon α 2b according consensus protocol approved on April 2020 by a Designed Commission by Ministry of Health. A negative test for COVID-19 is a prerequisite. Forty eligible patients meeting the inclusion criteria were recruited and maintained on hospital (i.e., history of fever and any respiratory symptom, e.g., cough or rhinorrhea); male or female aged 32-85 years old at the time of inclusion; within 3 weeks of negative PCR test; who did not participate in other clinical studies within the last three months. A detailed written explanation of the study process, researchers' names and institutions, possible benefits and potential adverse effects of the interventions were explained to the participants and additional information should be provided upon request. Informed participants were asked to sign a written informed consent. A convenient sample was divided into two groups: one group received BIOPLA®, the other BIOPLA® and RIO3, both groups in concomitant manner to continuous COVID-19 therapy. Both groups were treated and followed during one month. Participants were allocated at the time of inclusion and were subsequently identified throughout the application of O3 only by their allocated number, always assigned in chronological order. This was an open label application of BIOPLA® and O3. BIOPLA® was indicated 1 daily capsule of 400 mg during 30 days. RIO3 was indicated with increasing dose of ozone/oxygen. The first week, 150 mL of gas with 20 μg / mL of ozone concentration every 24 hours during five days, 150 mL at 25 μg / mL every 24 hours the second week (5 days), 150 mL at 30 μg / mL every 24 hours for the third week (5 days) and 200 mL at 35 μg / mL each / 24 h for the last fourth week (five days). Ozone generator used was OZOMED Plus® with inscription number 1029N and Cuban regulatory agency number I 10290002.

Twenty individual supposedly healthy (ISH) were enrolled and evaluated as control group.

At the initial visit all participants required to complete anthropometrics (weight, height), demographic and age data information, prior to the study. A comprehensive physical exam was performed, a complete medical history was appointed and specific lab tests were indicated by the trial clinician in turn. All participants were instructed to take their usual diet and physical activity during intervention period. Follow- up procedure was done by week questionnaire application that was included in individual clinical history of patients.

Measures of Compliance

The patients received their drugs daily by nurses. The compliance was determined as the proportion of unused capsules related to the total number of the capsules for the supplements.

Unused capsules number refers to capsule which were not taken by patients in the time schedule.

O2/O3 rectal insufflation was also recorded to evaluate compliance. The compliance was determined as the proportion of not application done relate to the total number of insufflation scheduled.

Biochemistry measurements

After an overnight fasting (10-12 hours) at 8:30 – 9:30 a.m. in the morning venous blood samples (20.0 mL) were collected. Some haematological and chemical indexes were assayed. The remaining blood is put into a dry tube for serum extraction and after analyzed for biochemical profile characterization. Sera are transferred into clean micro- tubes in aliquots for determine proposed schedule of indexes measurement.

This study was open, implying that both participants and the conductors of the trial (individuals providing drugs to patients, those performing physical examinations and taking histories in addition to those in charge of performing lab tests) were ware whether each patient is receiving BIOPLA® or BIOPLA® and RIO3T.

Redox plasmatic, hematological and chemicals indexes were obtained using validated analytical techniques for diagnosis in humans according to international recommendations. Reference values were established in a healthy supposedly population considered as a control. Two extractions have been considered the first at baseline and the second one month after interventions.

For assay of superoxide dismutase and catalase activity, hemoglobin was extracted from haemolysate. For the rest of the indexes, 3 mL of serum were employed. Serum samples were frozen at - 70°C and protected from light exposure until analyses were carried out.

All redox parameters were determined by spectrophotometric methods using Zuzi Spectro-photometer from Japan.

Glutathione Concentration

GSH from Sigma, St. Louis, M.O., USA was used to generate standard curves. Serum GSH concentrations were measured by the kinetics assay using the glutathione reductase reaction. Autoxidation of GSH to GSSG was prevented by addition of N-ethylmaleimide to the samples.[60]

Malondialdehyde Concentration

Malondialdehyde (MDA) concentrations were analyzed with the LPO-586 kit obtained from Calbiochem (La Jolla, C.A., USA). In this assay, stable chromophore production after 40 min of incubation at 45°C is measured at a wavelength of 586 nm by Pharmacia Spectrophotometer. To ensure that no lipid oxidation occurs during the assay, BHT [0.01% (v/v) of a 2% stock solution in ethanol] and EDTA (1 mM final concentration) were added to the sample prior to assay develop. Freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma, St. Louis, M.O., USA) assayed under identical conditions were used as reference standards. Concentrations of MDA in serum samples were calculated using the corresponding standard curve and values were expressed as nmol g-1 Hb.[61]

Superoxide dismutase (SOD)

SOD activities were assayed by a modified pyrogallol autoxidation method.[62]

Catalase (CAT)

CAT activity was measured according with the method of Clairbone. Using a molar extinction coefficient of 43.6 M-1 cm-1, the rate of the first 30 s was used to calculate the activity. Catalase activity was expressed as U mg-1 Hb. [63]

Advanced oxidation protein products (AOPP)

Serum AOPP was measured according to the methods of Witko-Sarsat et al, 1998.[64] The values were expressed in chloramine T equivalents and corrected by serum albumin concentrations.

Biochemical indexes

Blood parameters such as hematocrit, hemoglobin, and erythrocyte sedimentation rate (ESR) were screened by Hematological counter MICROS 60. Others as triglycerides, creatinine, cholesterol and alanine aminotransferase activity were performed by standard procedures in HITACHI analyzer 912, all in a specialized laboratory of Hospital.

Adverse reactions vigilance

After ozone application and BIOPLA capsule consumption patients were interviewed for identify any non-beneficial or negative symptomatic effect. A model

33 36 1 (official questionnaire from MINSAP) was used in case a positive reaction was identified to notify to medical staff and CECMED (Cuban regulatory agency). Equipment's and drugs were allocated as stock permanently to treat any adverse reactions identified.

Statistical analysis

All analysis was performed using SPSS software (Version 22, SPSS Inc., Chicago, IL, USA). Normality of data was tested with the Kolmogorov- Smirnov test. A two-sided P value less than 0.05 was considered statistically significant.

For descriptive statistics of continuous variables, means and standard deviations were calculated, whereas categorical variables were expressed as proportions. The normality of variables was evaluated by the Kolmogorov-Smirnov test and variance homogeneity was evaluated by Levene s test. Comparisons between the groups before and after interventions and respect control groups were assessed using Student T test for pared and non-pared samples followed by a post hoc Newman Keuls method.

It was analyzed if the values and the mean of the biochemical and hematological indexes were in the reference interval reported in the literature.

For each biochemical and hematological indexes were considered how many patients had significant change (difference between parameters at baseline and 1 month after treatment) according treatment respect reference values.

Simultaneous change in biochemical and hematological indexes were also analyzed for each treatment. A combinatorial variable evaluates beneficial change in antioxidant system and damage of biomolecules (simultaneous positive change in AOPP, CAT, SOD, GSH and MDA) considering indexes with significant difference in intra group comparison. For each patient global success was considered every time as a simultaneous change was positive, but if at least one of the parameters result in negative modification it was considered as a global failure. Frequency of global success was reported for each group. In addition to the null hypotheses test, a magnitude bases inference was performed. For inter and intra group comparisons the chance was calculated that the true modification of the mean of each studied group was beneficial-better, unclear, and detrimental-worse. The quantitative chances of beneficial/better or harmful effects were qualitatively accessed, using the following classification: <1% certainly not, 1–5% very unlikely, 5–25% unlikely, 25–75% possible, 75–95% probable, 95–99% most likely, and >99% almost certainly. If the chance of the evaluated effect being beneficial/better or harmful/worse was>5% for both situations, the differences were classified as "unclear."

An exploratory multivariate discriminate analysis was performed combining redox indexes values for each group at different moment of study.

RESULTS AND DISCUSSIONS

The COVID-19 pathological aspects are of utmost importance to better understand the extent and type of damage associated with this infection and its possible pathogenesis in order to adopt rationale-based clinical therapeutic strategies.[26,34]

The baseline characteristics of the 60 studied subjects are showed in Table 1. The supposedly healthy individuals (SHI) were considered as controls. There were no statistical significant differences between the groups at baseline according to demographics, gender and number of patients ($p>0.05$) except for body mass index (BMI) which is lower in both convalescent groups respect SHI, and 80 % of patients in convalescent groups (30/40) were classified as underweight range. As a real-world study 29/40 had preexistent conditions (Chronic heart disease, Asthma, Stroke, Hypertension, Diabetes) and 23/40 had toxic habits (Smoking, Alcoholism). Major percent of patients in two groups of convalescent were older than 32 years, skin color white and were on long term treatment due to history of previously diseases.

Previous clinical findings showed elevated levels of ROS strongly correlated with inflammation[14,65,66] oxidative injury.[11,24,57] as well as viral infection and replication which further exacerbates the immune response.[16,68-71] As a result, an increasing number of leukocytes are recruited; further releasing ROS and cytokines, resulting in hyperinflammation and cytokine storm syndrome in different diseases.[12,14,72]

ROS are a class of partially reduced metabolites of oxygen that possess strong oxidizing capability, which are generated as byproducts of cellular metabolism through the electron transport chains in mitochondria and cytochrome P450.[14,25,72] The other major source are oxidases (e.g., NAPDH oxidase), which are ubiquitously present in a variety of cells, particularly phagocytes and endothelial cells.[15]

Under a pathological condition, where ROS are excessively produced but antioxidant enzymes are insufficiently presented, H2O2 may accumulate locally or systematically, oxidizing proteins with sulfur-containing residues (cysteine and methionine) and reacts with transition metals (e.g., iron) previously demonstrated as altered in COVID-19 patients.[9,10,16] Also lipids, carbohydrate and nucleic acid could be oxidized and product might be identified by damage associated molecular patterns (DAMPs) triggering activation of NFkB producing inflammatory gene activation. Thus contribute to inflammatory persistent cycle and tissues damage. Oxidized biomolecules generate downstream ROS that are highly active.[65,73]

Oxidative stress generated in COVID-19 by innate immune response activation, inflammation and drug detoxification can influence the oxidation chemical reaction with the particular ability to induce both biomolecule functional alteration and redox regulated signal of cell death in different tissues.[8-10]

All patients took the total of capsule recommended and received ozone application during 1 month. The mean value of all biochemical and redox indexes evaluated for control and both studied groups are shown in Table 2 and 3.

MDA (marker of lipid peroxidation) and AOPP serum concentrations were significantly higher ($p<0.05$) in convalescent groups at baseline respect control group, without differences between them. GSH serum levels in convalescent groups at baseline were significantly lower ($p<0.05$) respect control. The activity of the erythrocyte antioxidant enzyme SOD and CAT were significantly higher in studied groups respect control at initial extraction ($p<0.05$). After 1 month of intervention serum levels of MDA, AOPP, GSH, and SOD activity showed significant difference ($p<0.05$) in group BIOPLA/RIO3 respect baseline values with a tendency to control group values. Also serum levels of GSH showed significant difference ($p<0.05$) in group with BIOPLA respect baseline. Serum GSH levels in group BIOPLA/RIO3T at final extraction showed significant differences between groups ($p<0.05$). In all final values for redox indexes persisted significant difference respect control group values (Table 2).

Simultaneous modification analyses of redox status permit to identify at baseline that almost patients presented alteration in CAT, SOD, MDA, AOPP and GSH in relation to control group. Hemoglobin, ESR, ALAT, UA and TP mean value of convalescent groups respect control group showed significant differences ($p<0.05$) at baseline. Also BMI is altered in almost patients presenting nonspecific symptoms as previously reported.[74,75]

Glucose, ASAT and creatinine mean values in both group showed no significant difference respect control ($p>0.05$) remained on interval considered as physiological-reference.

At final extraction Hemoglobin, ESR, ALAT and UA mean values in both studied groups showed significant modification ($p<0.05$) respect baseline and all in the interval considered as physiological-reference (table 3). Individual analyses identified beneficial chance globally in 55, 3 % of patients in Hemoglobin, 30% in Uric acid and 65, 8 % in TP.

Simultaneous analyses permit to identify the percent of patients who expressed beneficial modification in a set of redox variables resulting higher in BIOPLA/RIO3T group (85%) respect BIOPLA group (37%). (Figure 1). Otherwise of effects magnitude evaluated the qualitative evaluation permit to consider as probably and most likely associated to interventions the modifications observed in variables analyzed in both groups of interventions.

Also beneficial modification of BMI was identified in 86,2% of total patients obtaining normal status and the totally of patients referred no experiment any of symptoms described at baseline.

Multivariate discriminate analysis considering OS indexes in each group is showed in Figure 2.

Discriminant analysis revealed that 95,7% of the variation between groups was accounted by the two firsts discriminant functions ($p=0.000$) (Figure 2).

The biochemical redox indexes were related in these two functions with discriminant loadings of 0.982 and 0.814 respectively.

Interestingly, preliminary observations point out that antioxidant and ozonetherapy complementary application in Cubans COVID-19 convalescent patients promoted clinical and redox status improvement.

Related to antioxidant biomarkers 60% of patients in group with BIOPLA increased values were detected and 90% in BIOPLA plus RIO3T group. Thus results should be attributed accordingly to the analysis as probably to intervention applied. Almost redox indexes modified showed a simultaneous reduction of 47% of total with tendency to values of physiological conditions (control). It is possible that some patients couldn't reach beneficial values because of preexistent diseases and toxic habits associated also to oxidative stress alterations as hypertension, cardiovascular, asthma and diabetes.[15] Patients who not oxidative balance modifications were detected exhibit more than one chronic diseases and also toxic habit as smoking and alcoholism increasing oxidative pathways,[21,72] otherwise not detrimental indexes were observed. The results of beneficial modification were expected because BIOPLA had different components with secondary antioxidant properties and the major ozone targets are redox balance promoting also antioxidant genes expression as were observed in the study, and secondary reducing inflammation as previously demonstrated.[39,40] Others studies using rectal ozone insufflation detected improvement in redox balance and clinical status in diverse diseases.[51,53,54] One emphasizes had to be done respect GSH concentration that are a tripeptide resulting a co-sustrate in multiple biochemical reactions which present secondary antioxidant capacity. This biomolecule is depleted in diverse pathological conditions and also in COVID-19 infection. Results exhibit a GSH recuperation, and enzymatic activity increase that could be responsible among others aspects of redox circuits of protection to lipids and proteins observed in the study.[76,77] The reductions in circulation of oxidized

biomolecules is probably influencing to DAMPs exes, its reducing or non-activation in turn could reduce the induction of signal associated and reduce of inflammation observed in the context of disease and in convalescent condition.[78-81] The mechanism underlying COVID-19 inflammation and clinical diversity have not yet fully elucidated.[82] Otherwise interventions effects observed are closely related to medical ozone application as previously have been demonstrated in other pathological conditions.[48,51]

Also regulation of a cytokine pattern when mononuclear/macrophage cells in peripheral blood are activated by medical ozone has been previously demonstrated. Thus can developed responses that make it less reactive that certain challenge. It conduces to both tolerance, counteracting extensive tissue injury by enhancing Nrf2 genes expression and enhancing the cell response to improve tissue surveillance, diminishing inflammation response.[13,41,47,83-85] In that consist the ozonetherapy, a low stimulus promoting an enhance induction of antioxidant response, a reactivity of low grade to a subsequent stimuli.[47,83-85] Also ozone has the potentially of disrupted virion structure and could sterile rectal area and intestine, it is probably reducing the possibility of oral fecal transmission.[86]

Discriminant analysis considering progression and redox indexes works by conforming one or more linear combinations of predictors detected, creating a new latent variable for each function. These functions called discriminant functions could express accumulated variance contemning on data.

These analyses offer an integral observation and synthetic representation of multiple indicators and also summaries possible complex correlation. Redox metabolites and species interact very quickly with biomolecules in cellular and fluid microenvironment and also with antioxidant so relation between different redox indicators could be multifactorial and nonlinear. [14,72] The two functions obtained permit to visualize by synthetic manner the relation of baseline values groups and the effect after interventions and its expression by mathematical expression.

Taking into account that causes of polipathologies are complex and multifaceted, the recognition of molecular and cellular concert involved are crucial. A causal relationship between some elements such as oxidative macromolecules modifications and, chemistry-hematological status has emerged but the mechanism by which these molecular and biochemicals events occur remain to be established.[82]

The OS evaluations were therefore become potential useful to characterize infection, antiviral combinations effects, as well as the usefulness of antioxidant and alternative therapies for counteracts oxidative damage.[15,23]

Several previous studies have been showed that also antiretroviral treatment including Lopinavir-ritonavir has additional impact on pre-existing OS related to condition as observed in HIV infection Treatment. [25] Considering that some authors have been suggested the use of antioxidants as agents which might reduce the incidence of OS as consequences of infection or treatment and, also it could impact on development of other related diseases that interventions were assessed.[19,57,58]

Several findings pointed in the direction that an imbalanced redox system is on the causal chain from morbidity to mortality in patients with COVID-19 [8-10] and also in post COVID-19 sequels. Future studies should evaluate interventions against high oxidative stress in patients with COVID-19 and may use these biomarkers as end points.

The incidence of adverse effects of ozone therapy is very low (estimated at 0.0007%), and typically manifests itself as euphoria, nausea, headaches and fatigue. In general, it is a very safe therapy when administered correctly, with the recommended dose.[54,56] In the present study non adverse effects have been reported and non-biosecurity violation has occurred.

This proof of concept study points to the need of further research, with extended-designed, well-powered multicenter randomized clinical trial, to confirm presented findings and proves effect on sustainably treatment for more time, at least 6 months.

The present study has several limitations. First, the sample size of groups is small. Second, the 95% CIs for our adjusted estimates were wide, and do not exclude a 20–30% decrease in the coefficient for time (days) to clinical improvement. Third, as with most observational studies, we cannot exclude the possibility of residual unmeasured confounding. Fourth, it is a single-center study, limiting the external validity of our results. Finally, outcome assessors were not blinded to treatment arm assignment.

The strengths of this study include its pragmatic real-world COVID-19 population, use of objective primary clinical outcome and risk-adjustment using methods of regression modeling analyses.

The experimental study confirms the biological properties of ozone and BIOPLA and allows it to be an alternative or compassionate therapy for the effective management of SARS-CoV-2 post infection as recommended international organizations (PAHO, WHO).[87,88]

Table 1: Age, gender, ethnicity body mass index and others characteristic of studied groups at HEG-UCI, 2020.

Variables/Groups		Supposedly healthy volunteers N (%)	Hospital Ernesto Guevara –University of Informatics Science	
			BIOPLA N (%)	BIOPLA and Rectal ozone insufflation N (%)
Number of patients (N)		20	20	20
Age, years (median ± SD)		56.9 ± 10.55	60.55 ±13.04	58.71 ± 9.93
Gender	Male	18	18	18
	Female	2	2	2
Ethnicity	White	7	7	7
	Mixed race	5	4	4
	Black	8	9	9
Body mass index (kg/m^2)		23.41 ± 1.15	18.35 ± 4.62*	18.70 ± 3.01*
Diagnoses Chronic heart disease Asthma Stroke Hypertension Diabetes		-	 5 (25) 2 (10) 2 (10) 4 (20) 1 (5)	 5 (25) 2 (10) 2 (10) 5 (25) 1 (5)
Regular physical activity		20 (100)	17 (85)	18 (90)
Smoking status active Alcoholism		-	7 (35) 4 (20)	8 (40) 4 (20)
Length of hospital stay before intervention days, mean (SD),		-	d 15 (6.4)	d 13.5 (7.2)
Post–acute COVID-19 follow-up characteristics Days since PCR negative, mean (SD)		-	10.2 (3.4)	12.5 (4.8)
Persistent symptoms, 1 or 2 ≥3		-	 20 (100) 15 (75)	 20 (100) 17 (85)

Legend
SD: standard deviation
Source: clinical history archived in Medical Register Department of Hospital

Period of hospitalization from May to July 2020.

Biopla®: group receiving supplementation with a commercial dried bio-prepared from placenta of 400 mg during 1 month (June-July 2020)

Biopla®/R03I: group receiving supplementation with a commercial dried bio-prepared from placenta and ozone by rectal insufflation during 1 month (June-July 2020)

Note: No significant differences were detected in comparison between variables for the different groups ($p<0.05$) except for BMI
* Significant difference respect supposedly healthy volunteers

Table 2: Redox indexes data in different studied groups at different moment of study.

Indexes	Supposedly healthy volunteers (mean ± SD)	BIOPLA initial (mean ± SD)	BIOPLA final (mean ± SD)	BIOPLA/R03I initial (mean ± SD)	BIOPLA/R03I final (mean ± SD)
MDA (nmol/g Hb)	2.31 ± 0.33	5.16 ± 0.68 a	5.01 ± 1.32 a	5.81 ± 0.61 a	4.05 ± 0.72 ab
AOPP (μM Cloramine T)	13.70 ± 2.51	20.18 ± 2.27 a	17.63 ± 1.55 a	24.08 ± 3.47 a	15.56 ± 1.73 ab
GSH (μM/g Hb)	1215 ± 207.4	594.62 ± 12.04 a	651.74 ± 41.82 ab	576.4 ± 29.5 a	864.23 ± 62.4 abc
CAT (U/mg Hb min)	144.5 ± 22.29	360.0 ± 51.9 a	358.4 ± 113.28 a	300.0 ± 40.02 a	281.2 ± 99.58 a
SOD (U/mg Hb min)	2.82 ± 0.69	4.66 ± 1.03 a	3.55 ± 0.42 a	4.07 ± 0.5 a	3.56 ± 0.41 ab

Legend
SD: standard deviation
CAT: catalase, SOD: superoxide dismutase, MDA: malondialdehyde, GSH: glutathione, AOPP: advanced oxidation protein' product
Different letter represents significant differences ($p<0.05$):
a represents significant differences respect control (supposedly healthy volunteers)
b represents significant differences between initial and final value of each group
c represents significant differences between groups at final evaluation

Table 3: Hematologic and hemochemical indexes data in the both studied groups.

Hematologic and hemochemical indexes	Supposedly healthy volunteers (mean ± SD)	BIOPLA initial (mean ± SD)	BIOPLA final (mean ± SD)	BIOPLA/R03I initial (mean ± SD)	BIOPLA/R03I final (mean ± SD)
Hemoglobin (g/L) (RI: 11.0-16.0)	13.45 ± 0.64	10.35 ± 1.4 **a**	12.89 ± 1.14 **b**	10.6 ± 1.02 a	13.07 ± 2.01 b
Erythrocyte sedimentation rate (mm/h) (RI: M < 15 y W < 20)	13.52 ± 1.60	21,03 ± 3,0*** a**	15.82 ± 2.16 **ab**	22.14 ± 4.6 * a	14.82 ± 2.3 b
Glucose (mmol/L) (RI: 4.0 - 5.4)	4.3 ± 1.40	3.9 ± 2.00	4.0 ± 1.20	4.1 ± 1.35	4.0 ± 1.70
Albumin (g/L) (RI: 32.0 – 45.0)	38.42 ± 3.10	34.63 ± 3.11	36.55 ± 4.70	32.50 ± 4.32	35.55 ± 3.90
Alanine Amino Transferase (U/L) (RI: M < 33 y F < 25)	31.48 ± 1.18	36.27 ± 3.86 **a**	32.19 ± 2.40 **b**	37.28 ± 2.53 **a**	31.62 ± 2.71 **b**
Aspartate Amino Transferase (U/L) (RI: < 42 U/L)	39.53 ± 2.15	43.45 ± 2.07	40.52 ± 1.71	42.34 ± 2.60	41.50 ± 2.42
Creatinine (mmol/L) (RI: < 5.18)	3.86 ± 1.34	4.12 ± 1.23	3.31 ± 1.01	4.25 ± 1.46	3.64 ± 1.55
Uric acid (µmol/L) (RI: M = 202 – 416 y F = 142 - 339)	346.5 ± 37.63	461.2 ± 22.37 **a**	403.2± 30.2 **b**	440.6 ± 37.63**a**	400.51± 31.4 b
Total protein (g/L) (RI: 60 to 83)	75.7 ± 3.22	40.5 ± 1.60 **a**	69.3± 2.37	39.4 ± 3.16 **a**	70.2± 3.80

Legend:
SD: standard deviation
RI: reference interval
RIO3 rectal ozone insufflation
M males, F female
Different letter represents significant differences ($p<0.05$):
a represents significant differences respect control group
b represents significant differences between initial and final value of each group
c represents significant differences between groups at final evaluation

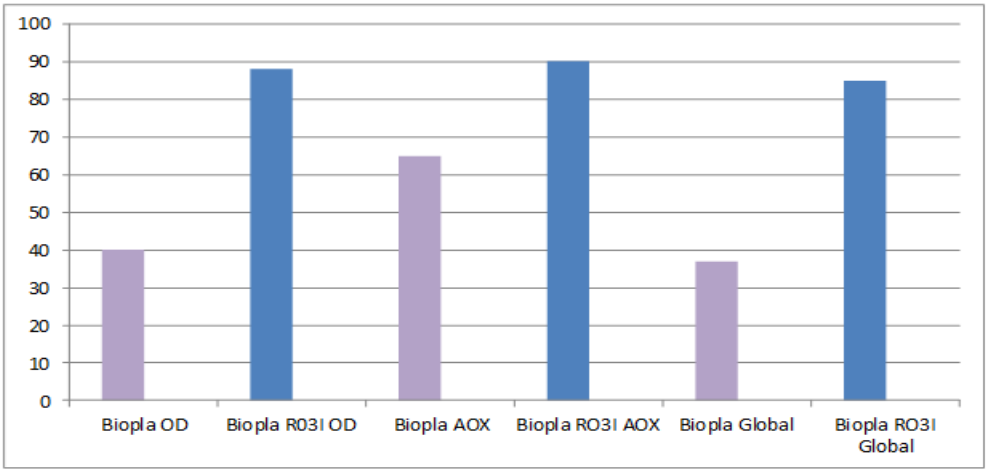

Figure 1: Number of patients that modify simultaneously oxidative damage indexes (AOPP, MDA) and antioxidant indexes (CAT, SOD, GSH) or global markers respect initial value.

Legend
CAT: catalase, SOD: superoxide dismutase, MDA: malondialdehyde, GSH: glutathione, AOPP: advanced oxidation protein' product, OD: oxidative damage, AOX: antioxidant, RO3I: rectal ozone insufflation.

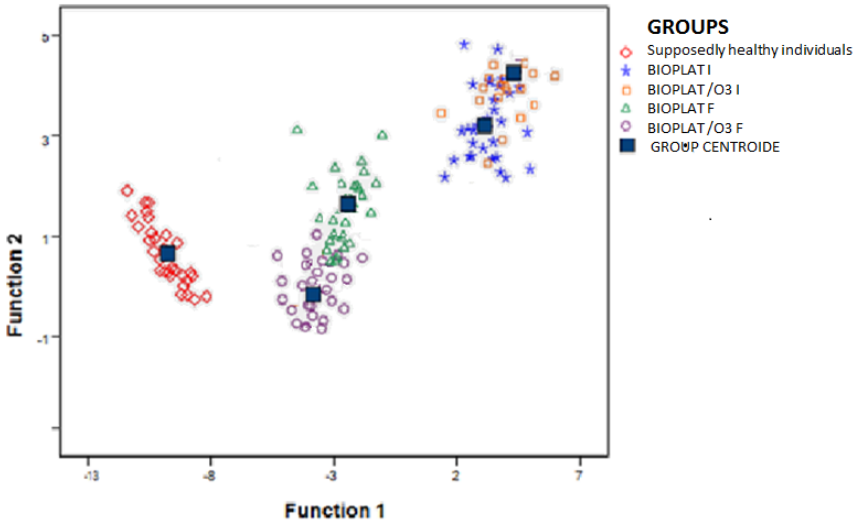

Figure 2: Canonical discriminate analysis representation related to redox indexes in both studied groups before and after interventions.

Legend

1. SHI: supposedly healthy individuals
2. Biopla 1. Initial values
3. Biopla 2. Final values after 1 month of supplementation
4. Biopla+R03I 1 Initial values
5. Biopla+R03I 2 Final values after 1 month of supplementation and ozonetherapy
6. Centroid of each evaluated group

Function 1: 0.608 MDA – 0.003 GSH + 0.004 CAT + 0.042 AOPP + 0.103 SOD – 15.093
Lambda de Wilks = 0.002 ($p<0.05$)
Function 2: -0.044 MDA + 0.007 GSH – 0.012 CAT + 0.034 AOPP + 0.027 SOD – 9.009
Lambda de Wilks = 0.125 ($p<0.05$)

CONCLUSIONS

Supplementation with BIOPLA and combination of BIOPLA plus RIO3T in COVID-19-recovered individuals improves the metabolic, nutritional status and oxidative stress parameters studied. Considering the complexity of metabolic and immunity alterations in SARS CoV 2-infected individuals, further clinical studies with different dosages and intervention times are needed to better understand the effects of proposed intervention in the context of post COVID-19 assistance.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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