Multiple Organ Injury Evolution in an Experimental Model of Burn Mice Treated with Ozone Therapy and Aloe b

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Abstract

Burn disease is related with sepsis, generalized systemic inflammation syndrome, multiple organ dysfunction syndrome, shock, etc. Taking into account the therapeutic properties of ozone and Aloe b, a study of the multiple organ injury evolution, in an experimental model of critically burn mice, using both medication was performed. Animals were divided into 5 groups: 1-no treated, hydration with saline solution after burn; 2-placebo, as group 1 but maintaining the hydration daily up to 14 days; 3-aloe b, 15 treatments of aloe b in saline solution after burn; 4-ozone, hydration after burn and then treated with 15 treatments of ozone (rectal); 5-aloe b+ozone, as groups 3 and 4. Respect to vitality, ozone was the only that presented active animals (75 %). Also presented an increase in the figures of catalase and histological studies demonstrated less damage, respect to the other groups. Ozone was capable to diminish mortality and regulate the body response.

Introduction

While many burn injuries are minor and require little or no intervention, approximately 70,000 persons are hospitalized for these injuries, and 20,000 of this number are burnt severely enough to require admission to a specialized burn unit. Burns are predisposed to infection by damaging the protective barrier function of the skin, thus facilitating the entry of pathogenic microorganisms, and by inducing systemic immunosuppression. It is therefore not surprising that infectious complications are the major cause of morbidity and mortality in a serious burn injury. Severe burns cause defects in both cellular and humoral immunity that have a major impact on infection. The increased levels of multiple cytokines detected in burn patients are compatible with the widely held belief that the inflammatory response becomes unregulated in these individuals. Therefore, burn disease is related with sepsis, generalized systemic inflammation syndrome, multiple organ dysfunction syndrome, shock, trauma, etc.

Multiple organ dysfunction syndrome (MODS), get importance in the eighty decade. It is defined as an acute threat of the homeostasis due to an intense physiological attack (2). In answer to this, a systemic inflammation response (SIR) is produced by the action of several mediators that give rise to a progressive organ and system dysfunction (3). A rapid and decisive treatment is necessary in order to avoid death (4,5). The number of morphological

alterations found in these death patients was named multiple organ injury (MOI) (6). Thus, MODS is a clinic diagnose, whereas, MOI is a morphologic diagnose. In MODS therapy, the use of immunomodulators (5,7,8) and oxygen supplementation (9), in order to prevent or attenuate the SIR is recommended.

Burn disease produces a loss in the homeostatic control (10,11). Loss of the cutaneous barrier facilitates entry of the patient´s own flora and of organisms from the hospital environment into the burn wound. The wound often contains devitalized or frankly necrotic tissue that quickly becomes contaminated with bacteria. Then, the inflammation mediators and proinflammatory cytokines amplify their effects to a systemic scale (12). The study of burn patient necropsies shows the high frequency of MOI (6)

Aloe b, with a recognize therapeutic use, is known by its immunomodulators properties, among others (13). Ozone (O3) has a great germicide power, improve oxygen metabolism, stimulates antioxidant defense systems and is also an immunomodulator. Beside the disinfectant properties, ozone appears to stimulate the phagocytic activity of neutrophils and to modify immunoglobulin levels (14-20).

Taking into account the therapeutic properties of aloe b and ozone, and considering that burn disease is the biggest challenge in intensive care (10), the aim of this paper is to study the histopathological aspects of the MOI in an experimental burn model, using aloe b and ozone.

Materials and Methods

Animals and experimental model

The study was performed with female balb/c mice, weighing between 20 ± 2 g, of 12 weeks old. Animals were maintained in an air filtered and temperature conditioned room (20-22 ^{o}C) with a relative humidity of 50-52 %. Mice were fed with standard commercial pellets and water *ad libitum*. Animals were acclimatized during 7 days. Evolution control was made 2 times per day. The studies were made in concordance with the European Union regulations for animal experiments.

The study was performed using an experimental burn model, previously elaborated (21), and consisted in a dry paravertebral burn, with 11 % of area, at 100 ^{o}C and 20 seconds of exposition.

Ozone was generated by an OZOMED equipment, manufactured by the Ozone Research Center (Cuba).

Experimental Design

Animals were divided, at random, in 5 groups of 24 animals each and another group (the negative control group, without burn) with 5 animals only:
Group 1- Control without treatment (No treated): burn lesion and after it, hydration with 1 ml of saline solution (SS) (0.9 %), intraperitoneally (IP).
Group 2- Treated with SS (placebo): burn lesion and after it, daily hydration with 1 ml of SS (0.9 %), IP, during 14 days.
Group 3- Treated with aloe b (aloe b): burn lesion and after it, aloe b IP, daily, during 14 days, at doses of 0.15 mg/kg in the first week and 0.10 mg/kg in the second one, prepared in 1ml of SS (0.9 %).
Group 4- Treated with ozone (O3): burn lesion and after it, hydration with 1 ml of SS (0.9 %) IP and then ozone, daily, by rectal administration, during 14 days (0.9 ml of ozone, with a concentration of 37 mg/l, dose=1.7 mg/kg)
Group 5- Treated with aloe b and ozone (aloe b + O3): burn lesion and after it, aloe b (as group 3) in 1 ml of SS (0.9 %) IP and then ozone (as group 4).

Each group was divided in 4 subgroups (n=6), corresponding to different times of euthanasia: 24 h, 72 h, 7 days and 14 days. The euthanasia used was the immersion on liquid nitrogen during 7 seconds and the necropsy was performed in situ (22,23). Fragments of lung, heart, brain, stomach, gut (1 cm up the ileocecal valve), kidney, suprarenal gland, liver and spleen were studied.

Determinations

Animal vitality was analyzed according to different criteria: dying animals, with few reflex, not eat or move; vital animals, with maintained reflex, that eat and move with limited activity; and active animals, with appropriated reflex and feeding and mobility since 24 h.

The biochemical parameters were determined by spectrophotometric methods using an Ultraspect Plus Spectrophotometer (Pharmacia LKB). In homogenates of the liver left lobule and of the left kidney were measured total superoxide dismutase (SOD) (24), catalase (25) and lipid peroxidation, as malondialdehyde (26).

MOI was evaluated by the presence of lesion in 3 or more organs. Splenitis and reactive hepatitis were considered as a response of the phagocytic-monocyte system. For each case, MOI was evaluated as: 0-negative (no lesions), 1-slight, 2-moderate and 3-intense (27).

The main studied variables were vitality and the presence of MOI. The dependent variables, also studied, were the morphological alterations and the biochemical determinations.

Histological study

Fragments of lung, heart, brain, stomach, gut, kidney, suprarenal gland, liver and spleen were taken and fixed in neutral 10 % formalin, processed and embedded in paraffin. The histological sections were stained with hematoxylin and eosin and evaluated by light microscopy. The histological study was qualitative, according to established criteria for each lesion evaluated in the different organ (27). The study was made, using a magnification of 100x and 400x, in fixed points of observation for each fragment. The count was made in a blind way by the researcher and corroborated by other 2 pathologists.

Statistical analysis

Student´s t test was performed for evaluation of significant differences between groups. G test of contingent tables was used in order to know the dependence between the mortality and vitality among groups, as well as the organ alterations. For the analysis of the oxidative stress parameters, the normality was compared and an analysis of bifactorial variance was performed, taking into account the liver and kidney results in the different groups, in animals died at 24 h (time with a major number of animals in each group). In addition, a multiple comparison test was used (Duncan test); values are expressed by the mean ± standard error of mean. Different letters indicate a statistical significance of at least $p<0.05$.

Results

Table I shows the mortality (spontaneous or by euthanasia) results in the different studied groups. When the percent is compared (t_s), an unfavorable result is achieved in the group treated with aloe b + O_3, with 100 % of spontaneous dead ($t_s=10.88$, $p<0.001$). In placebo, aloe b and O_3 no significant differences were obtained between spontaneous dead and euthanasia. The highest percent of euthanasia corresponded to the O_3 group.

Table I: Mortality (spontaneous or by euthanasia) results in the different studied groups.

Groups	Spontaneous %	Euthanasia %	t_s
No Treated	75	25	5.68 ***
Placebo	50	50	n. s.
Aloe b	54.2	45.8	0.87 n. s.
O3	37.5	62.5	1.7 n. s.
Aloe b+ O3	100	0	10.88 ***

*** p<0,001; n. s: no significative

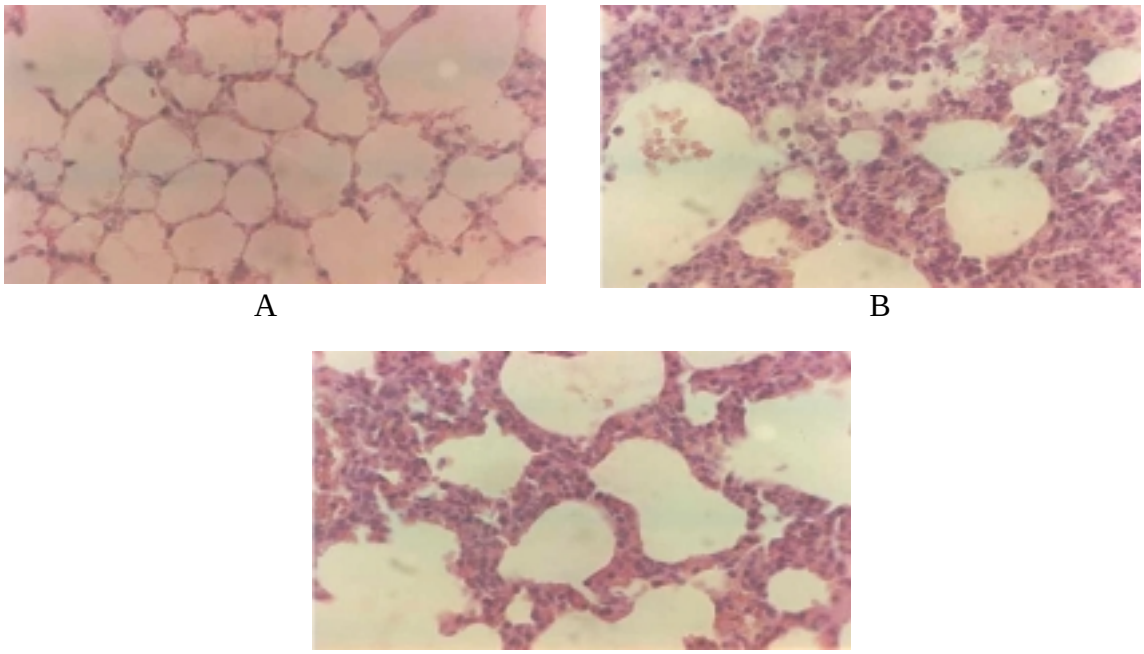
As shown in Table II, since 24 h after the burn, animals treated with ozone presented appropriated reflex and mobility (75 % active animals), with a significant difference between dying and active animals (G = 18.25, p<0.001). No active animals were found in the other groups.

Table II. Vitality behavior in the different studied groups, 24 h after the burn.

Groups (n = 24)	Dead animals (%)	Dying animals (%)	Vital animals (%)	Active animals (%)
No treated	16.7	75	8.3	0
Placebo	0	66.7[a]	33.3	0
Aloe b	0	0	100	0
O3*	8.33	19.5	4.17	75
Aloe b + O3	100	-	-	-

*G= 18.25, p<0.001, between dying and active animals.
[a]62.5 % of them died before the 48 h.

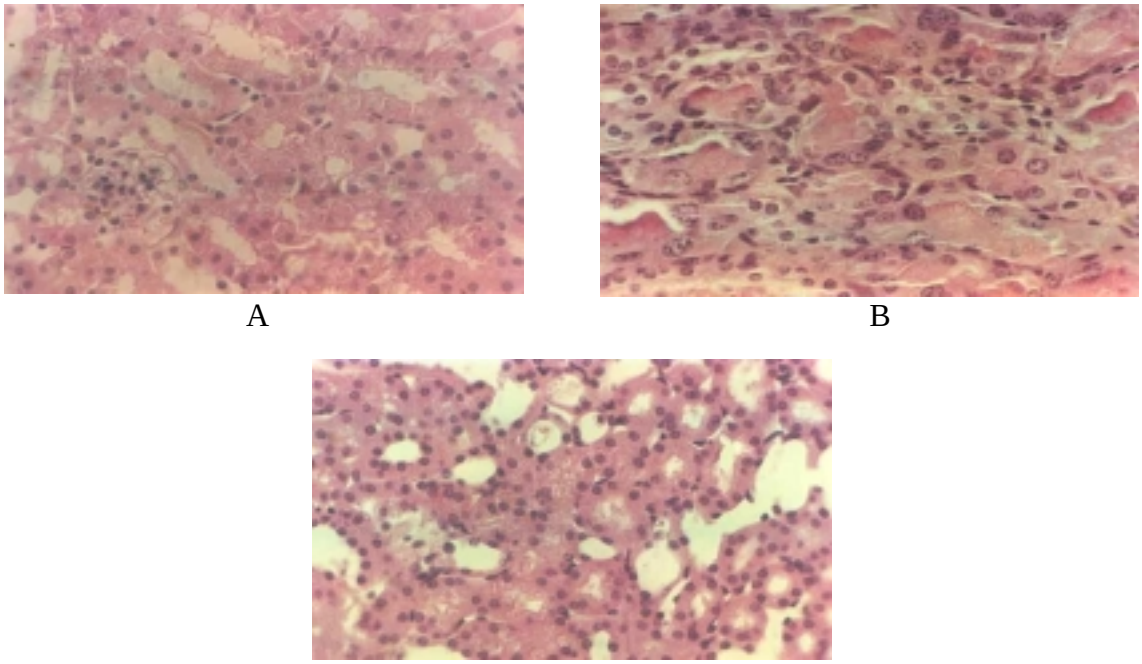
After the burn, different grades of organ damage were observed In no treated and placebo groups predominated slight and moderate lesions (G=126.0, p<0.001). In the ozone group, slight and no lesions were predominated (G= 146.06, p<0.001) (see Figure 1).



A B C

Figure 1. Lung histological study. A: Negative control group; B: No treated group, lung edema of permeability (moderate); C: Ozone group, lung edema of permeability (slight). 400x.

Animals treated with aloe b presented slight, moderate and no lesions (G=124.752, p<0.001) (see Figure 2). Reactive hepatitis and splenitis were found in all the groups submitted to the burn. Aloe b group showed a slight response in these parameters. Sepsis was found in placebo group.



A B C

Figure 2. Kidney histological study. A: Negative control group; B: No treated group, acute tubular necrosis (moderate); C: Aloe group, acute tubular necrosis (slight). 400x.

A variable behavior, between the MOI intensity and the different studied groups was observed (G= 45.11, p<0.001). In the percent analysis (t_s), animals of the treated group (placebo, aloe b and ozone) presented MOI in the slight grade, being favorable, with significance difference in the ozone group (t_s= 5.74, p<0.001). Even though, aloe group had more animals that presented MOI in the slight grade than in placebo group, their figures were not significatives (see Table III). No treated group presented 100 % of moderate MOI, slight grade was not observed.

Table III**.** Different grades of MOI in the different studied groups.

Groups	n	Slight MOI (%)	Moderate MOI (%)	t_s
No treated	6	0	100	-
Placebo	12	16.66	83.33	3.57***
Aloe b	11	36.36	63.63	1.29 n.s.
O3	15	93.33	6.66	5.74***

***p<0.001; (n.s.= no significative)

The oxidative stress parameters presented similar behavior in the studied organs (liver and kidney). SOD did not show important variations in the study. Liver MDA figures increased progressively in placebo group. In ozone group, MDA increase at 24 h and after that its figure were as that of negative control group. Respect to CAT, in ozone group, a significant increase was observed at 24 h. This increase in CAT (a scavenger of hydrogen peroxide) is in relation with the high vitality and MOI results (slight grade of damage) observed in a high per cent of animals treated with ozone. In aloe b group, no significative variation was found.

Table IV. Behavior of malondialdehyde (MDA) and catalase (CAT) in liver and kidney, 24 hours after the burn, in the different studied groups.

Groups	Organs	MDA (nmol/ml)	CAT (U/ml)
Negative control	Liver	1.33 ± 0.10^{c}	346.1 ± 32.1^{c}
	Kidney	0.76 ± 0.02^{c}	275.9 ± 15.9^{c}
No treated	Liver	3.45 ± 0.18^{b}	434.2 ± 45.2^{b}
	Kidney	2.79 ± 0.17^{b}	281.7 ± 18.3^{c}
Placebo	Liver	4.62 ± 0.15^{a}	342.3 ± 21.1^{c}
	Kidney	1.54 ± 0.09^{c}	382.9 ± 23.3^{c}
Aloe b	Liver	1.48 ± 0.07^{c}	578.9 ± 55.1^{b}
	Kidney	1.52 ± 0.06^{c}	273.1 ± 13.2^{c}
O3	Liver	3.16 ± 0.12^{b}	719.2 ± 55.3^{a}
	Kidney	3.09 ± 0.11^{b}	835.9 ± 57.8^{a}

Duncan test. Different letters indicate significant differences of at least $p<0.05$.

Discussion

The daily hydration, in the placebo group, made possible to extend animal life. It was reaffirmed that hydration is necessary in burn disease therapy (28). Aloe b treatment has not presented the expected response. However, a significant difference between aloe b group and placebo and no treated groups, in the grade of lesions was observed. Slight lesions were observed in aloe b and ozone groups. However, ozone group achieved an important survival, due to hydration and its beneficial effects, as for example a supplementary tissue oxygen (14,29) and immunomodulator capacity.

The therapeutic properties of aloe b and ozone (14-20,29,30), applied separately in the burn disease showed good results. Both shared immunomodulator properties (15,16,31-37). Ozone, being an oxidant, could promote organ stress inducing an enhancement of the endogenous protective mechanism in order to attenuate the burn injury. The phenomenon can be described

as an induction of tolerance to O3 and reactive oxygen species (ROS) generated by toxic agents and has been denominated as an oxidative preconditioning (17). Also, it is reported that aloe b stimulates the antioxidant defense system (38,39).

However, the combination of aloe b + O3, in the presence of the burn, was lethal for all the animals of this group. Taking into account the existed knowledge about both products and the rapid establishment of the lesions that produced the death, this phenomenon was explained by an excessive formation of free radicals. The burn disease ¨per se¨ produces a great quantity of free radicals, either by the direct effect of the burn or by the SIR that is involved (40,41). In the other hand, the aloe b injections are made from a macerate of cortical parts and plant gel. The cortical part is rich in anthraquinones (13,42), that in his chemical structure have various rings of benzene, with increased in hydroxyl groups, that can react and produce ROS. Ozone, by its mechanism of action, produces a controlled oxidative stress. In this situation (using aloe b + O3) can account a phenomenon of potentiation of ROS, that together with the free radicals that are produced in the burn, saturate the antioxidant defense system. This chain reaction, difficult to control, can produce the animal death in the first 24 h. Future studies in this line are necessary in order to prove this situation.

Respect to microvacuolar hepatic steatosis observed it was presented in 50 % of no treated, placebo and aloe b groups and only in 20 % of the animals treated with ozone and in a slight grade. Hepatic cellular tumefaction was observed in almost all animals, except in the ozone group, where 4 animals have not presented this damage and in 66.7 % the alterations were slight. It has been demonstrated that 15 sessions of ozone applied rectally, before a damage produced by intraperitoneal carbon tetrachloride, protected the liver, obtaining good results in the hepatic histology (17). The same protection was observed in kidneys of animals treated with ozone after the burn, as referred previously (19). In this study, ozone is used after the damage and in general, a favorable response is achieved in the burn animals treated with it.

The reactivity of the immunologic system was evaluated by the presence of reactive hepatitis and splenitis. It was presented in almost all animals, but in the ozone group was observed in 100 %, in slight grade. The results obtained of the reactivity of the phagocytic- monocyte system, evaluated in liver and spleen, corresponded with the SIR that burns produced as causal factor (27).

Whereas MODS define the progressive fall of the functions of different organs, MOI is the morphological change that produces the function failure. It constitutes the first cause of death and is the greatest complication in burn patients, provoking up to 70 % of mortality in intensive care units (43).

An increase in CAT figures was observed 24 h after the burn, in animals treated with O3. This was in relation with animal best evolution and less morphological alterations. It is known that hydrogen peroxide (H_2O_2) is a strong oxidant agent, with half life time superior to other ROS, and play a significative role in the oxidative stress injury associated to burn (44). In the presence of various transition metals, especially iron and copper, H_2O_2 is rapidly converted to the extremely reactive hydroxyl free radical. This is related with the capacity of H_2O_2 to

oxidize proteins and to cause DNA damage. CAT converts H_2O_2 to water and oxygen. Also, a significative decrease of lipid peroxides (measured as MDA) was observed in the ozone group, with respect to the rest of the groups, after 24 h. It has been proved (17-20), as in this study, the increase in the antioxidant defense system produced by ozone treatment.

In aloe b group the response, according to evolution, morphological changes and biochemical parameters, was stable, with few changes among the different variables. This behavior is in relation with the immuno response given by the hepatic and splenic reactivity observed in this group, as slight grade for both organs. The presence of oligosaccharides of mannose for the transport of intracellular proteins and glycoproteins, is important (45). The immunomodulator effect of aloe b is found in the gel of the plant, constituted fundamentally by polysaccharides of mannose, being its mechanism of action linked to this biochemical distribution. In this group, vital animals, with morphologic lesions, between slight and moderate, were found. The mechanisms of action that justified these results are not well clarified, but it appears that neither the lymphoid proliferation nor the stimulation of the antioxidant defense are involved, because both of them remained stables.

Conclusions

In the experimental model studied, with a mortality higher than 75 %, similar to an extreme critical burn patient, the application of aloe b and ozone, separately, showed favorable results in the animal evolution and morphology (slight and moderate grades). Also, both treatments have permitted a good survived with better quality of life. No side effects were observed. Comparing aloe b and ozone, better results were achieved in the ozone group: higher percent of active animals and slight MOI, as well as stimulation of the antioxidant defense system. It was reaffirmed that hydration is necessary in burn disease therapy. In no treated group, the evolution was the worst, with 100 % moderate MOI and 75 % dying animals.

Key Words

Ozone; aloe b; experimental burn model; multiple organ injury; systemic inflammatory response.

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