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Research Article,

Comparative Study on the Disinfectant and Sterilizing Effects between the Ozone Bag and Traditional Autoclavation: Pilot Study

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Abstract:

Introduction: Disinfection and sterilization are important biosafety measures used in health environments. The means routinely used to reduce the microbiota in these environments are chemical deferment's, autoclave and ozone. It is important to highlight that many of the utensils used by health professionals are no sensitive, leaving the autoclave unfeasible in the sterilization process.

Objective: To collect preliminary results about the efficacy of the ozone bag, as a sterilizing and disinfectant agent, comparing its effect with traditional autoclaving.

Methodology: An in vitro experimental analytical study was carried out using an ozone generator and autoclave. Three volunteers were selected, without restriction of gender and age, who contained oily skin and who reported not having done skin antisepsis until 2 hours before material collection. The materials used were 18 curettes and 18 Petri dishes, divided into 3 groups: a) Control, where no sterilization process was used; (b) Autoclave, carried out at a temperature of 121ºC for 30 minutes; and c) Ozone bag, which employed concentrations of 30μg/ml e 60μg/mland exposure times ranging from 5 to 30 minutes. The microbial agent extracted from the skin of the volunteers were mostly the *Staphylococcus aureus.*

Results: We found that ozone bags, normally used for the treatment of various aesthetic dysfunctions and skin healing, especially in cases of diabetic ulcerations, have the ability to sterilize in a similar way to autoclave, when used with a concentration of 30μg/ml for 30 minutes.

Conclusion: The ozone bag can also be used to sterilize health utensils in the presence of *Staphylococcus aureus*, provided that the materials are exposed to the gas with the concentration and exposure time suggested in this study.

Keywords: Autoclave, ozone therapy, bag ozone, Staphylococcus aureus.

Introduction:

Disinfection and sterilization are important processes for the sanitization of environments. Disinfection is a physical and chemical process capable of destroying or inactivating most microorganisms present in inanimate objects and surfaces, except bacterial spores. Sterilization destroys all life forms present on surfaces, from vegetative and sporulated forms to viruses and fungi. These processes can be performed by means of several substances that act on the structure and metabolisms of microorganisms, eliminating them (1).

Sterilization is part of biosafety procedures. This technique is performed through chemical or physical processes, capable of promoting the destruction of all microbial life forms (bacteria, spores, helminths, viruses, fungi and protozoa), with the objective of ensuring safety in medicines, food, instruments, medical-hospital devices and other areas. To choose the most appropriate sterilization technique some factors are

considered, such as the number, type and location of microorganisms, in addition to the type of material that must pass through the process and other conditions for the use of the method. Therefore, the criteria of quality, agility, efficiency and availability of space should be evaluated (2).

Barbosa *et al* (2018)(3) stated that *the microorganisms Staphylococcus aureus*, *Enterococcus spp., Clostridium difficile, Acinotobacter spp*., among other potentially pathogenic microorganisms, can survive for weeks on surfaces in the health service environment. The Disinfection and Sterilization guidelines in Health Facilities proposed by the Centers for Disease Control and Prevention consider that environmental surfaces are often touched by the hands and can potentially contribute to secondary transmission by infecting other health professionals or equipment, which are later used in patients (4).

In addition to these means used to reduce the microbiota in health services, autoclavation is one of the techniques for treating Health Service Waste (SSC) belonging to the biological risk group and most applied sharp in Brazil. However, one of the deficiencies in relation to treatment by autoclavation refers to the operational parameters and adequate frequency for monitoring the process, considering that there is little scientific evidence on the ideal parameters (5). For this procedure, an autoclave is used that functions as a greenhouse, which has a closed container, which under pressure and high temperatures promotes sterilization (6). It consists of a pressure câmara that operates from 1.8 to 2.0 bars (bar) and works by subjecting the pressurized saturated steam item to 121 Celsius (°C) The parameters of autoclaving of pressure, temperature and time range from 15 pounds per square inch (psi), 116°C to 127°C and from 10 to 60 minutes, respectively (7).

It is also important to highlight that bacteria are organisms that do not survive in the presence of ozone (O_3). This gas primarily acts on the bacterial membrane and causes loss of normal cellular enzymatic activity. As a result, there is a change in the permeability of the cell that leads to the death of this microorganism (8). This gas is able to oxidize the fatty acids (lipid peroxidation) that make up the cell barrier (9). It may also exert antiviral action by interfering with the replication phase of the virus; this characteristic is linked to the ozone's ability to oxidize cysteine residues through the formation of disulfide bridges present in the structures of the virus itself in large quantities (10), and it is also m can damage the viral capsid and disrupt the reproductive cycle of the viral pathogen (11).

Ozone has been used as a microorganism control chemical in several segments of the health area. The generating machines of this gas have an antimicrobial potential on surfaces and artificially acclimatized indoor air (12). This gas besides bactericide, is germicidal and can perform a chemical cleaning (13). In addition, it is a potent oxidizing agent, and is also considered an important disinfectant since the bactericidal power of the gas can reach 3,500 times the speed of chlorine (14).

Worldwide, in the various health care services (pathological or aesthetic), ozone is routinely used for various therapeutic purposes. For decades of experiments and clinical studies, several methods of application of ozone therapy have been demonstrated. Topical application by means of a "*bag"* consists of using a plastic material, similar to a "bag", which forms a closed system of circulation of ozone gas, and which can be connected to a catalyst or not This system has high bactericidal power and can be used in various types of affection, especially in open wounds on the skin (15).

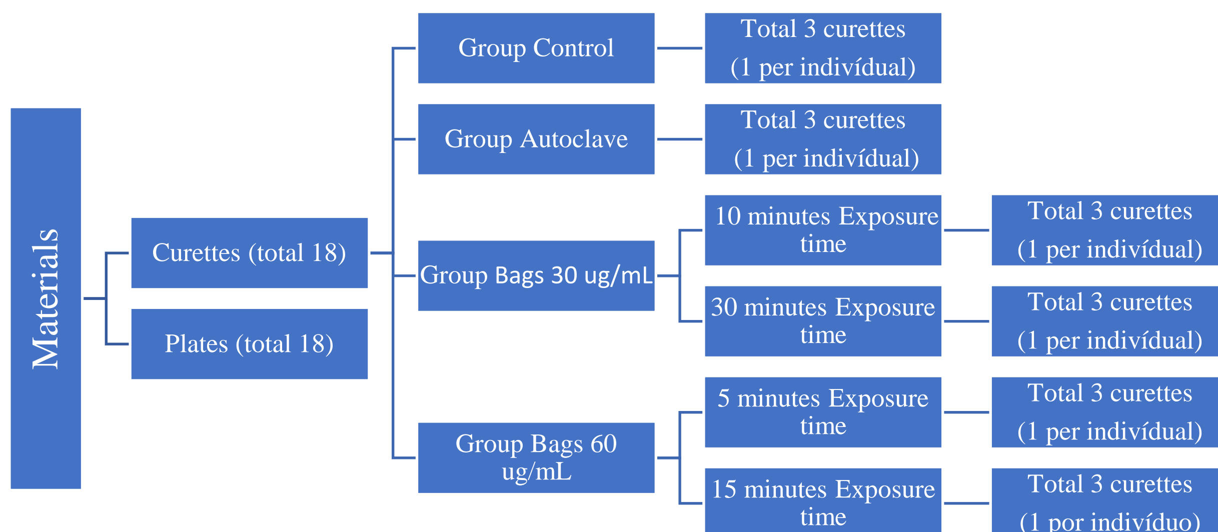
Thus, the aim of this pilot study is to collect preliminary results about the efficacy of the ozone bag as a sterilizing and disinfectant agent, comparing its effect with traditional autoclave, using some utensils normally used in aesthetic care, since many of these are termsable and therefore prevented from being placed in the autoclave.

Subjects and methods:

An experimental in vitro analytical study was *carried*out using an ozone and autoclave generator. The experiment was carried out at the Microbiology Laboratory of the Federal Institute of Rio Grande do Norte - IFRN, Macau campus.

Three volunteers were selected, without restriction of gender and age, who contained oily skin and who reported not having done asepsis of the skin until 2 hours before the evaluation.

To carry out the research, the materials used were 18 curettes and plates, distributed according to flowchart 1.



Flowchart 1: Materials used in the research.

Source: Survey data, 2022.

Preparation of curettes

All curettes used for collection and analysis were initially sterilized in the autoclave, with a temperature of 121ºC for 30 minutes. After this stage, these utensils were gently attenuated on the skin of the volunteers, with movements of back and going, repeated 5 times, in order to promote contamination to later perform the evaluation of the proposed means of sterilization. The areas of the skin that were anted were demarcated with a distance of 1 cm between the points (Figure 1), to avoid contamination of the utensils in the same region, since possibly previous friction with a sterilized utensil could cause a decrease in bacteria in the region.

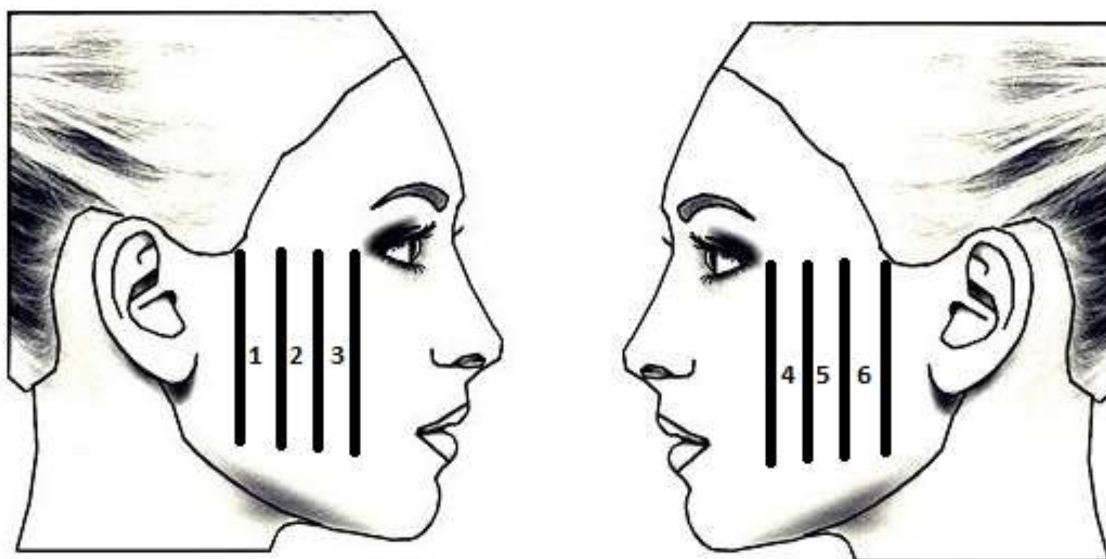


Figure 1: Areas that were delimited on the volunteers' faces for the collection of the material to be sterilized (the collection area was the space between the black lines).

Procedure performed for the control group.

After sterilization of curettes, three units were exposed to contact with human skin (one for each individual) and did not go through a new sterilization process. In this material was passed *the swab* and directed to the culture medium.

Procedure performed for the group submitted to autoclave.

After the preparation of the curettes, three of them were exposed to contact with human skin (one for each individual), and were placed again in the same autoclave equipment for sterilization, with a temperature of 121ºC for 30 minutes. After sterilization, the *swab i*n these materials were also passed and they were directed to the culture medium.

Procedure performed for the group submitted to *the bags* with ozone.

After the preparation of the curettes, twelve of these were exposed to human skin, and then submitted to ozone bag by different exposure times and ozone concentrations. The *bags* were labeled (Figure 2) in order to facilitate the identification and analysis of the materials.

Figure 2: Ozone bags containing curettes exposed to different exposure times and concentrations.

The ozone used in the bags was generated through the Oxetane Prime equipment®, manufactured by Tonederm Paganin e Cia. Four plastic bags were used*for each patient, each containing* a curette that was submitted to gas at different concentrations and for different times, according to chart 1. The bags were carefully closed so that there was no gas leak.

Quadro 1: Concentration and exposure time of each *bag*.

Material	Concentration	Exposure time
Bag 1	30µg/ml	10 minutes
Bag 2	30µg/ml	30 minutes
Bag 3	60µg/ml	5 minutes
Bag 4	60µg/ml	15 minutes

The volume of gas used to fill each *bag* was approximately 360 ml. The calculation was made by means of a graduated Becker cup, in which it was filled with a certain amount of water that was then transferred to the *bags* to evaluate its maximum filling capacity. It is important to highlight that all *these bags* were the same and had the following dimensions: 10cm (width) x 14.5cm (length).

After the end of the exposure time of the curettes to the ozone inside the bags, each curette was removed from *inside the bag* with a sterile glove and the *swab was then passed*. This care was taken so that *the swab* did not suffer ozone interference. Soon after each swab was directed to the culture medium.

Plating of samples in culture medium

The Agar Nutrient culture medium was prepared according to the manufacturer's recommendations and submitted to autoclave sterilization. After passing the sterility test, the culture medium was dwelled into sterile Petri dishes. 18 plates containing the culture media used in the tests were prepared.

The material was collected by sterile swab, which was soaked in sterile saline solution to facilitate sample collection. The wet swab was passed throughout the area of the curettes to be analyzed, observing which group (control, autoclave or bags) and individuals (1, 2 or 3) the sample was belonging to. After sample collection, the sowing technique was performed in stretch marks, in petri dishes, containing nutrient Agar culture medium. The sued plates were incubated inverted in a bacteriological greenhouse for 24 hours at 35°C. After incubation time the plates were analyzed and sub measures the count of colony-forming units and the results between the groups were compared.

Morphological analysis was observed that bacteria extracted from the skin of patients were mostly *Staphylococcus aureus.*

Data analysis

The results of the tests were submitted to statistical analysis following the variance analysis model (ANOVA). The program used in statistical analysis was GraphPad Prism version 5.0, 2014 (La Jolla, CA, USA).

Ethical considerations

In Brazil, research involving human beings follows The Cns National Health Council Resolution No. 466/2012, which clarifies that it is necessary to observe the principles of autonomy, not maleficence, beneficence and justice. The research followed this resolution and for the implementation of this study it was necessary to refer it to the Research Ethics Committee (CEP). The authorization opinion was 5,586,095. After this stage, the volunteers were asked to sign the free and informed consent form. This term made aware of the freedom not to participate in the research, without prejudice to its assistance.

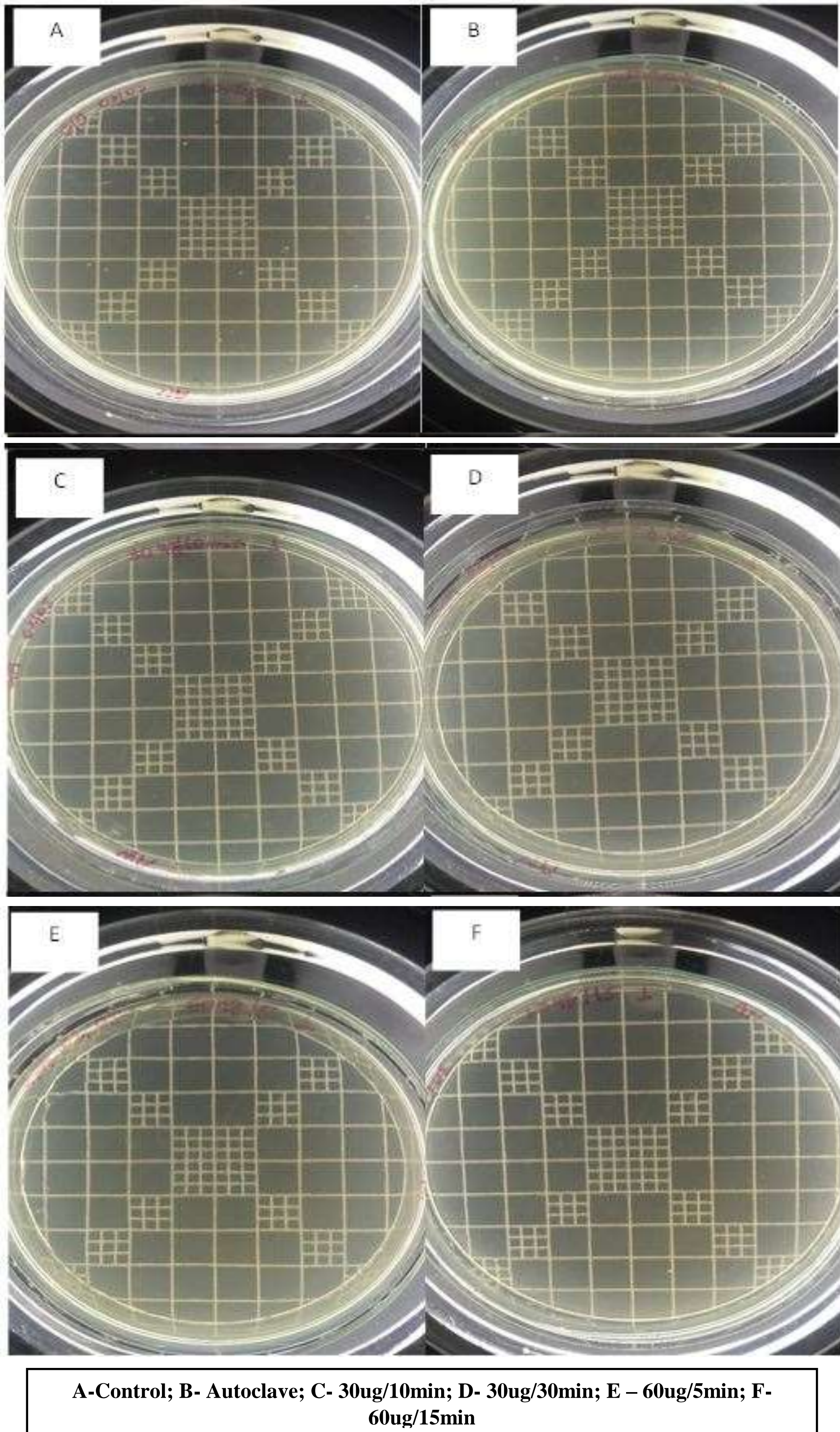
Results:

After performing all methodological stages, the bacteria count described in chart 2 was performed, and the comparison between the groups was performed.

Table 2: Count of bacteria present in the skin of volunteers before (control group) after (autoclave and bag) sterilization procedures. (*CFU*: Colony Forming Unit).

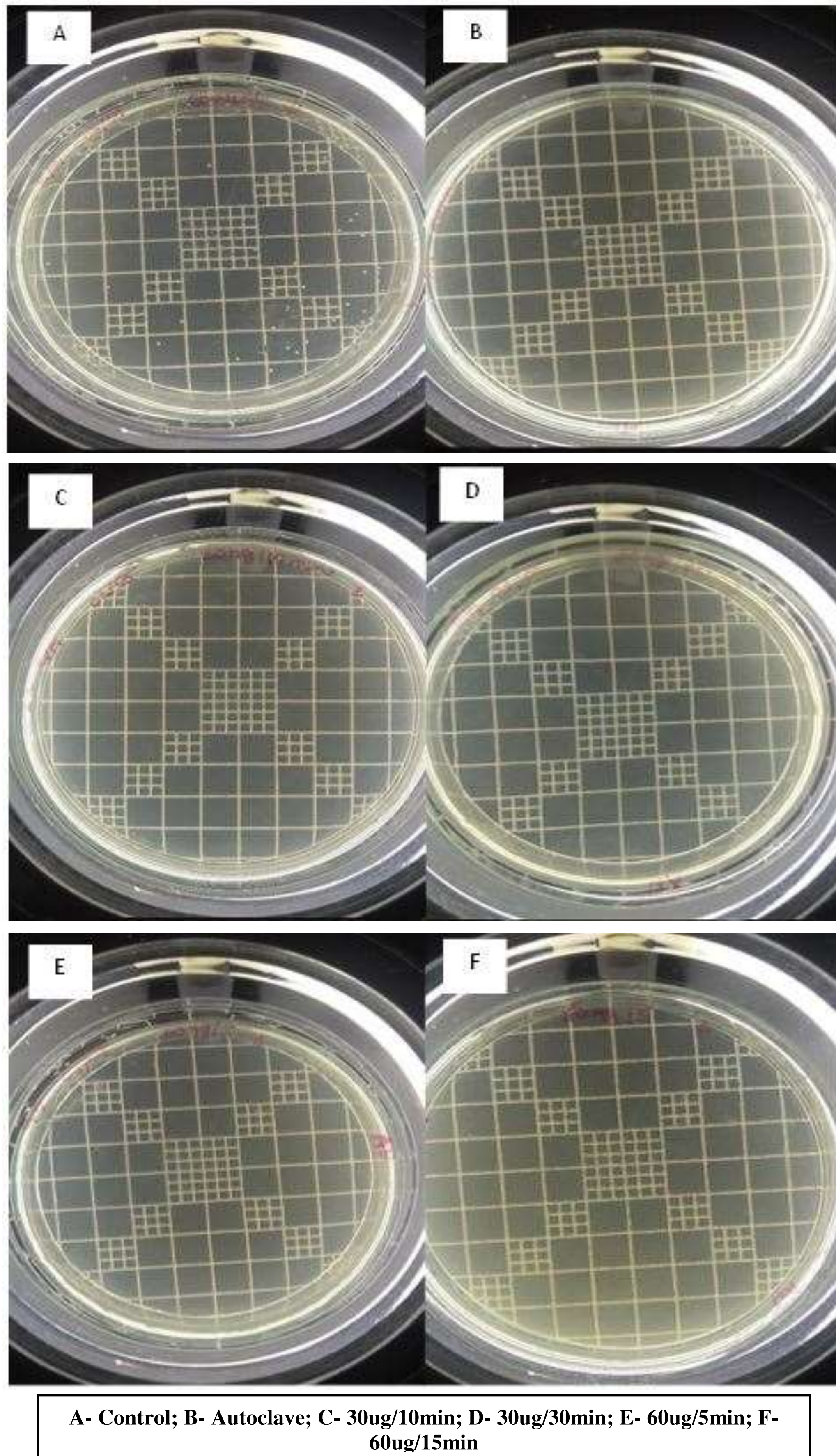
Grupos		Individual 1 (UFC)	Individual 2 (UFC)	Individual3 (UFC)
Group control		24 UFC	63 UFC	756 UFC
Group Autoclave		0 UFC	0 UFC	0 UFC
Group Bags 30 μg/mL	10 minutes	0 UFC	0 UFC	532 UFC
	30 minutes	0 UFC	0 UFC	0 UFC
Group Bags 60 μg/mL	5 minutes	0 UFC	0 UFC	784 UFC
	15 minutes	0 UFC	0 UFC	12 UFC

In the control group, the presence of bacteria extracted from the skin of the three individuals was initially observed. From the interpretation of the data contained in Chart 3, it was also possible to verify the susceptibility of bacteria submitted to autoclave and ozone *bags.* In the case of autoclaving, it was verified that there was no growth of bacteria collected from the skin of the three individuals, demonstrating to be a fully effective sterilization method. The attempt to sterilize the curettes with *bags with a concentration* of 30 μg/mL for 10 minutes, 60 μg/mL for 5 minutes, and 60 μg/mL for 15 minutes was effective only for the bacteria present in the curettes extracted from individuals 1 and 2, but not for individual 3. Despite this, the research demonstrated that the *application of the bag* with ozone at a concentration of 30 μg/mL for 30 minutes presented the same result as the autoclave, with the effective sterilization of bacteria extracted from the 3 volunteers, as can be observed in figures 3 to 5.



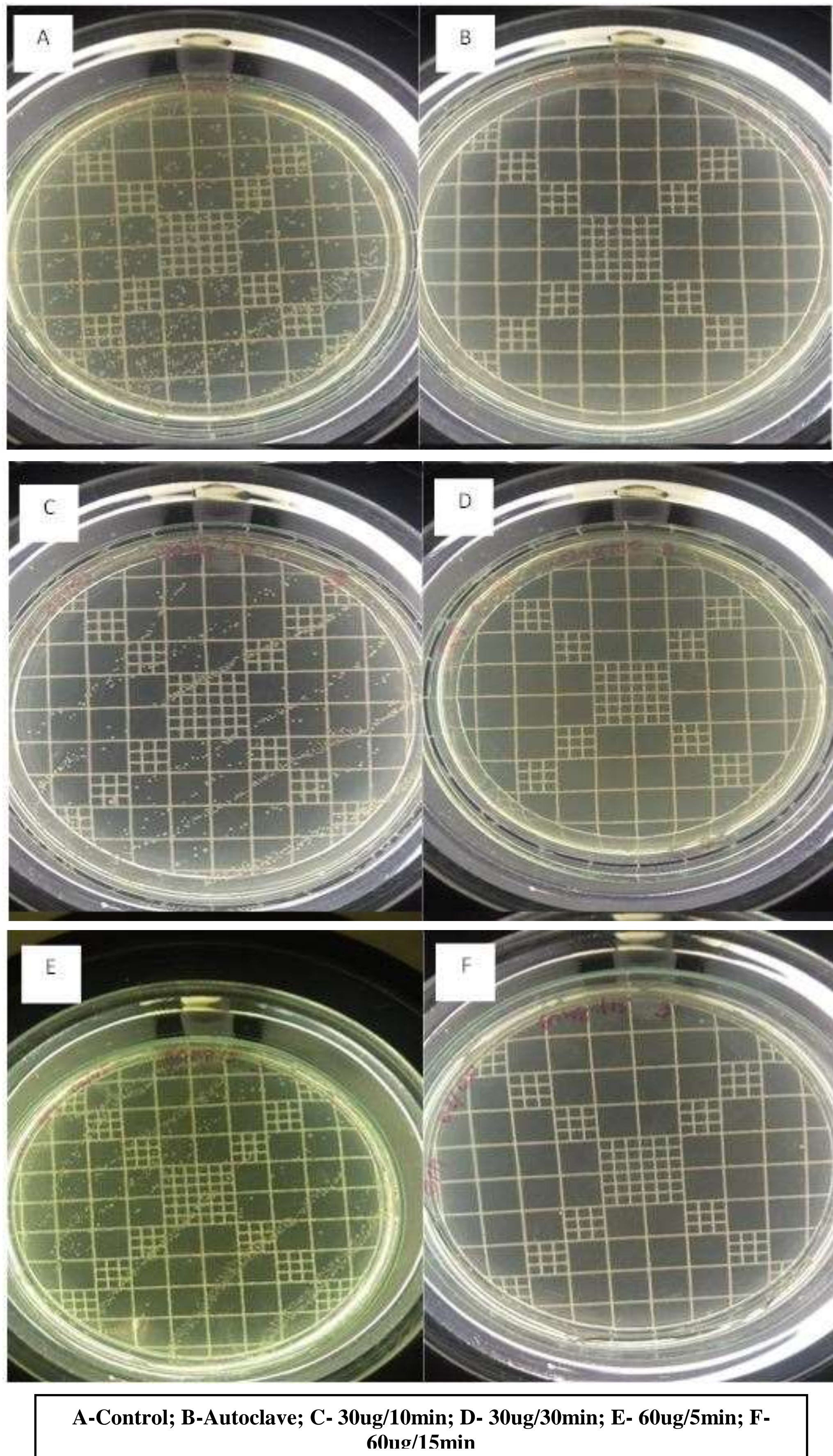
A-Control; B- Autoclave; C- 30ug/10min; D- 30ug/30min; E – 60ug/5min; F- 60ug/15min

Figure 3: Individual Results 1.



A- Control; B- Autoclave; C- 30ug/10min; D- 30ug/30min; E- 60ug/5min; F- 60ug/15min

Figure 4: Individual Result 2



A-Control; B-Autoclave; C- 30ug/10min; D- 30ug/30min; E- 60ug/5min; F- 60ug/15min

Figure 5: Individual result 3.

Discussion:

We attest in our results the sterilization power of the autoclave in the inactivation of *Staphylococcus aureus present* in the skin of the volunteers. This corroborates a study conducted by Hossain *et al* (2016) (16) in which it was observed that in clinical residues of body fluids containing this type of bacteria, as *well as Escherichia coliand Bacillus subtilis,* the process of autoclave sterilization, as well as by supercritical carbon dioxide (SC-CO2), proved to be effective in antimicrobial control.

Regarding ozone sterilization, it is important to highlight that in this pilot study concentrations of this gas commonly used in clinical practice in the use of bags were tested, and that the proposal was also to observe whether by doubling the concentration from 30 μg/mL to 60 μg/mL it would be possible to reduce the time to half of ozone exposure, from 10 and 30 minutes to 5 and 15 minutes respectively; and if in all cases the results would be similar to that of autoclave sterilization.

A study by Abuzairi *et al.* (2022) had a proposal very similar to the one developed in our research. The authors have developed a sterilization box containing ozone, intending to produce an antimicrobial effect on medical materials that contained bacterial samples of *Staphylococcus aureus*. The instruments used for analysis were stethoscopes and thermometers. In the first phase of the study, the ozone concentration inside the box was observed, after 5 minutes, reached 1.65ppm (1.65μg/mL), with 3 minutes for the decomposition of the gas. In 10 minutes, the ozone concentration was 4.01ppm (4.01μg/mL), and after turning off the ozone generator, it took 4 minutes to decompose all the gas. When turned on the generator for 20 minutes, the gas concentration gradually increased until it reached a concentration of 4.92 ppm (4.92μg/mL), and to decompose as a whole, it took about 6 minutes. The authors observed that the gas concentration would lend to linearity after this period.

It was also verified that these medical devices, , had bacterial colonies above 300 UFC, when not treated. In other words, it could be attested that they were too numerous to count (TNTC). In addition, it was observed that after exposing these materials to 20 minutes of ozone, the number of bacterial colonies was reduced to less than 50 CFU, which led to the conclusion that with this exposure time, there may be damage to the wall of this type of bacterium.

Similarly, Almeida (2017) carried out a study that aimed to determine parameters for the sterilization process using ozone, in addition to evaluating the effectiveness of the sterilizing agent for medical devices. The sterilization process was verified through the action of this gas on the spores of Geobacillusstearothermophilus ATCC 7953. Carriers inoculated with 106 of the spore were introduced into 3 mL syringes and tubes with different lengths and diameters, simulating hospital medical products. Such devices were subjected to half cycle and full cycle of the ozone sterilization process. The gas concentration used was high, above 7 g/N.m3, approximately 35,000 ppm (35,000μg/mL). It was observed that the time required for logarithmic reduction of the initial microbial load (initial value), submitted to the ozone sterilization process, were: Staphylococcus aureus, 3.42 minutes; Pseudomonas aeruginosas, 3.04 minutes; Escherichia coli, 4.08 minutes; Bacillus subtilis 20 x 106, 3.17 minutes; Salmonella, 3.66 minutes; Aspergillus brasiliensis, 4.34 minutes; and Candidaalbicans, 3.77 minutes.

Corroborating the idea that ozone is effective for sterilization, Alrafee et al (2022) (19) reported that ozone gas has high oxidizing power and a reliable bactericidal effect due to the destruction of cell walls and cytoplasmic membranes of bacteria. In addition, the authors showed significant efficacy in reducing the number of S.mutans in dental samples through a mechanism that involves the rupture of their membranes.

A study by Hu *et al.* (2022) (18) evaluated the effect of ozone on the gas and water phase for inactivation of bacteriophages φ6, φX174 and MS2. It was observed that the action of disinfection of ozone in the water phase increased as the treatment time increased. In the gas phase, there was a similar trend when comparing the application of ozone for 1, 2 and 3 minutes. It was observed that by increasing the exposure time to 3 minutes, there was a significant increase in the reduction of bacteriophage registration, so that the prolonged treatment applied to bacteriophages φX174 and MS2 were reduced in a tendency similar to φ6 in the aqueous and gaseous phase.

Although the type of microorganism is different from that analyzed in our experiment, these findings resembled our pilot study, since when bacteria extracted from the skins of volunteers were exposed to a longer exposure time (30 minutes, compared to 5, 10 and 15 minutes) there was a better effect on

antimicrobial action, with a result similar to that of the autoclave for the 3 individuals, in which there was no record of bacterial growth.

Similarly, a study conducted by Lamb *et al* (2019) (19) aimed to evaluate *the in vitro antimicrobial activity of the ozone gas for 5 bacterial* strains (*Pseudomonas aeruginosa*, *Enterococcus faecali*s, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, a fungus (*Aspergillus niger*) and a yeast (*Candida albicans*). Concentrations of 2.5 ppm (2.5 µg/mL) and 10 ppm (10 µg/mL) were used. Ozone in the concentrations used was effective in reducing most microbial counts. Ozone was applied for a time of 30, 60, 120 and 240 minutes. It was observed that the longer the time of application of the gas on some of the microorganisms, the greater the antimicrobial effect, being more efficient in the higher dose (10 ppm) and longer exposure time (4h).

Another similar study, conducted by Lamb *et al., (*2021) (1), analyzed the antimicrobial activity of ozone gas in media with a high degree of contamination, using two concentrations of gas 2.5 ppm (2.5 µg/mL) and 10 ppm (10 µg/mL), at times of 30, 60, 120 and 240 minutes in five bacterial and fungal strains (*Enterococcus faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa, Salmonella Typhimurium, Staphylococcus aureus, Aspergillus niger* and *candida albicans*yeast). The authors concluded that ozone, in the concentrations used, was effective in reducing most microbial counts, being totally efficient in the concentration of 10ppm. It was also observed that the longer the time of application of the gas on some of the microorganisms, the greater the effectiveness of this microorganism scan.

Final considerations:

Biosafety measures in health care services are of paramount importance to ensure a set of actions aimed at preventing, minimizing or eliminating risks inherent to the provision of these services, which can compromise the health of patients.

One of the problems encountered by professionals in this area is that many of the utensils used are nonsensitive leaving the autoclave unfeasible in the sterilization process. Despite this, this study was able to *verify that ozone bags*, extracted from the machines used in these health services, have the ability to sterilize the autoclave equally when using a concentration of 30ppm for 30 minutes.

We suggest the continuation of this study using the concentration of 60 µg/mL for 20 minutes or 100 µg/mL for 10 min to verify whether the sterilization effect will be maintained and consequently professionals may reduce the execution time of this process.

Thus, it is intended to contribute to this growing area of research, exploring the role of gaseous ozone in the sterilization of utensils used in the health area through bags.

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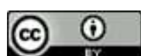

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