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SUPPLEMENTATION WITH OZONIZED WATER DOES NOT ALTER THE CLINICAL, ANTHROPOMETRIC, AND OXIDATIVE STRESS PROFILE IN APPARENTLY HEALTHY INDIVIDUALS

Marilia Esteves Alves de Souza, Maria Beatriz Tiezzi Vergara, Letícia Freire, Patrícia Pinto Saraiva, Amanda Creste Martins da Costa Ribeiro Risso, Rafaela Alponti Vendrame, Claudio Lera Orsatti

Universidade do Oeste Paulista - UNOESTE, Faculdade de Medicina, Jaú, SP. E-mail: orsatti@unoeste.br

Abstract
To evaluate the effect of isolated supplementation of low-concentration ozonized water on the clinical, anthropometric, and inflammatory profile in apparently healthy individuals.
A randomized, double-blind, placebo-controlled clinical trial study to evaluate the effects of ozonized water supplementation in two concentrations (10µg/day and 16µg/day) in short-term (7days). Were performed with 66 male and female, over 18 years old. Anthropometric and clinical parameters were measured, serum levels of nitric oxide, manganese superoxide dismutase, glutathione peroxidase were assessed in serum using an immunoassay – ELISA.
With average age of 24.2 years, composed of 54.2% males, 35.4% were undergoing hormone therapy and only 31.2% knew ozonized water. Anthropometrics, clinical and oxidative stress characteristics of study participants did not difference significant to time by group interaction (p>0.05). Supplementation of ozonated water in apparently healthy individuals has no short-term effects on evaluated parameters on anthropometric, clinical, and oxidative stress parameters.
Keywords: ozonetherapy; oxidative stress; ozonized water; clinical profile; anthropometric profile.

Suplementação de água ozonizada não altera o perfil clínico, antropométrico e estresse oxidativo em indivíduos aparentemente saudáveis

Resumo
Avaliar o efeito da suplementação isolada de água ozonizada com baixa concentração no perfil clínico, antropométrico e inflamatório de indivíduos aparentemente saudáveis.
Um estudo clínico randomizado, duplo-cego, controlado por placebo para avaliar os efeitos da suplementação de água ozonizada em duas concentrações (10µg / dia e 16µg / dia) em 7 dias. Inclusos 66 homens e mulheres, maiores de 18 anos. Os parâmetros antropométricos e clínicos foram medidos, os níveis séricos de óxido nítrico, superóxido dismutase de manganês e glutationa peroxidase foram avaliados no soro por ELISA.
Com idade média de 24,2 anos, composto por 54,2% do sexo masculino, 35,4% faziam terapia hormonal e apenas 31,2% conheciam água ozonizada. As características antropométricas, clínicas e inflamatória dos participantes do estudo não apresentaram interação do tempo por grupo (p> 0,05). A suplementação de água ozonizada em indivíduos aparentemente saudáveis não tem efeitos nos parâmetros antropométricos, clínicos e de estresse oxidativo.
Palavras-chave: ozonoterapia; estresse oxidativo; água ozonizada; perfil clínico; perfil antropométrico.

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INTRODUCTION

The use of natural components, whether herbs or medicinal gases with nutritional value and capacity oxidative elimination can provide metabolic benefits in people's health [1]. Due to the COVID-19 pandemic, the potential effectiveness of some vitamins [2], micronutrients [3], natural products [4,5] and ozone therapy (O_3) [6,7] was put into practice. However, the mechanisms underlying the effects of O_3 in physiological homeostasis are not yet fully understood [8]. In addition, research about the effects of ozonated water on health, is still limited.

Physically, the O_3 is dissolved in pure water, depending on the temperature, pressure, and concentration of the gas [9]. Thus, its stability changes, with part of the molecules decomposed in seconds and part remaining stable for hours [10]. As a result, the half-life of the stable portion is around 10 hours (at a pH = 7 and at 20 ^{o}C) and can remain in the water for a few days if stored in a tightly closed amber glass container [11]. O_3 reacts with any electron donor that undergoes oxidation, generating O_3- (radical anion), which decomposes into a hydroxyl radical, also forming a dioxygen molecule. Thus, this reaction makes O_3 a potent oxidant that can act as a precursor to a series of radicals, with actions both *in vitro* and *in vivo* [12,13].

O_3 *in vivo* is safe, regardless of administration[11]: topical, infusions and drinking water, using the techniques of autohemotransfusion, intramuscular, intradiscal, paravertebral, rectal insufflation, and skin exposure [13–16], being characterized as systemic. O_3 can be prepared in a therapeutic concentration without significant side effects [13,14]. It has been reported that the use of minimal doses of O_3 (10–80g/ml of gas per ml of blood or 0.21–1.68 mol/ml) is free of side effects [17–19].

Multiple studies evaluating pathological conditions have provided evidence that O_3 therapy promotes the balance of blood biomarkers of oxidative stress [20], endothelial function [21] by stimulate transmembraneous flow of oxygen, inducing enzymes such as superoxide dismutase, catalase or peroxidases [22], demonstrating to be an effective therapeutic agent in several diseases [20]. Meantime, few studies have demonstrated the effect of O_3 on healthy individuals, and your findings are inconsistent. Inal *et al* in 2011, evaluating healthy people, demonstrated that the activity of superoxide dismutase was significantly increased in the ozone groups, indicating that ozone activates the antioxidant defense system [23]. The MOSES study, showed no effects of O_3 in the primary markers of systemic inflammation when healthy subjects were exposed to O_3 for 3 hours. The authors did not identify any changes mechanistic basis for the most common markers of systemic inflammation after exposure to O_3 in healthy adults and suggests further studies with different O_3 concentrations or prolonged exposure should be carried out [24].

Bocci et al in 2015 recommended that clinical studies take an interest in verifying the therapeutic validity of ozone in an integrated, randomized, international placebo-controlled trials [20]. As far as we know studies evaluating the implementation with ozonated water were only performed under pathological conditions[25]. Thus, the objective of this study was to evaluate the effects of short-term supplementation with ozonized water on apparently healthy individuals.

METHODS

Study design and sample selection

This was a randomized, double-blind, placebo-controlled trial to assess the effects of supplementation of ozonized water in two different concentrations (10 µg/mL and 16 µg/mL). The sample size calculation was based on the study by Niu *et al* de 2018 [16], with a 95% confidence coefficient and a 5% margin of error that supports a sample size composed of at least 18 individuals (figure 1).

Individuals of both genders who were at least 18 years-old and who accept to participate in the initial interview were included. The non-inclusion criteria were presence of current or previous coronary artery disease; chronic kidney disease; insulin-dependent diabetes; liver disease; autoimmune diseases; addiction to either alcohol or illicit drugs. The study was performed according to the declaration of Helsinki. Informed consent was obtained from all participants and the study was approved by the Research Ethics Committee (process number: 3.418.606-CEP).

Randomization and supplementation protocol

After initial screening, all participants were given a number (1 – 66) according to their order of inclusion in the study. Central computer randomization was conducted using specific

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software (SAS 9.2 for Windows using Procedure Plan). The participants were randomly assigned to three groups in a pre-determined sequence: H_2OZ-A, with supplementation of ozonized water at a concentration of 10µgO_3/day (n= 22) H_2OZ-B, with supplementation of ozonized water at a concentration of 16µgO_3/day (n= 22); H_2O-PL, individuals who were given placebo (potable water). The research follow-up time was for 7 days. From each participant, 10 ml of venous blood was sampled into biochemistry tubes with gel separator for measurement. Blood samples were collected, at the initial moment (D0), and then at day 4 (D03) and day 8 (D07) after the beginning of the ozonized water and placebo ingestion (figure 2). The 44 individuals received weight-proportional concentration (µgO_3/day) [18,26] (22 individuals received ozonized water at the concentration of 10 µgO_3/day and 22 individuals received ozonized water at the concentration of 16 µgO_3/day – diluted in 10 mL), once a day, for 7 days. The other 22 individuals received placebo:10 mL, once a day for 7 days. The investigators and the patients were unaware of the group allocation (blinding); only the pharmacist responsible for the manipulation of the placebo knew to which group the patients belonged. The containers were identical, packaged and numbered in code.

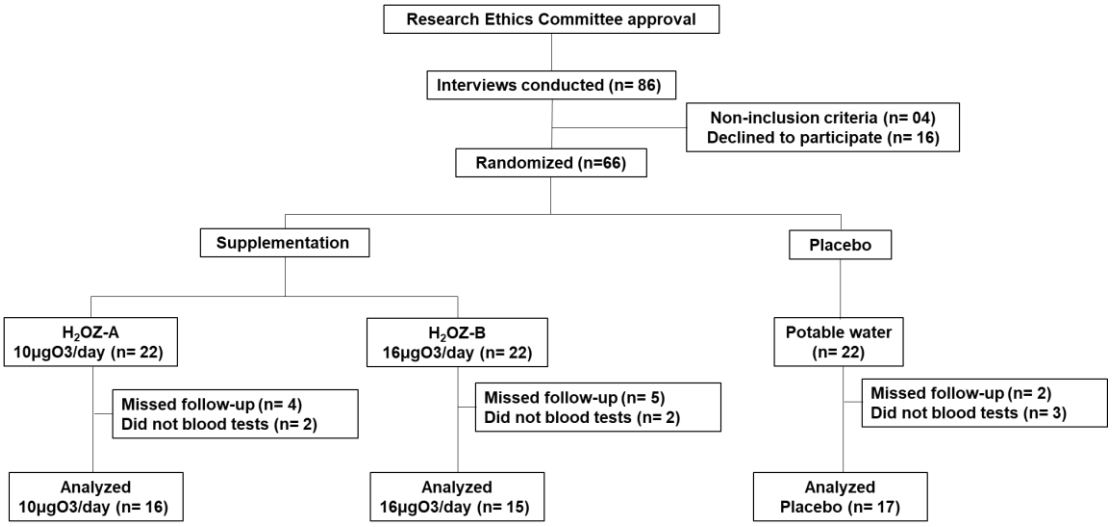

Figure 1. Study design

Ozonated water production and measurement

O_3 Generator (OZONLIFE®) used in this research, has a production capacity of 0.3 grams of O_3 per hour (g/h), and was coupled to an oxygen generator (ever flo model – White Martins®), with a flow rate of 1 L/min. In this system, the O_3 is generated by the action of electric current over the oxygen molecules of high purity, supplied by the oxygen generator. It was used 100 mL of sterile distilled water (25°C), placed in a beaker (sterile). A silicone hose, with a porous stone at one end was connected to the O_3 generator and placed in contact with the water promoting a fine bubble diffuser system. This system remained on for 15 minutes ozonizing the water [27]. After those 15 minutes of ozonation, the final concentration of O_3 in water – residual O_3 – was monitored, with the help of a CHEMest® KIT Ozone colorimetric set (K-7404), following the manufacturer's instructions.

Clinical and anthropometric measurements

All participants underwent individual interviews in which the following data were collected: sex, age (when female, if applicable: age of menopause, time of menopause and parity), use of hormonal therapy, current smoking, history of chronic diseases (hypertension, diabetes, cardiovascular disease), medication use, physical activity, blood pressure, weight, and height. The blood pressure measurement was taken on the left arm with the forearm supported at precordium level, palm up, using sphygmomanometer standard aneroid, with the individuals in a seated position. Individuals were defined as smokers with the habit of smoking daily, regardless of the number of cigarettes smoked.

The following information were obtained for anthropometric evaluation: weight (kg), height (meters) and body mass index (BMI =

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weight/height^2). For the measurement of the waist, the smaller circumference between the last rib and the upper anterior iliac crest was considered, and the reading was toked at the expiration moment. It was considered increased if it were superior to 88 cm for women or superior to 94 cm for men [28].

Laboratory Tests

Blood samples were collected from each subject, after 12 hours of fasting. After centrifugation to remove the clot, samples underwent biochemical analysis immediately and a serum aliquot was frozen and kept at −80°C for the analyte's determinations. Triglycerides (TG), total cholesterol (TC) and glucose measurements were processed by an automated analyzer, Model Vitros 950®, by the colorimetric dry chemistry method (Johnson & Johnson®, Rochester, NY, United States). The optimal values were TC < 200 mg/dl, TG < 150 mg/dL, and glucose <100 mg/dL. The method is linear up to 800 mg/dL for TG and 900mg/dL for TC [28].

Nitric oxide (NO) quantification was performed, which is evaluated by the Griess colorimetric method in a 96-well plate, 50 µL of the plasm, 50 µL of 2% sulfanilamide (in 5% of phosphoric acid) and 50 µL of the solution were added 0.1% NED (dissolved in 5% phosphoric acid). Subsequently, the plate was incubated at 37°C, with shaking for 3 hours. Then a curve containing 0.39-12.5 µM sodium nitrite solution was prepared, containing 50 µL of each point on the curve, plus the sulfanilamide and the NED solution. The plate was read in a spectrophotometry reader with absorbance at 535nm, and the absorbance values are proportional to the amount of nitrite present in the samples [29].

Manganese superoxide dismutase (MnSOD) (R&D® Systems, Minneapolis, USA) and glutathione peroxidase (GPx-1) (ab193767 - Abcam®) were evaluated, by ELISA technique (Enzyme-Linked ImmunoSorbent Assay), obeying the manufacturer's recommendations. The coefficient of variation within and between assays was less than 10% according to the description of the kits. All dosages of each analyte were performed in a single moment and by a single researcher to minimize inter-assay variations. All evaluations were carried out at the Biochemistry Laboratory of the Faculty of Medicine of Jaú - UNOESTE.

Statistical analysis

Starting from data, tables of clinical variables and evaluated parameters were constructed. The variables were analyzed for normal distribution using the Shapiro-Wilk test and homogeneity using the Levene test. For data analysis, mean and standard deviation were calculated for quantitative variables and frequency and percentage for qualitative variables. For comparison between the groups concerning the initial characteristics (clinical, anthropometric and laboratory) the Student t-test and the Gamma distribution (asymmetric) were used. Comparison of anthropometric and laboratory variables among the moments (D0, D3 and D7) and among the groups, were analyzed the delineation in repeated measures in time (ANOVA) followed by the Tukey's multiple comparison test adjusted for interaction between group x moment. And for the asymmetric variables, the same design was used in repeated measures through the Gamma distribution followed by Wald's multiple comparisons. In all tests, the significance level of 5% or the corresponding *p*-value was adopted. The analysis was performed using the program IBM SPSS Statistics, version 9.2.

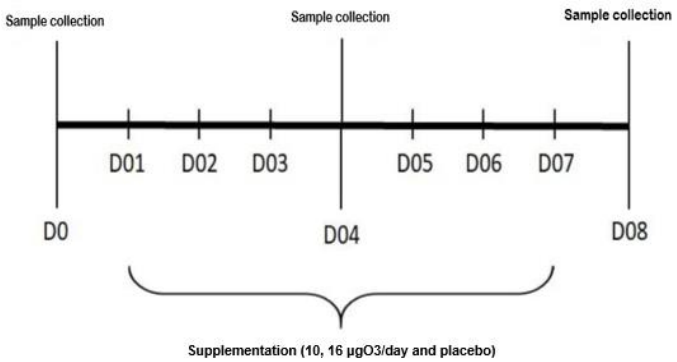

Figure 2. Supplementation of groups

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RESULTS

The general characteristics of the population in this study comprised of 48 participants with a proportion of 45,8% female to 54,2% male, are described in Table 1. From the interview, it was observed that 31.2% of participants knew ozonized water, but no more than 2.1% had consumed it, 95.8% of our population were non-smokers and 35.4% used hormone therapy. The population sample randomization was distributed so that 35.4% of the population received placebo, 33.3% received supplementation with O_3 10 μgO_3/day and 31.3% received the treatment with $O_3$16 μgO_3/day.

Table 1. General characteristics of the study population

Variables	Frequency	Percentage
Gender (f/m)	22/26	45.8/54.2
Hormonal therapy (n/y)	34/17	64.6/35.4
Number of children (n/y)	47/1	97.9/2.1
Smoking (n/y)	46/2	95.8/4.2
Use of medication (n/y)	20/28	41.7/58.3
Know ozonized water (n/y)	33/15	68.8/31.2
Ozonized water consumption (n/y)	47/1	97.9/2.1

(f/m): female/male; (n/y): no / yes.

Analysis of clinical and anthropometric characteristics are shown in table 2. There was no significant association between time by group interaction in systolic and diastolic blood pressure, waist circumference, body mass index, total cholesterol, triglycerides, and glucose after 3 and 7 days of ozonized water supplementation regardless of concentration.

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Table 2. Clinical and anthropometric characteristics of study participants between groups before and after 7 days of supplementation

Variables	Basal	3 days	7 days	P† (group)	P† (time)	P† (time by group interaction)
SBP (mmHg)						
Placebo	117 (11.80)	121 (13.04)	118 (14.79)			
$O_3$10µg/mL	115 (11.55)	126 (12.42)	126 (14.42)	0.490	0.057	0.073
$O_3$16µg/mL	122 (11.43)	121 (12.85)	121 (14.52)			
DBP (mmHg)						
Placebo	74.2 (7.72)	77.1 (11.11)	78.7 (9.95)			
$O_3$10µg/mL	76.3 (7.97)	76.7 (11.23)	78.9 (9.44)	0.925	0.059	0.071
$O_3$16µg/mL	75.4 (7.79)	82.4 (11.01)	75.1 (9.57)			
WC (cm)						
Placebo	88.2 (12.54)	88.8 (12.85)	88.4 (12.77)			
$O_3$10µg/mL	83.5 (12.48)	84.3 (12.66)	84.0 (12.61)	0.056	0.069	0.867
$O_3$16µg/mL	80.0 (12.32)	80.4 (12.52)	79.7 (12.48)			
BMI (kg/m^2)						
Placebo	26.8 (4.64)	26.6 (4.28)	26.6 (4.41)			
$O_3$10µg/mL	25.3 (4.08)	25.4 (3.83)	25.5 (3.90)	0.094	0.484	0.080
$O_3$16µg/mL	23.6 (4.16)	23.6 (3.96)	23.8 (4.03)			
TC (mg/dL)						
Placebo	156 (41.29)	149 (43.24)	170 (33.72)			
$O_3$10µg/mL	142 (41.44)	151 (37.12)	174 (40.09)	0.852	0.001	0.649
$O_3$16µg/mL	134 (40.43)	147 (37.39)	172 (40.02)			
TG (mg/dL)						
Placebo	191 (79.67)	179 (65.29)	189 (74.99)			
$O_3$10µg/mL	146 (81.62)	130 (60.90)	162 (80.07)	0.076	0.073	0.841
$O_3$16µg/mL	147 (78.69)	111 (62.38)	146 (78.41)			
Glucose (mg/dL)						
Placebo	74.6 (20.3)	103 (30.5)	95.1 (14.1)			
$O_3$10µg/mL	83.5 (38.5)	105 (27.3)	87.5 (32.3)	0.138	0.620	0.181
$O_3$16µg/mL	80.2 (16.7)	83.9 (16.8)	78.4 (16.3)			

Mean values (± standard deviation). SBP. systolic blood pressure; DBP. diastolic blood pressure; BMI. body mass index; WC. waist circumference; CT. total cholesterol; TG. triglycerides.
*Significant difference (P<0.05) from baseline.
‡ Significant difference (P<0.05) from placebo.
† = *p*-value adjusted to gender.

The inflammatory profile in table 3 shows the concentrations of NO, MnSOD and GPx-1 during the timeline and the interaction from pre to post supplementation of the groups did not demonstrate statistical significance. Cytokine analysis (IL-6, IL-10 and TNF-alpha) for ELISA were performed but levels of these cytokines were below the detection threshold of the commercial kits used (data not shown).

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Table 3. Oxidative stress mediators of study participants between groups before and after 7 days of supplementation

Variables	Basal	3 days	7 days	P† (group)	P† (time)	P† (time by group interaction)
NO (µM)						
Placebo	37.6 (16.59)	31.3 (23.70)	37.5 (34.69)	0.628	0.966	0.943
$O_3$10µg/mL	34.1 (19.83)	38.4 (23.79)	40.6 (34.67)			
$O_3$16µg/mL	45.5 (23.42)	42.9 (23.47)	39.6 (33.16)			
MnSOD (ng/mL)						
Placebo	0.892 (0.39)	0.987 (0.49)	0.970 (0.75)	0.445	0.920	0.844
$O_3$10µg/mL	0.884 (0.59)	0.760 (0.44)	0.699 (0.63)			
$O_3$16µg/mL	1.020 (0.58)	0.891 (0.44)	1.107 (0.66)			
GPx-1 (ng/mL)						
Placebo	0.237 (0.64)	0.329 (0.76)	0.179 (0.48)	0.528	0.568	0.126
$O_3$10µg/mL	0.050 (0.46)	0.077 (0.52)	0.044 (0.36)			
$O_3$16µg/mL	0.057 (0.44)	0.056 (0.51)	0.146 (0.34)			

Mean values (± standard deviation). NO. Nitric oxide; MnSOD. manganese superoxide dismutase; GPx-1. glutathione peroxidase.
*Significant difference (P<0.05) from baseline.
‡ Significant difference (P<0.05) from placebo.
† = P value adjusted to gender.

DISCUSSION

In this study, we aimed to explore the effects of short-term ingestion of ozonized water on apparently healthy individuals to verify if it could be useful for improvement of the anthropometric, clinical profile and markers of oxidative stress. To our knowledge, we are the first to investigate supplementation of ozonized water with two concentrations in supposedly healthy individuals. However, the supplementation of ozonized water used herein showed no significant difference between placebo and control groups.

Previous studies have been suggested that low doses of O_3 has beneficial and are attributed mainly to its bactericidal and oxidant properties in pre-existing disease [10,19,20,30,31], O_3 stimulates the production of enzymes that act as free radical and cell wall protectors: GPx-1, NO and MnSOD [24,32]. These findings are inconclusive when observed in young healthy subjects. In a double-blind, randomized, crossover study, young healthy subjects were exposed to ozone for 3h compared those exposed to filtered air, the study found no significant effects of ozone on measure of cardiac or vascular function [33]. Rich *et al* exposed healthy volunteers to low levels of ozone. They found no significant effects of ozone exposure on any of the measures vascular function (systolic blood pressure and flow-mediated dilation)[34]. Short-term exposure to ozone does not impair of vascular function or affect heart rate variability in healthy young men was demonstrated [35].

Although ozone is a gas capable of inducing oxidative stress and inflammation in apparently healthy individuals [24], evidence for the effects is not clear. Ozone reacts rapidly with circulating low molecular weight antioxidants, is unlikely to cause systemic oxidant and

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inflammatory effects when the antioxidants are not present in a enough systemic amount, not exerting their oxidative effect [20]. Barath *et al* related that ozone exposure does not have a direct and rapid effect on the cardiovascular system, and although we cannot exclude an effect of chronic exposure [36]. Although our results cannot be directly compared with those of previous studies, as it is not ozonized water supplementation, the design of our study was based on a short term and a previously described minimum concentration that does not affect cell proliferation or death, does not alter nuclear domains despite identifying an increase in the rate of transcription at nucleoplasmic (mRNA) and nuclear (rRNA) levels, without altering cell viability [26].

One of the main processes involved in the development of diseases and their progression is the alteration of cellular redox homeostasis. The ability of an organism to restore homeostasis and increase oxidative levels can be critical to survive and avoid possible cellular damage [37]. We understand that the sample size is small and, on the other hand, we cannot exclude an oxidative effect possible in use of chronic or higher concentrations of O_3. Our group believes that these data are promising, and that new studies should be carried out to understand the effect of O_3 *in vivo*.

CONCLUSION

Short-term ozonized water supplementation in individuals without any pre-existing disease does not influence the anthropometric, clinical, and oxidative stress mediators: GPx-1, NO and MnSOD. New studies are necessary in order to the effect of ozone supplementation comparing healthy individuals and those in pathological conditions.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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