Effects of ozone oxidative preconditioning on radiation-induced organ damage in rats

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Because radiation-induced cellular damage is attributed primarily to harmful effects of free radicals, molecules with direct free radical scavenging properties are particularly promising as radioprotectors. It has been demonstrated that controlled ozone administration may promote an adaptation to oxidative stress, preventing the damage induced by reactive oxygen species. Thus, we hypothesized that ozone would ameliorate oxidative damage caused by total body irradiation (TBI) with a single dose of 6 Gy in rat liver and ileum tissues. Rats were randomly divided into groups as follows: control group; saline-treated and irradiated (IR) groups; and ozone oxidative preconditioning (OOP) and IR groups. Animals were exposed to TBI after a 5-day intraperitoneal pretreatment with either saline or ozone (1 mg/kg/day). They were decapitated at either 6 h or 72 h after TBI. Plasma, liver and ileum samples were obtained. Serum AST, ALT and TNF-α levels were elevated in the IR groups compared with the control group and were decreased after treatment with OOP. TBI resulted in a significant increase in the levels of MDA in the liver and ileal tissues and a decrease of SOD activities. The results demonstrated that the levels of MDA liver and ileal tissues in irradiated rats that were pretreated with ozone were significantly decreased, while SOD activities were significantly increased. OOP reversed all histopathological alterations induced by irradiation. In conclusion, data obtained from this study indicated that ozone could increase the endogenous antioxidant defense mechanism in rats and thereby protect the animals from radiation-induced organ toxicity.

Keywords: Ozone oxidative preconditioning; oxidative stress; radiation

INTRODUCTION

Whole body exposure to ionizing radiation in humans and animals may trigger multiple organ dysfunctions directly related to an increase of cellular oxidative stress due to overproduction of reactive oxygen species (ROS) from molecular ionization [1, 2]. When cells or tissues are exposed to ionizing radiation, the water molecules undergo dissociation (radiolysis) and produce free radicals and related species in the form of ROS. These, in turn, can act on biomolecules such as DNA, lipids and proteins, and cause oxidative damage [3, 4]. Subsequent to the radiation-induced oxidative stress, intracellular lipid peroxidation increases as a result of the oxidative transformation of multi-unsaturated lipid acids to malondialdehyde (MDA) and nitric oxide (NO) [5]. ROS also negatively impact the antioxidant defense mechanisms, reduce the intracellular concentration of glutathione (GSH) and decrease the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) [6]. The exposure of the human body to ionizing radiation leads to depletion of these endogenous antioxidants [7–9] and ultimately to the development of systemic disease. Recently, research has focused on finding effective and reliable antioxidants that can maintain the pro-oxidant vs. antioxidant redox balance and protect tissues against radiation-induced damage.

Ozone is made up of three oxygen atoms and is known chemically as O₃. Ozone is applied in medical therapy...
using a gas mixture composed of oxygen and ozone. Clinical studies have shown that ozone therapy appears to be useful in diseases including gastroduodenal ulcers, peri-
tonitis, colitis and chronic skin ulcers [10–12]. The term ‘ozone oxidative preconditioning (OOP)’ refers to the ad-
ministration of ozone at repeated atoxic doses that provide an adaptation to oxidative stress. It has been demonstrated that controlled ozone administration increases the activity of antioxidant enzymes such as GSHP, SOD and CAT, and prepares the host for physiopathological conditions mediated by ROS [13, 14]. OOP has proven to be useful in inhibiting inflammation and apoptosis and has been shown to induce a sort of cross-tolerance to free radicals released after hepatic and renal ischemia reperfusion in experimental studies [15, 16]. It has also been demonstrated that OOP can induce an adaptation to oxidative stress in the hepatocytes of rats following carbon tetrachloride (CCl₄) poisoning [17].

Exposure to ionizing radiation exposure involves the development of potentially serious health conditions. Acute effects mainly include hematopoietic cell loss, immune suppression, mucosal damage and potential injury to the liver and other tissues. Because radiation-induced cellular injury is attributed mainly to ROS, it is anticipated that OOP should prepare the host for the radiation-induced oxidative stress and tissue injury. Therefore, the aim of the present study was to examine the effects of OOP against oxidative damage and organ injury induced by ionizing radiation in the rat liver and ileum tissue after a 6 Gy single dose of total body irradiation (TBI).

MATERIALS AND METHODS

The experimental protocols were conducted with the approval of the Animal Research Committee at Bulent Ecevit University, Zonguldak. All animals were maintained in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Animals and experiments

Thirty female Wistar rats weighing 200–230 g were housed individually in cages and were allowed free access to standard rat chow and water before and after the experiments. The animal rooms were windowless with temperature (22 ± 2°C) and lighting controls. The animals were fasted overnight before the experiments but were given free access to water. They were anesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine by body weight, which was administered intraperitoneal (ip). The rats were divided into five equal groups.

Control group (Group 1): the animals daily received ip injection of 0.9% saline for 5 days. Saline-treated and irradiated (IR) groups (Groups 2 and 3): animals received daily ip injections of 0.9% saline for 5 days. One hour after the last injection of saline, the animals were exposed to a dose of 6 Gy TBI. Rats were decapitated at 6 h (Group 2) and 72 h (Group 3) after the exposure to radiation. Ozone oxidative preconditioning (OOP) and IR groups (Groups 4 and 5): An ozone/oxygen O₂/O₃ mixture was administered ip at a dose of 0.7 mg/kg. The volume of gaseous mixture administered to each animal was approximately 2.3 ml. OOP was performed using five applications (one daily) of the ozone/oxygen mixture. One hour after the last injection, the rats were irradiated with 6 Gy TBI in a single fraction. The rats were decapitated at 6 h (Group 4) and 72 h (Group 5) after the exposure to radiation.

Ozone production

Ozone was generated by an ozone generator, which allowed control of the gas flow rate and ozone concentration in real time using a built-in UV spectrometer, and was administered immediately at a dose of 0.72 mg/kg daily via an ip route. The volume of the injected mixture was approximately 2.3 ml. Oxidative preconditioning was performed using five applications (once daily) of the O₂/O₃ mixture. The ozone flow-rate was kept constant at 3 l/min, representing a concentration of 60 mg/ml and a gas mixture of 97% O₂ + 3% O₃. Tygon polymer tubes and single-use silicon-treated polypropylene syringes (ozone resistant) were used throughout the experiment to ensure containment of O₃ and consistency of concentration [18–19].

Total body irradiation

TBI was delivered to anesthetized (ketamine 100 mg/kg intramuscular injection) rats in the prone position with a single non-lethal dose of 6 Gy using a 6-MV linear accelerator at a dose rate of approximately 1 Gy/min with source axis distance (SAD) technique and a 1.0-cm bolus material on the surface. Computerized tomography simulation of a rat was performed with 1-mm slices, and a dose calculation was performed with the Eclipse treatment planning system version 8.9 (Varian Medical Systems, Palo Alto, CA, USA). Animals were returned to their home cages following irradiation. Control animals were aneasthetized but were not exposed to radiation. All irradiations were performed between 07:00 and 08:30.

Sample collection

At the end of the experimental period, all animals were sacrificed. Trunk blood was collected, and the serum was separated to measure the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels as indicators of liver function. Tumor necrosis factor alpha (TNF-α) was also assayed in serum samples for the evaluation of general-ized tissue damage. Tissue samples from the liver and ileum were fixed in formaldehyde for histological analysis,
while additional samples were stored at −80°C for the determination of MDA levels and SOD activity.

**Biochemical analysis**

Serum ALT and AST levels were measured with commercially available kits on an Advia 2400 automated analyzer (Siemens Healthcare Diagnostics, Tarrytown, New York, USA). Serum TNF-α levels were measured using rat commercial enzyme-linked immunoassay (ELISA) reagents (eBioscience; San Diego, CA, USA) by following the manufacturer’s protocol. Hepatic and ileal tissues were homogenized in ten volumes of 150 mM ice-cold KCl using a glass teflon homogenizer (Ultra Turrax IKA T18 Basic) after cutting the tissues into small pieces with scissors (for 2 min at 5000 rpm). The homogenate was then centrifuged at 5000 g for 15 min. The supernatant was used for analysis. High performance liquid chromatographic (HPLC) analysis was performed with the isocratic method using an Agilent 1200 HPLC system (San Jose, CA, USA) with a commercial MDA kit (Immundiagnostik AG, Bensheim, Germany). The first step in determining MDA is a sample preparation with a derivatization reagent that transforms MDA into a fluorescent product. Afterwards, the pH was optimized and the reaction mixture (20 ml) was chromatographed on a reversed phase C18 column (18.5 mm, 125 × 4 mm) at 30°C. The flow rate was 0.8 ml/min. Fluorometric detection was performed with excitation at 515 nm and emission at 553 nm. The detection limit was 0.15 mmol/l, and linearity was up to 100 mmol/l. Protein concentrations of the supernatants were determined using the method of Lowry et al. [20]. Total SOD activity was determined according to the method of Sun et al. [21]. The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the liver homogenate after a 1.0-ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of the hemolysate and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. The results are expressed as nmol/g protein for MDA and U/g protein for SOD.

**Histopathological analysis**

Tissue samples were fixed in 10% buffered p-formaldehyde and prepared for routine paraffin embedding. Tissue sections (5 μm) were then stained with hematoxylin and eosin and examined under a light microscope (Olympus-BH-2) by an experienced histologist, who was unaware of the treatment conditions.

**Statistical analysis**

Statistical analysis was performed with SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). All values are expressed as the mean ± standard error of mean. Continuous variables were compared with the Kruskal–Wallis test. P values < 0.05 were considered statistically significant for all tests.

**RESULTS**

Plasma AST and ALT levels, which were used as indices of hepatic injury and of generalized tissues damage, were significantly higher in both IR groups decapitated at 6 or 72 h after irradiation when compared with those of the control group (P = 0.001 and P < 0.001, respectively; Fig. 1A and 1B), while OOP prevented these elevations in AST in groups 4 and 5 (P = 0.001; Fig. 1A), and in ALT in group 5 (P < 0.001; Fig. 1B). In the saline-treated IR groups, TNF-α levels were significantly increased (P < 0.001) when compared with the control group, while this irradiation-induced rise in serum TNF-α level was decreased with OOP (Table 1). MDA is an index of hepatic damage associated with lipid peroxidation. Compared with those of the control group, MDA levels in the hepatic and ileal tissues were significantly higher in the irradiated group that had received vehicle treatment (P = 0.001 and P < 0.001, respectively, Table 1), while treatment with ozone significantly prevented lipid peroxidation in both tissues (P = 0.001 and P < 0.001 respectively, Table 1). SOD activity indicates the generation of oxidative stress, and an early protective response to oxidative damage in the liver and ileal tissues of saline treated rats was significantly decreased at 6 h and 72 h following irradiation compared with the control group (P < 0.001, Table 1). However, treatment with ozone significantly prevented the alterations in the SOD activity in all the tissues (P < 0.001, Table 1).

On histopathological examination, no abnormalities were seen in the liver or ileum of the control group (Group 1, Figs 2A and 3A). Sinusoidal dilatation, and congestion and dilatation in the central veins as well as minimal mononuclear inflammatory cell infiltrate in the portal triads were observed in the liver tissues of the saline-treated IR group at 6 h (Group 2, Fig. 2B). Aberrant congestion and dilatation in both the sinusoids and central veins and mononuclear inflammatory cell infiltrate in the portal triads were more prominent in the saline-treated IR group at 72 h (Group 3, Fig. 2C). Moderate hepatocellular degeneration also occurred in this group. In the OOP and IR groups at 6 h (Group 4, Fig. 2D) and 72 h (Group 5, Fig. 2E), hepatocellular degeneration, inflammation, and congestion and dilatation in both the sinusoids and central veins were reduced when compared with the saline-treated IR groups at 6 h (Group 2) and 72 h (Group 3). Focal degeneration and loss of the epithelium in the villi and mononuclear inflammatory cell infiltrate in the lamina propria were observed in the ileal mucosa of the saline-treated IR group at 6 h (Group 2, Fig. 3B). Disordered villous structure, focal degeneration and loss of the epithelium in the villi,
reduced goblet cells in the epithelium, subepithelial congestion and marked mononuclear inflammatory cell infiltrate in the lamina propria were noted in the saline-treated IR group at 72 h (Group 3, Fig. 3C). In the OOP and IR group at 6 h (Group 4, Fig. 3D), inflammatory cell infiltrate in the lamina propria was reduced when compared with the saline-treated IR group at 6 h (Group II). Regular villous structure, abundant goblet cells in the epithelium and reduced inflammatory cell infiltrate in the lamina propria occurred in the OOP and IR group at 72 h (Group 5, Fig. 3E). There was no subepithelial congestion in this group.

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**Fig. 1.** (A) Serum aspartate aminotransferase (AST) and (B) alanine aminotransferase (ALT) levels in serum samples of control and saline- or ozone-treated groups decapitated at 6 h or 72 h after irradiation. Each group consisted of six rats. *P=0.001, +P<0.001: compared with control group; **P=0.001, +P<0.001: compared with saline-treated group.

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### Table 1. Liver and ileal tissues MDA (nmol/g) levels and SOD (U/g) activities, and serum TNF-α (pg/ml) levels in control, and saline- or ozone-treated irradiated groups decapitated at 6 h or 72 h after irradiation (IR). Each group consists of six rats.

|                | Control (6 h) | IR 6 h | IR 72 h |
|----------------|---------------|--------|---------|
|                | Group 1       | Group 2 | Group 3 | Group 4 |
|                | Saline-treated Group 4 | Ozone-treated Group 5 | Saline-treated Group 3 | Ozone-treated Group 5 |
| Ileum          |               |        |         |
| MDA (nmol/g)   | 1.76 ± 0.40   | 5.60 ± 0.48* | 2.35 ± 0.38* | 6.16 ± 0.63* | 2.78 ± 0.54* |
| SOD (U/g)      | 24.09 ± 1.24  | 7.67 ± 0.39* | 18.26 ± 2.39* | 9.25 ± 1.09* | 24.97 ± 1.39* |
| Liver          |               |        |         |
| MDA (nmol/g)   | 4.33 ± 0.12   | 6.42 ± 0.28** | 4.42 ± 0.12** | 6.72 ± 0.21** | 4.16 ± 0.56** |
| SOD (U/g)      | 42.69 ± 1.94  | 20.04 ± 2.328* | 37.60 ± 3.16* | 20.24 ± 0.78* | 46.31 ± 2.38* |
| Serum          |               |        |         |
| TNF-α (pg/ml)  | 20.96 ± 0.70  | 28.66 ± 0.40* | 19.51 ± 0.62* | 27.03 ± 0.77* | 17.91 ± 0.47* |

*P <0.001, **P = 0.001 compared with control group; +P <0.001, ++P = 0.001 compared with saline-treated irradiated groups.
Radiotherapy is an important modality for cancer treatment, and it is estimated that approximately 50% of cancer patients derive benefits from it [22]. Although it is a common and important tool for cancer treatment the radiosensitivity of normal tissues adjacent to the tumor limits the therapeutic gain. Tissue injury from ionizing radiation ultimately begins with oxidative stress from radiolytic hydrolysis and the formation of ROS [23]. ROS can induce oxidative damage to vital cellular molecules and structures including DNA, lipids, proteins and membranes [24]. These species can also induce the cellular antioxidant defense enzymes such as SOD, GSH and CAT [25]. However, ionizing radiation decreases the total antioxidant capacity of the organism and results in an imbalance between pro-oxidants and antioxidants [8, 9]. It is hypothesized that if oxidative stress is involved in the origin of radiation-induced tissue damage then successful antioxidant treatment should delay or prevent the onset of that damage.

Ozone therapy has had beneficial effects for various diseases, such as caustic esophageal burn, diabetic foot and...
radiation-induced cystitis [26–30]. It can be administered via topical, rectal, peritoneal (animal studies) or oral (ozonated water or oil) routes. In addition, it has recently been discovered that ozone is able to induce an adaptation to oxidative stress and promote oxidative preconditioning through the increase and preservation of antioxidant endogenous systems in animal models of hepatotoxicity, which can be induced by CCl₄. It also can produce protection against hepatic and renal ischemia reperfusion injury [15–17]. Ajamieh et al. demonstrated that a controlled number and dose of ozone treatments conferred protection against different physiopathological processes mediated by ROS [17, 24]. They called this phenomenon OOP. OOP protected the host from the damage produced by ROS and induced improvement of antioxidant and pro-oxidant balance and the concomitant preservation of the cell redox state.

In this study, we specifically investigated whether OOP would provide a protective effect against radiation-induced oxidative damage in the liver and ileum tissue and in the

Fig. 3. Effect of OOP on rat ileum morphology. (A) Control group; (B) Saline-treated and IR 6 h: epithelial degeneration and loss of the villi (thin arrow) and mononuclear inflammatory cell infiltrate in the lamina propria (thick arrow); (C) Saline-treated and IR group at 72 h: disordered villous structure, epithelial degeneration and loss of the villi (thin arrow), reduced goblet cells in the epithelium, subepithelial congestion (thick arrow) and marked mononuclear inflammatory cell infiltrate in the lamina propria; (D) OOP and IR group at 6 h: reduced inflammatory cell infiltrate in the lamina propria (arrow); (E) OOP and IR group at 72 h: regular villous structure, abundant goblet cells (arrow) in the epithelium, reduced inflammatory cell infiltrate in the lamina propria. (hematoxylin and eosin, A, B, C, D and E; ×200).
serum. Our results showed that TBI of rats at 6 Gy causes increased levels of lipid peroxidation in hepatic and ileal tissues as demonstrated by MDA levels, which is a good indicator of the degree of lipid peroxidation [31]. It has been reported that whole-body exposure of rats to high-energy radiation from Co-60 causes tissue damage in several organs as assessed by increased lipid peroxidation at 2, 12 and 72 h after irradiation [8, 9]. Thus, radiation-induced damage might result in adverse health effects within hours to weeks and delayed effects may be observable many months after exposure [23]. In our study compared with those of the control group, the MDA levels in the hepatic and ileal tissues in the saline-treated IR groups significantly increased at 6 h and 72 h following IR, whereas almost the same MDA values were recorded in both early and later phases after IR (Table 1). OOP prior to radiation was found to decrease lipid peroxidation. It seems likely that ozone ameliorates radiation-induced oxidative injury in part by activating metabolic pathways that maintain an equilibrated redox balance. Similarly, it was shown that OOP protected the liver and kidney tissues of rats exposed to ischemia reperfusion [15, 16]. In the study by Ajamieh et al., both ischemic preconditioning and OOP provided similar protective effects in liver ischemia reperfusion injury [15]. Moreover, Guven et al. demonstrated that medical ozone therapy reduced ileal MDA levels in a pup model of necrotizing enterocolitis [18]. Our results agree with previous studies that found that ozone administration attenuated protein and lipid oxidation in ROS-mediated liver and ileal damage [15, 16, 18]. Lipid peroxidation can cause severe impairment of membrane function by increasing membrane permeability and membrane protein oxidation [29]. In the present study, TBI induced a significant increase in the activities of ALT and AST. This increase in serum aminotransferase activities by IR may be attributed to the damage of cellular membranes of the hepatocytes by excessive production of ROS, which can cause the leakage of cytosolic enzymes outside the cells leading to an increase in the aminotransferase activities in blood serum. OOP significantly restores the changes of enzyme activities (AST and ALT) due to its ability to preserve the cell redox state and to ensure that toxic aldehydes are not formed.

In the present study, both the liver and ileal antioxidant systems were clearly affected by TBI. A considerable reduction in SOD activity was observed in the saline-treated IR groups. This could be due to an enhanced utilization of the antioxidant system as an attempt to detoxify the free radicals generated by radiation. OOP increased total SOD activity in both the hepatic and ileal tissues with regard to the saline-treated IR groups. Our results are in agreement with those of former studies reporting increased activities of SOD, CAT and peroxidase after repeated atoxic doses of ozone exposure [13–17]. OOP was apparently able to enhance the antioxidant system, and the effect of ozone on the liver and ileum was similar to the effect on the antioxidant enzymes. The ozone-treated groups had less hepatocellular degeneration than the saline-treated groups. Moreover, reduced inflammatory cell infiltrate in the lamina propria occurred in the ileal tissues of the OOP groups. Ozone appears to minimize the radiation-induced tissue injury by restoring the oxidant balance.

Tissue damage and repair initiated by irradiation are associated with the production of important biological mediators, including cytokines [30], which perpetuate the inflammatory and fibrogenic processes associated with irradiation injury [31]. Both clinical and experimental studies have shown that any noxious event is perceived by tissue macrophages and monocytes, which in turn secrete cytokines such as interleukin-1 (IL-1) and TNF-α [32, 33]. As evidenced in the present study, IR resulted in increased serum TNF-α, indicating the role of this cytokine in radiotherapy-induced toxicity, while OOP depressed the TNF-α response. Thus, it seems likely that the inhibitory effects of ozone on TNF-α levels in the serum of rats that received IR are a consequence of the stimulation of the antioxidant defenses induced by OOP. Recently, Zamora et al. analyzed the effect of OOP on serum TNF-α levels and hepatic tissue in an endotoxic shock model [33]. In accordance with our results, they found that ozone oxidative preconditioning exerts inhibitory effects on TNF-α production.

Numerous studies have demonstrated that OOP is able to provide an adaptation to oxidative stress [13–19, 26–28]. The present study confirms previous findings that ozone exerts influence on the antioxidant and pro-oxidant balance for the preservation of the cell redox state by the increase of the endogenous antioxidant systems. The mechanisms underlying OOP remain unknown, and several studies tried to evaluate them. In the study by Leon et al. [17], OOP provided protection against CCl4 exposure of the liver. This result was supported by several findings such as increased activities of SOD, catalase and peroxidases after chronic O3 exposure. Plants can also express a protective role of nitric oxide synthetase (NOS) expression, which promotes NO formation in the required concentrations.
for protecting against liver ischemia-reperfusion injury. Similarly, Punjabi et al. [37] and Pendino et al. [38] have shown that exposure to ozone causes NO production in macrophages and type II cells of the rat, whereas Haddad et al. [39] demonstrated inducible nitric oxide synthetase (iNOS) induction in rats. More recently, it was demonstrated that intrarectal application of ozone reduced ROS by stimulation and/or preservation of the endogenous antioxidant systems in experimental models of liver and renal ischemia-reperfusion, respectively [17, 39, 40]. Additionally, there is evidence of an increase in the activity of antioxidant enzymes such as SOD and GSHPx and a decrease of MDA after OOP in patients with cardiopathy and in rats suffering ischemia-reperfusion injury [27, 41]. Finally, Martinez et al. evaluated therapeutic efficacy of ozone in patients with diabetic foot, and they concluded that one of the mechanisms underlying OOP could be the induction of SOD activity through the stimulation of SOD gene expression. Previous studies mostly used the ischemia reperfusion to evaluate the effect of OOP on tissue damage, whereas our study is the first to demonstrate the protective effect of OOP on radiation-induced oxidative damage of the hepatic and ileal tissues, as well as of the serum TNF-α levels. However, in the present study, we did not assess the mechanisms underlying OOP, and we demonstrated the effects of ozone treatment on radiation-induced organ damage. The main limitation of our study was the inadequate evaluation of the mechanisms underlying OOP. We could have made clear whether ozone could protect antioxidant enzymes from radiation or induce antioxidant enzyme gene expression. However, previous studies provided some explanation of the mechanisms underlying ozone treatment in various experimental models. Therefore, we designed our study according to previous findings, and we did not evaluate the active mechanism underlying ozone treatment in IR tissues.

In summary, the radioprotection observed with OOP is attributed to an increase in the endogenous antioxidative defense enzymes and the inhibition of lipid peroxidation. However, further studies are needed to elucidate the mechanism underlying the beneficial effect of OOP on radiation-induced damage.

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