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Photodynamic Effect on Photosensitized *Candida albicans* with Hematoporphyrin Derivative Using Low Power Diode Laser *In Vitro*

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ABSTRACT

In this study, the Photodynamic Effect (PDE) of Hematoporphyrin derivative (HpD) combined with irradiation from low power laser on *Candida albicans* was investigated. Four isolates of *C. albicans* were diagnosed in seven samples collected from oral cavity of adults with dental caries. Laser irradiation used in this study has been emitting from Diode laser at a wavelength equal to 630 nm (red region of the spectrum), while the output power was approximately 10mW as a continuous wave. The exposure time of irradiation was between (30-240) seconds, irradiation area was (0.125) cm^2. *C. albicans* were exposed to irradiation after its exposure to several concentrations of HpD these concentrations were varied from (16 -128) µg/ml. The results clearly showed the Photodynamic effect in killing *C. albicans* which was photosensitized by HpD in combination with laser irradiation. There was no determined effect on yeast cells viability neither by the using HpD concentrations nor irradiation doses separately. It could be concluded that PDE of HpD by low power laser irradiation at wavelength 630 nm on *Candida albicans* was a powerful technique in growth inhibition of that pathogenic yeast *In vitro*.

Keywords: photodynamic effects**,** *Candida albicans***,** hematoporphyrin**,** diode laser.

التأثير الحركي الضوئي على خميرة *Candida albicans* المحسسة ضوئيا بمشتق الهيماتوبورفرين باستعمال ليزر الثنائي ذي القدرة الواطئة خارج الجسم

الملخص

تم التحري في هذه الدراسة عن التأثير الحركي الضوئي لمشتق الهيماتوبورفرين (HpD) بالاقتران مع اشعة ليزر ذات قدرة واطئة على خميرة الكانديدا البيضاء *Candida albicans*. اربع عزلات مـن خميـرة *C. albicans* شخصت في سبع عينات تم جمعها من تجويف الفم للبالغين المصابين بتنخر الاسنان. اشـعاع الليزر المستخدم في هذه الدراسة ينبعث من ليزر الثنائي بطول موجي 630 نانومتر(منطقة الطيف الحمراء)،

اما الخرج الليزري فكان 10 ملي واط تقريبا وهو ليزر مستمر الموجة. فترات التعرض للتشعيع تراوحـت بين (30- 240) ثانية على مساحة تشعيع قدرها (0,125) سنتمتر مربع. تم تطبيق هذه الاشعة الليزرية على خلايا الخميرة بعد تعريض الاخيرة الى عدة تراكيز من مـشتق الهيمـاتوبورفرين متدرجـة ابتـداءاً مـن (16 – 128) مايكروغرام/مل. اثبتت النتائج بوضوح التأثير الحركي الضوئي على قتـل خلايـا الخميـرة المحسسة ضوئيا بمشتق الهيماتوبورفرين مقترنا بليزر الثنائي. في حين لم يكن هناك تـأثير قاتـل لخلايـا الخميرة عند استعمال مشتق الهيماتوبورفرين او اشعة ليزر الثنائي كلا على حدة. تم الاستنتاج بـان التـأثير الحركي الضوئي لمشتق الهيماتوبورفرين بواسطة ليزر الثنائي وبطول موجي 630 نانوميتر علـى خلايـا خميرة *C. albicans* هو تقنية ذات كفاءة عالية في تثبيط نمو هذه الخمائر الممرضة خارج الجسم.

الكلمات الدالة: التأثير الحركي الضوئي، خميرة الكانديدا البيضاء، الهيماتوبورفرين، ليزر الثنائي.

INTRODUCTION

Photodynamic Effect (PDE) is providing a technique of killing microorganisms in localized and topical infections (Malik *et al*., 1990; Wilson, 1993). It can be defined as destruction of photosensitized cells by free radical ions such as singlet oxygen ($^1O_2$) produced by binary mechanism of a photosensitizing agent and irradiation of an appropriate wavelength (Dougherty *et al*., 1998). Despite the PDE or what is called Photodynamic Action (PDA) is widely accepted for clinical application to treat neoplastic cells, but is also accepted for antimicrobial inhibition dynamically. A large number of microorganisms (including oral species) have been reported to be killed *In vitro* by this technique (Paardekooper *et al*., 1995; Wilson *et al*., 1992). PDE includes delivering irradiation of the appropriate wavelength to excite the photosensitizing agent (photosensitizer) from ground or triplet state to singlet state. This excited state may then undergoing intersystem crossing to the slightly lower energy, which may then react further by two processes known as Type I and Type II photochemical reactions, both of them require molecular oxygen (Ochsner *et al*., 1997). The Type I photochemical reaction includes electron-transfer reactions from the triplet state of photosensitizer to produce free radical ions which can react with oxygen to produce cytotoxic species, such as singlet superoxide anions (Athar and Bickers, 1988). The Type II photochemical reaction includes transfer of energy from the triplet state of photosensitizer to ground state molecular oxygen (triplet) to produce excited-state singlet oxygen ($^1O_2$), which can oxidize many biological molecules, such as proteins, nucleic acids and lipids, and then lead to photocytotoxic effects (Redmond and Gamlin, 1999). The mechanism of the generation of singlet oxygen ($^1O_2$) formed by the exposure to Hematoporphyrin Derivative (HpD) is considered as one of the important photodynamic techniques. There is no history of PDT can be mentioned without referring to HpD. Hematoporphyrin Derivative. They are referred to as first-generation sensitizers. HpD is the most extensively studied and clinically used photosensitizer. Recently, HpD has been used as an antimicrobial photosensitizer due to its capabilities to inhibit both gram-positive, gram-negative bacteria (Merchat *et al*., 1996). This photosensitizer has been successfully applied as broad spectrum antimicrobial compounds. However, in the most of studies

determinate the techniques of pathogenic yeast photodynamic inhibition, the anionic hematoporphyrin and its derivatives, are used as photosensitizers to sensitize these microorganisms such as *Candida albicans* (Bliss *et al*., 2004; Bocking *et al*., 2000). *C. albicans* may be present in different sites on the body of human due to its opportunistic behavior, sometimes invading surface tissues causing candidosis (El Azizi *et al*., 2004; Sen *et al*., 1997). The presence of *C. albicans* had already been determined in caries lesions, in addition to its capability to infect oral tissues. It has usually known with their ability for antimicrobial resistance (Akdeniz *et al*., 2002 ; Baena *et al*., 2005). The growing resistance against antimicrobial agents has generated a search for alternative antimicrobial treatments. Particularly, the use of topical antibiotics is under discussion since it has been suggested that such an approach induces antibiotics resistance faster than the use of antibiotics. A lack of effectiveness of some of the antibiotics used may be due to development of drug-resistant strains. To overcome the problems caused by the emergence of resistance, alternative antimicrobial approaches need to be developed. One potential alternative approach is photodynamic therapy (PDT), which could provide a means of killing microbes in localized, topical infections (Kandela *et al.,* 2004).

MATERIALS AND METHODS

Isolation and identification of yeast

Saliva samples for *C. albicans* counts were taken from saliva sublingually, from cheek and dorsum of the tongue with sterile cotton swabs. After gently rubbing, the samples were immediately inoculated onto Sabouraud dextrose agar (Difco, USA) added with 0.1 mg/ml of Chloramphenicol (Williams and Lewis, 2000). The samples were incubated at 37^{o}C for 2 days and at room temperature for 5 days. The counting of CFU/plate was carried out after the growth of characteristics yeast colonies.

Inoculate preparation for the PDE

Inocula were prepared from 4 isolates of *C. albicans* obtained with the abovementioned method. They were standardized to a final concentration of 1.5×10^6 cells/ml, and then colony was counted on Sabouraud medium, yeast colonies growing on Sabouraud dextrose agar plate were carefully collected by a sterile wire loop, collected colonies were inoculated individually in 5 ml of Tryptic Soy Broth (Difco, USA) contained in test tubes, they were incubated overnight at 37 °C. After the incubation, yeast cells were harvested by centrifugation at 5000 rpm for 10 min and resuspended in sterile phosphate buffer saline (PBS), the cell concentration inside the test tube was equivalent to 1.5×10^6 cells/ml, the standardization of the inoculua could be performed with a McFarland standard; the turbidity of the inocula was equal to no. 2 McFarland standard.

Laser irradiations

The source of laser used in this study was diode Laser (Ever Light Co., Taiwan – it was providing by Iraqi Centre for Cancer and Medical Genetic Research). The wavelength of light that emitted from this laser and output power were determined by using a portable power meter (Edmund Optics Inc., Portland, USA), according to the measurements, the wavelength was 630 nm (Mang *et al*., 2006), and output power was approximately 10mW as a continuous wave. The irradiation area was (0.125) cm^2 while the exposure time of irradiation was (30-240) seconds (Table 1).

Preparation of photosensitizer solutions

Photosensitizer solutions were prepared in dark chamber as stock solutions of a 1mg/mL Hematoporphyrins derivatives (HpD) (Sigma Aldrich, Germany) in phosphate buffer and Ethanol, then shaken by vortex mixer for 3 min at room temperature (25 ◦C), and stored in the dark.

Spectral analysis of HpD

Peaks of absorbance for HpD were detected by a Double beam spectrophotometer (Varian Cary 100UV-Vis Spectrophotometer, Australia- providing by Department of Biology/ College of Science/ University of Baghdad). UV-Visible absorption spectra of photosensitizer were determined as a plot of absorbance against wavelength (300–750 nm) for both stock solutions of HpD**.**

Photosensitization: sixteen experimental conditions had been tested, applied by crossing the four concentrations of HpD (16, 32, 64, 128) µg/ml with four different exposure periods of irradiation (30, 60, 120, 240) second, by the using a modified microtiter plat (Al-Khafaji, 2002; Kandela *et al.,* 2004). 24-wells from 96-wells plate were selected to be a matrix for crossing tests, each well was covered by irradiation area which was (0.125) cm^2, aliquots of 100 µl of the cell suspensions were photosensitized with different above concentrations of HpD. The wells containing the resulting suspensions were left to rest in the dark (because of HpD is a photosensitive compound) for half hour (to allow the HpD for entering the yeast cells). This method was determined the effect of irradiation on photosensitized yeast cells (L^+P^+). Furthermore, positive and negative controls of each irradiation effect alone (L^+P^-) and HpD effect alone (L^-P^+) were respectively evaluated too. In addition, control without any treatment (L^- P^-) has been used as a reference control (Table 1). After treatments, aliquots (100 µL) were plated from each well onto Sabouraud Dextrose Agar with chloramphenicol and allowed to grow for 24 - 48 h at 28 - 37^{o}C. The number of colonies were counted by direct plate enumeration and expressed as colony forming units (CFU/mL).

Statistical analysis

t-test was performed to investigate whether there were significant differences between the variable test conditions, and the difference was considered to be significant when $p < 0.05$.

RESULTS

The morphological characteristics of *C. albicans* colonies were microscopically confirmed. Gram staining was utilized in order to recognize other types of cells. The colonies that presented gram-positive budding cells of yeasts were sub-cultured and identified. After incubation at 37^{o}C for 2 days, the control plates (L^-P^-) of the four *C. albicans* isolates showed abundant growth of viable colonies. It was observed that the sensitizing effect of the four concentrations of HpD (L^-P^+) did not significantly alter the number of CFU/ml, in comparison with the values detected in the control groups ($p>0.05$). On the other hand, the four treatment of irradiation effect (L^+P^-) showed a little significantly higher CFU/mL values ($p<0.05$) at an exposure time equal to 240 sec in comparison with those detected in the control group. For all the treatments, a significant reduction in the number of CFU/mL was showed after photodynamic application. The effectiveness of the PDE in reducing cell viability had varied as a function of *C. albicans* and the association between HpD and the irradiation (Fig. 1). *C. albicans* showed no growth at 37^{o}C for 2 days

after PDT, indicating the complete inactivation of these microorganisms. Bearing in mind that certain associations of the conditions (L^+P^+) for *C. albicans*, showed no survival, it was impossible to make statistical interference. The survaival curve of photosensitized *C. albicans* showed a minimum influence by 30 sec of laser irradiation associated with concentration 16 µg/ml of HpD photosensitizer, these were necessary to achieve a minimal inhibition of *C. albicans* to approximately 10%. (Fig. 2). As the concentration of HpD increased and the exposure time of irradiation from the Diode laser have also increased; these were resulted in correspondingly greater killing of the *C. albicans*. 128 µg/ml concentration of HpD combined with exposure time 240 sec were resulted a total growth inhibition of yeast cells. With the same exposure, more than 90% reduction in the viable counts of yeast cells sensitized with 9.6 µg/ml of HpD was showed. *C. albicans* had been shown more sensitivity to inhibition by photosensitization with HpD dissolved in ethanol than dissolved in PBS.

The results of absorption spectra showed that the absorption spectrum of 5µg/ml of HpD in ethanol included a Soret band at 401 nm and Q bands at 554, and 598 nm, while the absorption spectrum of 5µg/ml of HpD in PBS involved a Soret band at 400 nm and Q bands at 550 and 591 nm (Table 2: A and B). The Q bands of absorption spectrum were larger when ethanol was used as a solvent than when PBS was used. The value of absorption at 600 nm was also observed, the absorbance of HpD-ethanol sample was increased and become higher than of HpD-PBS sample (Figure 3: A and B).

DISCUSSION

This study investigated the *in vitro* sensitivity of *Candida albicans* to Photodynamic effect by combination of different concentrations of HpD and several doses of Diode laser irradiation. One of the most significant findings was the complete inhibitory of the viable yeast cells of *C. albicans* using low power of irradiation promoted photosensitization with optimum concentrations of HpD. These results were considered relevant, since a few investigations observed the total elimination of *C. albicans* after PDE (Souza *et al*., 2006). Our investigations are approximately similar to those obtained for the PDE of microbial cells and other *Candida* species with HpD and the PDE of *C. albicans* with hematoporphyrin (Bertoloni *et al*., 1987 and Bliss *et al*., 2004). This study evaluated the *In vitro* susceptibility of *C. albicans* to PDE by means of the association of four different concentrations of HpD and four different energy doses of laser irradiation.

Bearing in mind that the PDE requires an association of HpD and light, the application of light without the presence of the HpD is not capable of promoting the formation of singlet oxygen. It is fundamental to have the presence of a substance to intermediate the process of reactive species formation. However, the results of condition L^+P^+ showed statistically significant differences, when compared with the control groups, suggesting a possible toxic effect of Diode laser irradiation (Lambrechts *et al*., 2005). The photochemical processes mediated by HpD, initially appear to promote an alteration in the cytoplasmic membranes of the microorganism. This effect possibly occurs through oxidative alterations in lipids and proteins present in those membranes (Teichert *et al*., 2002). Afterwards, HpD is able to penetrate into the cell and cause irreversible damage to the intracellular organelles, which leads to cellular inhibition. The increase in the permeability of the membrane during the initial photochemical processes is fundamental for accentuating the photodynamic effect

on cell viability, as inactivation effectively occurs after the uptake of HpD (Al-Khafaji *et al*., 2010).

On other hand, *C. albicans* was more susceptible to PDE by HpD dissolved in ethanol, than by HpD dissolved in PBS. This could be due to more absorption of HpD-ethanol sample than the absorption of HpD-PBS sample at 630 nm (Fig. 3: A, B), where the light emission of Diode laser was closely to absorbance of Q- bands at red region of electromagnetic spectrum at 630 nm which has considered as effective wave length using in treatment of oral lesions (Mang *et al*., 2006). According results which had been shown, the potential explanation might be the Ethanol has a lower dielectric constant than water, 25.3 and 80.1 (at 293.2 K), respectively (Wohlfarth, 1995). and this explains why the absorption peaks of Q-bands are better resolved in ethanol than in PBS (Figs. 3:A and 3:B), it has also been assumed that increase in absorption is due to the potential increasing of HpD solubility in ethanol solution (as organic solvent) comparing with PBS.

CONCLUSION

The results of present study *in vitro* using low power diode laser to activated photosensitive compounds to specifically target and destroy pathogenic yeast and reduce their viability may open the door widely to new approach of photo disinfection by applying this technique topically to the intended target; at that point, the treatment site was disinfected by Diode laser at 630 nm and energy dose settings combined with HpD dissolving in ethanol as a suitable solvent. These results may also serve as a parameter for future investigations, so the further studies of effectiveness of PDE on experimental animals (*In vivo*) extremely necessary to serve as a promising therapy of periodontitis, and if that happened will be also open the door for using against yeast infections of *C. albicans* that associate with oral cavity cancer.

Table 1: Design of photodynamic treatments.

(D,C) T_n / C_n	T_0	T_1	T_2	T_3	T_4	
C_0	L^-,P^- 0,0	30	60	120	240	$L^+ P^-$ Control $^+$
C_1	0,16	30,16	60,16	120,16	240,16	$L^+ P^+$
C_2	0,32	30,32	60,32	120,32	240,32	
C_3	0,64	30,64	60,64	120,64	240,64	
C_4	0,128	30,128	60,128	120,128	240,128	
	$L^- P^+$ Control$^-$	$L^+ P^+$				**Test**

$T_{n(0-4)}$: The exposure time of irradiation (secon), n: number of treatment.
$C_{n(0-4)}$: Concentration of HpD (µg/ml), n: number of treatment.
$L^- P^-$: yeast cells without Photosesitizer HpD and without Laser irradiation (reference control).
$L^- P^+$: yeast cells Photosensitized with HpD and without Laser irradiation (negative control).
$L^+ P^-$: yeast cells without Photosesitizer HpD and irradiated with Laser (positive control).

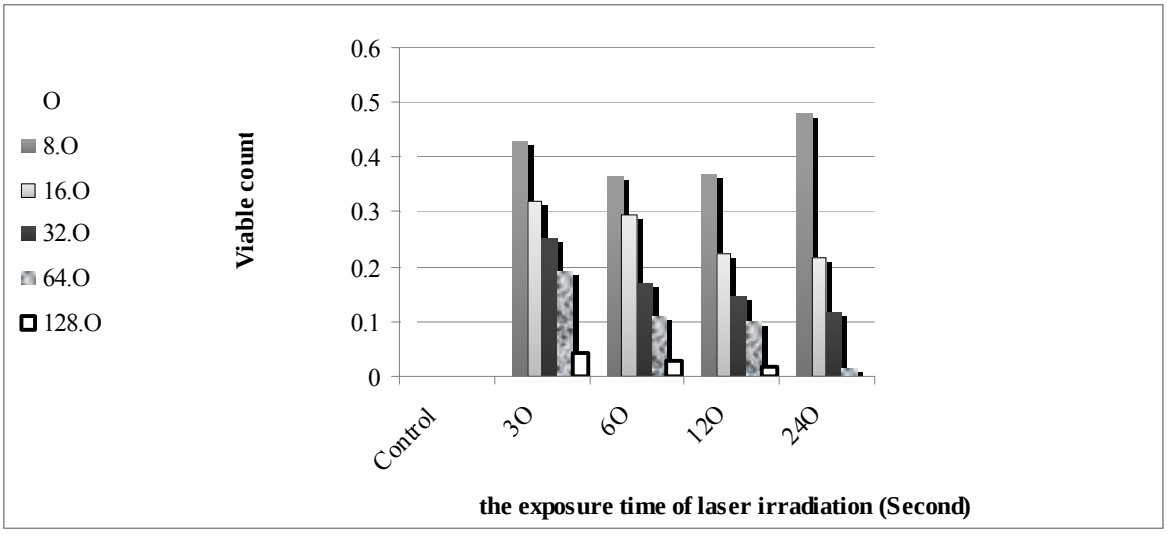

Fig. 1: Photodynamic effect on the viable count of yeast cells.

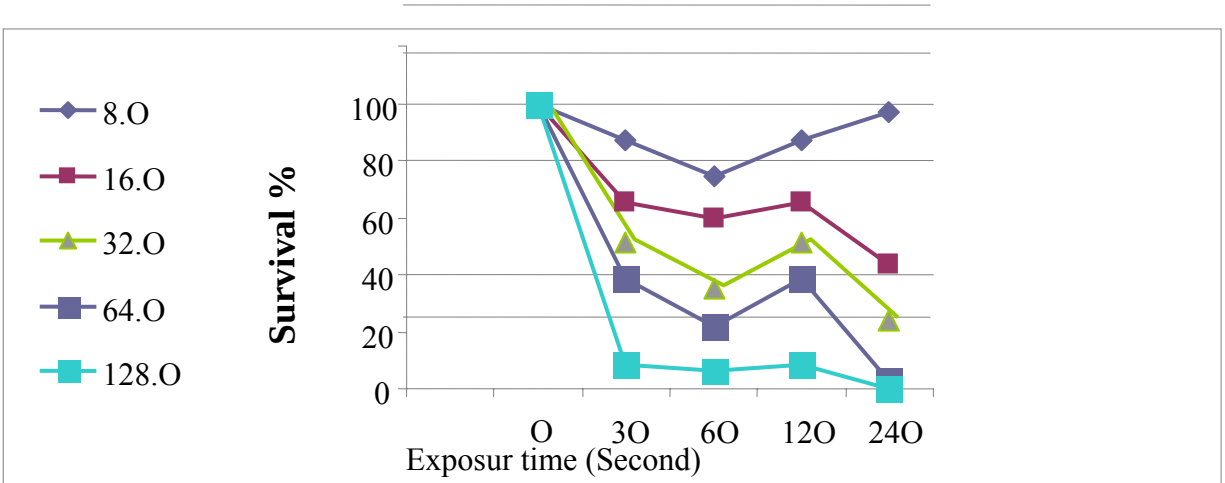

Fig.2: Survival cs of phonsitized yeast cells with HpD by diode laser

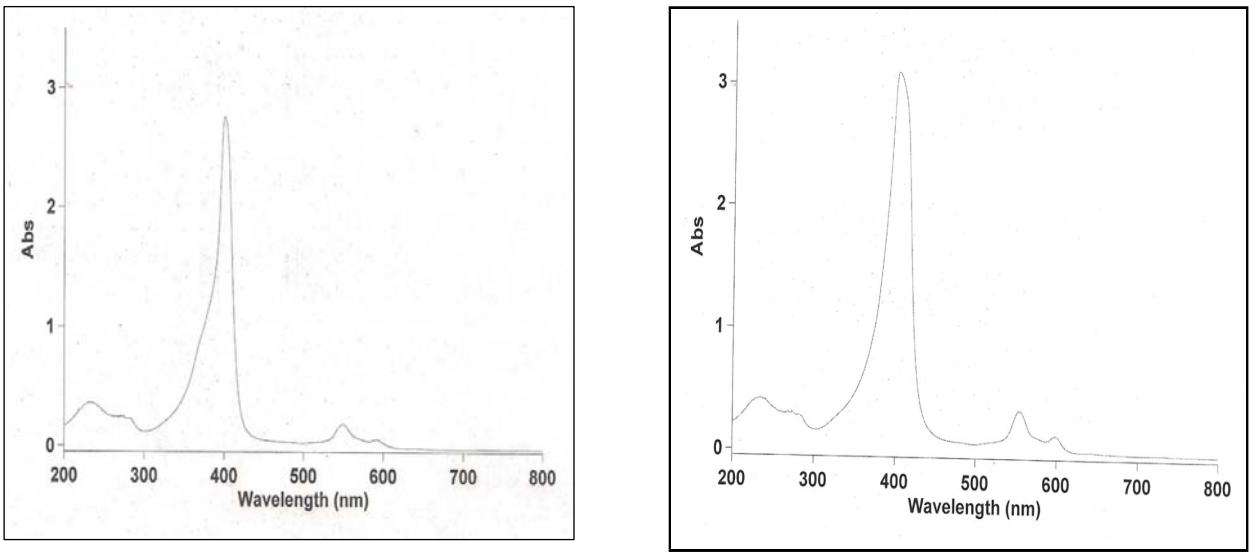

Fig. 3 :Absorption spectra

(A) Absorption spectrum of HpD dissolved in PBS.

(B) Absorption spectrum of HpD dissolved in ethanol.

Table 2: Peaks of HpD absorption

(A) Peaks of absorption spectrum of HpD dissolved in PBS.

Wave lenght (nm)	Absorbance	Type of band
591.00	**0.059**	Q-band
550.00	**0.185**	Q-band
400.00	**2.764**	Sort band
273.00	0.253	
271.00	0.252	
232.00	0.363	

(B) Peaks of absorption spectrum of HpD dissolved in ethanol.

Wave lenght (nm)	Absorbance	Type of band
598.00	**0.144**	Q-band
554.00	**0.345**	Q-band
401.00	**3.102**	Sort band
273.00	0.311	
268.00	0.312	
233.00	0.421	

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