# Voluntary Wheel Running in Rats Receiving Doxorubicin: Effects on Running Activity and Cardiac Myosin Heavy Chain

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**Abstract.** *Background: The clinical use of the highly effective chemotherapeutic agent doxorubicin (DOX) is limited by its dose-dependent cardiotoxicity. This cardiotoxicity is associated with a cardiac myosin heavy chain (MHC) isoform shift from the α isoform to the β isoform. Exercise prior to DOX treatment has been shown to attenuate the MHC shift associated with DOX, but little is known about the cardioprotective nature of exercise during DOX treatment. Materials and Methods: DOX-treated rats were assigned to normal cage activity (sedentary, SED+DOX) or 24-hour voluntary wheel running access (WR+DOX). All animals received weekly 2.5 mg/kg DOX injections for 6 weeks (15 mg/kg cumulative) and hearts were subsequently excised for determination of MHC isoform expression using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Results: At baseline, WR+DOX rats on average ran 62±4 km, and at week 6 ran 30±5 km, which was significantly lower than baseline ($p<0.05$). SED+DOX hearts expressed 57±7% of MHC as the α-MHC isoform and 43±7% as the β-MHC isoform. WR+DOX hearts expressed 76±4% as the α-MHC and 24±4% as the β-MHC isoform, which was significantly different from that of SED+DOX ($p<0.05$). Conclusion: DOX treatment significantly reduced wheel running activity, but this reduced running distance deemed to be cardioprotective as hearts from WR+DOX rats contained significantly greater levels of the favorable α-MHC isoform than SED+DOX.*

Doxorubicin (DOX; trade name Adriamycin) is an anthracycline antibiotic used in the treatment of a wide variety of cancer types. Although DOX is highly effective at treating a range of systemic cancer types and solid tumors, its clinical use is limited by a dose-dependent cardiotoxicity. This cardiotoxicity is evident only days after administration (1), and this early-onset cardiotoxicity can develop into a chronic cardiotoxicity which manifests itself as dilated cardiomyopathy or congestive heart failure months (2) or years (3) after the cessation of treatment. The nature of DOX-induced cardiomyopathy is highly complex and the causes have been attributed to, among other things, oxidative stress (4), myocardial bioenergetic alterations (5), and apoptosis (6).

Because of its value as an antineoplastic agent, numerous adjunct therapies aimed at minimizing DOX-induced cardiotoxicity have been investigated which include iron chealators (7, 8) and antioxidants (9-11). Another intervention gaining attention involves endurance exercise in conjunction with DOX administration. Previously, it was shown that weeks of exercise preconditioning attenuates early-onset DOX-induced cardiotoxicity (12-15). Likewise, one bout of treadmill running prior to DOX administration has been shown to minimize early onset cardiotoxicity in previously untrained rats (16). These strategies (*i.e.* exercise prior to DOX treatment) may have limited application clinically as cancer patients receiving DOX may not have previous endurance training experience and a high intensity exercise bout may not be advantageous to cancer patients (17-19). Therefore, approaches incorporating low intensity exercise during DOX treatment may be more clinically relevant.

Motorized treadmill running during DOX treatment has been used as a successful means of ameliorating cardiotoxicity (20), however, this mode of training required DOX-treated animals to run at identical loads as controls, and therefore, training intensity and volume relative to the DOX-treated animals' physical condition could not be taken into consideration. One way of employing an endurance training stimulus to animals receiving DOX treatment is through the use of voluntary wheel running (WR) which has been shown to be effective at minimizing cardiac dysfunction associated with hypertension (21, 22), myocardial infarction (23) and hormone deprivation therapy (24). Besides allowing animals

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to run at an intensity and duration appropriate to their physical condition, WR allows for an analysis of the effects of disease, disorders, and, in this case, drugs. Therefore, the first purpose of this study was to investigate the effects of DOX treatment administered over the course of six weeks on WR activity. It was hypothesized that, due to the cardiotoxicity associated with DOX, running distances would be substantially reduced.

In addition to its effects on the diseased heart, exercise training has a positive impact on the healthy heart (25-27). Limited data, however, are available regarding the impact of exercise on the heart during DOX treatment. The study of the exercise and DOX interaction has typically focused on oxidative stress and antioxidants (12, 14, 20, 28). These profiles can be somewhat transient over the course of weeks depending on when DOX and/or exercise was administered, and thus may not always be the best representation of DOX-induced cardiotoxicity or exercise-induced cardioprotection. One consistent DOX-induced cardiac maladaptation is an up-regulation in β-myosin heavy chain (MHC) with a corresponding down-regulation in α-MHC (15, 29). This α-MHC to β-MHC shift may be the result of numerous DOX cardiotoxicity mechanisms (*i.e.* oxidative stress, DNA interference, metabolic disruption), but this isoform shift results in reduced myosin ATPase activity and sliding filament velocity (30), which ultimately leads to impaired cardiac function (31-33). Although 10 weeks of exercise preconditioning has been shown to protect against DOX-induced MHC alterations (15), it is unknown whether exercise during DOX treatment can protect against such alterations. Therefore, the second purpose of this study was to examine the effects of voluntary WR on MHC distribution during six weeks of DOX treatment. It was hypothesized that WR during DOX treatment would minimize the up-regulation of β-MHC and the corresponding down-regulation of α-MHC.

## Materials and Methods

*Animals and animal care*. All protocols were approved by the University of Northern Colorado Institutional Animal Care and Use Committee and were in compliance with the Animal Welfare Act guidelines. Female Sprague-Dawley rats (200-220 g) were housed in an environmentally controlled facility on a 12:12-h light:dark cycle and provided chow and water *ad libitum*. Rats were randomly assigned to either a sedentary DOX group (SED+DOX, n=8) or the voluntary WR DOX group (WR+DOX, n=9). SED+DOX animals were housed 2 per cage in standard rat cages, and WR+DOX animals were housed individually in cages outfitted with stainless steel, commercially available rat running wheels (Mini Mitter, Bend, OR, USA).

*Doxorubicin and wheel running*. Animals received 2.5 mg/kg DOX weekly *via i.p.* injections for 6 weeks to a cumulative DOX dose of 15 mg/kg. Animals in the WR+DOX group were introduced to running wheel cages one week before the start of DOX treatment in order to obtain baseline WR data. WR+DOX rats remained in these cages 24 hours per day (with the exception of the weekly DOX injection) for the remainder of the treatment period. Running distances were recorded using a VitalView data acquisition system (Mini Mitter). Animals in the SED+DOX group remained in standard rat cages throughout the 6-week treatment period. Seven days following the final DOX injection, animals were anesthetized with an *i.p.* injection of heparinized (500 U) sodium pentobarbital (50 mg/kg), and when a tail pinch reflex was absent, the heart was rapidly excised. Each heart was then flushed of coronary blood, weighed, dissected, and the left ventricle was isolated, frozen in liquid $N_2$, and stored at –80˚C for ensuing analysis of MHC.

*Myosin heavy chain isoform expression*. Left ventricular homogenates from SED+DOX and WR+DOX rats were analyzed for MHC isoform expression according to the techniques described by Talmadge and Roy (34) and as used elsewhere (15, 24). In addition, left ventricle homogenates from age-matched, non-treated, non-wheel run female rats were analyzed to serve as controls (CON, n=10). Approximately 100 mg of ventricular tissue was minced in 10 volumes of 4˚C homogenizing buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris-Base, pH 6.8) and homogenized using a glass tissue grinder. Cell membranes were further disrupted using sonication. Homogenates were centrifuged at 1000 × g using a microcentrifuge for 10 min at 4˚C. The resulting supernatant was discarded, and the pellet was resuspended in 4˚C washing buffer (175 mM KCl, 0.5% Triton® X-100, 2 mM EDTA, and 20 mM Tris-Base, pH 6.8) at an identical volume as the homogenization buffer. The sample was centrifuged using a microcentrifuge at 1000 × g for 10 min at 4˚C. The resulting supernatant was discarded, and the pellet was again resuspended in washing buffer (same volume as before). The sample was again centrifuged using a microcentrifuge at 1000 × g for 10 min at 4˚C. The resulting supernatant was discarded, and the pellet was resuspended in final resuspension buffer (150 mM KCl and 20 mM Tris-Base, pH 7.0) at a high concentration (1/12 previous volume).

Total protein concentration was determined using the method of Bradford (35). Protein (9.75 μg) was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis on an 8% polyacrylamide separating gel with a 4% polyacrylamide stacking gel at 100 V in a Novex Sure-Lock electrophoresis unit (Invitrogen Corporation, Carlsbad, CA, USA) until the tracking dye ran to the bottom of the gel. Gels were stained using Coomassie blue. Stained gels were scanned and protein bands were analyzed using densitometry.

*Statistical analysis*. All results are expressed as mean±SEM. Student's *t*-tests were used to analyze body, heart, soleus, and extensor digitorum longus (EDL) mass differences between SED+DOX and WR+DOX groups. Repeated measures ANOVA with Dunnett's *post hoc* testing was performed to analyze weekly WR distances. One-way ANOVA with Tukey's *post hoc* testing was performed to analyze differences in MHC distribution between SED+DOX, WR+DOX, and CON. For all procedures, significance was set at $\alpha$=0.05.

## Results

*Animal characteristics*. Body mass, heart mass, and skeletal muscle mass data are presented in Table I. No body mass differences were observed immediately prior to the initial DOX injection, and no body mass differences were observed

Table I. *Animal characteristics*.

| | SED+DOX | WR+DOX |
|---|---|---|
| Initial BM (g) | 209±3 | 218±4 |
| Sacrifice BM (g) | 243±2 | 249±5 |
| Heart mass (g) | 1.00±0.02 | 1.03±0.02 |
| Rel heart mass (g/kg) | 4.12±0.10 | 4.13±0.06 |
| Soleus mass (mg) | 99±2 | 104±4 |
| EDL mass (mg) | 101±2 | 100±1 |

Values are mean±SEM. SED+DOX, Sedentary doxorubicin-treated; WR+DOX, wheel run doxorubicin-treated; BM, body mass; Rel heart mass, heart mass per kg sacrifice body mass; EDL, extensor digitorum longus. No significant differences were observed.

at the time of sacrifice ($p>0.05$). Similarly, absolute heart mass and heart mass expressed in terms of body mass were not found to differ between groups ($p>0.05$). Additionally, no significant difference was found in soleus or EDL mass between SED+DOX and WR+DOX groups ($p>0.05$).

*Wheel running distance*. Total weekly running distance is illustrated in Figure 1. One week prior to the start of DOX treatment, WR+DOX rats ran on average 62±4 km. In the week following the first DOX injection, there was an 11% decline in running distance, but this difference was not found to be significant ($p>0.05$). Significantly lower running distances, however, were observed at 2, 3, 4, 5, and 6 weeks as animals ran 25%, 33%, 44%, 55%, and 53% less, respectively, than baseline ($p<0.05$). Figure 2 illustrates the effect of each 2.5 mg/kg DOX injection on the daily running distances. Each DOX treatment tended to reduce the subsequent daily running distance, but running distances tended to rebound toward pre-DOX levels following the initial lag in activity. Even after administering a cumulative 15 mg/kg dose of DOX over the course of six weeks, animals still ran a mean of 30±5 km during the final week, at an average daily distance of 4.3±0.4 km.

*Myosin heavy chain distribution*. Figure 3 illustrates α- and β-MHC isoform expression in left ventricles from CON, SED+DOX, and WR+DOX rats. In CON left ventricles, 93±2% of MHC was expressed as the α-MHC isoform and 7±2% as the β-MHC isoform. In SED+DOX left ventricles, 57±7% as the α-MHC isoform and 43±7% as the β-MHC isoform was expressed which was found to be significantly different from that of CON ($p<0.001$). Left ventricles from WR+DOX animals expressed 76±4% as α-MHC and 24±4% as β-MHC, which was also found to be significantly different from that of CON ($p<0.01$). However, WR+DOX α-MHC and β-MHC expression was found to be significantly different from that of SED+DOX ($p<0.05$).

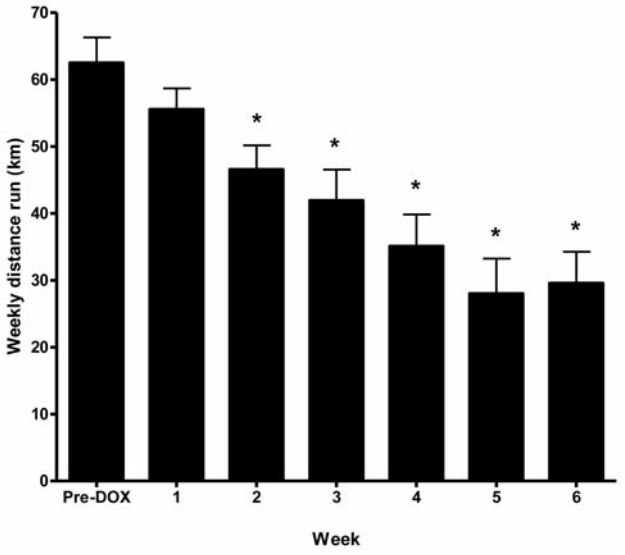


Figure 1. *Weekly running distances for wheel-run rats receiving doxorubicin. DOX, Doxorubicin; Pre-DOX, one week prior to the beginning of doxorubicin injections; *p<0.05 vs. Pre-DOX running distances.*

## Discussion

To our knowledge, this is the first report investigating the effects of weekly DOX injections on voluntary WR activity. Overall, DOX treatment resulted in significantly lower distances beginning at week 2 and continued through week 6. Daily running distances in response to the injections revealed that each injection was associated with depressed daily activity which eventually increased until the next DOX injection. Although a placebo WR control group was not used in this study, we demonstrated previously that control female rats progressively increase WR activity from weeks 1 through 4 at which time running distances plateau through week 8 (24). In the present study, however, this was not the case as running distances progressively declined from weeks 1 through 6. Nonetheless, during the final week of the present study, animals ran ~30 km. This finding is promising since one of the purposes of this study was to investigate the effects of DOX treatment on running activity, and although DOX is highly cardiotoxic, animals were able to maintain running activity throughout the course of the observation period.

As exercise has been shown to be cardioprotective prior to DOX treatment (12-15), the question of cardioprotection due to exercise during DOX treatment is of clinical concern. Chicco *et al*. (20) demonstrated that forced treadmill running during two weeks of DOX treatment resulted in cardioprotection, and although the exercise intensity employed was relatively low, it is difficult to extrapolate how that intensity relates to the conditions experienced by the

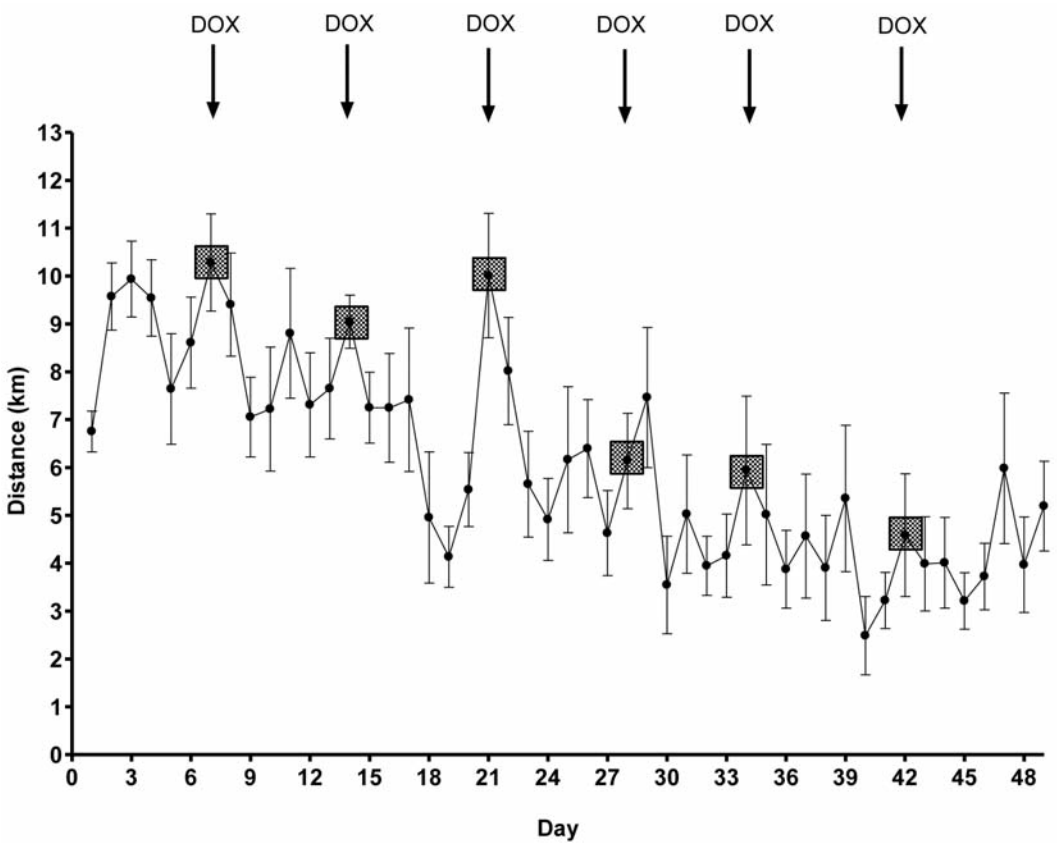


Figure 2. *Daily wheel running activity in rats receiving doxorubicin. Arrows with corresponding shaded boxes indicate when doxorubicin was administered intraperitoneally.*

DOX-treated animals. Employing a voluntary WR model, as in the present study, allows for animals to run at-will based on the severity of DOX toxicity experienced on any given day. When compared to forced treadmill running, voluntary WR has been shown to elicit a lower stress response (36). This low-stress exercise model is consistent with the clinical recommendation of prescribing low intensity exercise to cancer patients (17-19), as high intensity exercise may compromise immune function (37, 38).

The fluctuations in daily running distances in response to DOX injections demonstrates that the animals' willingness to run was reduced on the days following each treatment, but running activity tended to rebound in the ensuing days until the next injection. This variation in WR activity corresponds to serum DOX and DOX metabolite (doxorubicionol and 7-deoxydoxorubicinolone) concentrations being elevated up to 2 days following administration; however, DOX and its metabolite levels are undetectable 7 days post (39). Although DOX is known to accumulate in cardiomyocytes, the effect of increased physical activity on myocardial DOX concentrations has yet to be determined. There is evidence, however, that exercise training up-regulates cardiac multidrug-resistance protein 1 (Mrp1) which is involved in extruding DOX out of the cell (40). Even if there is potential for exercise reducing levels of DOX in the heart, Jones *et al*. (41) demonstrated that exercise training during DOX treatment did not interfere with its antineoplastic effect.

With our observation that female rats receiving DOX will continue to run voluntarily, albeit to a lesser extent, the next purpose of this study was to analyze cardiac MHC distribution to gain a better understanding of the cardioprotective nature of voluntary WR. Animals receiving DOX that remained sedentary during the 6 week treatment period expressed significantly lower α-MHC and significantly greater β-MHC than sedentary controls, which is consistent with other reports (15, 29). Hearts from DOX animals allowed free access to running wheels also expressed significantly lower α-MHC and significantly greater β-MHC than did sedentary controls, but also expressed significantly greater α-MHC and lower β-MHC than did sedentary treated animals. Although WR did not completely ameliorate the cardiac MHC shifts induced by DOX, the increased α-MHC expression and reduced β-MHC expression in wheel run animals demonstrates the cardioprotective nature of exercise during DOX treatment.

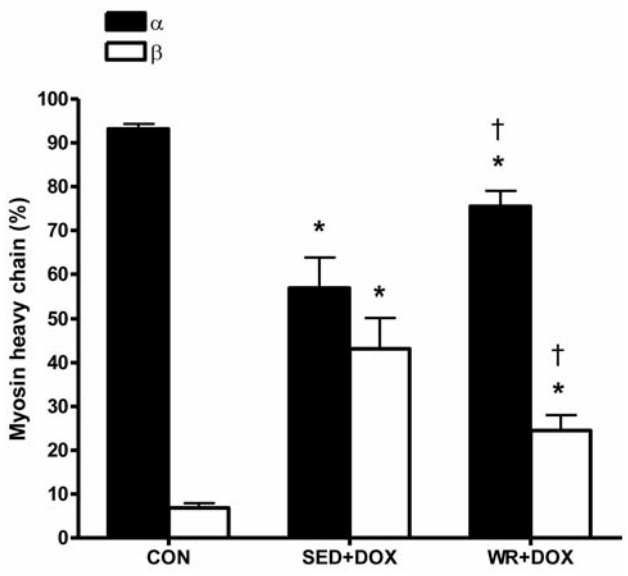


Figure 3. *α- and β-myosin heavy chain expression in left ventricular homogenates from control rats, sedentary rats receiving doxorubicin, and wheel-run rats receiving doxorubicin. CON, Control; SED, sedentary; WR, wheel run; DOX, doxorubicin; *p<0.05 vs. CON. †p<0.05 vs. SED+DOX.*

The cardiotoxic effect of DOX has been primarily attributed to myocardial oxidative stress as DOX accumulates in cardiac mitochondria and undergoes redox cycling at complex I of the electron transport chain. This redox cycling then leads to the generation of reactive oxygen species (ROS), and free radical formation can lead to the up-regualtion of β-MHC with corresponding down-regulation of α-MHC (42). In addition to ROS, myocardial bioenergetics disruption has been reported as another mechanism behind DOX cardiotoxicity (43), which may also lead to disruption in cardiac MHC expression. The ATPase associated with the α-MHC isoform has a two-fold greater ATP hydrolysis rate than that for the β-MHC isoform (30), and thus the reduced expression of α-MHC and increased expression of β-MHC in the DOX-treated heart may be an attempt to conserve ATP while compromising myocardial performance. Nonetheless, changes in the cardiac MHC profile have a dramatic impact on cardiac function, and as such, up-regulation of β-MHC with corresponding down-regulation of α-MHC has been reported as one mechanism responsible for cardiac dysfunction associated with heart failure (44), diabetes (45), and hypothyroidism (46). Increased β-MHC and reduced α-MHC isoform expressions have been shown to impair power output of isolated cardiocytes (31, 46) and systolic function in the isolated perfused heart (33). Interestingly, even a relatively small disruption in the MHC profile can significantly affect cardiac performance (32).

The protective effect of exercise on DOX-induced MHC alterations was demonstrated previously in a treadmill preconditioning model (15), and voluntary WR has also been shown to protect against cardiac MHC disruption during sex hormone ablation (24). The primary mechanism behind the exercise-induced cardioprotection in the present study may very well be due to the quenching of free radicals, as exercise training has been shown to increase myocardial antioxidant status (47). However, exercise training also has a direct impact on contractile performance and MHC profile. Natali *et al*. (26) reported that myocytes isolated from wheel run rats demonstrated improved contractility following only 6 weeks of voluntary running wheel access. Moreover, in analyzing gene expression changes following exercise, Jin *et al*. (25) reported that of the genes analyzed, only *α-MHC* mRNA was found to increase following a 13-week progressive treadmill training protocol. Wheel running also has a positive impact on the diseased heart as Konhilas *et al*. (48) reported that voluntary WR up-regulated *α-MHC* mRNA in a hypertrophic cardiomyopathy model. In the present study, it is possible that the positive effects of WR on MHC may have superseded the negative effects of DOX on MHC thereby attenuating the MHC shift.

The present study demonstrates that while DOX administration results in depressed running wheel activity, animals will still continue to voluntarily run even while undergoing treatment. Specifically, our data show that even after receiving a cumulative dose of DOX at 15 mg/kg over a 6-week period, animals maintained an average voluntary running distance in excess of 4 km/day. Although running distance was significantly depressed starting at week 2, this reduced running distance was sufficient to attenuate the cardiac MHC shifts associated with DOX. The significance of the attenuated MHC isoform shifts observed in the present study lies in its importance in contractile performance. Voluntary WR-induced cardioprotection in the current study also provides insight as to how to enhance the clinical management of DOX cardiotoxicity. Because of increased fatigue, DOX treated cancer patients may lack the energy and motivation to exercise for long durations (*i.e*. long distance walking, running, cycling), but it is promising that the lower running distances observed in DOX-treated animals in the present study was cardioprotective in the form of attenuated MHC shifts. It is possible that cancer patients whose therapy includes DOX may benefit from short duration exercise (*i.e*. short distance walking, running, cycling) during treatment.

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