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AVALIAÇÃO HISTOLÓGICA DA BIOCOMPATIBILIDADE DE OTÓLITOS *CYNOSCION ACOUPA* EM RATOS

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RESUMO

O objetivo deste estudo foi avaliar a biocompatibilidade de otólitos de *Cynoscion acoupa* por ensaios in vivo realizados em ratos *Wistar*. O material utilizado foi preparado com 2 g de pó de otólitos de *Cynoscion acoupa* e 0,5 g de colágeno hidrolisado, diluídos em água destilada. O ensaio biológico consistiu na utilização de 24 ratos *wistar* nos quais foram implantados tubos de polietileno contendo otólitos (OI) no lado direito do dorso e tubos vazios (CI) no lado esquerdo. Após a cirurgia, os ratos foram medicados e sacrificados após 3, 7 e 30 dias. Após cada período os tubos foram removidos com o tecido circundante e submetido a processamento histológico clássico. As alterações inflamatórias foram avaliadas em uma escala de 0 a 4 e submetidas a análise estatística (nível de significância 5%). No CI após o 3º dia, foi observado um infiltrado inflamatório de moderado a severo. No 7º dia, formação de tecido de granulação maduro e com 30 dias presença de um tecido conjuntivo mais organizado. Em relação à OI, moderados leucócitos, infiltrado crônico sem necrose ou reação granulomatosa foi observada no dia 3. No 7º dia, a severidade da reação inflamatória foi atenuada, e um tecido de granulação bem desenvolvido foi observado. No dia 30, o tecido conjuntivo ao redor dos implantes era composto de novos fibroblastos e fibras colágenas onduladas. Não foram observadas diferenças significativas entre os grupos com relação à intensidade da resposta inflamatória em nenhum tempo experimental (p>0,05). Como conclusão, a preparação otólitos foi considerada biocompatível, pois não houve diferenças significativas de reação tecidual entre os grupos experimentais.

PALAVRAS-CHAVE

Otolitos. Teste de Biocompatibilidade. Ensaio Biológico. Murinos.

ABSTRACT

The purpose of this study was to evaluate the biocompatibility of *Cynoscion acoupa´s otoliths* by in vivo assays performed in Wistar rats. The material was prepared using 2g of powded *Cynoscion acoupa's otoliths* and 0.5g of hydrolyzed collagen diluted in distilled water. The biological tests consisted of the use of 24 Wistar rats, which were implanted in polyethylene tubes containing otoliths (HI) on the right side of the back, empty tubes (IC) on the left. The animals were euthanized 3, 7 and 30 days after the surgical procedures. After each period the tubes were removed with surrounding tissue and the subjected classic histological processing. The inflammatory changes were assessed on a 0-4 scale and submitted to statistical analysis (significance level of 5%). IC groups presented moderate to severe inflammatory infiltrate after the 3rd day. On the 7th day there was formation of mature granulation tissue, and 30 days a well-organized fibrous tissue was observed surrounding the implant area. Regarding HI, moderate leukocyte infiltration without necrosis or chronic granulomatous reaction was observed on 3rd day. On the 7th day, the severity of the inflammatory response was attenuated and a well-developed granulation tissue was observed. On day 30, the tissue surrounding the implants was composed of fibroblasts and new collagen fibers wavy. There were no significant differences between the groups regarding the intensity of the inflammatory response in no time trial (p>0.05). In conclusion, the *otoliths* preparation was considered biocompatible with the dermal tissues, since there were no significant differences of tissue reaction between the experimental groups.

KEYWORDS

Otoliths. Biocompatibility Test. Biological Assay. Murine

RESUMEN

El objetivo de este estudio fue evaluar la biocompatibilidad de los otolitos de *Cynoscion acoupa* por ensayos in vivo realizados en ratones Wistar. El material se preparó usando 2 g de polvo de otolito de *Cynoscion acoupa* y 0,5 g de colágeno hidrolizado diluido en agua destilada. Para el ensayo biológico se utilizaron 24 ratones en el cual fueron implantados tubos de polietileno que contienen los otolitos (OI) en el lado derecho de la espalda del animal y tubos vacíos (CI) en el lado izquierdo. Después de la cirugía, los ratones fueron medicados y sacrificados después de 3, 7 y 30 dias. Después de cada período los tubos fueron retirados con el tejido circundante y sometidos al procesamiento histológico clásico. Los cambios inflamatorios se evaluaron en una escala de 0 a 4 y se sometieron a análisis estadístico (nivel de significación del 5%). En los CI se observó después del tercer día, un infiltrado inflamatorio de moderado a severo. En el séptimo día, la formación de tejido de granulación maduro y a los 30 días la presencia de un tejido más organizado. En cuanto a los OI, moderada infiltración leucocitaria sin necrosis o reacción granulomatosa crónica se observó a los 3 días. En el séptimo día, la gravedad de la respuesta inflamatoria fue atenuada, y un tejido de granulación bien desarrollado tambien fue observado y a los 30 dias, el tejido que rodeaba los implantes era compuesto de fibroblastos y nuevas fibras de colágeno onduladas. No hubo diferencias significativas entre los grupos con respecto a la intensidad de la respuesta inflamatoria en ningún tiempo experimental (p> 0,05).En conclusión, la preparación de los otolitos se considera biocompatible, ya que no hay diferencias significativas en la reacción del tejido entre los grupos experimentales.

PALABRAS CLAVE

Otolitos. Prueba de Biocompatibilidad. Ensayo Biológico. Ratones.

1 INTRODUÇÃO

Recently, many studies have been peformed in attempt to improve bone regeneration by using acellular or cellular implantable materials (FINKMEIR, 2002). Biominerals are an alternative class of biomaterials able to induce or improve bone formation in substitution to the autogenic grafts when applied into large bone defects (SERVICE, 2000; BORELLI et al., 2003). They consist of an inorganic phase (or phases) (usually simple salts or oxides) and a range of biomolecules that are often proteins, but may be carbohydrates, lipids or low-molecular-weight (<1 kDa) molecules such as polyamines. These biominerals are widely formed by bacteria, single-celled protists, plants, invertebrates and vertebrates including humankind (PERRY et al., 2009).

Otoliths are crystalline structures, comprised primarily of calcium carbonate, located in the inner ear of bony fishes, which function as balance organs (CAMPANA, THORROLD, 2001). Studies have indicated that otoliths are rich in calcium carbonate, metallic elements (Sr, Ba, Mg, Cd, Co, Cu, Zn, Na, K, etc) and non-metallic compounds (Si, P, S, B, etc.) (CAMPANA; NEILSON, 1984), as well as in a high molecular weight collagenous protein called otolin (MURAYAMA et al., 2002; MURAYAMA et al., 2004; TOHSE et al., 2008). Therefore, despite otolith applications strayed so far across taxonomic and methodological boundaries, due to its chemical composition it is possible to suppose that this material might work as a biomineral. However, both organic and inorganic biomaterials are characterized by biocompatibility and atoxicity, in addition to mechanical resistance, elasticity, and chemical and biological stability. Therefore, new materials demand research to evaluate their properties and commercial viability (HAN et al., 2007; MORETTI-NETO et al., 2008).

Biocompatibility evaluations can be peformed in vitro by cytotoxicity or genotoxicity tests, or *in vivo* studies using animals. Besides, subcutaneous implants in rats are regarded as one of the first screening *in vivo* evaluations, although afterwards more complex implants in larger animals simulating the real conditions of the implant material as grafts or prostheses must be used (MAIZATO et al., 2008).

Thus, the goal of this study was to assess the biocompatibility of otoliths of *Cynoscion acoupa* by *in vivo* assays performed in *Wistar* rats.

2 MATERIAL AND METHODS

The material in this study was prepared with 2 g powder of otolith of *Cynoscion acoupa* with particle size 60 mesh and addiction 0,5 g of hydrolyzed collegen, diluted in distilled water. The final product was packaged in dishes Petri and sterilized in rays 60 gama/cobalt.

In vivo assay was performed with 24 male *Wistar* rats (250 ± 50 g), supplied with food and water *ad libitum* in a temperature and humidity-controlled environment. The animals were anesthetized with intraperitoneal ketamine-xylazine (100mg/kg - 5mg/kg) and had the dorsal region submitted to trichotomy. Polyethylene tubes with 1.5-mm inner diameter, 2.0-mm outer diameter and 10.0 mm long were sterilized in autoclave prior to use. The otoliths preparation was placed into the polyethylene tubes and implanted into the subcutaneous tissue of the rats. Empty tubes were used as control. The implants were placed subcutaneously into pockets created by a blunt dissection

through 10 mm incision of the skin. Two pockets were created in the back of each animal, in the right side (otoliths implants – OI) and left side (control implants – CI). The samples were stitched to the dorsal muscle and the incision sutured by a monofilament wire (Mononylon5.0 Ethicon, Johnson and Johnson, Brazil). After surgery, the rats received intramuscular antibiotic cefazolin sodium (ABL, Brazil) 4 mg/kg and were kept under observation until recovered from the anesthesia. Eight rats were assigned to each time interval (3, 7 and 30 days), sizing up 24 animals. At the end of the experimental periods, the animals were euthanized in CO2 chamber. The skin overlaying the implants was shaved and tubes with surrounding tissue were removed from the rats, immersed in formalin solution and fixed for 24 hours. The specimens were processed for paraffin embedding, serially sectioned into 5-µm cuts and stained with hematoxylin and eosin.

The conditions of the tissue surrounding the implants, the occurrence and location of fibrous tissue, the types of inflammatory cells present, calcification and the vascular changes were assessed and graded between 0 (zero) and 4, as follows as follows:

- 0 (lack): absence of changes in the tissue examined;
- 1(mild): changes presented in 1–10% of the tissue examined;
- 2 (moderate): changes presented in 11–50% of the tissue examined;
- 3 (severe): changes presented in more than 50% of the tissue examined;

Statistical analysis was performed by Kruskall-Wallis test and Dunn post-hoc test to determine significant differences among the groups. Significance level was set at 5%.

In accordance to the institution's guidelines outlined in "Guide for the Care and Use of Laboratory Animals", it is hereby assured that all animals received humane care during all the steps of the experimentation. Furthermore, the study protocols were approved by our National Research Council prior to the beginning of the experiments (register 171205).

3 RESULTS

After surgery the cicatrization process showed no intercurrences, and no rat presented overinduced inflammatory processes or apparent contamination.

Regarding the analysis of CI, on the 3rd day, a moderate to severe infiltrate of chronic cells and some few neutrophils was observed, but there were no giant cells or foci of necrosis (figure 1a). The intensity of the inflammatory infiltration was milder on the 7th day, and blood vessels formation and fibroblastic proliferation were quite apparent, forming a mature granulation tissue (figure 1c). On the 30th day, it was possible to observe a more organized connective tissue with predominance of collagen fibers, fibroblasts and an uncompletely formed capsule surrounding the implants (figure 1e).

In relation to OI, moderate chronic leukocytes infiltrate without necrosis or granulomatous reaction was observed on the 3rd day. The inflammatory reaction was characterized by presence of Lymphocytes, macrophages, and some scanty neutrophils. No granulomatous reaction was found. In addition, fibroblasts and few delicate collagen fibers were also seen (figure 1b). On the 7th day, the severity of the inflammatory reaction was attenuated, and a well-developed granulation tissue was noticed (figure 1d). On the 30th day, the connective tissue surrounding the implants was composed of new wavy fibroblasts and collagen fibers forming a thin capsule. Foci of dystrophic calcification were also seen (figure 1f).

The table 1 presents the results of the semiquan-

titative analysis of the intensity of the inflammatory infiltrate evaluated in both groups. No statistical difference between the groups was observed during the experimental periods (p>0.05).

Figure 1. Histological evaluation of the biocompatibility of the control (CI) and otoliths (OI) implants groups. Moderate chronic inflammatory reaction (CI) observed surrounding the polyethylene tube (pt) in both CI (a) and OI (b) on the 3rd day. Well-developed granulation tissue (gt) was seen in both groups (c and d), but only in OI there was signs of dystrophic calcification (arrow) (d). Fibroblastic reaction (fr) was verified surround the polyethylene tubes (pt) in CI (e) and OI (f), whereas calcified globules were observed in the latter (arrow) (f) (HE, 100x).

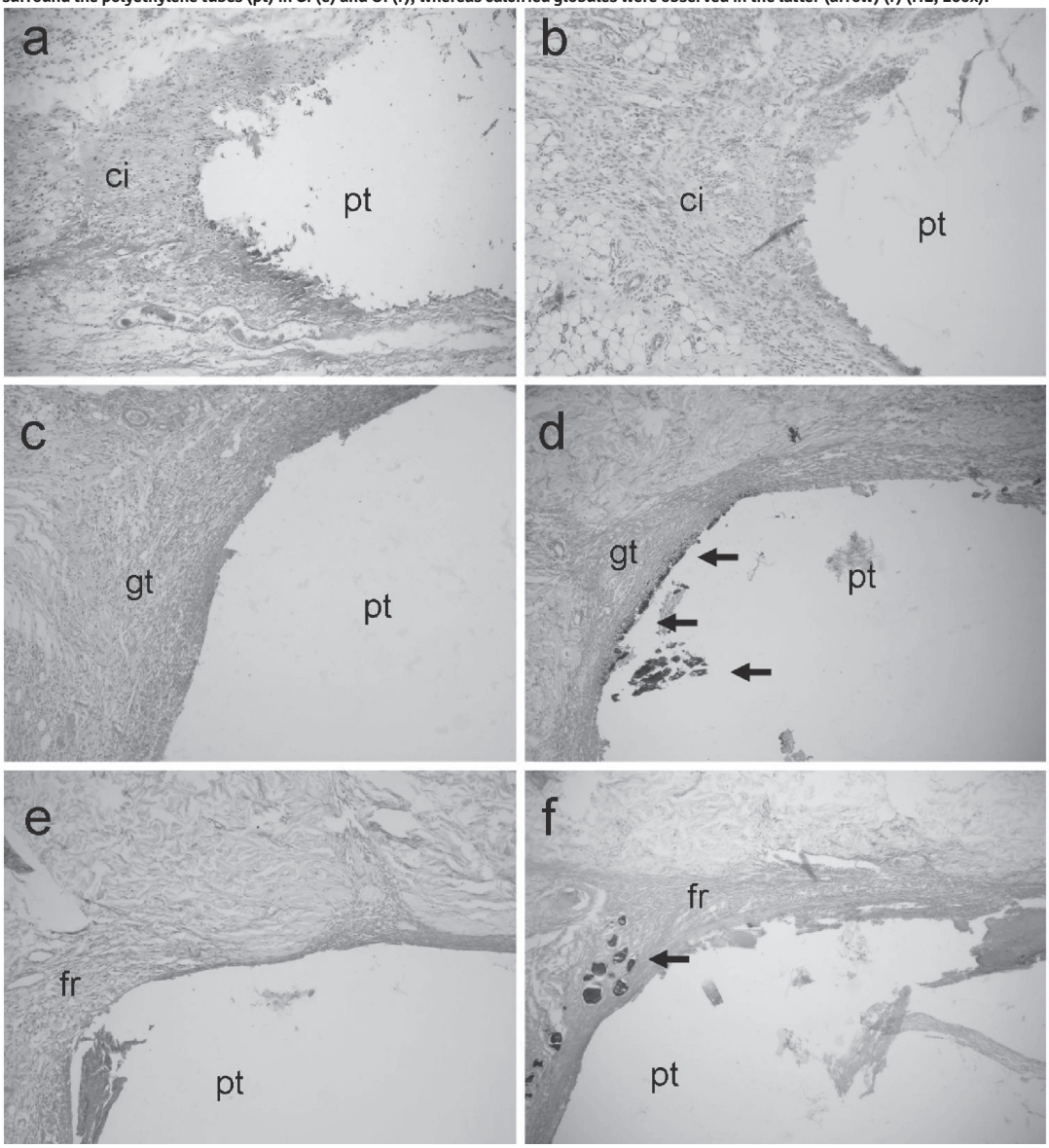

Table 1. Assessment of the intensity of the inflammatory infiltrate surround the open extremity of the polyethylene tubes in both CI (control implants) and OI (otoliths implants) groups.

Experimental Periods	Implant Materials	Animals							
		R1	R2	R3	R4	R5	R6	R7	R8
Day 3	Control implants (CI)	3	2	3	2	2	2	2	2
	Otoliths Implants (OI)	3	3	2	2	3	2	2	2
Day 7	Control implants (CI)	1	1	1	1	2	2	1	2
	Otoliths Implants (OI)	2	2	1	1	2	1	2	1
Day 30	Control implants (CI)	0	1	0	1	1	1	1	0
	Otoliths Implants (OI)	1	2	1	1	1	0	1	0

4 DISCUSSION

Every material for biological use is supposed to be biocompatible (HAN et al., 2007; MORETTI-NETO et al., 2008). In this study, the biocompatibility of otoliths preparation materials was assessed by examining their inflammatory reactions in 3, 7 and 30 days after subcutaneous implantation in the dorsal area of rats. This type of subcutaneous implant is widely used and easily reproduced (HAN et al., 2007; MORETTI-NETO et al., 2008; MAIZATO et al., 2008).

One of the most relevant factors in the analysis of a material's biocompatibility is its ready acceptance by the host tissues, eliciting an inflammatory response that should resemble that of the normal healing process. In this regard, *in vivo* implants are preferable, for providing more realistic information than *in vitro* systems about the host's responses at the interface between the implanted material and the surrounding biological environment (MRUE et al., 2004).

The results showed that independently from the presence of not of the otoliths preparation within the polyethylene tubes, the inflammatory response predominantly chronic and decreasing. The attenuation of the intensity of the inflammatory infiltrate along the time, as well as the absence, in all the samples, of substantial infiltrated/neutrophilic exudate, necrosis, and bacteria reinforces this implies the satisfactory evolution of the healing process (GOISSIS; PARREIRA, 2003). This fact is further reinforced by the absence of clinical signs of overinduced inflammation, such a as abscess, edema or fistulation, as suggested by Maizato et al (2008). The lack of granulomatous inflammation, as indicated by the absence of giant cells and epithelioid macrophages, is suggestive that no foreign body reaction developed as a response of the implantation of the biomaterial (HAN et al., 2007; MAIZATO et al., 2008). In addition, the pattern of the inflammatory evolution was similar in both groups, showing a gradual decrease in the intensity along the time. This fact attests the atoxicity and biocompatibility of the otoliths preparation.

As regards the allergenicity of this product, it should be emphasized that this biomaterial contains no toxic product, which is conventionally used in the rubber industry, nor any other adjuvants or preservatives (CAMPANA, NEILSON, 1984; MURAYAMA et al.,

2002; BORRELI et al., 2003; TOHSE et al., 2008). The lack of allergenic properties was confirmed not only by the absence of any immediate or long-term clinical response (Type I and type IV hypersensitivity, respectively), but also by the lack of eosinophils infiltrate and granulomatous reaction in the paraffin-embedded tissue samples.

Dystrophic calcification represents the deposition of calcium salts in areas of degenerated or necrotic tissue, so that the absorption of the minerals is facilitated and stimulated by their high-affinity for fatty acids derived from the external membrane of injured cells (RUSSELL et al., 1986). The lack of calcification in the control group, in addition to a decreasing chronic inflammatory infiltrate, is suggestive that the injury promoted by the surgical process of the tubes implantation was well supported by the host tissues. Nevertheless, the presence of calcification foci in some animals treated with otoliths preparation should not be misinterpreted as a result of tissue damage associated to the biomineral implantation. In fact, it is probably related to the high content of minerals in the preparation, as described in previous studies (CAMPANA, NEILSON, 1984; CAMPANA, THORROLD, 2001). The gradual resorption of the hydrolyzed collagen matrices which composed the otoliths preparation might have led to a slow but continuous release of a wide sort of minerals involved in the dynamics of the calcification process, thus facilitating their deposition in the tissue. Despite dystrophic calcification is usually considered a sign of tissue damage, in this case it might be regarded as a good property, since this biomaterial is supposed to work as a calcification inducer biomineral. However, since this study focused in the analysis of biocompatibility of the otoliths preparation, further investigations will be required in attempt to assess its real role in the bone regeneration process.

The results indicated that the preparation of *Cynoscion acoupa´s* otoliths does not induce deleterious biological response after implantation in dermal tissues of rats. This experimental model proved to be suitable for *in vivo* evaluation of biocompatibility. The following step will be the quantitative analysis of the calcification by atomic emission spectrometry in order to assess the total calcium incorporated in the connective tissue surround the implanted materials.

5 CONCLUSION

The results of the histological analyses of subcutaneous implantation in *Wistar* rats of the otoliths preparation was considered biocompatible, as long as it has not induced, in the conditions described, significant differences in tissue reaction as compared to the control groups.

REFERENCES

BORELLI, G.; MAYER-GOSTAN, N.; MERLE, P.L.; DE PONTUAL, H.; BOEUF, G.; ALLEMAND, D.; PAYAN P. Composition of biomineral organic matrices with special emphasis on turbot (*Psetta maxima*) otolith and endolymph. **Calcif Tissue Int**. v.72, n.6, p.717-725, 2003.

CAMPANA, S.E.; NEILSON, J.D. Microstructure of fish otholiths. **Can J Fish Aquat** Sci. v.42, n.5, p.1014-1032, 1984.

FINKMEIR, C.G. Bone-grafting and bone-graft substitutes, **J Bone Joint Surg**, v.84, n. 3, p.454-465, 2002.

GOISSIS, G.; PARREIRA, D.R. A study on the biocompatibility and integration of acellular polyanionic collagen: elastin matrices by soft tissue. **Rev Bras Eng Biomed**, v.19. n.3, p.197-173, 2003.

HAN, Y.J.; LOO, S.C.J.; LEE, J.; MA, J. Investigation of the bioactivity and biocompatibility of different glass interfaces with hydroxyapatite, fluorohydroxyapatite and 58S bioactive glass. **BioFactors**, v.30, n.4, p.205-216, 2007.

MAIZATO, M.J.; PIRES, M.D.; CANZIAN, M.; HIGA, O.Z.; PITOMBO, R.N.; LEIRNER, A.A. Histological evaluation of biocompatibility of lyophilized bovine pericardium implanted subcutaneously in rats. **Artif Organs**, v.32, n.4, p.268-271.

MANN, S. The study of biominerals by high resolution transmission electron microscopy. **Scan Electron Microsc**, v.2, p.393-413, 1986.

MORETTI-NETO, R.T.; MELLO, I.; MORETTI, A.B.; ROBAZZA, C.R.; PEREIRA, A.A. In vivo qualitative analysis of the biocompatibility of different cyanoacrylate-based adhesives. **Braz Oral Res**, v.22, n.1, p.43-47, 2008.

MRUE, F.; NETTO, J.C.; CENEVIVA, R.; LACHAT, J.J.; THOMAZINI, J.A.; TAMBELINI, H. Evaluation of The Biocompatibility of a New Biomembrane. **Materials Research**, v.7, n.2, p.277-283, 2004.

MURAYAMA, E.; TAKAGI, Y.; OHIRA, T.; DAVIS, J.G.; GREENE, M.I.; NAGASAWA, H.L. Fish otolith contains a unique structural protein, otolin-1. **Eur J Biochem**, v.269, n.2, p.688-696, 2002.

MURAYAMA, E.; YTAKAGI, Y.; NAGASAWA, H. Immuno**histochem**ical localization of two otolith matrix proteins in the otolith and inner ear of the rainbow trout, Oncorhynchus mykiss: comparative aspects between the adult inner ear and embryonic otocysts. Histochem, v.121, n.2, p.155-166, 2004.

PERRY, C.C.; PATWARDHAN, S.V.; DESCHAUME, O. From biominerals to biomaterials: the role of biomolecule–mineral interactions. **Biochem Soc Trans**, v.37, p.687-691, 2009.

RUSSELL, R.G.G.; CASWELL, A.M.; HEARN, P.R.; SHARRARD; R.M. Calcium in mineralized tissues and pathological calcification. **British Medical Bulletin**, v.42, p.435-446, 1986.

SERVICE, R.F. Tissue engineers build new bone. **Science**, v.289, p.1498-1500, 2000.

TOHSE, H.; TAKAGI, Y.; NAGASAWA, H. Identification of a novel matrix protein contained in a protein aggregate associated with collagen in fish otoliths. **FEBS J**, v.275, n.10, p.2512-2523, 2008.

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