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Role of Bone Marrow Derived Autologous Mesenchymal Stem Cells in Fracture Healing in Rabbits

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Abstract

Introduction: In patients with fractures, the bone marrow derived autologous stem cells have a therapeutic potential to accelerate the healing process and treat nonunion. It has become a clinical necessity to develop the novel therapeutic approaches to accelerate fracture healing. In our study we evaluated the effect of bone marrow derived autologous mesenchymal stem cells in fracture healing in rabbits by injecting them locally at the fracture site.
Method: For this study we osteotomised the mid shaft of the ulna in 28 rabbits and divided them into two groups. One group was injected with bone marrow derived autologous mesenchymal stem cells at the fracture site, whereas another group was not given any injection and served as control group. Both the groups were assessed radiologically, morphologically, volumetrically and histologically at 4, 8, 12 and 20 weeks.
Result: The analysis of the different parameters of our study showed that MSCs injected group showed enhanced fracture healing at 4, 8 and 12 weeks as compared to the control group, however, the results were similar at 20 weeks in both the groups.
Conclusion: Hence, it can be concluded that the injection of the bone marrow derived autologous mesenchymal stem cells locally at the fracture site hastens the process of fracture healing in the rabbits at earlier phases.
Keywords: autologous stem cells, osteotomised, fracture healing.

Introduction

In 10-20% of the fractures, the fracture healing process is impaired which leads to non union and delayed union [1, 2]. Such factors have led to the quest for modalities which can enhance fracture healing. Fracture healing takes place through various stages as hematoma formation, inflammation, angiogenesis, callus formation, and bone remodeling. The healing cascade is coordinated by the interaction of different set of molecules with the local and circulating cells i.e. mitogens (TGF B), Insulin like growth factor (IGF), morphogens (BMPs), effectors of inflammation(IL-1 , IL-6, COX -2), fibroblast growth factor(FGF), platelet derived growth factor(PGDF) and angiogenic factors(VEGF and angiopoietins). These factors affect the proliferation and differentiation of MSCs [3].

MSCs are the pluripotent mesodermal cells which have the potential to differentiate into the different tissues. In the presence of the lineage specific growth factors these MSC have been induced to differentiate into bone in tissue culture [4, 5]. The fracture repair process is initiated by the Mesenchymal stem cells which results in the formation of callus i.e. cartilaginous template .This callus is thereafter replaced by the new bone which repairs the gap[6]. In various experimental studies it has been

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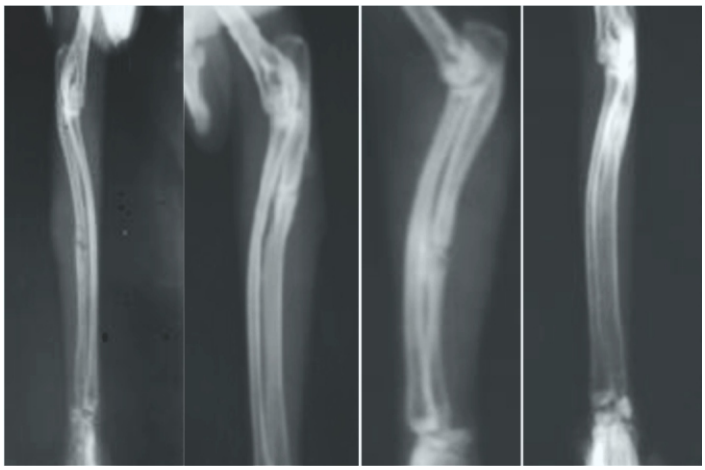


Figure 1: X-Ray photograph of the limb (Control group) at 4,8,12 and 20 weeks showing complete union

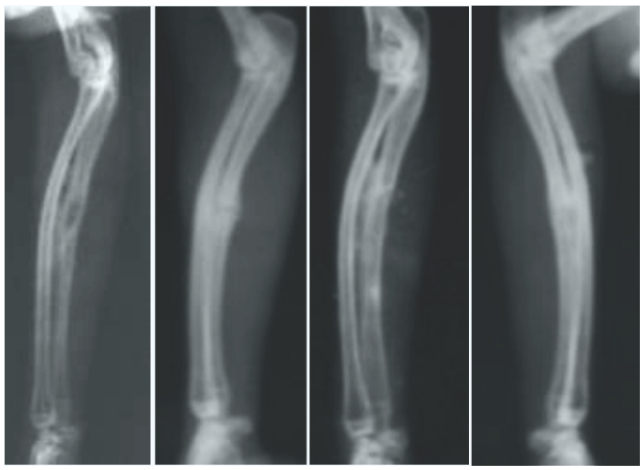


Figure 2: X-Ray photograph of limb (Test Group- MSCs injected group) at 4,8,12 and 20 weeks showing complete bony union and remodeling starting at 12 weeks

established that the MSCs isolated from the bone marrow of rats [7, 8], dogs [9] and humans [4, 5] can differentiate into osteoblasts. In one animal study it had been demonstrated that Mesenchymal Stem Cells (MSCs) accelerates the bone formation [10].

Our study has also been performed to evaluate the role of autologous bone marrow derived mesenchymal stem cells in fracture healing in rabbits.

*The work was carried out in the Experimental Medicine and Surgery Research Laboratory Section of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India in collaboration with Blood Bank and Pathology Department, IMS, BHU, Varanasi.

Material and methods

This study has been performed to assess the effect of stem cells in fracture healing. This work has been carried out in the experimental medicine and surgery research laboratory section in IMS, BHU in collaboration with blood bank. Autologous stem cells injection was given in animals with surgically created transverse midshaft ulnar osteotomy and describes the healing observed clinicoradiologically and histologically.

Animals:

The study was conducted on healthy mature rabbits irrespective of sex, weighing approx 1.5 kg or more. The experiments are protected under section of prevention of cruelty for Animals Act (1960). Approval was obtained by the ethical committee of Institute of Medical Sciences, BHU.

Experimental Design:

The study was conducted on 28 adult rabbits. Each rabbit was anaesthetized by a dose of 20 mg/kg of ketamine + 0.4 mg/kg of Midazolam I/M. Both upper limbs were shaved and cleaned with chlorhexidine, spirit and betadine. Rabbit was placed in prone position on operating table. Length of ulna was measured from tip of olecranon to wrist. Mid shaft of ulna was exposed through an incision over ulnar aspect of forearm. Few drill holes were drilled with 1 mm k wire to create fracture and minimal periosteal

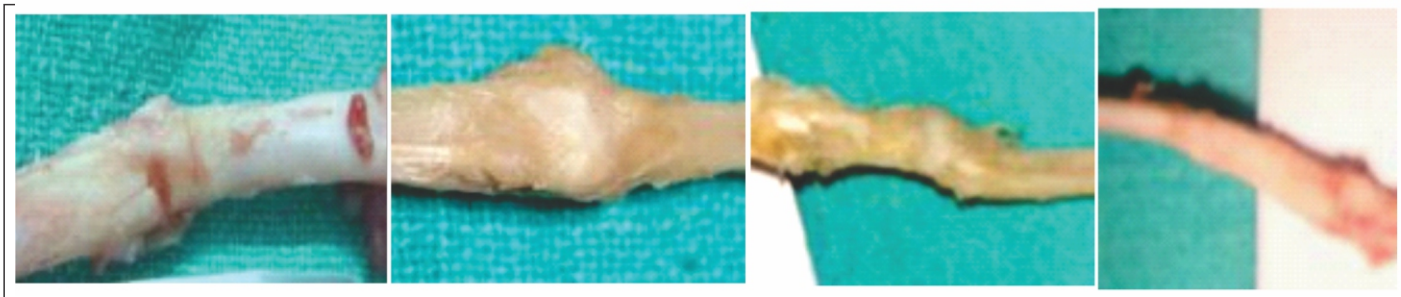
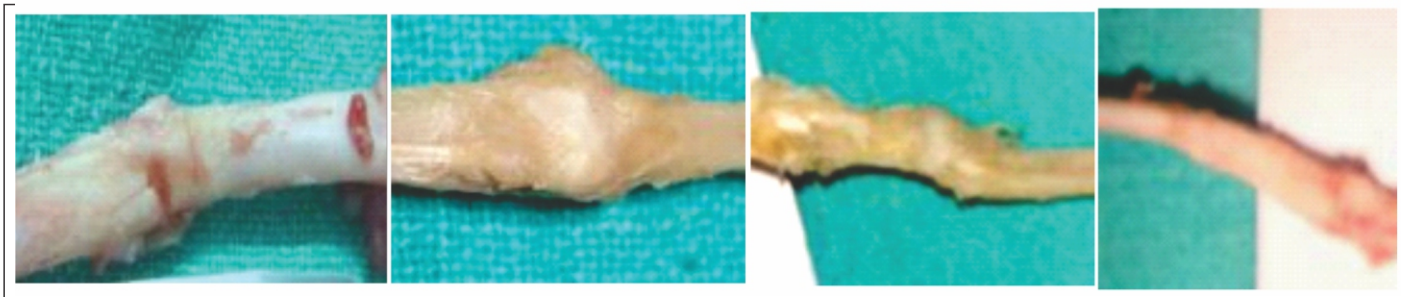


Figure 3: Gross specimen of the control group at 4weeks(a) showing cartilaginous callus, at 8 weeks(b) showing organized osteocartilaginous callus, at 12 weeks(c) showing osteocartilagenous callus with nearly complete bony union and at 20 weeks(d) showing decreased amount of callus with complete bony union and remodeling.



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stripping was taken care
of. Transverse osteotomy of mid shaft of ulna was created using osteotome and hammer. Local antibiotic (Chloramphenicol) was instilled before closure. Wound was closed by using interrupted 3-0 vicryl and skin closure done using prolene 3-0. Sterile dressing was done. Skin sutures were removed 10 days after the operation. Post operative intramuscular amikacin was given at dose of 20 mg/kg body weight and ceftriaxone at dose of 40 mg/kg body weight was given for 5 days.
After operation animals were divided into two groups-
(A) TEST GROUP (20 rabbits) - 1ml of final stem cell suspension was given after 10 days of fracture.
(B) CONTROL GROUP (8 rabbits) - With no stem cells injection
Proposed Plan: Both the groups were sacrificed at 4, 8, 12 and 20 weeks comprising 2 rabbits in each of the control group and 5 rabbits in each of the test group.
Marrows stem cell harvesting:
After giving adequate anaesthesia painting and draping was done for bilateral proximal tibia and bone marrow aspiration needle 16G was introduced into tibia. Trocar was removed and 1ml of heparin containing 2500 IU was taken into 10ml syringe and 10ml of bone marrow was aspirated and specimen was immediately transported to the laboratory.

Stem Cell Isolation:
Ficoll-Hipaque (sigma) density 1.077 15ml solution was taken in sterile centrifuge tube and then 10ml marrow aspirate was diluted with 20ml of normal saline. Centrifugation was done at spin speed 1500 rpm at 220C temperature for 30 minutes using heraeus centrifuger (6000i). After centrifugation plasma layer was carefully aspirated and discarded without disturbing the plasma Ficoll interface. Mono Nuclear Cell (MNC) layer was transferred into another sterile tube using sterile pipette and Phosphate Buffered Saline (PBS) was added to the cell suspension and washed thrice by centrifugating at a spin speed of 1500 rpm at 220C temperature for 10 minutes before a final re-suspension in phosphate buffered saline to make 1ml of final stem cell suspension.

Post-experimental schedule:
Animals were kept in wire cages and fed on standard rabbit diet containing about 6% protein and 60% non nitrogenous extract. All animals were observed daily for 1 week for any signs of wound infection. Wound healing was observed for 6-10 days for any signs of infection and

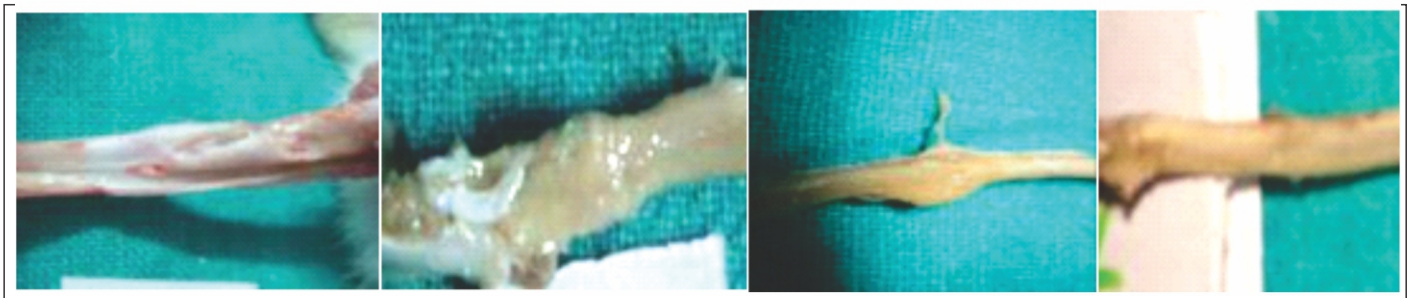

Figure 4: Gross specimen of the MSCs injected group at 4 weeks(a) showing osteocartilaginous callus , at 8 weeks(b) showing increased osteocartilagenous callus with nearly complete bony union, at 12 weeks(c) showing complete bony union with good amount of callus and at 20 weeks(d) showing complete bony union and remodeling.

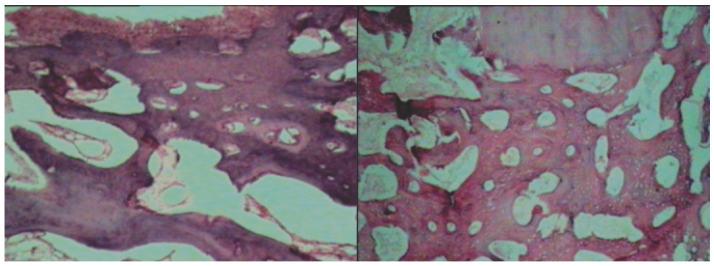

Figure 5: Photomicrographs showing(i)control group at 12 weeks showing healing with the presence of cartilages at places(H&E X25) (ii) complete healing at 12 weeks in MSCs injected group (H&E X40).

the suture removal was done on the 10th day after operation. Test rabbits were given 1.0 ml of stem cells injection over the fracture site on 10th day post-operation. In Control group stem cells injection was not given.

Parameters of study:

1. Radiological examination:

Rabbits were examined radiologically at 0, 4, 8, 12 and 20 weeks by using a standard anteroposterior and lateral skiagram of the operated limb. Fracture gap, bridge bone formation, degree of callus formation and fracture end sclerosis, if any, was observed and noted.

2. Gross examination at autopsy:

Rabbits were sacrificed by giving overdose of ketamine and succinyl choline intramuscularly. Mobility at fracture site was tested. Specimen was obtained and gross findings were observed like type and amount of callus, status of bony union and any abnormal mobility after removing the soft tissue as much as possible from the specimen.

3. Volumetrically measuring the size of callus:

Callus size was measured by taking multiple readings with the help of VERNIER CALIPER and assuming the cross section to be ellipsoid. Major and minor radii were measured at fracture callus site in test group and control group.

4. Histological examination:

Five and two rabbits each from the control group and the MSCs injected groups respectively were sacrificed at 4, 8, 12 and 20 weeks interval. The specimens for histological examination were decalcified using decalcification solution and studied for fracture union. The specimens were washed with water and dehydrated by passing for 12 hrs in each of the graded increasing concentrations of ethanol separately of 30%, 50%, 70%, 90% and through absolute alcohol. The specimen was subsequently treated with xylol solution mounted in liquid paraffin wax, cut into thin sections of 5-8 microns thickness which were made by cutting with microtome and were transferred to xylol to remove the paraffin. Next they were kept on bovine albumin coated slides. They were finally stained with haematoxyline and eosine. Thereafter, the fracture callus was assessed for degree of cellularity, amount of callus, cartilage, bone matrix formation, woven bone and mature bone formation and medullary repair and any remodeling and cortical repair etc. Rankit score, a five degree scoring system based on the observation of Allens et al [11] was used to score the degree of fracture repair and healing. As per this scoring system the GRADE 4 : Complete bone union (Fracture site bridged by well formed bony trabeculae); GRADE 3 : Less than complete bony union as evidenced by presence of small amount of cartilage in fracture callus; GRADE 2 : Complete cartilaginous union(Well formed plate of hyaline cartilage uniting the fragments); GRADE 1 : Incomplete cartilaginous union (as evidenced by retention of fibrous elements in plate); GRADE 0 : Pseudoarthrosis formation or non union seen as inconvertible cavity within cartilage plate between fracture fragments containing blood or other fluid and/ or lined by low cuboidal mesothelia.

Results

Post-operatively fracture healing was assessed in both groups at regular intervals of 4wks, 8wks, 12 wks and 20wks by radiological examination, gross examination at autopsy (morphological examination), volumetric study measuring callus size and histopathological examination. Within 6 to 10 days the wound was healed in all the cases and as absorbable sutures were used for wound closure hence there was no need of suture removal.

Radiological Examination

Radiographs were taken to assess callus formation, fracture gap, fracture end sclerosis, bridging bone formation, remodeling in both the groups. At 4 weeks - In the control group, minimal amount of bridging callus was seen with fracture gap clearly visible in all the rabbits. In the MSCs treated group, there was mild to moderate amount of bridging callus with fracture gap faintly visible in all the rabbits. As compared to the control group the amount of bridging bone callus appeared more in the test group radiologically and also quantitatively which have been detailed further. At 8 weeks – In the control group, the minimal amount of callus increased in all the rabbits.

The fracture line was faintly visible with minimal fracture end sclerosis. In the MSCs treated group, after 8 weeks of treatment there was complete bony union with fair amount of bridging callus. No fracture gap was visible in most of the rabbits with faintly visible fracture line in all the rabbits. In control group the healing was better than the control groups. At 12 weeks – There was minimal amount of callus in all the rabbits. The fracture line was still visible with faintly visible fracture end sclerosis. In the MSCs treated group, good amount of callus formation was seen; no fracture gap was visible with complete bony union in all the rabbits. Remodeling and repair of the medullary canal was observed better in this group. At 20 weeks – in control group, the fracture union & variable amount of remodeling was observed (Fig. – 1).

In the MSCs treated group at 20 weeks very good amount of callus with complete bony union was present. Remodeling and repair of medullary canal was observed better than the control group.(Fig.- 2)In both the groups bridging bone formation was increased with the time though remodeling was started earlier in MSCs treated groups .

Morphological Examination (Gross examination at autopsy) AND VOLUMETRIC ANALYSIS

All rabbits at the end of experiment were sacrificed as planned by giving lethal dose of succinyl choline and bilateral ulnas were harvested and morphological changes at the fracture site were observed. At 4 weeks – In the control group, the fracture callus was immature and soft. Also the fracture callus was sufficient in amount. In MSCs treated group, minimal to sufficient amount of bridging callus was seen in all the rabbits. Union was mostly cartilaginous and incomplete. At 8 weeks – In both the groups the amount of callus and maturity of callus was increased. In the control group, there was average amount of osteocartilagenous callus. In the MSCs treated group, all the rabbits showed good amount of osteocartilagenous callus with mostly nearly complete bony union. No abnormal mobility was seen in any of the samples. At 12 weeks, in the control group, there was average amount of osteocartilagenous callus in both the rabbits with nearly complete bony union. In MSCs treated group there was good amount of bony callus with complete bony union. At 20 weeks - there was decrease in amount of callus in both the groups compared to amount of callus seen at 12 weeks – probably due to maturation of callus and remodeling process (Fig.-3, 4).

Histological Analysis

Histological sections were taken and grading of fracture healing was done on five degree grading system, Rankit score. At 4 weeks, MSCs treated group showed much higher stages of healing and lesser amount of fibrous tissue as compared to the control groups. New bone formation was more as compared to the control group. Similar observations were made at 8 and 12 weeks (Fig. - 5) . At 20 weeks there was no difference among the MSCs treated group and the control groups with respect to the histological evidence of healing was observed.

Discussion

The clonogenic, multipotential precursor cells in the bone marrow were first described in the original work by Friedenstein at al [12]. These cells were termed as colony forming unit fibroblasts (CFU – Fs).They are currently termed as Mesenchymal stem cells and denote the pluripotent mesenchymal progenitor cells[13]. The bone marrow mainly consists of two types of cells i.e. stromal cells and hematopoietic cells. Due to their ability to diffentiate as mesenchymal or stromal cells, the stem cells for non hematopoietic tissues are referred as mesenchymal stem cells [14]. Mesenchymal stem cells can be isolated from the bone marrow and other adult tissues [15]. In bony compartment, they can be found in the bone marrow, endosteum, periosteum, thin connective tissue linings of the surface of the bones, and the mineralized bones. Mesenchymal stem cells play a significant role in bone healing [16].

Many studies have been done to establish the role of MSCs in fracture healing. In a study, Niemeyer et al[17] used xenogenic based bone marrow derived MSCs and autologous ovine bone marrow derived MSCs and implanted into a 3cm long sheep tibia bone defect. The results showed improved bone healing in the autologous cells as compared to the human cells. Also, Ai et al [18] did a study on bone healing rate after the xenograft of mineralized bone and together with an

allograft of bone marrow derived MSCs in the tibial bone defect of a rabbit model. Comparative study showed that stem cells produced more bone like tissue in the defect area than controls. MSCs have also been injected at the injured site in many of the studies to evaluate the effect of MSCs on fracture healing, similar method have been used in our study. Shao et al [19] injected the bone marrow derived MSCs to the experimentally induced gap of the callus in a model of rabbit. The results of bone union were better in the test group than the control group. The use of MSCs resulted in increased bone formation and bone density at the distraction site. The objective of this study was to further elucidate the role of bone marrow derived MSCs which has been claimed to enhance the role of fracture healing process. Our study also demonstrates that bone marrow derived autologous mesenchymal stem cell when locally administered at the fracture site enhances the bone healing. Our results showed that bone marrow derived MSCs injected animals had increased radiological, morphological, volumetric and histological differences in fracture healing at 4,8 and 12 weeks as compared to the control groups. The results were found similar at 20 weeks in both the groups. Our results are in agreement with the earlier observation of Shao et al [19]. Other studies have also been performed to see the efficacy of MSCs in tissue regeneration. Giannotti et al [20] in a case series treated the upper limb fractures showing pseudoarthrosis and delayed consolidation with the use of bone marrow derived MSCs.

The site of non union had been revitalized in all the cases by micro fractures and drilling, and a rigid plate had been used for synthesis. All the cases were healed and the same was confirmed radiographically.

In another study, Qu et al [21] investigated the effect of MSCs derived from human cord blood on bone nonunion of the femoral and tibial fracture. The results evaluated the accelerated bone healing with use of the human cord blood MSCs.

In stem cell therapy the most standard methods are the direct injection or cell seedling (Cell + Scaffold) and transplantation of the graft. Similar standard method has been followed in our study by injecting bone marrow derived autologous Mesenchymal stem cells at fracture site with favorable results.

In view of increasing the osteogenic potential of the stem cells, different methods have been conducted to differentiate the stem cells. In another study done by Qi et al [22], they investigated the fracture healing of an osteotomy in a model in a rat tibia, the effects of simvastatin locally applied from calcium sulphate combined with a MSC sheet were observed. In the cell treated group complete bone union was obtained after 8 weeks whereas there was no bone union in the control group at this time. These studies and their results opens a new avenue in the acceleration of fracture healing process , but further studies are needed in this area to demonstrate a beneficial effect for therapeutics for human beings.

Though the number of the animals used in our study in both the groups were small, on the basis of the findings it may be concluded that the bone marrow derived MSCs favorably hastens the process of fracture healing, especially in the early stages. The biological response of the use of MSCs on humans may vary, so caution must be exercised when extrapolating these data on human beings. Also, further investigations are required to establish the role of MSCs in fracture healing in human beings.

As conclusion our study showed that the stem cells injected group showed better healing at four, eight and twelve weeks of treatment as compared with the control group. Though, the number of control rabbits in each group was smaller than the test group and the overall number of rabbits per group was smaller, still on basis of findings, it can be concluded stem cells hastens the process of fracture healing.

Clinical Relevance

Role of autologous BMD –MSCs as an enhancer of fracture healing could be extended to humans, it would be of great benefit to all fracture patients as healing time would be shortened, which would eliminate the disadvantages of long-term immobilization of the involved bone and morbidity relating to long-term immobilization can be reduced by early fracture healing.

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