Khallaf and Kehinde, J Trauma Treat 2016, 5:2
DOI: 10.4172/2167-1222.1000299

Journal of Trauma & Treatment

Research Article Open Access

The Effect of Serum from Acute Traumatic Brain or Spinal Cord Injury Patients on the Growth of Human Bone Marrow-Derived Mesenchymal Stem Cells

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Abstract

Background: Accelerated osteogenesis associated with traumatic brain injury TBI or spinal cord injury SCI is inconclusive and its cause remains obscured. The purpose of this study was to ensure a clinical evidence of its presence and to reveal the possible underlying mechanism.

Methods: Healing indicators of diaphyseal femoral fractures in 20 patients with TBI and 20 patients with SCI were compared to 20 patients with femoral fracture only. The effect of sera of blood samples withdrawn from these patients on cell count proliferation rate of human bone marrow-derived mesenchymal stem cells MSCs (ATCC-USA) were measured and compared to sera from 20 patients with TBI only, 20 patients with SCI only, and a control group.

Results: The study showed that femoral fractures with TBI or SCI heal more expectedly, faster with exuberant callus ($p<0.0001$) and showed statistically significant increased cell count and mean growth rate of MSCs with sera from TBI and SCI patients with or without femoral fractures of 82.34 ± 6.93%, 83.9 ± 8.57%, 81.46 ± 5.37%, 81.5 ± 6.49% versus 52.96 ± 5.11% in the control and 59.77 ± 5.98% in patients with femoral fractures only ($p<0.0001$).

Conclusion: These results suggested enhancement of fracture-healing secondary to TBI and SCI due to the presence of factors in the serum that have a mitogenic effect on MSCs.

Keywords: Traumatic brain injury (TBI); Spinal cord injury (SCI); Long bone fractures; Acceleration of bone healing; Undifferentiated mesenchymal stem cells (MSCs)

Introduction

There is some clinical evidence to suggest that fractures of long bones heal more rapidly in patients with severe head injury or acute traumatic spinal cord injury. The mechanism underlying this orthopedic phenomenon is not well understood. Early clinical reports that researched the correlation between accelerated bone healing and acute traumatic nervous tissue damage in head or spinal cord injuries were inconclusive and demonstrated no evidence of accelerated union or increased callus formation but it revealed just increased incidence of heterotopic ossification without any effect on the clinical or radiological union [1-10].

The current understanding of bone healing event showed that the process involves the participation of orchestra of many growth factors and cytokine molecules and cells, primarily undifferentiated mesenchymal stem cells (MSCs) and blood inflammatory cells to induce formation of union callus at the fracture site [11-25].

The primary objective of this prospective controlled study was to ensure the accelerating effect of severe TBI and SCI on the healing of concomitant diaphyseal femoral fractures and the secondary objective was to test the effect of sera taken from patients with severe head or spinal cord injuries with concomitant long bone femoral diaphyseal on the growth rate of bone marrow derived mesenchymal stem cells on stem cells culture to elucidate the mechanism of accelerated bone healing in such patients.

Patients and Methods

Recruited patients in this current study, were non-smokers, between 18 and 60 years old, and had no history of chronic illness or systemic diseases. Patients on permanent medications and therapy for chronic disease such as diabetes mellitus, ischemic heart diseases, chronic renal failure, or endocrine diseases, or patients on corticosteroids for bronchial asthma, rheumatoid arthritis, other inflammatory arthritis, and autoimmune diseases were excluded from the study.

The patients were divided into five groups: Group A consisted of 20 patients with acute severe post-traumatic head (brain) injuries (TBI) who were admitted to the intensive care unit (ICU) with a Glasgow coma scale (GCS) of 8 or less (to define severe injury), Group B consisted of 20 patients with severe head injury (TBI) and concomitant long bone diaphyseal femoral fractures, Group C consisted of 20 patients with acute post-traumatic spinal cord injuries (SCI) with complete quadriplegia or paraplegia after the spinal shock stage, Group D consisted of 20 patients with SCI and femoral shaft fractures, and Group E consisted of 20 patients with femoral diaphyseal fractures only. All femoral fractures in patients of Group (B), (D), and (E) were treated surgically, by closed static reamed intra-medullary locking nail and followed-up weekly for three month and then, every three weeks for another three months (end-point of the study of fracture union, delayed union, or non-union), and every two months for another six to eight months. The patients' biodata and characteristics of injuries of all groups were shown in Table 1.

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Received March 06, 2016; **Accepted** April 05, 2016; **Published** April 09, 2016

Citation: Khallaf FG, Kehinde EO (2016) The Effect of Serum from Acute Traumatic Brain or Spinal Cord Injury Patients on the Growth of Human Bone Marrow-Derived Mesenchymal Stem Cells. J Trauma Treat 5: 299. doi:10.4172/2167-1222.1000299

Groups		A	B	C	D	E
No. recruited patients		**20**	**20**	20	20	20
Mean ± SD (Range) Age Years		32.5 ± 12.5, range (18-60)	29.1 ± 10.2 (range 18-59)	34.8 ± 9 range (18-52)	33.8 ± 8.5 (range 21-51)	32.8 ± 11.3 (range 20-60)
Sex	M	16	17	16	18	16
	F	4	3	4	2	4
Mean ± SD (Range) GCS*		6/15 ± 2/15, range (3-8)/15	6/15 ± 2/15, with a range of (3-8)/15	15/15	15/15	15/15
Cause of Injury	RTA**	20	20	12	17	17
	Fall from height	0	0	8	3	3
Type of injury	Head injury	yes	yes	0	0	0
	Spine injury	0	0	yes	yes	
	Quadriplegia			9	8	0
	Paraplegia	0	0	11	12	0
No of femoral shaft fracture		0	21	0	21	20
Status of Patient	Alive / Dead	20/0	20/0	20/0	20/0	20/0
*GCS = Glasgow Coma Scale **RTA = Road Traffic Accident						

Table 1: Patients' biodata and characteristics of injuries

Assessment of radiological healing of fractures is difficult and controversial, but mostly, radiological union is defined by the presence of bridging callus, disappearance of fracture line or the continuity of cortex in at least in three of the four bone cortices appear in the antero-posterior and the lateral X-ray views, so a score of 3-4 points of basically bridging callus defines fracture union. The healing of femoral shaft fractures in this study has been followed up by radiological assessment of the fracture in antero-posterior and lateral X-ray views weekly and once the plain X-ray showed fracture union according to the aforementioned radiological criteria, we use CT to assess the maximal amount of union callus formed at the fracture site. Delayed union was defined as absence of radiological union criteria 3 months after the occurrence of the femoral fracture, while non-union was defined as no bridging callus and radiologically visible fracture line 6 months after the injury with atrophic or hypertrophic fracture ends. The healing rate of femoral fracture was defined as the maximal thickness of union bridging callus in millimeters as observed in CT scan, divided by the time to healing in weeks. Time to radiological union (TRU), the maximal thickness of the amount of union callus (MTUC) formed, and healing rate (HR) of fractures were compared in three Groups of patients: (B), (D), and (E).

Blood samples were withdrawn from the injured patients of all groups from (A) to (E) at one week from the time of injury. 10 ml of blood was withdrawn only once, to test its effect and response with an *in vitro* cell assay.

Method

1) Blood samples were processed by centrifugation and separation of the sera which were preserved at -85°C.

2) Cell cultures of bone marrow-derived mesenchymal stem cells (BMDMSC) from ATCC, USA, were established as follows:

Components

1) 1 vial of Mesenchymal cells (1 × 106)

2) Mesenchymal stem cell basal medium

3) Supplements: FBS, rh FGF-b, rh IGF-1, L-alanyl-L-Glutamine, Penicillin, Streptomycin

4) Reagents for Subculture: DPbs, Trypsin-EDTA, Trypsin neutralizing solution

Preparation of complete growth medium

1. Obtain 1 mesenchymal stem cell growth kit from the freezer. Make sure the caps are tight.

2. Decontaminate the surfaces of all growth kit and basal medium with 70% ethanol or methanol.

3. Thaw the components of the growth kit prior to adding to the basal medium.

4. Take one bottle of basal medium.

5. Transfer the indicated volume of the kit component to the basal medium.

6. Swirl gently to assure homogenous solution.

7. Store at 2-8°C in the dark.

Procedure

1. Mesenchymal stem cells were grown until they were confluent using basal medium with supplements.

2. Cells were then trypsinized and counted the viable cells.

3. Aliquoted 10000 cells/ml to each 21 flasks.

4. To 5000 per ml of BMDMSC cells growing in small tissue culture flasks, 100 µl of sterile serum from the following groups of patients, were added: Group A of brain injury only, Group B of brain injury and long bone fracture, Group C of spinal cord injury only, Group D of spinal cord injury and long bone fracture, Group E of long bone fracture only, and one flask remains without serum as control.

5. Place the seeded flask in the 5% CO_2 incubator at 37°C for 72 hours.

6. Cells were trypsinized and viable cells counted using Vi-Cell XR cell viability analyzer (Beckman Coulter).

7. After 72 hours, growth inhibition or stimulating effect of serum from different patients' groups on BMDMSC in culture was assessed by counting the number of cells.

8. The experiment was repeated per category of patient, 20 times, using serum from 20 different patients per category.

9. The mean ± SD of the viable cell count in patients of each group was determined for the five groups and the results were compared between the groups and statistically analyzed.

Post-operative rehabilitation and follow up

Patients in the three Groups (B), (D), (E) were subjected to intensive program of physiotherapy including continuous passive motion CPM exercises whether in comatose, Group (B) patients or in paralyzed, Group (D) patients or patients with long bone fractures only, Group (E). Patients with head injury, when they became awake were allowed to be mobilized on a wheel chair. Patients with spinal cord injury were also allowed to be mobilized on a wheel chair when all their fractures were fixed, while patients in Group (E) actively exercised their limbs and were allowed to walk partial or full weight bearing with crutches or walker, once their femoral or tibial fractures were fixed. The mean period of follow up for patients in the three groups was 15 and the range (13-18) months.

Statistical analysis

Results were analysed with statistical package for the social sciences SPSS for Windows (Version 16). Means and standard deviations were determined. Mean scores between the two groups of patients were compared using chi square and the Student t-test. p value <0.05 was considered statistically significant.

Results

The biodata of patients in the study are shown in Table 1. 20 patients have been recruited in Group (A). The mean age in this group was 32.5 ± 12.5, range (18-60) years. The patients were 16 (80%) males and 4 (20%) females. The accidents in which, these patients were involved were high-velocity road traffic accidents RTA. 11 (55%) patients in this group had associated chest injuries and 4 (20%) had abdominal injuries. The mean GCS in the patients of this group was 6/15 ± 2/15, range (3-8)/15. Four (20%) patients required neurosurgical procedures such as craniotomy, evacuation of hematoma or elevation of depressed skull fractures. The findings of head CT scan in the patients of this group have been mentioned in Table 2.

20 patients were included in Group (B) and had a mean age of 29.1 ± 10.2 (range 18-59) years. There were 17 (85%) males and 3 (15%) females in this group. All patients in this group have been involved in RTA (Table 1). 9 (45%) patients in this group had associated chest injuries and 5 (25%) had abdominal injuries. The mean GCS in group (B) patients was 6/15 ± 2/15, with a range of (3-8)/15. Five (25%) patients required neurosurgical procedures. The findings of head CT scan in the patients of this group have been mentioned in Table 2. 21 closed diaphyseal femoral fractures were in the 20 patients in group (B), 11 (55%) of them were comminuted. These fractures were treated by closed or open reduction and internal fixation by static reamed interlocking intramedullary nail after a mean time of 7 and range of (5-9) days and were followed up for mean of 14.8 ± 1.7 and range of (12-18) months.

CT scan head findings	Group A		Group B	
	No of patients	%	No of patients	%
Skull fractures	17	85	18	90
Cranial bones only	16	80	15	75
Facial bones only	1	5	3	15
Cranial and facial fractures	1	5	3	15
Fracture base of the skull	0	0	0	0
Subdural hematoma	16	80	15	75
Subarachnoid haemorrhage	8	40	9	45
Diffuse brain oedema	15	75	17	85
Lobe and intra-cerebral hemorrhagic contusion	12	60	13	65
Midline shift	3	15	3	15
Extra-dural hematoma	5	25	4	20
Pneumocephalus	3	15	4	20
Impending conization	1	5	0	0
Diffuse axonal brain injury	3	15	3	15
Intra-ventricular haemorrhage	3	15	2	10

Table 2: Findings on CT of the skull in groups A and B patients.

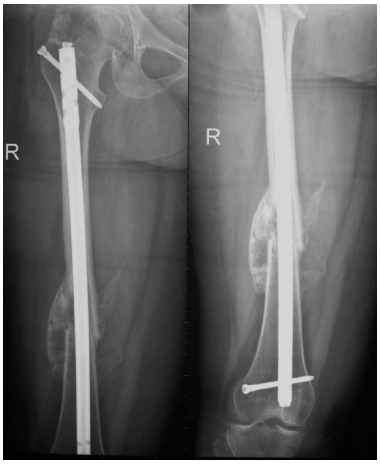

Figure 1: Showing x-ray of femur with accelerated fracture healing and abundant callus formation 5.8 weeks post-injury in a patient with severe head injury and long bone femoral diaphyseal fracture group (B) patient.

The mean TRU in this group was 7.6 ± 1.2 (range 5.8-10) weeks. There were no cases of non-union of the diaphyseal femoral fractures in this group. The mean MTUC was 31.5 ± 10.1 (range 15-48) mm. The mean HR of fractures of long bones in this group of patients was 4.3 ± 1.6 (range 2-7.2) mm/week, as shown in Figure 1.

20 patients have been recruited in Group (C). The mean age of the patients in this Group was 34.8 ± 9 range (18-52) years. These 20 patients included 16 (80%) males and 4 (20%) females. All patients have been involved in high velocity accidents, 12 patients (60%) have been involved in RTA and 8 patients (40%) in falling from height accidents. 7 (35%) patients in this group had associated chest injuries and one (5%) had abdominal injuries. Cervical spine injuries of fracture-dislocation with complete quadriplegia have occurred in 9 patients (45%) of this Group and burst vertebral body fracture or fracture-subluxation of dorsal spine and complete paraplegia have occurred in the remaining 11 patients (55%). The details of spine injuries seen in this group were shown in Table 3. In all Group (C) patients spine surgery procedures of open reduction, decompression, cage, plate fixation, trans-pedicular screws posterior fixation, and or fusion have been done in cervical and dorsal spine injuries. In this group, five quadriplegic and four paraplegic patients recovered incompletely after a mean of 3.8 ± 0.7 (range 2.1-4.8) months of follow-up.

20 patients were included in Group (D) and had a mean age of 33.8 ± 8.5 (range 21-51) years. There were 18 (90%) males and 2 (10%) females in this group. 17 (85%) of patients have been involved in RTA and 3 (15%) in falls from height accidents, as shown in Table 1. 5 (25%) patients in this group had associated chest injuries and 2 (10%)

had abdominal injuries. In this group, 8 (40%) patients had cervical spine injuries and complete quadriplegia and 12 (60%) patients sustained dorsal spine injuries and complete paraplegia. The details of spine injuries seen in this group were shown in Table 3. Spinal surgery procedures of open reduction, decompression, trans-pedicular screws posterior fixation, and/or fusion were performed in the thirteen patients of this group with dorsal spine injuries and in the eight patients with cervical spine injuries. Different procedures of reduction and plate fixation, anterior corpectomy, and/or fusion with instrumentation, were carried out.

21 closed diaphyseal femoral fractures were in the 20 patients in Group (D), 11 (52.4%) of them were comminuted. These fractures were treated by closed or open reduction and internal fixation by static reamed interlocking intramedullary nail after a mean time of 5 and range of (3-7) days and were followed up for mean of 14.1 ± 1.5 and range of (12-17) months. In this group, three quadriplegic and two paraplegic patients recovered incompletely after a mean of 4.1 ± 0.9 (range 3.2-4.5) months of the follow-up but the rest of the patients remained the same till the end of their follow up.

Type of spinal injury	Group C No of patients	Group D No of patients
Cervical spine injuries	**9**	**8**
Hangman fracture of C2	1	
C4-C5 fracture dislocation	3	2
C5-C6 fracture dislocation	3	2
C6-C7 fracture dislocation	1	2
C7-T1 fracture dislocation	3	2
Unstable burst fracture	1	1
Compression fracture	1	1
Fracture pedicle or lamina		
Traumatic disc protrusion		1
Dorsal spine injuries	**11**	**12**
Unstable burst fracture dorsal vertebra	6	5
Stable compression fracture dorsal vertebra	3	2
Dorsal spine fracture-dislocation	5	7

Table 3: Types of spine injuries seen in groups C and D patiernts.

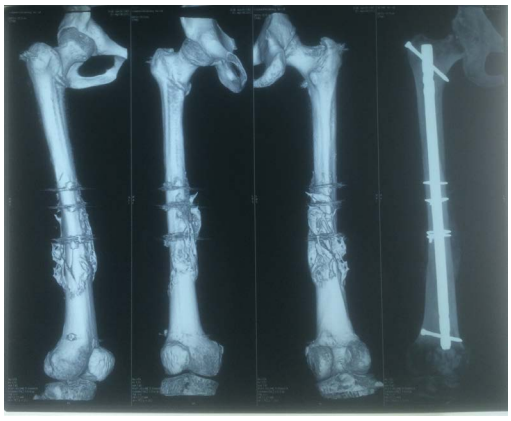

Figure 2: 3D CT scan and X-ray of femur with accelerated union of diaphyseal fracture with abundant callus formation 5.4 weeks post-injury in a group (D) patient with cervical spine fracture-dislocation and quadriplegia.

Patients group	No of patients finished follow-up	No of long bone femoral shaft fracture	No of fractures non-union	Mean ± SD (range) of healing time in weeks	Mean ± SD (range) of maximal thickness of union callus in mm	Mean (range) of healing rate in mm/week
B	20	21	0	7.6 ± 1.2 (range 5.8-10)	31.5 ± 10.1 (range 15-48)	4.3 ± 1.6 (range 2-7.2)
D	20	21	0	6.6 ± 0.66 (range 5.4-7.8)	31.8 ± 9 (range12-48)	4.9 ± 1.5 (range 2.2-7.2)
B+D	40	42	0	7.1 ± 1.1 (range 5.4-8.8)	31.6 ± 9.7 (range 12-48)	4.6 ± 1.6 (range 2-7.2)
E	20	20	3 (15 %)	27.3 ± 7.1 (range 13-41)	8.3 ± 3 (range 4-13)	0.32 ± 0.11 (range 0.13 - 0.52)

Table 4: Comparison of healing indicators of femoral fractures in patients in groups B, D, and E.

The mean TRU of the diaphyseal femoral fractures in Group (D) was 6.6 ± 0.66 (range 5.4-7.8) weeks. There were no cases of non-union of the femoral fractures in this group. The mean MTUC was 31.8 ± 9 (range 12-48) mm. The mean HR was 4.9 ± 1.5 (range 2.2-7.2) mm/week, as shown in Figure 2 and Table 4.

20 patients were included in Group (E) and had a mean age of 32.8 ± 11.3 (range 20-60) years. There were 16 (80%) males and 4 (20%) females in this group. The type of accident was high energy in all patients, RTA in 17 (85%) and falling from height in 3 (15%), as shown in Table 1. Only one patient (5%) in this group developed associated chest and abdominal injuries.

20 closed diaphyseal femoral fractures were in the 20 patients in Group (E), 8 (40%) of them were comminuted. These fractures were treated by closed or open reduction and internal fixation by static reamed interlocking intramedullary nail after a mean time of 7 and range of (3-11) days and were followed up for mean of 16.8 ± 1.6 and range of (14-20) months.

Among the 20 femoral fractures in the 20 patients in this group, 17 (85%) fractures united and 3 (15%) had delayed union, with union occurring 32-41weeks after the fractures occurred. Three (15%) fractures ended up by atrophic nonunion and required secondary procedures, as shown in Figure 2. Two (10%) of these non-united femoral fractures developed metal failure of broken nails with the osseous failure of union. The mean THU in this group of patients was 27.3 ± 7.1 (range 13-41) weeks. The mean MTUC in the united fractures in this group was 8.3 ± 3 (range 4-13) mm. The mean HR was 0.32 ± 0.11 (range 0.13-0.52) mm/week, as shown in Table 4 and Figure 3.

A comparative and statistical analysis of the 3 groups revealed that 20 patients were recruited into each Group of B, D and E respectively. The 3 groups were comparable with respect to mean age, gender, type of accidents, type of fracture, and method of skeletal stabilization. The mean (range) TRU of femoral fractures in Groups B, D and E was 7.6 ± 1.2 (range 5.8-10), 6.6 ± 0.66 (range 5.4-7.8) and 27.3 ± 7.1 (range 13-41) weeks respectively; (B or D versus E: $p<0.0001$). The mean (range) MTUC formed at fracture sites of femoral fractures in Groups B, D and E was 31.5 ± 10.1 (range 15-48), 31.8 ± 9 (range12-48) and 8.3 ± 3 (range 4-13) mm respectively; (B or D versus E: $p<0.0001$). The mean (range) HR of long bone fractures in Groups B, D and E patients was 4.3 ± 1.6 (range 2-7.2), 4.9 ± 1.5 (range 2.2-7.2) and 0.32 ± 0.11 (range 0.13 – 0.52) mm/week respectively; (B or D versus E: $p<0.0001$), as shown in Table 4.

Measuring the cell count of cell line of human bone marrow-derived mesenchymal stem cells MSCs (ATCC-USA) treated with control and patients' sera from different groups after 72 hrs incubation showed high statistically significant cell count and growth and viability rate in patients with severe TBI with or without long bone fractures Groups (A) and (B) and patients with SCI with or without long bone fractures Groups (C) and (D) in comparison to the control and to the effect of the sera from long bone fracture only Group (E). The mean growth rate in Groups (A), (B), (C), and (D) was 82.34 ± 6.93%, 83.9 ± 8.57%, 81.46 ± 5.37%, 81.5 ± 6.49% respectively, versus 52.96 ± 5.11% in the control and 59.77 ± 5.98% in Group (E) with long bone fractures only ($p<0.0001$), as shown in Figure 4 and Table 5.

Moreover, we found a positive correlation between fracture union time and thickness of union callus on one side and proliferation of bone marrow-derived mesenchymal stem cells in the tissue cultures on the other side. On the contrary, we found no correlation between the severity of Glasgow coma scale (GCS) with either the acceleration of fracture healing or the percentage of proliferation and viability of cells in the MSCs tissue cultures.

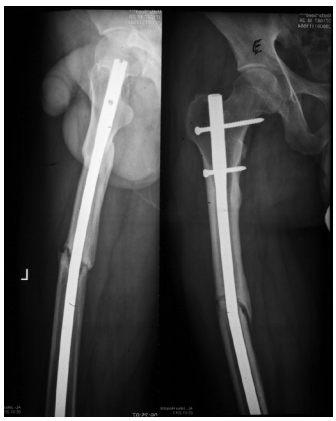

Figure 3: X-ray of fracture of the femur with osseous failure of atrophic nonunion and metal failure of broken nail 28 weeks post-injury in a group (E) patient with long bone fracture only.

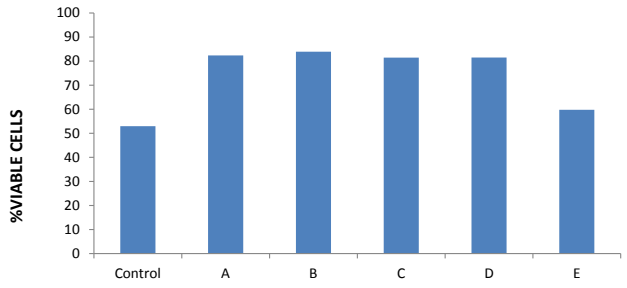

Figure 4: % cell growth viability chart of cell line MSCs treated with control and all groups A to E patients' sera after 72 hrs incubation.

Groups	Mean ± SD		% viable cells
	Total no. of cells/ml	Viable cells/ml	
Control (n=20)	$1.66 \times 10^5 \pm 7.7 \times 10^4$	$8.8 \times 10^4 \pm 2.25 \times 10^4$	52.96 ± 5.11
A (n=20)	$1.17 \times 10^5 \pm 1.5 \times 10^4$	$9.6 \times 10^4 \pm 1.5 \times 10^4$	82.34 ± 6.93
B (n=20)	$1.8 \times 10^5 \pm 2.34 \times 10^4$	$1.5 \times 10^5 \pm 2.13 \times 10^4$	83.9 ± 8.57
C (n=20)	$1.15 \times 10^5 \pm 1.79 \times 10^4$	$9.4 \times 10^4 \pm 1.44 \times 10^4$	81.46 ± 5.37
D (n=20)	$1.44 \times 10^5 \pm 2.88 \times 10^4$	$1.17 \times 10^5 \pm 1.86 \times 10^4$	81.5 ± 6.49
E (n=20)	$1.27 \times 10^5 \pm 2.43 \times 10^4$	$7.5 \times 10^4 \pm 1.91 \times 10^4$	59.77 ± 5.98
Control: healthy subjects: A: brain injury only; B: brain injury + long bone fracture; C: spinal cord injury only; D: Spinal cord injury + long bone injury; E: long bone fracture only			

Table 5: Summary of Cell count of cell line MSCs treated with control and patient samples after 72 hrs incubation.

Discussion

The current study is a prospective controlled study that compared the time of union, amount of union callus, and rate of healing of 42 diaphyseal femoral fractures in 40 patients associated with severe central nerve tissue damage (21 fractures in 20 patients with associated TBI and 21 fractures in 20 patients with SCI) to 20 femoral shaft fractures in 20 patients without head or spinal cord injuries with matching of the variables of age, type of accident, type of fracture, the fractured long bone, associated injuries, and method of skeletal stabilization. Furthermore, in this study we excluded patients with chronic illness or systemic diseases, thus further reducing confounders that may affect fracture healing.

From the results of the two groups of patients with diaphyseal femoral fractures associated with head injury in Group (B) and with spinal cord injury in Group (D) compared to the group of patients with only femoral fractures Group (E), we observed that fracture union occurred faster over a short period of time (TRU) in Groups (B) and (D) compared to Group (E). Femoral fractures in Group (B) and (D) patients united within mean of 7.1 ± 1.1 (range 5.4-8.8) weeks compared to mean of 27.3 ± 7.1 (range 13-41)weeks in Group (E) patients, a statistically significant difference ($p<0.0001$).

Another important finding of our study is that diaphyseal femoral fractures in head or spinal cord injury patients healed with more exuberant and florid callus formation compared with patients with femoral fractures only. The mean MTUC in Groups (B) and (D) was 31.6 ± 9.7 (range 12-48), compared to 8.3 ± 3 (range 4-13) mm in Group (E) ($p<0.0001$). Accordingly, the mean HR was also statistically significantly faster in Groups (B) and (D) compared to Group (E) 4.6 ± 1.6 (range 2-7.2) mm/week versus 0.32 ± 0.11 (range 0.13-0.52) ($p<0.0001$).

The study also showed that femoral fractures in patients with TBI or SCI all united and healed without a single case of nonunion or delayed union. However, 3 (15%) femoral fractures in 3 patients of Group (E) had atrophic nonunion, two (10%) of them developed metal failure of broken nails. Furthermore, 3 (15%) femoral fractures in Group (E) had delayed union which united in 38, 39, and 41 weeks post-injury.

One of the points of weakness in this study was the determination of the exact time of union whether clinical or radiological and the controversies of definition of radiological union, that may require to be exact, to have daily X-ray which is impossible for very overt reasons. Another point of weakness was the significance of the meaning of the healing rate as a healing indicator which may give a delusive impression that fracture healing is consistent through the whole process of osteogenesis, for which we do not have any proof. The third point of weakness was our dependence, basically on the bridging callus as radiological criterion among other criteria to assess union, but this may be justified by our method of fractures treatment, using interlocking intramedullary fixation, which mostly lead to secondary fracture union with bridging callus formation and unlike if we would have treated these fractures by open anatomical reduction and internal fixation by compression dynamic plates CDP, which mostly, may lead in this case to primary bone healing with radiological continuity of the cortex at the fracture site with no callus formation.

Some articles in the literature were found to support the results of the current study. Newman et al. [26] performed a retrospective study and demonstrated an unusually rapid healing of 13 closed long bone fractures in patients with concomitant severe head injuries. A study that included 17 patients with head injury and associated femoral shaft fractures and 50 patients without head injury (25 treated with reamed and 25 with unreamed nailing technique), and these authors reported a significantly shorter mean time to fracture union (TRU) in patients with head injury than either the reamed or the unreamed nailing groups without head injury [26]. A retrospective study and compared the healing of femoral shaft fractures in 20 patients with associated TBI to 54 patients without brain injury, and these authors confirmed that an injury to the brain may be associated with accelerated healing and enhanced callus formation in femoral shaft fractures [27,28].

Investigating the underlying mechanism of this accelerated osteogenesis, the results of the current study showed a high statistically significant proliferation and growth rate of cell line of human bone marrow-derived mesenchymal stem cells MSCs (ATCC-USA) treated with control and patients' sera from different groups after 72 hrs incubation in patients with severe head injury or spinal cord injury with or without long bone fractures Groups (A) to (D) in comparison to the control and to the effect of the sera from long bone fracture only Group (E). The mean growth rate in groups with central nervous tissue damage in Groups (A), (B), (C), and (D) was 82.34 ± 6.93%, 83.9 ± 8.57%, 81.46 ± 5.37%, 81.5 ± 6.49% respectively, versus 52.96 ± 5.11% in the control and 59.77 ± 5.98% in Group (E) with long bone fractures only ($p<0.0001$). These results indicate that sera from TBI or SCI patients with or without long bone fractures are mitogenic *in vitro* and induce a statistically significant proliferation of the cell line of human bone marrow-derived mesenchymal stem cells MSCs in the stem cell tissue culture. We understand that the *in vitro* mitogenic effect of these sera may be due to increased levels of growth factors and cytokines in the blood of the patients with severe head injury or spinal cord injury patients with or without long bone fractures or due to humoral substance, which has been produced in the damaged brain or spinal cord and crossed the blood brain barrier to peripheral circulation to enhance MSCs proliferation at the fracture site to induce acceleration of fracture healing.

In previous studies by the same authors of the current one, we investigated the levels of Insulin like growth factor- II (NGF-II); platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), Activin-A transferring growth factor β, and Cytokine Interleukin-1 (IL-1) in patients with TBI and SCI with concomitant long bone fractures and compared these to its levels in the control group of patients with long bone fractures only. We found that levels of PDGF, VEGF, Activin-A and IL-I cytokine were persistently high with statistical significance during the whole period of follow-up in TBI and SCI patients with associated long bone fractures compared to its values in the control group of patients with long bone fractures only [29,30].

The results of in- vitro studies of the effect of serum from patients with TBI on cultured cells have not been uniformly conclusive. Although few studies documented activation of osteoblasts on exposure to serum from patients with a brain or spinal cord injury, that finding was not substantiated in other subsequent studies and no substance or protein has been identified as the causative agent [11-25].

Despite the current lack of conclusive results, a study by Binder et al. found strong evidence that patients with a traumatic brain injury possess a humoral mechanism for accelerated fracture healing by demonstrating a mitogenic effect of the serum taken from those patients on cultured osteoblasts. Boes et al. proposed an influence of unknown factors released by injured brain tissue, which exert their proliferative effect specific to mesenchymal stem cells [31]. In their *in vitro* analysis, they showed that the serum of rats with a fracture and concomitant TBI stimulated a multipotent mesenchymal stem cell line ($C_3H_{10}T$-cells) to proliferate at a significantly higher level, resulting in a 76% increase in cells in the fracture/TBI group compared to the fracture-only group. A human fetal osteoblastic mesenchymal stem cell line (hFOB1.19 cells) in an early stage of its differentiation [32]. They saw that the cerebrospinal fluid of patients with a traumatic brain injury had an osteoinductive potential and therefore they expected that any osteoinductive factor in the serum of patients with TBI would have a stimulating effect on the hFOB1 cells *in vitro*. They also observed an increased proliferation rate of osteoblasts exposed to sera from patients with TBI during the first week after injury. A similar effect in patients with SCI and heterotopic ossification. However, there is no evidence that the in-vitro culture changes, which the serum of patients produce can lead to clinically significant changes in fracture healing or fracture methods of management [33-36].

Conclusions

We conclude according to the results of this study that union of diaphyseal femoral fractures is ensured, augmented and accelerated in patients with concomitant TBI or SCI which may be due to MSCs proliferation, accelerating bone healing based on the *in vitro* mitogenic effect of sera taken from these patients on the cell line of human bone marrow-derived mesenchymal stem cells MSCs cultures as shown in the study. Growth factors, cytokines, and damaged brain or spinal cord releasing humoral substance may the underlying cause of this mitogenic effect which needs further research to reveal it.

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