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High concentrations of several metabolites and growth factors in patients with delayed lower limb fracture healing

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Purpose To study concentrations of metabolites and growth factors as well as hematological parameters in patients with delayed lower limb fracture healing **Material and methods** Concentrations of several metabolites and growth factors as well as hematological parameters were studied in 13 patients with a delay in healing of femoral and tibial fractures after 7 to 11 months following injury. Seven patients with consolidated diaphyseal femoral and tibial fractures examined 10 to 12 months after the injury were a control group. Ten healthy subjects that did not have any history of fractures were a reference group. **Results** Unlike individuals of control and reference groups, patients with delayed fracture healing showed significantly higher concentrations of lactate, triglycerides, TGF-α and TGF-β2 in their serum while IGF-1 levels were significantly lower. Hematology tests did not show differences between the groups. **Conclusion** Local hypoxia, acidosis and expression of GFs that support osteolysis were the main pathophysiological processes that could cause the delay in long-bone fracture healing.

Keywords: femur, tibia, fracture, delayed union, growth factors, hematological parameters

INTRODUCTION

Delayed bone healing may occur after long bone fractures and remains a challenging issue for orthopaedic surgeons. The risk factors in the development of the pathology are the nature of the injury and fracture pattern, systemic status of the patient before injury, local response to the injury, postoperative care, as well as pharmacologic medication [1-3].

No doubt, any of the factors listed above may provoke osteogenesis delay. However, regardless of the causes of delayed fracture union, the molecular mechanisms of the delayed bone healing pathogenesis would be similar. It is obvious that the first manifestations of the pathological changes that result in osteogenesis retardation would be the changes in the concentrations of metabolites and biologically active substances in the blood serum [4-6].

In fact, it was shown that delayed long-bone fracture healing was associated with characteristic changes in several specific regulatory polypeptides found in the blood serum such as growth factors (GFs) and cytokines if no congenital or endocrine pathology was diagnosed in the patients [7-12], or alterations in the expression of some genes [13]. In the opinion of several authors, the levels of metabolites and GFs that show significant changes in case of delayed limb fracture healing could be used for prognosis of such a delay in the clinical settings [14-17]. Their studies underline the relevance and prospective value of studying the molecular mechanisms of the pathogenesis of this condition and would be valuable both for understanding the fundamental issues of osteogenesis and for developing the solutions that could be applied in the management of patients with delayed fracture healing.

Our objective was to estimate the levels of several metabolites, growth factors and hematological parameters in patients with delayed fracture healing in the lower limb long bones.

MATERIAL AND METHODS

Thirteen patients with delayed healing of closed diaphyseal fractures (AO/ASIF type A) of the femur (n = 4) and tibia (n = 9) were studied (index group) seven to 11 months after the injury (exceeded the regular consolidation time for this type of fractures by 1.5–2 times. There were eight males and 5 females in the mean age of 42.0±7.6 years (range: 23–59) that did not have any associated diseases. All patients were

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referred to our clinic from other hospitals where their fractures had been treated with the Ilizarov method. The analysis of the medical records by our surgeons revealed that the main cause of delayed consolidation was poor fracture reduction. Their clinical examination showed that there was swelling and painful mobility in the fracture area within 30 to 45 degrees. Crepitation was absent while distal arteries were pulsating when palpated. The patients were not weight-bearing and used crutches. Radiographic study revealed osteoporotic bone fragment ends; their bone callus was very weak and fracture lines were well visualised; the periosteal responses were weak and interrupted.

Control group were seven patients, four males and three females, in the mean age of 37.2 ± 5.1 years (range: 24–59), that had no associated diseases and had passed the treatment with the Ilizarov apparatus for closed diaphyseal fractures (AO/ASIF type A) of the femur (n = 3) and tibia (n = 4) that consolidated on time within two to four months. The time-point for the study in the control group was between 10 to 12 months after the injury.

Reference group included ten healthy individuals (5 males and 5 females) in the age from twenty six to 50 years (mean age 38.2 ± 6.1 years) that did not have any history of fractures.

All the subjects included into the study had a single blood extraction for laboratory tests.

Biochemical analysis Blood serum biochemical levels of the following molecules were determined: total protein, albumin, urea, C-reactive protein, total cholesterol, triglyceride, glucose, lactic acid, total calcium, non-organic phosphate, magnesium, potassium, chlorides, alkaline phosphatase (ALP), bone (tartrate-resistant) isoenzyme of acid phosphatase, creatine phosphokinase, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase. Their levels and enzymes activity were defined with the use of a biochemical automated analyser Hitachi/BM 902 (F. Hoffmann-La Roche Ltd/Roche Diagnostics GmbH) with a reagent kit of Vital Diagnostics (Russia).

Hematology tests Erythrocytes, leucocytes, thrombocytes, hemoglobin, hematocrit value and leucocyte formula were counted in the subjects of each group with the use of hematology analyzer ABX Pentra60 (Horiba, Japan).

Estimation of growth factors Concentrations of the following GFs were estimated in the subjects of three groups: stem cell factor (SCF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth factors (TGF-α, TGF-β1, TGF-β2), insulin-like growth factor 1 (IGF-1). Their levels were evaluated with the immune enzyme method on ELX808 (BIO-TEK Inc., USA) using a reagent kit of R&D Systems (USA).

The significance of difference between the groups studied was determined with the Kruskal-Wallis non-parametric method followed by multiple comparisons with the Dunn's test.

Clinical tests were performed on permission of the Ethics Board of the institution. All the individuals signed informed consents.

RESULTS

The majority of blood serum biochemical tests showed no significant differences between the index groups and two other groups studied (data not presented), except three parameters (Table 1). In particular, the levels of lactate and triglyceride in the group of delayed fracture healing were significantly higher than in the control and reference groups. The ALP activity was significantly decreased but as compared to the control group.

Hematology tests did not show significant differences between the groups and were within the normal levels (Table 2).

Table 1

Biochemical parameters in the groups that showed significant difference (median, 0.25÷0.75 percentile)

Parameter	Reference group	Index group	Control group
alkaline phosphatase, U/l	80 54÷103	73# 58÷92	115 89÷149
lactate, mmol/l	1.80 1.41÷2.02	2.21*# 2.00÷2.39	1.88 1.70÷2.01
triglycerides, mmol/l	0.81 0.44÷0.96	1.27*# 0.93÷1.48	0.70 0.51÷0.84

Note: * – significant difference with the reference group ($p < 0.05$); # – significant difference as compared with the control group ($p < 0.05$).

Table 2

Findings of hematology tests in the groups studied (min÷max)

Parameter	Reference group	Index group	Control group
RBC, 10^9/l	3.90÷5.70	3.90÷4.65	3.97÷4.60
Hb, g/l	120÷157	121÷134	125÷139
HCT, %	38.0÷51.0	38.0÷48.0	40.0÷47.0
PLT, 10^9/l	210÷390	215÷370	231÷380
WBC, 10^9/l	4.8÷8.1	5.5÷8.4	4.9÷7.5
LYM, %	28.0÷40.0	26.0÷38.0	27.0÷37.0
MONO, %	4.10÷10.00	7.00÷1000	6.50÷10.00
EOS, %	030÷3.50	1.50÷4.00	2.00÷4.50
BASO, %	0.00÷1.00	0.00÷1.00	0.00÷1.00
ESR, mm/hour	2.0÷17.0	1.0÷15.0	0.0÷11.0

Levels of TGF-α and TGF-β2 were significantly higher while IGF-1 concentration was two-fold lower in the blood serum of the patients with delayed fracture healing as compared with the other groups (**Fig. 1** and **Fig. 2**). EGF levels were significantly higher than its concentrations in the reference group but not in the control group (**Fig. 1**). There was no significant difference in the levels of SCF and VEGF between the groups.

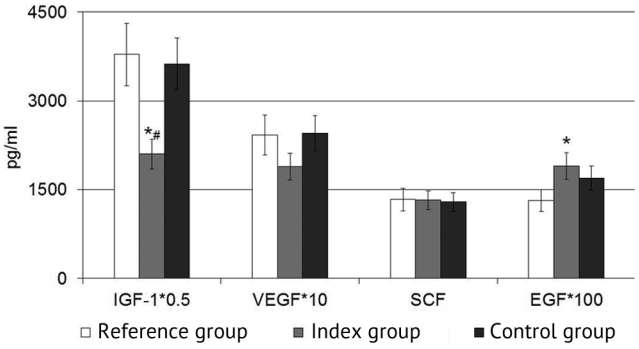

Fig. 1 Concentrations of some GFs (pg/ml) in the serum of the patients in three groups studied. Note: * – significant difference with the reference group ($p < 0.05$); # – significant difference as compared with the control group ($p < 0.05$)

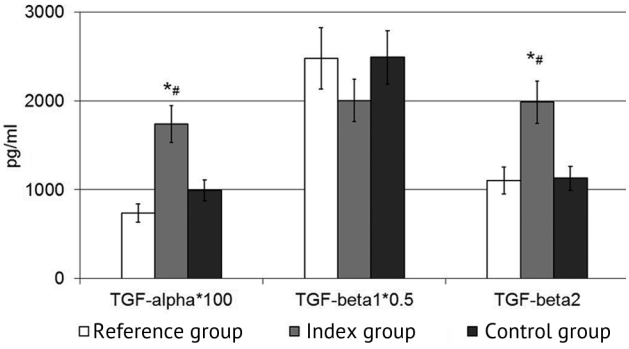

Fig. 2 TGFs concentrations (pg/ml) in the serum of the patients in three groups studied. Note: * – significant difference with the reference group ($p < 0.05$); # – significant difference as compared with the control group ($p < 0.05$)

DISCUSSION

Our results of studying the serum samples of the patients with delayed fracture healing in the period from 7 to 11 months after the injury have shown that they had higher levels of several metabolites and GFs. Those changes could be linked to the retardation of fracture repair through several molecular mechanisms.

In particular, high levels of TGFs in patients with delayed union could result in their immune system modulation (inhibition effect) and hemopoiesis suppression [18]. In the opinion of several authors, anemia caused by disorders in hemopoiesis is the main risk factor of delay in the fracture union [19]. However, this mechanism could not have been realized in our patients as far as their hematology test parameters were within the normal values.

However, high levels of TGF-α and TGF-β2 in the patients with delayed fracture healing could have had a direct inhibition impact on bone reparation. It is known that those transforming GFs activate osteoclasts and increase bone resorption [20]. High EGF levels discovered in the serum of the index group could support the increased resorption as far as EGF stimulates the release of calcium from bone tissue through its interaction with osteoclasts and promotes osteolysis [21].

It can be supposed that metabolic acidosis that could be caused by disturbed oxygen supply to the injured tissues aggravates bone resoption. This supposition is confirmed not only by the increased level of lactate in the main patients' group serum but also by the rise in the triglycerides concentrations. It was shown that the

disorders in the oxygen supply in the patients with skeletal trauma could be accompanied by the mobilization of several substrates of lipid metabolism in the posttraumatic period [22]. Such metabolic disturbances following fractures could be predictors of osteogenesis disorders that had been shown previously [23].

Another possible reason of the acidosis that was observed in the group of delayed union would be an impaired blood supply to the bone due to the lack of proper fixation stability of bone fragments. In fact, bone fragments instability and disorders in angiogenesis that follow thereby result in acidosis and hypoxia were found to be the main causes of bone formation delay in the posttraumatic period [24].

The decrease in the serum IGF-1 concentration contributed to the disturbance of osteoreparation in the group of delayed fracture healing. It was confirmed that the reparative capability of bone tissue was considerably worse if this GF level was low in blood serum [25, 26]. Thereby, the decreased IGF-1 level that results in the osteogenesis deceleration could be caused by the insufficient protein nutrition [27].

Reduction in the ALP activity in the serum of the group with delayed fracture healing, although it is a limited parameter due to its low grade association with bone tissue, could be also referred to a proof of decreased osteogenesis activity. Our supposition correlates to the findings of an experimental study that discovered the correlation between the ALP activity and deceleration of osteogenesis [28].

The combination of the biochemical changes that were observed in our patients with delayed fracture healing and the findings reported in the available literature allow us to suppose that the disturbance in the bone callus angiogenesis due to improper fracture reduction and impaired systemic blood flow to the fractured bone, subsequent hypoxia, acidosis and overexpression of the GFs that support osteolysis are the main pathophysiological processes that provoke the delay in long-bone fracture healing.

However, the key problem in the discussion of the findings obtained is the issue whether these alterations would be secondary and develop due to biomechanical issues that contribute to the delay, as was stated by the clinicians, or those are primary changes that would directly affect osteogenesis. We would accept that the delay in fracture healing is the combination of two factors: insufficient reduction of the fracture and possible physiological predisposition of some individuals to such retardation through biochemical processes. The latter could be, as we suppose, a complex of hereditary (or acquired) characteristic peculiarities of the metabolic processes and humoral regulation that can be responsible for the conditions in which a decreased osteoreparative process runs.

CONCLUSION

We conclude that the molecular changes that support (or possibly can precondition) resorption activity in the fractured bone are present in the patients that suffered a delay in lower limb long bone fracture repair in the period of more than seven months following the injury. Those are: 1) high levels of GFs that cause and support osteolysis; 2) reduction in the level of IGF-1 that stimulates bone regeneration; 3) local metabolic acidosis.

Only those biomarkers can be referred to the predictive factors of delayed fracture healing that statistically differed in the patients of the index group when compared to the control and reference groups. High levels of lactate, EGF, TGF-α and TGF-β2, as well as a decreased level of IGF-1 in blood serum could be predictors of delayed bone healing following long bone fractures.

REFERENCES

1. Hayda R.A., Brighton C.T., Esterhai J.L.Jr. Pathophysiology of delayed healing. *Clin. Orthop. Relat. Res.*, 1998, no. 355 Suppl., pp. S31-S40.
2. Rodriguez-Merchan E.C., Forriol F. Nonunion: general principles and experimental data. *Clin. Orthop. Relat. Res.*, 2004, no. 419, pp. 4-12.
3. Calori G.M., Albisetti W., Agus A., Iori S., Tagliabue L. Risk factors contributing to fracture non-unions. *Injury*, 2007, vol. 38, no. Suppl. 2, pp. S11-S18.
4. Keramaris N.C., Calori G.M., Nikolaou V.S., Schemitsch E.H., Giannoudis P.V. Fracture vascularity and bone healing: a systematic review of the role of VEGF. *Injury*, 2008, vol. 39, no. Suppl. 2, pp. S45-S57. DOI: 10.1016/S0020-1383(08)70015-9.
5. Barnes G.L., Kostenuik P.J., Gerstenfeld L.C., Einhorn T.A. Growth factor regulation of fracture repair. J. Bone Miner. Res., 1999, vol. 14, no. 11, pp. 1805-1815. DOI: 10.1359/jbmr.1999.14.11.1805.
6. Marsell R., Einhorn T.A. The biology of fracture healing. *Injury*, 2011, vol. 42, no. 6, pp. 551-555. DOI: 10.1016/j.injury.2011.03.031.

7. Street J., Bao M., deGuzman L., Bunting S., Peale F.V. Jr., Ferrara N., Steinmetz H., Hoeffel J., Cleland J.L., Daugherty A., Van Bruggen N., Redmond H.P., Carano R.A., Filvaroff E.H. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc. Natl. Acad. Sci. USA*, 2002, vol. 99, no. 15, pp. 9656-9661. DOI: 10.1073/pnas.152324099.
8. Sarahrudi K., Mousavi M., Thomas A., Eipeldauer S., Vécsei V., Pietschmann P., Aharinejad S. Elevated levels of macrophage colony-stimulating factor in human fracture healing. *J. Orthop. Res.*, 2010, vol. 28, no. 5, pp. 671-676. DOI: 10.1002/jor.21048.
9. Sarahrudi K., Thomas A., Mousavi M., Kaiser G., Köttstorfer J., Kecht M., Hajdu S., Aharinejad S. Elevated transforming growth factor-beta 1 (TGF-β1) levels in human fracture healing. *Injury*, 2011, vol. 42, no. 8, pp. 833-837. DOI: 10.1016/j.injury.2011.03.055.
10. Hankenson K.D., Zimmerman G., Marcucio R. Biological perspectives of delayed fracture healing. *Injury*, 2014, vol. 45, no. Suppl. 2, pp. S8-S15. DOI: 10.1016/j.injury.2014.04.003.
11. Kobbe P., Vodovotz Y., Kaczorowski D.J., Mollen K.P., Billiar T.R., Pape H.C. Patterns of cytokine release and evolution of remote organ dysfunction after bilateral femur fracture. *Shock*, 2008, vol. 30, no. 1, pp. 43-47. DOI: 10.1097/SHK.0b013e31815d190b.
12. Pountos I., Georgouli T., Bird H., Kontakis G., Giannoudis P.V. The effect of antibiotics on bone healing: current evidence. *Expert. Opin. Drug Saf.*, 2011, vol. 10, no. 6, pp. 935-945. DOI: 10.1517/14740338.2011.589833.
13. Dimitriou R., Kanakaris N., Soucacos P.N., Giannoudis P.V. Genetic predisposition to non-union: evidence today. *Injury*, 2013, vol. 44, no. Suppl. 1, pp. S50-S53. DOI: 10.1016/S0020-1383(13)70012-3.
14. Pountos I., Georgouli T., Pneumaticos S., Giannoudis P.V. Fracture non-union: Can biomarkers predict outcome? *Injury*, 2013, vol. 44, no. 12, pp. 1725-1732. DOI: 10.1016/j.injury.2013.09.009.
15. Zimmermann G., Müller U., Wentzensen A. The value of laboratory and imaging studies in the evaluation of long-bone non-unions. *Injury*, 2007, vol. 38, no. Suppl. 2, pp. S33-S37.
16. Zimmermann G., Henle P., Küsswetter M., Moghaddam A., Wentzensen A., Richter W., Weiss S. TGF-beta1 as a marker of delayed fracture healing. *Bone*, 2005, vol. 36, no. 5, pp. 779-785. DOI: 10.1016/j.bone.2005.02.011.
17. Goebel S., Lienau J., Rammoser U., Seefried L., Wintgens K.F., Seufert J., Duda G., Jakob F., Ebert R. FGF23 is a putative marker for bone healing and regeneration. *J. Orthop. Res.*, 2009, vol. 27, no. 9, pp. 1141-1146. DOI: 10.1002/jor.20857.
18. Heldin C.H., Miyazono K., ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature*, 1997, vol. 390, no. 6659, pp. 465-471. DOI: 10.1038/37284.
19. Heppenstall R.B., Brighton C.T. Fracture healing in the presence of anemia. *Clin. Orthop. Relat. Res.*, 1977, no. 123, pp. 253-258.
20. Ibbotson K.J., Harrod J., Gowen M., D'Souza S., Smith D.D., Winkler M.E., Derynck R., Mundy G.R. Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. *Proc. Natl. Acad. Sci. USA*, 1986, vol. 83, no. 7, pp. 2228-2232.
21. Carpenter G., Cohen S. Epidermal growth factor. J. Biol. Chem., 1990, vol. 265, no. 14, pp. 7709-7712.
22. Lepistö P.V. Post-traumatic blood lipid changes and fat embolism. Relation of post-traumatic blood lipid changes and fat embolism syndrome. *J. Trauma*, 1976, vol. 16, no. 1, pp. 52-57.
23. Stogov M.V., Luneva S.N., Tkachuk E.A. Biochemical parameters in the prediction of the course of osteoreparative processes in skeletal injury. *Klin. Lab. Diagn.*, 2010, no. 12, pp. 5-7.
24. Lu C., Saless N., Hu D., Wang X., Xing Z., Hou H., Williams B., Swartz H.M., Colnot C., Miclau T., Marcucio R.S. Mechanical stability affects angiogenesis during early fracture healing. *J. Orthop. Trauma*, 2011, vol. 25, no. 8, pp. 494-499. DOI: 10.1097/BOT.0b013e31822511e0.
25. Bernstein A., Mayr H.O., Hube R. Can bone healing in distraction osteogenesis be accelerated by local application of IGF-1 and TGF-beta1? *J. Biomed. Mater. Res. B. Appl. Biomater.*, 2010, vol. 92, no. 1, pp. 215-225. DOI: 10.1002/jbm.b.31508.
26. Fowlkes J.L., Thrailkill K.M., Liu L., Wahl E.C., Bunn R.C., Cockrell G.E., Perrien D.S., Aronson J., Lumpkin C.K. Jr. Effects of systemic and local administration of recombinant human IGF-I (rhIGF-I) on de novo bone formation in an aged mouse model. *J. Bone Miner. Res.*, 2006, vol. 21, no. 9, pp. 1359-1366. DOI: 10.1359/jbmr.060618.
27. Day S.M., DeHeer D.H. Reversal of the detrimental effects of chronic protein malnutrition on long bone fracture healing. *J. Orthop. Trauma*, 2001, vol. 15, no. 1, pp. 47-53.
28. Komnenou A., Karayannopoulou M., Polizopoulou Z.S., Constantinidis T.C., Dessiris A. Correlation of serum alkaline phosphatase activity with the healing process of long bone fractures in dogs. *Vet. Clin. Pathol.*, 2005, vol. 34, no. 1, pp. 35-38.

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