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MODULATORY EFFECT OF GASTRIC PENTADECAPEPTIDE BPC 157 ON ANGIOGENESIS IN MUSCLE AND TENDON HEALING

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Angiogenesis is a natural and complex process controlled by angiogenic and angiostatic molecules, with a central role in healing process. One of the most important modulating factors in angiogenesis is the vascular endothelial growth factor (VEGF). Pentadecapeptide BPC 157 promotes healing demonstrating particular angiogenic/angiomodulatory potential. We correlated the angiogenic effect of BPC 157 with VEGF expression using *in vitro* (cell culture) and *in vivo* (crushed muscle and transected muscle and tendon) models. Results revealed that there is no direct angiogenic effect of BPC 157 on cell cultures. On the other hand, immunohistochemical analysis of muscle and tendon healing using VEGF, CD34 and FVIII antibodies showed adequately modulated angiogenesis in BPC 157 treated animals, resulting in a more adequate healing. Therefore the angiogenic potential of BPC 157 seems to be closely related to the healing process *in vivo* with BPC 157 stimulating angiogenesis by up-regulating VEGF expression.

Key words: *angiogenesis, pentadecapeptide BPC 157, cell culture, healing, muscle, tendon, vascular endothelial growth factor*

INTRODUCTION

Numerous peptide growth factors are supposed to influence angiogenesis, and thereby wound healing and tissue regeneration. The healing process is particularly interesting in two closely related tissues: muscle and tendon.

Muscle usually heals remarkably well due to highly developed vasculature network and existence of satellite cells and muscle derived stem cells. Tendons, which are during development rich in cells, metabolically active and containing high number of blood vessels (1), are eventually maturing to hypocellular, hypovascular and hyponeural structures (2-4). Tendons do not heal as well as muscle, but the healing process in both is very complex and under regulation of many different factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), tumor necrosis factor (TNF) and NO (5, 6).

Pentadecapeptide BPC 157 is a peptide effective in muscle and tendon healing (7-12), applied alone, without any carrier. In tendon and muscle healing its functional, biomechanical, and pathohistological beneficial effect is accompanied by angiogenic action. BPC 157 may directly protect endothelium (13), influence NO-system, counteract the effect of both NOS-inhibitor and NO-precursor (14, 15) as well as over expression of endothelin (16). BPC 157 has a particular wound healing effect, also in response to vessel injury and coordination of the expression of multiple genes involved in the pathogenesis of vascular disease; it stimulates the expression of the early growth response 1 (EGR-1) gene and expression of the EGR-1 repressor, nerve growth factor 1-A binding protein-2 (nab2) (17). From these results BPC 157 shows a particular angiogenic/angiomodulatory potential, worthy of further investigation.

In this study, we correlated the angiogenic effect of BPC 157 with VEGF expression using *in vitro* and *in vivo* models.

MATERIALS AND METHODS

BPC 157

Synthetic pentadecapeptide BPC 157, GEPPPGKPADDAGLV, M.W. 1419, (Diagen, Ljubljana, Slovenia) is a part of protein sequence isolated from gastric juice and after high pressure liquid chromatography purification theprotein is obtained with 99% purity, having 1-des-Gly peptide as impurity. It is very stable and applied dissolved in sterile saline, without any additional carriers.

Cell culture

As an angiogenesis model TCS CellWorks AngioKit (TCS CellWorks, Buckingham, United Kingdom) was used. Following manufacturer's instructions we treated cells with pure medium (TCS CellWorks, Buckingham, United Kingdom), or with VEGF as positive control (2 ng/ml), Suramin as negative control (20 uM) and BPC 157 in two different final concentrations (10 ug/ml and 2 ug/ml). Medium was changed on the first, fourth,

seventh and ninth day. Cell cultures were cultivated in usual conditions on 37°C and 5% CO_2. After 11th day cultures were fixed and tubules were visualized using CD31 antibody (TCS CellWorks, Buckingham, United Kingdom). Using digital camera, tubules were transferred into ISSA- special software for morphometrical analysis (Vamstec, Zagreb, Croatia). Angiogenic effect was evaluated by counting the number of the tubule branching on 5 visual fields (microscope objective x 20) (modification of Jones RA, 18).

Animal models

All experimental protocols were approved by the Ethics Committee at the University of Zagreb School of Medicine. Three different, already established and published models were used (10-12). Animals used were male Wistar Albino rats, weighting from 280 to 320 g. Six animals per each experimental group and time interval were used. Animals were anaesthetized prior to any trauma. Animals were treated either with BPC 157 (10 ug/kg) dissolved in saline, or with equivalent volume of saline alone (5 ml/kg).

1. Muscle crush injury

Right hind limbs were shaved, and using special system a force of 0.727 Ns/cm^2 was delivered to a maximum diameter of gastrocnemius muscle complex 2 cm proximal to the insertion of the Achilles tendon (12). The therapy was applied intraperitoneally immediately after injury and once a day until one day before sacrificing on 1st, 4th, 7th and 14th day after trauma.

2. Quadriceps muscle transection

The right quadriceps muscle was isolated and transected 1 cm proximal to patella. The therapy was applied intraperitoneally 30 minutes after injury and once a day until one day before sacrificing on 4th, 14th, 21st and 28th day after trauma (11).

3. Achilles tendon transaction

Skin incision in the length of 3 cm was performed above the right Achilles tendon which was transected 0.5 cm proximal to calcaneus's insertion. After transection, only skin was sutured. The therapy was applied intraperitoneally 30 min after surgery and once a day until one day before sacrificing on 1st, 4th, 7th, 10th and 14th day after transection (10).

Pathohistological analysis

After sacrificing the respective tissue was dissected, fixed in buffered formalin (pH 7.4) for 24 hours and embedded in paraffin using standard procedures. For pathohistological evaluation transversal sections of crushed muscle, longitudinal section of transected muscle and longitudinal section of transected tendon were used. Tissue samples were cut semiserially, stained with hematoxylin and eosin, or immunohistochemically for FVIII, CD34 and VEGF (Dako, Glostrup, Denmark) using Dako Autostainer and manufacturer's protocols. Vascular elements on hematoxylin and eosin stained slides as well as immunohistocemically positive elements were examined in a blinded fashion using hot-spot assessment and ISSA program (VAMSTEC, Zagreb, Croatia). From the area of maximal tissue damage detected using semiserial sections, five high power fields were randomly selected for analysis.

Stastistical analysis

For analysis of distribution normality *in vivo* and *in vitro* acquired data Kolmogorov-Smirnov test was used. Since no group had normal distribution Mann-Whitney U test was applied. Statistical analysis was performed using SPSS 11.5 for Windows and all values of $p<0.05$ were considered statistically significant.

RESULTS

Cell culture

The number of branching points of newly formed tubular structures was without significant difference ($p>0.05$) between cell cultures treated with pure medium or with BPC 157 in both concentrations (10 ng/ml and 2 ng/ml). As expected, VEGF as positive control had proangiogenic effect, as opposed to negative control Suramin which practically completely inhibited branching (*Fig. 1*).

Animal models

1. Muscle crush

After crush injury (*Fig. 2*) in control animals angiogenic response with all tested parameters (number of VEGF, CD34 and FVIII positive elements (data obtained on hematoxylin-eosin stain were equal to FVIII presentation and are not presented)) consistently reached its peak at day 7. Increase in CD34 and VEGF positive elements was obvious since 2 hours, while FVIII showed

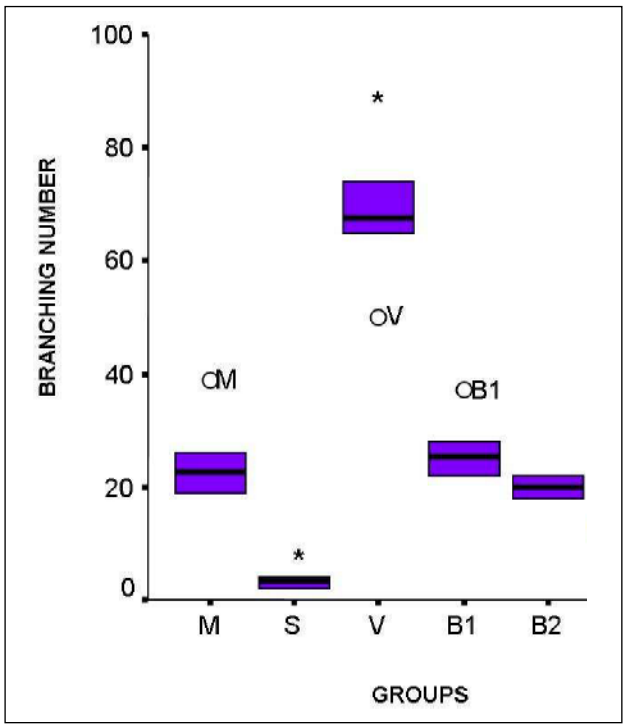

Fig. 1. The number of branching tubules in cell cultures demonstrated the absence of positive and negative angiogenic effect of BPC 157 in *in vitro* conditions.
M-pure medium; S-Suramin (negative control); V-VEGF (positive control); B1-BPC 157 10 µg/ml; B2-BPC 157 2 µg/ml; *- statistically significant difference ($p<0.05$)

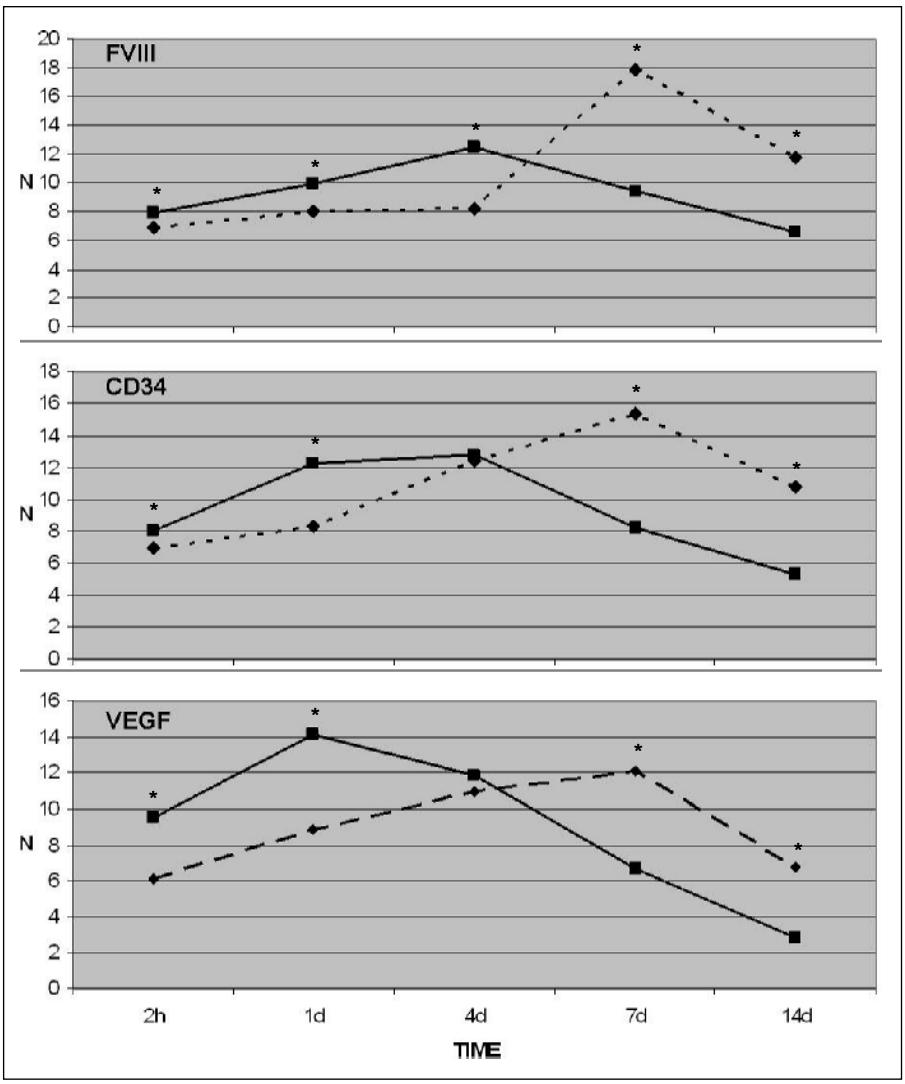

Fig. 2. Results of immunohistochemical analysis after muscle crush injury, demonstrating positive angiomodulatory effect of BPC 157.
Legend: N- number of positive elements, full line- BPC 157 treated animals; broken line- controls; time-time after injury; *- statistically significant difference ($p<0.05$).

an increase after day 4. On the other hand, angiogenic response after BPC 157 application showed a shift toward left, implying at the early interval (2h-4th day) increased blood vessels formation (VEGF>CD34>FVIII) with gradual decrease in later period (4th - 14th day). VEGF reached its peak the 1st day, as the earliest breakpoint, while CD34 and FVIII reached their peaks at 4th day.

2. Muscle transection

In concordance with the severity of injury (complete transection) we should note that angiogenic response was generally higher in both groups than after crush injury (measured with the number of VEGF, CD34 and FVIII positive elements), but without a consistent peak (*Fig. 3*). Interestingly, curves in both groups are of the same shape, but with the statistically significant difference- higher numbers of positive vascular elements in the BPC 157 treated group. The angiogenic activity in both groups was obviously prolonged. The highest activity in this model demonstrated CD34 positive elements with earliest peak at 4th day, followed by VEGF (peak at 14th day) and FVIII (peak at 21 day).

3. Transected Achilles tendon

After tendon transection (*Fig. 4*), in both groups, angiogenic response demonstrated with all tested parameters (the number of VEGF, CD34 and FVIII positive elements) consistently reached its peak already at day 4. In control group the numbers in the first time periods remained within relatively low values, but toward the end reaching higher values than BPC 157 animals. The number of FVIII positive elements showed a second peak only in control animals, retaining this number of vascular structures till the end. BPC 157 treated animals showed pronounced decrease in all parameters after their peak, resulting in low number of positive vascular elements at the end of investigated period.

DISCUSSION

This study evaluated the angiogenic potential of BPC 157. In early post-injury periods, BPC 157 therapy induced a prominent increase of angiogenesis in rats with transected Achilles tendon or quadriceps muscle and in rats with crushed muscle. This was consistently visualized with different endothelial cell antigens, FVIII (involved in platelet adhesion and aggregation, present on endothelial cells of mature blood vessels) and CD34 (involved in leukocyte adhesion and endothelial cell migration during angiogenesis, present on capillary endothelial cells), as well as with VEGF presentation (main factor in angiogenesis, expressed on endothelial cells, mitogen for vascular endothelial cells). Generally, BPC 157 increased the number of VEGF, CD34 and FVIII positive vascular elements, and angiogenic response was

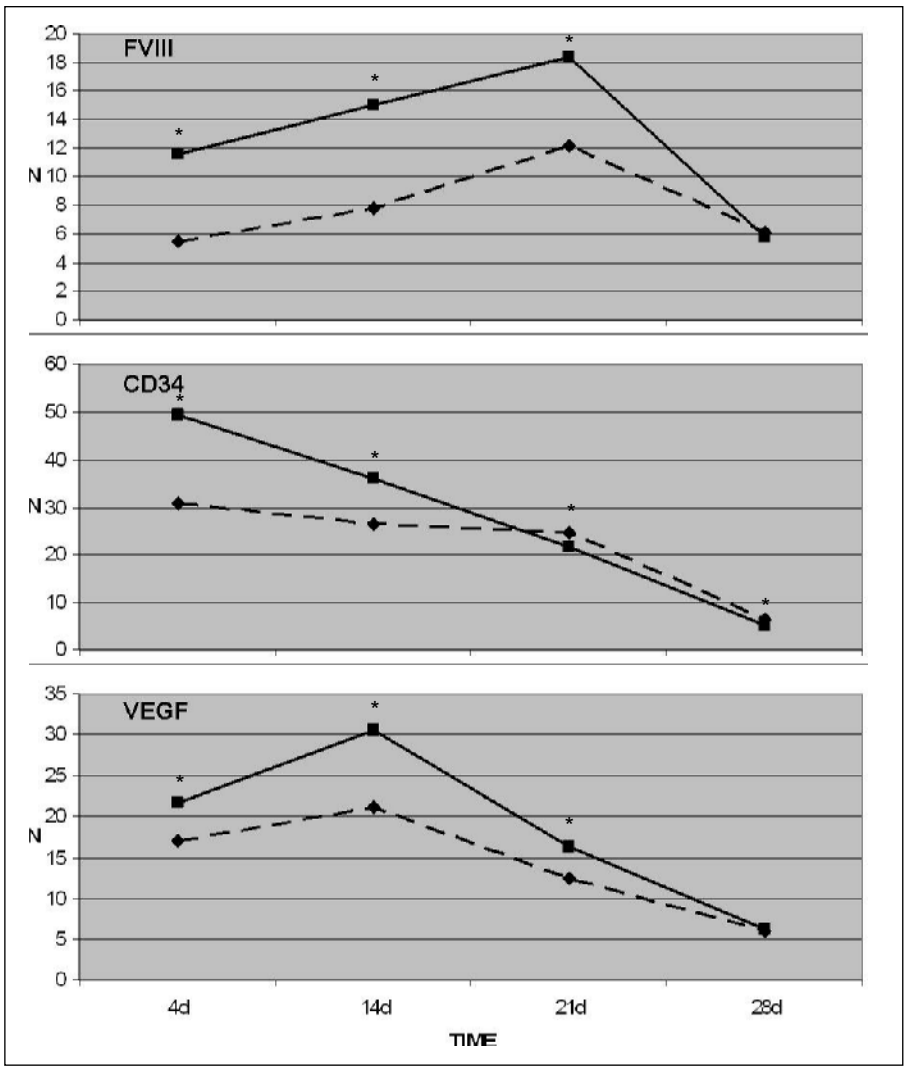

Fig. 3. Results of immunohistochemical analysis after muscle transection, demonstrating positive angiomodulatory effect of BPC 157.
Legend: N- number of positive elements, full line- BPC 157 treated animals; broken line- controls; time- time after transection; *- statistically significant difference ($p<0.05$).

regularly augmented and shifted toward the left. On the other hand, results obtained in *in vitro* conditions, using human endothelial cells, showed that there is no direct angiogenic effect of BPC 157 on cell cultures. The angiogenic potential of BPC 157 seems to be closely related to the healing process *in vivo* with BPC 157 stimulating angiogenesis by up-regulating VEGF expression.

In general, angiogenesis, the formation of new vessels from pre-existing ones, consists of an orderly sequence of events triggered by growth factors secreted from the surrounding hypoxic tissue (19). It is a complex process controlled by angiogenic and angiostatic molecules resulting in ideal revascularization of the wound bed, and providing oxygen, nutrients, and inflammatory cells to the newly growing/regenerating as well as tumor tissue (20, 21). Conditions less than ideal (*e.g.* hypoxia, wound fragments misadaptation, infection) tend to hamper this process resulting in scar formation or delayed healing, and probably induce VEGF formation by more than one mechanism (22). Our study demonstrated in all investigated models of tendon and muscle injury that BPC 157 induces higher VEGF and CD34 positivity, preceding the increase in actual number of blood vessels as demonstrated on HE and FVIII stains. Such a particular activity in angiogenesis and healing is concordant with the previous evidence that BPC 157 may directly protect endothelium (13), influence NO-system, counteract the effect of NOS-inhibitor and NO-precursor (14, 15), as well as over expression of endothelin (16). Beside the stimulation of expression of the EGR-1 gene, BPC 157 also stimulated expression of nab2 (17). Coordinated regulation of this transcription factor and its repressor suggests that this system may play a role in maintaining vascular homeostasis. It is possible that BPC 157 - nab2 interaction is part of a feedback mechanism which serves to regulate EGR-1-mediated gene transcription. The BPC 157 effects on rats with crushed muscle, transected muscle and tendon suggest appropriate angiogenic response that results in better healing (10-12). We assessed the angiogenic response in connection to different extent of tissue damage (muscle transection *vs*. muscle crush) and different tissue healing capacity (muscle *vs*. tendon). The detrimental consequence of muscle transection outweighs crush injury, and after quadriceps muscle transection more VEGF and CD34 positive elements were present than after blunt trauma. Consistently, after Achilles tendon transection the same parameters remained far below the values noted after muscle transection.

However, after either muscle or tendon had been completely transected, the commonly negligible tissue repair clearly shows that without therapy the described angiogenic response may still be inadequate. In tendon transection model control animals had two peaks of FVIII positivity, announced by the higher number of CD34 and VEGF positive elements on the seventh day, in comparison to the BPC 157 animals. As previously published, there is no longer visible detachment between Achilles tendon

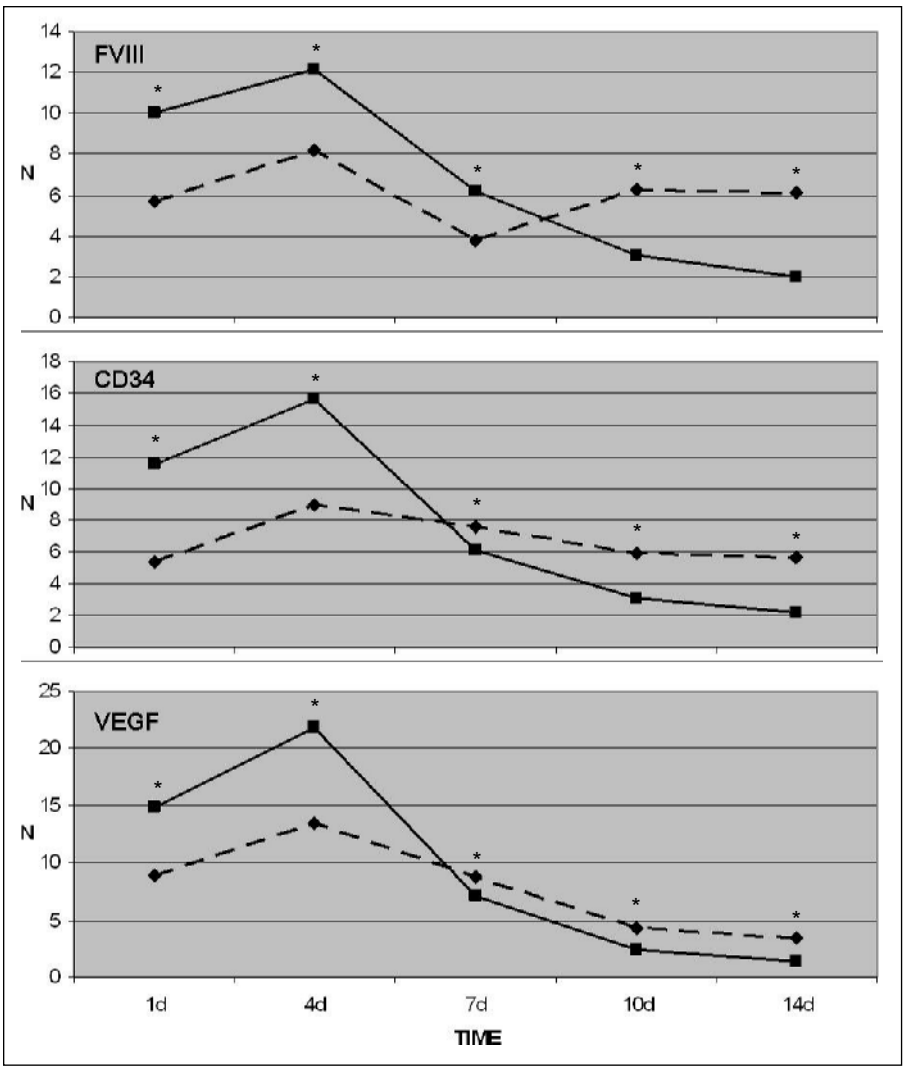

Fig. 4. Results of immunohistochemical analysis after Achilles tendon transection, demonstrating positive angiomodulatory effect of BPC 157.
Legend: N- number of positive elements, full line- BPC 157 treated animals; broken line- controls; time- time after transection; *- statistically significant difference ($p<0.05$).

ends in BPC animals after seventh day, while it disappears only after tenth day in control group (10). This space is filled with granulation tissue with active angiogenic process. Although angiogenesis is very important for tendon healing, prolonged angiogenesis may result in prolonged/impaired healing and leads to chronic tendon disease. Thus, regardless of particularities in angiogenic response(s), different tissues and different healing stage, a more generalized and more powerful angiogenic response induced by BPC 157, with adequately improved angiogenesis leads consistently to better healing conditions and thereby eventually to more adequate healing.

It is important to stress that the positive influence of BPC 157 on healing can not be attributed to angiogenesis alone, and that one should bear in mind its effects on inflammatory reaction where it decreases leukotriene B4, tromboxan B2 and myeloperoxidase concentration in injured tissues (23). Tkalcevic *et al*. have recently demonstrated a better modulatory effect on granulation tissue in excisional wounds in genetically modified diabetic mice db/db in comparison to PDGF-BB (which is the only approved medication for diabetic ulcers treatment) (17). Interestingly, PDGF in wounds directly induces VEGF-A mRNA (24). Tkalcevic *et al*. also showed that in *in vitro* conditions BPC 157 stimulates mRNA EGR-1 (17) having the same effect as VEGF in cell cultures (25, 26). Interactions of BPC 157 with the same growth factors and cytokines are under investigation.

In conclusion, we have demonstrated that, although BPC 157 doesn't have any direct angiogenic effect in cell culture, it has a positive angiomodulatory effect in animal models of muscle and tendon healing, resulting in faster and better healing, which could be helpful in further therapy development.

Conflict of interests: None declared.

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