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Identification of Small Peptides of Acidic Collagen Extracts from Silver Carp Skin and Their Therapeutic Relevance

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. Low-temperature techniques that prevent protein denaturation are being used to extract collagen from fish skin for cosmetic purposes. These extracts contain collagen with its triple helix structure preserved, as well as a number of other proteins.
Objectives. The aim of the study was to investigate collagen extracts from the skin of silver carp for the presence of small-molecule peptides.
Material and Methods. Liquid chromatography-mass spectrometry (HPLC-MS) was performed to analyze collagen extracts from silver carp skin for the presence of small-molecule peptides.
Results. A large number of different peptides were detected in the silver carp skin collagen extracts analyzed. Among the smaller peptides, the most abundant were those of 7–29 aminoacids originating from the following proteins: collagen Iα1, collagen Iα2, collagen Iα3, collagen VIα3, decorin, lumican, histone H2A, histone H2B and histone H4.
Conclusions. The study demonstrated that, in addition to high-molecular-weight collagen proteins, acidic collagen extracts acquired from the skin of silver carp at temperatures up to 16°C also contain considerable amounts of small 7–29 amino-acid peptides. The application of these peptides could therefore be expected to result in beneficial clinical effects in patients in need of reconstructive treatment (**Adv Clin Exp Med 2016, 25, 2, 227–235**).

Key words: collagen, histones, matrikines, HPLC-MS/MS, silver carp skin peptides.

For a few years now, low-temperature techniques that prevent protein denaturation has been used to extract collagen from fish skin for cosmetic purposes. These extracts contain collagen with its triple helix structure preserved, as well as a number of other proteins. An interesting question concerning composition of those extracts is the presence of small-molecule peptides.

Preparations of collagen are known to have a stimulatory influence on some cellular and tissue-specific processes – effects that are caused only by active compounds of low molecular weight, which are capable of penetrating into the skin and deeper tissues [1]. Reports [2–6] on the beneficial effects of collagen-containing products on wound healing were confirmed in the authors' pilot study on five patients with varicose leg ulcers from the Plastic Surgery Department at the Medical University of Gdańsk (Poland) using collagen extracted from the skin of silver carp [7]. These ulcers are wounds that are very difficult to heal. A few effects were seen: wound closure, accelerated granulation, anti-inflammatory effect, decreased evaporation of water from the wound. The collagen gel also protected the wound edges from the effects of toxic substances produced by the infected wound (proteolytic enzymes, etc.) [7].

Figure 1 shows examples of the treatment effects of collagen from the skin of silver carp – the subject of present article – on difficult-to-heal wounds in leg ulceration. The ulceration remained

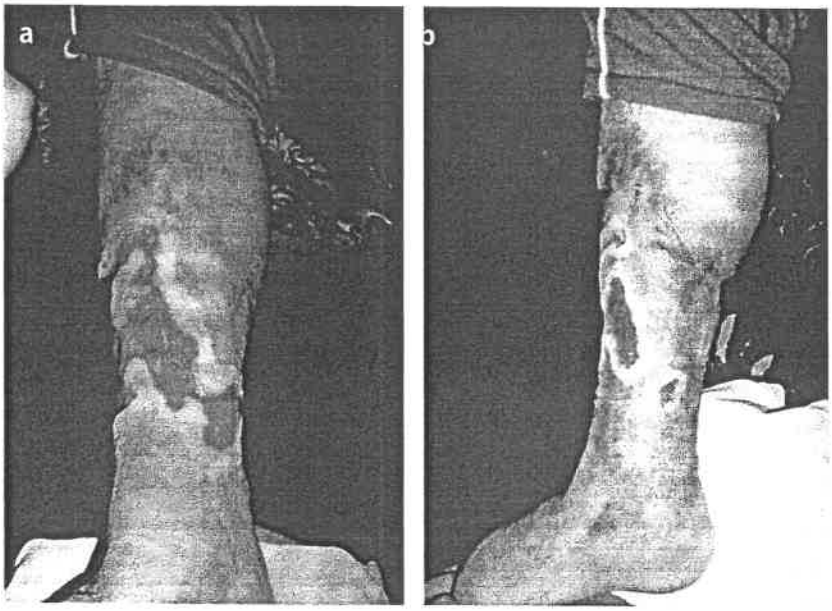

Fig. 1. Leg ulceration with the use of collagen from the skin of silver carp, a: before, b: after 3 months of collagen gel application directly to the wound twice a day [7]

unhealed for one year when other methods were used, but significant effects were achieved after three months of collagen gel applied directly to the wound twice a day.

There are also a few reports that confirm the biological activity not only of collagen extracts derived at low-temperatures without denaturation, but also of gelatin [8, 4]. Clinical studies suggest that the ingestion of 10 g daily of pharmaceutical-grade collagen hydrolysate obtained by hydrolysis of pharmaceutical gelatin reduces pain in patients with osteoarthritis of the knee or hip [8]. The first known description of the beneficial effects of gelatin ingestion in humans is from 1175, when St. Hildegard wrote that eating gelatin improved joint conditions by reducing pain [8]. In cases of individuals affected with osteoporosis, studies of the effects of calcitonin with and without a collagen hydrolysate-rich diet suggested that calcitonin plus collagen hydrolysate more effectively inhibited bone collagen breakdown than calcitonin alone [8]. Ausar et al. found that "orally administered bovine tracheal type-II collagen in the treatment of rheumatoid arthritis... induced clinical benefits in 90% of the patients" [9]. Oral administration of collagen peptide increased bone mineral density in rats and mice [10–12]. Other researchers have found that oral administration of type-II collagen decreases autoimmune response in rheumatoid arthritis and reduces joint inflammation in mice [13, 14]. The influence of collagen hydrolysate intake (including fish collagen hydrolysate) on skin has been demonstrated [15].

A few years ago, the term matrikines was proposed [16] for peptides liberated in the process of partial proteolysis of extracellular matrix macromolecules and capable of regulating cell functions. Many proteins of the intercellular matrix contain matrikines, which influence such phenomena as wound healing, malignant transformation and the atherosclerotic process. Some matrikines exert an inhibitory influence on these processes, while others augment them. Examples of source proteins for known matrikines, along with their stimulatory (+) and inhibitory (–) effects on malignant transformation and growth, are presented in Table 1.

As illustrated in Table 1, the effects of matrikines are selective. Also, the same proteins may be the source of both stimulatory and inhibitory matrikines in certain proportions. Therefore, it is important to bear in mind that extracts from the intercellular matrix may have either a beneficial or a detrimental effect with respect to malignant transformation or other processes, depending on the method of preparation of the extract.

The small leucine-rich proteoglycans decorin, lumican, biglycan and fibromodulin are secreted extracellular matrix molecules that associate with fibrillar collagens and regulate collagen fibrillogenesis [17]. A study on ligament proteoglycans yielded the following results: Proteoglycans represent less than 3% of the dry weight of ligaments, which is consistent with the levels found in other dense fibrous connective tissues; approximately 90% of the total proteoglycans in fresh ligament was decorin; and approximately 23% of the decorin detected in the matrix was degraded [18]. Intact decorin and decorin fragments similar to those observed in the matrix were also found in the medium of ligament cultures; similarly, with versican, biglycan and aggrecan, the co-appearance of intact proteoglycans and of large fragments was observed; also,

Table 1. The origins of known matrikines: Their source proteins and effects on malignant transformation and growth

Matrikines originating from	Tumor progression	Tumor proliferation	Tumor angiogenesis	Tumor metastasis
Collagen IV	–	0/–	–	+/–
Collagen VIII		–	–	
CollagenXV		0	–	–
CollagenXVIII	–	–	–	–
Elastin	+/–	–	+	+
Fibronectin	+/–	0/–	–	–
Laminins				+/–
Perlecan		+	+/–	
Thrombospondin -1	–		–	
Collagen Iα2			+	
Decorin		–	–	–
Lumican				–

(+) – activation of malignant transformation and growth; (–) – inhibition of malignant transformation and growth; (+/–) – activation or inhibition, different effects for different fragments of the source protein; (0) – no effect of a particular fragment on a given process, with concomitant activity towards another malignancy-related process [51–60].

type XII collagen appeared in both intact and degraded forms [11]. In the case of decorin, an electrophoretic technique utilizing antibodies applied to the ligament of 1- to 2-year-old steers revealed after-deglycosylation; in addition to full-length protein 43 kDa, smaller fragments of 32, 30, 21, 18 and 13 kDa were also present, in relative amounts ranging from 2.5% do 10% of the total amount of the full-length protein [18].

This report focuses on decorin, lumican and histone peptides which were detected in the extracts, as these may be particularly important for the therapeutic properties of crude collagen extracted in low-temperature conditions.

Material and Methods

Collagen Extracts

Collagen extracts were prepared as follows: The skin of silver carp (*Hypophthalmichthys molitrix*) was dissected from fat and muscle tissues, and 40 g/L of the skin was immersed in 1% solution of lactic acid for 24 h at 16°C. The gel obtained was filtered through a silk cloth filter to assure the homogeneity of the extract. For the analysis of native small peptides, the extracts were diluted 1 : 10 with 1% solution of lactic acid or acetonitrile and subjected to ultrafiltration through a filter with a molecular weight cut-off of 5 kD. Analyses were then performed for each of the two extracts.

Mass Spectrometry

The HPLC-MS/MS analysis was performed at the Laboratory of Mass Spectrometry at the Polish Academy of Sciences in Warsaw. Peptides were separated by nanoscale reverse phase high-performance liquid chromatography followed by electrospray ionization. Tandem mass spectra were obtained on a LTQ FT mass spectrometer and the ion generator used was a Finnigan Nanospray (both from Thermo Finnigan, Ringoes, NJ, USA). The separation conditions on the C18 nanocolumn were: acetonitrile gradient 0–40% in 0.05% solution of formic acid; separation time 60 min (division into approx. 2000 fractions).

A preliminary interpretation of the results was done with Mascot software (Matrix Science Inc, Boston, MA, USA). Only results with Mascot scores for proteins above 30 were included in the analysis; protein scores are related to the consistency of the measured masses of protein fragments with those obtained with theoretical calculations for a given mass, and therefore only indirectly related to the magnitude of the signal. All the results fell within the range 0–130.

Due to the lack of direct information on the ionic currents from fragments of different peptides, a scoring system was adopted in this study, in which the total numbers of occurrences of particular sequences were calculated in the measurements of longer or shorter peptides – that is, the sum of repetitions of the same peptide in a num-

ber of adjacent chromatographic fractions representing a wider peak was calculated, as well as occurrences of different peptides representing the same protein fragment, but scattered over a period of time in the chromatographic assessment. Additionally, points for samples of a given extract filtered in acetonitrile and lactic acid were added up.

Results

HPLC-MS/MS Analysis

A large number of different peptides were detected in the analyzed silver carp skin collagen extracts. Among the smaller peptides, the most abundant were those of 7–29 aminoacids, originating from the following proteins: collagen Iα1, collagen Iα2, collagen Iα3, collagen VIα3, decorin, lumican, histone H2A, histone H2B and histone H4.

Table 2 presents the results of the assessment of silver carp skin preparations. The extracts were prepared from skin samples obtained from fish originating from three different fish cultures in Hungary and collected in different seasons of the year. Four different extracts from the skin preparations were investigated. One of them was pale and transparent (marked as A). Two of them contained some quantities of melanin, which resulted in their having a dark tint (marked as B and C). The fourth sample (D) was degraded collagen obtained from extract A (a preparation additionally kept at 40°C for a period of 48 h was marked as Adeg for degraded.). Most of the proteins in preparation D were degraded by proteolysis. The degradation was revealed by SDS-polyacrylamide electrophoresis; the data is not presented.

Next to the peptide sequence the score is given reflecting the estimated quantitative differences in peptide content (italic characters were used for peptides found in the degraded sample only). The sequences presented in Fig. 2 represent the places within the proteins identified as the sites of origin of some families of shorter peptides.

Similar results demonstrating the presence of decorin, lumican and histones peptides were obtained in a series of verifying measurements with a slightly modified protocol (data not included).

HPLC/UV-VIS Analysis

Separate measurements (data not included) of the content in the filtrates of substances absorbing at 280 nm, performed with use of an RP 18 analytical column (*i.e.*, of markedly lower resolution) under separation conditions similar to those in the HPLC-MS/MS method, revealed the presence of about 40 highly distinct absorption peaks.

For extracts A, B and C there were over 300% differences in peak magnitudes for 12 peaks. Analogous comparisons done for separately prepared skin extracts from ventral and melanin-containing dorsal parts of the same fish revealed a difference in peak magnitude of over 300% for one peak only. These results show, therefore, that differences in peptide content between extracts A, B and C are not related to fish skin melanin content and the resulting degree of protection against UV, but rather to other environmental factors, fish age or genetic differences.

Discussion

The authors subscribe to the hypothesis that the biological activity of collagen extracts depends on the content of biologically active peptides be-

Decorin (*Danio rerio*)

```
  1 MKSACLSLLL VSVCWALPFR QSGFMDFVME DEPASGDGPG PELPTTRKPH
 51 VERLPMMPEG PEVPFCPFRC QCHLRVAQCS DLGLKTVPEK IPLDTTLLDL
101 QNNKITEIKE NDFKGLKGLQ TLILVNNKIT IIHAKAFSSL INLERLYLSK
151 NLLKEVPANI PKSLQELRIH ENQINKIKKS SFAGMANVIV MELGSNPLSS
201 SGVDNGAFAD LKRVSYIRIA DTNLTSIPKG LPSSLFELHL DGNKITKVTA
251 DSLKGLKNLS KLGLSHNEIS VVENGSLANV PHLRELHLEN NALTAVPAGL
301 ADHKYIQVIY LHSNKIAAVG TEDFCPPGYN TKKAMYSGIS LFSNPVPYWE
351 VQPITFRCVF DRSAIQLGNY RKK
```

Protein similar to vertebrate lumican (*Danio rerio*)

```
  1 FLSRVLGPHT TVPEEREAGR FNLSCAAMFA LGSILLAGLL SLSLAQYDYY
 51 DEYYIPSAPL EGVSSPSCAQ ECECPINFPT AMYCNERNLK FIPIVPTGIK
101 YLYLQNNFIE EIKAGVFDNA TDLRWLVLDN NNITSDKIQA GTIDKLGSLE
151 KLLFSHNKLT KPPGSLSKSL DELKLIGNKL TSFPANTLAG MENLTTVHLS
201 KNKLTTESLT GAFKGLKSLI LLDVSENKLK KLPSGVPASL LMLYADNNDI
251 DSIPNGYLAK LPLLQYLRIS HNKLVDSGVP AGVFNVSSLL ELDLSFNKLK
301 TIPEINESLE HLYLQVNEIN KFELTNICRF SSPVNYSRLR TLRLDGNNIT
351 HSSMPDDTAN CLRQASEIIF E
```

Fig. 2. The distribution of peptides found in two example protein sequences

Table 2. The most abundant peptides from the skin of silver carp

Protein	Found peptide	A	B	C	A deg
Collagen Iα1 (*Danio rerio, Oncorhynchus mykiss*)	FIAQPQEKAPDPFRHFRA LKMCHPDWKSGEYWIDPDQGCNQD IAQPAQEKAPDPFRHF SGLPGPIGPPGPRGRSGEMGP GMPGERGAAGLPGLKGD	15 1 1	18 1	4 1	
Collagen Iα2 (*Danio rerio*)	TSGGYDEYR LRGHPGLQGMPGPNGPSGDSGPAGI MPGPNGPSGDSGAAGIAGPSGPRGPAGPN *PGPVGVKGDSGVKGE*		1 1 1	1	 3
Collagen Iα3 (*Danio rerio*)	GPDPLRGGY FPGPKGT *PGLQGPKGD* *AGKEGQRGARGEKGPAGRPGEAG* *GKTGDRGEAGPAGPAGPSGPAGARGALGPA*		4 1	 1	3 4 8 1
Collagen IVα1 (*Danio rerio*)	*PGLQGIKGD* *PGIPGTKGD* *PKGDRGDQGQPGERGATGEQGPPGIP*			 1	2 2
Protein homologous with collagen VIα3 (*Tetraodon nigroviridis*) (six repetitions in the gene of the sequence: LLDGSDGTRSGFPAMRDF)	LLDGSDGTRSGFPAMRDF PRGKDVVFLLDGSDGTRSGFPAMRDF DRVSVVQYSRD RGGAPVRTGAALQYVRD		16	8 4 1	
Protein similar to collagen VII (*Danio rerio*)	*GEQGEKGPAGPQGPTGRAIGERGPEGP*			1	
Procollagen VIIIα2 (*Mus musculus*)	*GPRGDRGLKGD*				1
Decorin (*Danio rerio*)	ELGSNPLSSSGVDNGAFADLKRVSYIR FSNPVPYWEVQPIT HLDGNKITKVTAD ILVNNKITIIHAKAFSSLINL	 1	3 3 2 2	5 1 2 3	
Protein similar to *vertebrate lumican* (*Danio rerio*)	DLSFNKLKTIPEINESLEHL LDVSENKLKKLPSGVPASLLML	6 6	13 3	16 7	
Histone H2A (*Danio rerio*)	AVLLPKKTEKPAKS ILELAGNAARDNKKTR AVRNDEELNKLLGGVTIAQGGVLPNIQA	6	7 1	10 1 1	
Histone H2B (*Rattus norvegicus*)	SSTAAVLAQRLVPEYNMPEPTKSVPAPK KVLKQVHPDTGISSKAMGIMNS		1 1		
Histone H4 (*Mus musculus*)	YTEHAKRKTVTAMD			2	

longing to the four groups described above. In the extracts obtained in this study a large number of peptides – several hundred – originated from protein cleavage. The most abundant were decorin, lumican, histone 2A and four types of collagen. Reports from other studies confirm that, apart from their well-explored structural function, all four types of proteins, in the form of both intact molecules and smaller peptide derivatives, exhibit properties of signaling and protective molecules related to tissue repair processes.

The results obtained in this study demonstrate a certain discrepancy in the peptide composition of collagen extracts from skin containing different quantities of melanin. The degraded specimen was not found to contain decorin, lumican and his-

tone peptides, although it should be admitted that, considering the greater amount of peptides originating from collagen breakdown in the degraded specimen as compared with non-degraded extracts (Mascot score 100–130 vs. 30–70, respectively), the decorin, lumican and histone peptide signals could have been outweighed by signals for collagen peptides – in this method only the peptide with the strongest signal is selected from each chromatographic fraction by the software analysis system.

As for collagen type VI alpha 3, among the obtained peptides there were some containing six repetitions of the sequence LLDGSDGTRSGFPAMRDF found in this protein, representing classic examples of a matrikine cleaved from the protein in multiples.

This investigation of collagen extracts from silver carp skin demonstrated the presence of large dominant amounts of decorin, lumican and histone peptides, along with a number of single or low-quantity peptides from different types of collagen. Considering the total length of the particular proteins, decorin, lumican and histone peptides accounted for an even greater proportion of those proteins. Although the method of mass spectrometry used in this study does not have the capacity to reveal the presence of all the peptides present due to major sequence-related differences in the degree of their ionization, and also because of certain limitations resulting from the method of chromatographic separation, and therefore all quantitative estimations without an internal standard must be taken as rough approximations, the very fact of the presence of large quantities of decorin, lumican and histone peptides in the analyzed extracts can be recognized as an indicator of their important biological role for homeostasis in the fish skin. The amino acid sequences for the peptides that were detected in the skin of silver carp were largely inconsistent with those described in other reports. There were only two peptides for which a partial overlap was found between the sequences detected in the present study and those reported by other researchers.

Using decorin, lumican and histone for treatment and prevention seems to be a promising perspective. Therefore, the authors of the current study investigated available references and collected information on their effects, which may prove helpful in the course of further clinical investigations. The significant properties of these three proteins and their smaller derivatives are summarized below.

Decorin is a naturally occurring antagonist of scar formation; it promotes adult sensory neuron axon growth across spinal cord scar tissue [19, 20]. Direct infusion of human recombinant decorin into acute stab injuries of an adult rat spinal cord resulted in major reductions in astrogliosis, macrophage accumulation and deposition of the axon growth inhibitors neurocan, brevican and phosphacan within the spinal cord scar tissue [19]. Decorin has been successfully employed to reduce tissue fibrosis in different disease models in the kidney, lung, and vascular structures. The antifibrotic properties of decorin have been confirmed in a mouse model of pulmonary fibrosis, in which fibrosis was induced by transient overexpression of active TGF-β using adenoviral gene transfer and followed by 21-day overexpresion of decorin, also by adenoviral vector [21]. Decorin is required for the proper fibrotic evolution of myocardial infarction [22] and plays a role in limiting the inflammation process [23]. It is also a natural inhibitor of fibroblast proliferation, its main function probably being to repress the action of transforming growth factor-β (TGF-β), which is involved in wound healing [24–26]. Decorin is a natural anticancer agent; it significantly suppresses the growth of rat breast carcinoma cells and could also inhibit metastases to the lungs [27]. Decorin induces both *in vitro* and *in vivo* tumor cell apoptosis [28]. The antitumoral effect of decorin is mediated inter alia by epidermal growth factor receptor EGFR and TGF-β [27, 29, 30]. Decorin also suppresses tumor cell-mediated angiogenesis both *in vitro* and *in vivo* [31]. As Sulochana et al. wrote, "not only purified decorin but also the 26-residue leucine-rich repeat 5 of decorin core protein functions as angiogenesis inhibitor by inhibiting both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-induced angiogenesis" [32, 33].

As Chakravarti et al. wrote: "Lumican is a major constituent of the corneal stroma, where it plays a significant role in the acquisition of corneal transparency by regulating collagen fibril diameter and interfibrillar spacing" [34]. It is also present in the dermal extracellular matrix of the skin [34]. Lumican deficiency leads to increased cell proliferation, down-regulation of p53, down-regulation of the CDK inhibitor p21 and increased expression of G1-S cyclins; lumican also plays an important role in Fas-mediated regulation of apoptosis. Lumican-deficient mice show decreased apoptosis of stromal keratocytes [35]. Lumican inhibits melanoma progression. After subcutaneous injections of transfected B16F1 melanoma cells in syngenic mice, lumican expression significantly decreased subcutaneous tumor formation *in vivo* [36]. Lumican core protein expression in growing tumors inhibited the expression of cyclin D1, which is a major cyclin controlling Cdk activation and the regulation of cell cycle progression. These results

suggest that the mechanism of action of lumican on melanoma cells is different from that of decorin [36]. Lumican, as well as leucine-rich repeat peptide from human lumican, inhibits melanoma cell migration [37, 38].

Peptides containing 20–50 amino acids originating from the N-terminal tail of histone H2A possess antimicrobial activity. Examples of such peptides are hipposin from the skin mucus of Atlantic halibut [39, 40], buforin I [41] and buforin II [42], isolated from the stomach tissue of *Bufo bufo gargarizans* (an Asian toad), and parasin from the mucous layer of wounded catfish skin [43, 44]. Parasin I is generated from histone H2A in the skin mucus of catfish by the action of cathepsin D activated by a procathepsin D processing enzyme – matrix metalloproteinase 2 (MMP2) – induced upon epidermal injury [45, 46]. Intact histone H2B possesses antimicrobial and antifungal activity in catfish, rainbow trout and sunshine bass [47]. Histone H2B is a functional polypeptide of the antimicrobial defense in human colon mucosa. It has also been suggested that histones H2A and H2B participate in the host defense of the fetus by being produced in the placenta and then secreted into the amniotic fluid [48]. They also possess hormone-releasing activity [49]. Buforin I also possesses anticoagulant activity [50]. Bone marrow regeneration is associated with a marked increase in the serum levels of a 14-amino acid osteogenic peptide identical to the C-terminus of histone H4 [49].

Given the fact that investigations on matrikine peptides are still not very advanced, the potential role of the peptides contained in fish skin collagen extracts, as well as in other collagen preparations, in such important functions and reactions as regulation of proliferation, angiogenesis, metastasis, apoptosis, wound healing, fibrosis, immunological response and hormonal response deserves serious attention and seems to cast some light on why collagen-containing and collagen-derived products have become so popular over the last few years.

An issue of special interest is the possibility of using fish skin peptides in the form of preparations for oral use. The capacity of peptides to penetrate into tissues is the reason they outperform proteins with the same regulatory actions in practical medical applications. Moreover, it is known that peptides may be absorbed from the digestive tract, which makes them easy to use orally. Although it is frequently stated that proteins such as gelatin taken in oral form are enzymatically digested to their amino acid components in the intestinal tract, gelatin peptides are only digested to a certain degree within the gastrointestinal tract, with a proportion of intact high-molecular-weight proteins reaching the serum subsequent to passing through the intestinal wall at a level of approximately 10% [8].

References

[1] **Benson HA, Namjoshi S:** Proteins and peptides: Strategies for delivery to and across the skin. J Pharm Sci 2008, 97, 3591–3610.

[2] **Burget A, Nathan P, Holder IP, Macmillan BG:** The effect of a collagen dressing on contaminated surgical wounds in rats. Langenbecks Arch Chir 1976, 343, 69–73.

[3] **Purna SK, Babu M:** Collagen based dressing – a review. Burns 2000, 26, 54–62.

[4] **Fleck CA, Simman R:** Modern collagen wound dressing: function and purpose. J Am Coll Certif Wound Spec 2010, 2, 50–54.

[5] **Bello TR:** Practical treatment of body and open leg wounds of horses with bovine collagen, bisynthetic wound dressing and cyanoacrylate. J Equine Vet Sci 2002, 22, 157–164.

[6] **Elgharably H, Ganesh K, Dickerson J, Khanna S, Abas M, Ghatak PD, Dixit S, Bergdall V, Roy S, Sen CK:** A modified collagen gel dressing promotes angiogenesis in a preclinical swine model of chronic ischemic wounds. Wound Repair Regen 2014, 22, 720–729.

[7] **Frydrychowski AF:** Pilot study of collagen gel wound treatment on patients with varicose leg ulcers (results not published).

[8] **Moskowitz RW:** Role of collagen hydrolysate in bone and joint disease. Semin Arthritis Rheum 2000, 30, 87–99.

[9] **Ausar SF, Beltramo DM, Castagna LF, Quintana S, Silvera E, Kalayan G, Revigliono M, Landa CA, Bianco ID:** Treatment of rheumatoid arthritis by oral administration of bovine tracheal type II collagen. Rheumatol Int 2001, 20, 138–144.

[10] **Wu J, Fujioka M, Sugimoto K, Mu G, Ishimi Y:** Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. J Bone Miner Metab 2004, 22, 547–553.

[11] **Nomura Y, Oohashi K, Watanabe M, Kasugai S:** Increase in bone mineral density through oral administration of shark gelatin to ovariectomized rats. Nutrition 2005, 21, 1120–1126.

[12] **Guillerminet F, Beaupied H, Fabien-Soule V, Tome D, Benhamou CL, Roux C, Blais A:** Hydrolyzed collagen improves bone metabolism and biomechanical parameters in ovariectomized mice: An *in vitro* and *in vivo* study. Bone 2010, 46, 827–834.

[13] **Park KS, Park MJ, Cho, ML, Kwok SK Ju JH, Ko HJ, Park SH, Kim HY:** Type II collagen oral tolerance; mechanism and role in collagen-induced arthritis and rheumatoid arthritis. Mod Rheumatol 2009, 19, 581–589.

[14] **Ju JH, Cho ML, Jhun JY, Park MJ, Oh HJ, Min SY, Cho YG, Hwang SY, Kwok SK, Seo SH, Yoon CH, Park SH, Kim HY:** Oral administration of type-II collagen suppresses IL-17-associated RANKL expression of CD4+ T cells in collagen-induced arthritis. Immunol Lett 2008, 117, 16–25.
[15] **Zagne V:** A new view concerning the effects of collagen hydrolysate intake on skin properties. Arch Dermatol Res 2008, 300, 479–483.
[16] **Maquart FX, Pasco S, Ramont L, Hornebeck W, Monboisse JC:** An introduction to matrikines: extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. Crit Rev Oncol Hematol 2004, 49, 199–202.
[17] **Matheson S, Larjava H, Hakkinen L:** Distinctive localization and function for lumican, fibromodulin and decorin to regulate collagen fibril organization in periodontal tissues. J Periodontal Res 2005, 40, 312–324.
[18] **Ilic MZ, Carter P, Tyndall A, Dudhia J, Handley CJ:** Proteoglycans and catabolic products of proteoglycans present in ligament. Biochem J 2005, 385, 381–388.
[19] **Davies JE, Tang X, Denning JW, Archibald SJ, Davies SJ:** Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. Eur J Neurosci 2004, 19, 1226–1242.
[20] **Minor K, Tang X, Kahrilas G, Archibald SJ, Davies JE, Davies SJ:** Decorin promotes robust growth on inhibitory CSPGs and myelin *via* a direct effect on neurons. Neurobiol 2008, Dis 32, 88–95.
[21] **Kolb M, Margetts PJ, Sime PJ, Gauldie J:** Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung. Am J Physiol Lung Cell Mol Physiol 2001, 280, 1327–1334.
[22] **Weis SM, Zimmerman SD, Shah M, Covell JW, Omens JH, Ross J, Jr Dalton N, JonesY, Reed CC, Iozzo RV, McCulloch AD:** A role for decorin in the remodeling of myocardial infarction. Matrix Biol 2005, 24, 313–324.
[23] **Xaus J, Comalada M, Cardo M, Valledor AF, Celada A:** Decorin inhibits macrophage colony-stimulating factor proliferation of macrophages and enhances cell survival through induction of p27(Kip1) and p21(Waf1). Blood 2001, 98, 2124–2133.
[24] **Stewart JE, Wheatley DN, Holmes JD, Muir IF:** Purification and identification of a human dermal extract component inhibitory to fibroblast proliferation. Cell Biol Int 2001, 25, 607–612.
[25] **Wheatley DN, Graham E, McMaster RS, Muir IF, Holmes JD, Davies M:** Recovery of the Decorin-Enriched Fraction, Extract (D), From Human Skin: An Accelerated Protocol. J Biomed Biotechnol 2004, 2004, 211–218.
[26] **Zhang Z, Li XJ, Liu Y, Zhang X, Li YY, Xu WS:** Recombinant human decorin inhibits cell proliferation and downregulates TGF-beta1 production in hypertrophic scar fibroblasts. Burns 2007, 33, 634–641.
[27] **Reed CC, Waterhouse A, Kirby S, Kay P, Owens RT, McQuillan DJ, Iozzo RV:** Decorin prevents metastatic spreading of breast cancer. Oncogene 2005, 24, 1104–1110.
[28] **Tralhao JG, Schaefer L, Micegova M, Evaristo C, Schonherr E, Kayal S, Veiga-Fernandes H, Danel C, Iozzo RV, Kresse H, Lemarchand P:** *In vivo* selective and distant killing of cancer cells using adenovirus-mediated decorin gene transfer. FASEB J 2003, 17, 464–466.
[29] **Stander M, Naumann U, Dumitrescu L, Heneka M, Loschmann P, Gulbins E, Dichgans J, Weller M:** Decorin gene transfer-mediated suppression of TGF-beta synthesis abrogates experimental malignant glioma growth *in vivo*. Gene Ther 1998, 5, 1187–1194.
[30] **Santra M, Reed CC, Iozzo RV:** Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. J Biol Chem 2002, 277, 35671–35681.
[31] **Grant DS, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV:** Decorin suppresses tumor cell-mediated angiogenesis. Oncogene 2002, 21, 4765–4777.
[32] **Sulochana KN, Fan H, Jois S, Subramanian V, Sun F, Kini RM, Ge R:** Peptides derived from human decorin leucine-rich repeat 5 inhibit angiogenesis. J Biol Chem 2005, 280, 27935–27948.
[33] **Fan H, Sulochana KN, Chong YS, Ge R:** Decorin derived antiangiogenic peptide LRR5 inhibits endothelial cell migration by interfering with VEGF-stimulated NO release. IJBCB 2008, 40, 2120–2128.
[34] **Chakravarti S, Stallings RL, SundarRaj N, Cornuet PK, Hassell JR:** Primary structure of human lumican (keratan sulfate proteoglycan) and localization of the gene (LUM) to chromosome 12q21.3-q22. Genomics 1995, 27, 481–488.
[35] **Vij N, Roberts L, Joyce S, Chakravarti S:** Lumican suppresses cell proliferation and aids Fas-Fas ligand mediated apoptosis: Implications in the cornea. Exp Eye Res 2004, 78, 957–971.
[36] **Vuillermoz B, Khoruzhenko A, D'Onofrio MF, Ramont L, Venteo L, Perreau C, Antonicelli F, Maquart FX, Wegrowski Y:** The small leucine-rich proteoglycan lumican inhibits melanoma progression. Exp Cell Res 2009, 296, 294–306.
[37] **Zeltz C, Brezillon S, Perreau C, Ramont L, Maquart FX, Wegrowski Y:** Lumcorin: A leucine-rich repeat 9-derived peptide from human lumican inhibiting melanoma cell migration. FEBS Lett 2009, 583, 3027–3032.
[38] **Brezillon S, Radwanska A, Zelts C, Malkowski A, Ploton D, Bobichon H, Perreau C, Malicka-Blaszkiewicz M, Maquart FX, Wegrowski Y:** Lumican core protein inhibits melanoma cell migration *via* alterations of focal adhesion complexes. Cancer Lett 2009, 283, 92–100.
[39] **Birkemo GA, Luders T, Andersen O, Nes IF, Nissen-Meyer J:** Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus L.*). Biochim Biophys Acta 2003, 1646, 207–215.
[40] **Birkemo GA, Mantzilas D, Luders T, Nes IF, Nissen-Meyer J:** Identification and structural analysis of the antimicrobial domain in hipposin, a 51-mer antimicrobial peptide isolated from Atlantic halibut. Biochim Biophys Acta 2004, 1699, 221–227.
[41] **Park CB, Kim MS, Kim SC:** A novel antimicrobial peptide from *Bufo bufo gargarizans*. Biochem Biophys Res Commun 1996, 218, 408–413.

[42] **Park CB, Yi KS, Matsuzaki K, Kim MS, Kim SC:** Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. Proc Natl Acad Sci U S A 2000, 97, 8245–8250.
[43] **Park CB, Kim HS, Kim SC:** Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem Biophys Res Commun 1998, 244, 253–257.
[44] **Park IY, Park CB, Kim MS, Kim SC:** Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, Parasilurus asotus. FEBS Lett 1998, 437, 258–262.
[45] **Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC:** Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J 2002, 16, 429–431.
[46] **Cho JH, Park IY, Kim MS, Kim SC:** Matrix metalloproteinase 2 is involved in the regulation of the antimicrobial peptide parasin I production in catfish skin mucosa. FEBS Lett 2002, 531, 459–463.
[47] **Kawasaki H, Isaacson T, Iwamuro S, Conlon JM:** A protein with antimicrobial activity in the skin of Schlegel's green tree frog *Rhacophorus schlegelii* (*Rhacophoridae*) identified as histone H2B. Biochem Biophys Res Commun 2003, 312, 1082–1086.
[48] **Tollin M, Bergman P, Svenberg T, Jornvall H, Gudmundsson GH, Agerberth B:** Antimicrobial peptides in the first line defence of human colon mucosa. Peptides 2003, 24, 523–530.
[49] **Brown OA, Sosa YE, Goya RG:** Thyrotropin-releasing activity of histone H2A, H2B and peptide MB35. Peptides 1997, 18, 1315–1319.
[50] **Chu AJ, Chen BM, Lin H, Beydoun S:** Antimicrobial peptide buforin I inhibits tissue factor-initiated coagulation. Arch Biochem Biophys 2001, 392, 3–7.
[51] **Labat-Robert J:** Age-dependent remodeling of connective tissue: role of fibronectin and laminin. Pathol Biol (Paris) 2003, 51, 563–568.
[52] **Maquart FX, Pasco S, Ramont L, Hornebeck W, Monboisse JC:** An introduction to matrikines: extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. Crit Rev Oncol Hematol 2004, 49, 199–202.
[53] **Pasco S, Ramont L, Maquart FX, Monboisse JC:** Control of melanoma progression by various matrikines from basement membrane macromolecules. Crit Rev Oncol Hematol 2004, 49, 221–233.
[54] **Duca L, Floquet N, Alix AJ, Haye B, Debelle L:** Elastin as a matrikine. Crit Rev Oncol Hematol 2004, 49, 235–244.
[55] **Bellon G, Martiny L, Robinet A:** Matrix metalloproteinases and matrikines in angiogenesis. Crit Rev Oncol Hematol 2004, 49, 203–220.
[56] **Maquart FX, Bellon G, Pasco S, Monboisse JC:** Matrikines in the regulation of extracellular matrix degradation. Biochimie 2005, 87, 353–360.
[57] **Tran KT, Lamb P, Deng JS:** Matrikines and matricryptins: Implications for cutaneous cancers and skin repair. J Dermatol Sci 2005, 40, 11–20.
[58] **Sulochana KN, Fan H, Jois S, Subramanian V, Sun F, Kini RM, Ge R:** Peptides derived from human decorin leucine-rich repeat 5 inhibit angiogenesis. J Biol Chem 2005, 280, 27935–27948. (Epub 2005, May 27).
[59] **Karagiannis ED, Popel AS:** Identification of novel short peptides derived from the alpha 4, alpha 5, and alpha 6 fibrils of type IV collagen with anti-angiogenic properties. Biochem Biophys Res Commun 2007, 354, 434–439. (Epub 2007, Jan 16).
[60] **Zeltz C, Brézillon S, Perreau C, Ramont L, Maquart FX, Wegrowski Y:** Lumcorin: a leucine-rich repeat 9-derived peptide from human lumican inhibiting melanoma cell migration. FEBS Lett 2009, 583, 3027–3032. DOI: 10.1016/j.febslet.2009.08.012. (Epub 2009, Aug 15).

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An Estimation of the Biological Properties of Fish Collagen in an Experimental *In Vitro* Study

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The principal sources of medical collagen are pork, calf skin and bone. There are now more studies on a much safer, alternative source of active collagen, mainly from aquatic life. Active collagen and its peptides FCP (fish collagen peptides) have already been extracted from the skin of salmon, cobia, hoki, tilapia, zebrafish, ling, shark, silver carp and also jellyfish.

Objectives. The aim of the study is to evaluate the effect of fish collagen on human fibroblasts from gingiva. The cytotoxicity of the new formulation and induction of endogenous collagen was estimated by means of the collagen derived from fish skin.

Material and Methods. Fish collagen was extracted from the skin of silver carp at 16 degrees Celsius. To compare the biocompatibility and endogenous collagen production Geistlich Bio-Gide® membrane was ordered in Geistlich Biomaterials (Geistich AG, Wolhusen, Switzerland). The culture of human fibroblasts was performed acc. to Saczko et al. The fibroblasts were treated 96 hours with 1.0%, 0.5% and 0.1% experimental collagen formulation to induce endogenous collagen production. The Sircol collagen assay was done to measure amount of collagen. Cell viability was assessed by measuring mitochondrial activity in MTT assay after 24 h followed by 24 h of incubation with experimental collagen formulation. Qualitative analysis was performed by immunocytochemically staining of collagen type I and III.

Results. Preparations of fish collagen are not cytotoxic at concentrations below 1%. Cells cultured in the presence of this product are characterized by a large number of endogenous collagen, which is comparable to the control. In case of porcine collagen membrane was noticed decreased to 83% production of endogenous collagen and reduction of cell viability to 69%.

Conclusions. Our study showed that experimental fish collagen is an innovative product which may induce expression of endogenous collagen in fibroblasts (**Adv Clin Exp Med 2015, 24, 3, 00–00**).

Key words: biomaterials, fish collagen, endogenous collagen.

Collagen is one of principle proteins of extracellular matrix and is still being investigated. It is the main component of the connective tissue, links tendons, bones, teeth and cornea, being in effect a stable structure supporting bodies of all vertebrates [1]. Its main constituent is cross-linked tropocollagen taking fibrous form and it has an unusual structure that comprises of three polypeptide alpha-chains consisting of over 1000 amino-acids, mainly glycine, proline, hydroxyproline and hydroxylysine. Every chain takes the form of left-handed triple helix [2]. Peptide spiral is a well-designed and tightly packed structure. In the presence of e.g. ascorbic acid and amino-acid hydroxylation, collagen is transformed into its proper forms during collagen formation in fibroblasts, chondrocytes and keratinocytes.

Collagen is a biomaterial commonly used in medicine, dentistry, pharmacology, cosmetology and tissue engineering applications because of its

excellent biocompatibility, low antigenicity, high biodegradability, and good mechanical, haemostatic, and cell-binding properties [3]. It can be used in various forms, e.g. gels, sponges, membranes, scaffolds or powder [1]. That diversity of forms allows collagen to be efficient in various fields, including but not limited to scaffolding of cell and growth factors, wound healing, scar correction, soft tissue augmentation or closure of extraction sites [4]. The most popular collagen type seems to be biomaterial. Using collagen as a biomaterial for tissue regeneration introduces some disadvantages versus other materials. These include its low biomechanical stiffness and rapid biodegradation. Further, the high rate of enzymatic degradation of natural collagen in vivo makes stabilization of collagen-based biomaterials necessary. This stabilization can be achieved by physical and/or chemical cross-linking, which provide biomaterials with the desired mechanical and degradation kinetics for in vivo applications. Despite these disadvantages and due to their cell-binding properties [5], there are available pure collagen membranes for guided tissue regeneration or soft tissue augmentation [6, 7]. The other very important issue in surgery is the problem of tissue healing. Mostly all available surgical dressing consists only of chlorhexidine, herbs or cements. The aim of proper healing is the stimulation of epithalization, which is why collagen could be a very promising product, especially in the form of gel.

In medicine the main sources of industrial collagen is calf skin and bone [3]. Triple-helical conformation of collagen and its peptides were extracted from various animals including bovine skin, porcine skin, bird feet, frog skin, shark skin, rat tail tendons [8]. There have been many attempts to find an alternative source of equally efficient and in some opinion safer collagen and another form [3, 8, 9]. One of the prospective sources comes from marine organisms (sea urchins, fish scale and skin, jellyfish, shark skin). Many studies indicated that bird feet, frog skin, sea urchin and shark skin collagen have a molecular structure different than domestic animals [10–12]. Their amino acid composition, peptide constitution, glycosaminoglycan content and thermal behavior are significantly different from land animals. On the basis on Lin et al. [8] study, it has been established that bird feet and porcine skin collagen should be used as a suitable material utility because of their better biostability. In the course of other research, it was established that fish collagen and its peptides have stimulatory influence on some cellular and tissue specific processes, e.g. procollagen synthesis, wound healing and reduction of scar tissue.

The aim of the study was to evaluate the effect of fish collagen on human fibroblasts from gingiva. The cytotoxicity of the new formulation and induction of endogenous collagen was estimated by means of the collagen derived from fish skin.

Material and Methods

Breeding Silver Carp (*Hypophthalmichthys molitrix*) were caught in sweet Polish waters and transported into the laboratory. To compare the biocompatibility and endogenous collagen production Geistlich Bio-Gide® membrane was ordered in Geistlich Biomaterials (Geistich AG, Wolhusen, Switzerland). These membranes consist of I and III pure collagen and are well known and examined scaffolds commonly used in dentistry and tissue engineering technique.

Chemicals

DMEM and In Vitro Toxicology Assay – MTT from Sigma (St. Louis, MO), fetal bovine serum (FBS) from Bio-Whittaker (Walkersville, MO); Sircol collagen assay kit from Biocolor Ltd. (United Kingdom), phosphate-buffered saline (PBS) was purchased from IITD (Wroclaw, Poland). Other chemicals came from POCH S. A. (Gliwice, Poland).

Extraction of Collagen from Silver Carp

Collagen extracts were prepared as follows: skin of silver carp was dissected from fat and muscle tissues. Skin in the amount 40 g/L was immersed into 1% solution of lactic acid for 24 h at the temperature of 16°C. The obtained gel was filtered through a silk cloth filter to assure homogeneity of the extract.

A mass spectrometry analysis HPLC/MS/MS was performed at the Laboratory of Mass Spectrometry Polish Academy of Sciences in Warsaw. Peptides were separated by nanoscale reverse phase high-performance liquid chromatography followed by electrospray ionization. Tandem mass spectra were obtained on an LTQ FT (Thermo Finnigan). The ion generator used was Finnigan Nanospray. Separation conditions on C18 nanocolumn were: acetonitrile gradient 0–40% in 0.05% solution of formic acid; separation time 60 min. (division into ca. 2000 fractions).

Preliminary interpretation of the results was done with the Mascot software. Included in the analysis were only the results with Mascot-scores for proteins above 30, which is related to the consistency of the measured masses of protein

fragments with those obtained with theoretical calculations for a given mass, and therefore only indirectly related to magnitude of the signal. All results fell within the range 0–130.

A large number of different peptides were detected in the analyzed silver carp skin collagen extracts. Among the smaller peptides, the most abundant were those of 7–29 aminoacids, originating from the following proteins: collagen I a1, collagen I a2, collagen I a3, collagen VI a3; decorin; lumican; histone H2A, histone H2B, histone H4.

Cell Culture

A primary culture of fibroblasts was obtained from 2 mm^2 of human gingiva taken during oral surgery. The gingival biopsies were obtained from the patients of Dental Surgery Department of Medical University of Wroclaw, Poland. The experiments were conducted in accordance with the requirements of the Bioethics Committee of Wroclaw Medical University (no 864/2012). Gingival biopsy, isolation and culture of human gingival fibroblasts were done as described by Saczko et al. [13] with modification. Before surgery, the tissue was washed with boric acid; a sample of gingiva (2 × 1 × 1 mm) was placed in a nutritional medium (Dulbecco's modified eagle medium, DMEM) containing 10% fetal bovine serum (FBS), and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL, and amphotericin B 100 µg/mL). The tissue was rinsed in sterile phosphatebuffered saline (PBS, Ph = 7.4) and transferred to a Petri dish containing DMEM. The tissue was minced mechanically. The suspension of tissue fragments and fibroblast cells was centrifuged (200 × g for 5 min). The pellet was resuspended in in culture medium (DMEM) supplemented with 20% FBS, penicillin/streptomycin and amphotericin B 100 µg/mL and put in cell culture flask. Monolayer of fibroblasts was allowed to grow in DMEM medium with 20% FBS and glutamine in a humidified CO_2 atmosphere at 37°C. The cells were adherent to the culture support and detached by trypsinization (trypsin 0.025%, EDTA 0.02%).

Evaluation of Collagen Content

Fibroblasts were cultured 24 h in multiwell plates (3.8 cm^2 of cell growth area) and grown 96 h in the presence or absence of the Bio-Gide® membrane as a comparative experimental model. The Sircol collagen assay, a quantitative dye-binding assay which measures total collagen (types I–V) for *in vitro* and *in vivo* analyses, was used to determine the total collagen content in collected culture medium. 250 µL of medium and 50 µL collagen isolation reagent were added into microcentrifuge tubes and incubated in 4°C for all night. Then 100 µL of Sircol dye reagent was added to all tubes. Tubes were capped and the contents were mixed by using a mechanical mixer at room temperature for 30 min. Tubes were transferred to a microcentrifuge and centrifuged at 14000 g for 25 min to obtain collagen-dye pellet at the bottom of the tubes. The supernatants were drained off and discarded. To remove the unbound dye solution Acid-Salt reagent was added, the tubes were centrifuged in the same way as describe above. To the collagen-dye pellet, 100 µL of the alkali reagent was added, then the tubes were capped and contents mixed by a mechanical mixer at room temperature for 5 min and the collagen bound dye was dissolved into alkali reagent. Finally, the dye solutions were added into 96-well plate and the absorbance was determined using the Multimode Plate Reader at 550 nm (EnSpire, PerkinElmer).

MTT Reduction Assay

The *in vitro* cytotoxicity of silver carp collagen was investigated by the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT test), which relies on the mitochondrial activity of vital cells and represents a parameter for their metabolic activity. The cytotoxicity was compared with that of commercially available naturally derived biomaterials such a bovine collagen (Bio-Gide® membrane).

The cells were seeded into 96-well microculture plates (Nunc, NunclonTM Surface, Thermo Fisher Scientific, Biokom, Janki k/Warszawy, Poland) at the concentration of about 4 × 10^5 cells/well, next day the medium was replaced with collagen formulation in concentration 1.0, 0.5, 0.25 i 0.01% and incubated for 24 h at 37°C and 5% CO_2 and then experimental formulation was replaced by culture medium for 24 h. Then the medium of each well was replaced with 100 µL of 0.05 mg/mL MTT stock solution (dimethylthiazol-diphenyltetrazoliumbromide thiazolyl blue; Sigma-*In Vitro* Toxicology Assay). After 2 h of incubation, isopropanol with 0.04 M HCl was added (100 µL/well). The absorbance was determined using the Multimode Plate Reader at 570 nm (EnSpire, PerkinElmer). Mitochondrial function was expressed as a percentage of viable cells under treatment relative to control cells.

Immunohistochemical Method

Cells were grown on glass slides for 3 days in the presence of 1%, 0.5%, 0.25% and 0.125% fish collagen. After that they were fixed with 4%

paraformaldehyde and Real Peroxidase-Blocking Solution (Dako) for 10 min and Protein Block (Dako) for 15 min was applied. Then the solutions of antibodies were spotted: goat policlonal anti-collagen type I (Santa Cruz BT COL1A1) at a dilution of 1 : 50, and goat policlonal anti-collagen type III (Santa Cruz BT COL3A1) at a dilution of 1 : 50. Incubation was carried overnight at 4°C in a humid glass chambers. Then the procedure was performed using the DAKO LSAB kit + System-HRP, successively with Biotynylated Link Universal, Streptavidin-HRP, DAB + substrate buffer with DAB + Chromogen, and following the general method guidelines. Delafield hematoxylin was used as the counterstain. Preparations were carried out by the ascending series of alcohol (50–100%) and the slides were immersed and closed with the glass coverslip using DPX (Aqua Medica).

Statistical Analysis

Cell proliferation in the aspect of various concentrations of fish collagen was analyzed by Kruscal-Wallis test. Results of MTT test and Sircol for cells cultured in the presence of Bio-Gide and without were analyzed by Mann-Whitney test. Statistical significance was $p < 0.05$. For the analysis two programs were used: the R statistical package for Windows and MedCalc for Windows.

Results

The Effect of Experimental Collagen Formulation Treatment on Ffibroblasts

The cytotoxicity of the experimental collagen formulation was studied by MTT assay as an evaluation of the oxidoreductive mitochondrial function. The results showed that using 1% concentration of fish collagen decreases mitochondrial function up to the value 79.93% ± 16.8% compared with control cell level (Fig. 1). However, statistical analysis has not shown that the concentration of collagen was statistically different from the control cells.

Effect of Bio-Gide Cultivation on Fibroblasts

The cytotoxicity and induction of endogenous collagen by Bio-Gide® collagen membrane was studied. After 96 h of incubation of fibroblasts with collagen membrane, the production of endogenous collagen decreased to 83% in comparison to control (Fig. 2A). The cytotoxicity effect of Bio-Gide® was evaluated after 24 h of incubation with membrane by MTT assay. We noticed a reduction of mitochondrial work to 69% compared to the control group (Fig. 2B). Statistical tests, however, showed no significant difference between control cells and those cultured in the presence of BioGide, both in terms of survival and quantities of produced collagen.

It was noticed that the endogenous fibrillar (type I and III) collagen or procollagen in the fibroblasts cultured in the presence of fish collagen. The staining intensity appeared to be related to the amount of fish collagen added to the culture medium and it was increased with the rise of added fish collagen formulation (Fig 3B, Fig 3K, Fig. 3N). However, it was observed that the more fish collagen in the medium the more cells have altered morphology. This suggests that fish collagen may stimulate cells for expression of endogenous collagen.

Discussion

The growing demand for collagen in various fields of medicine forces researchers to find other

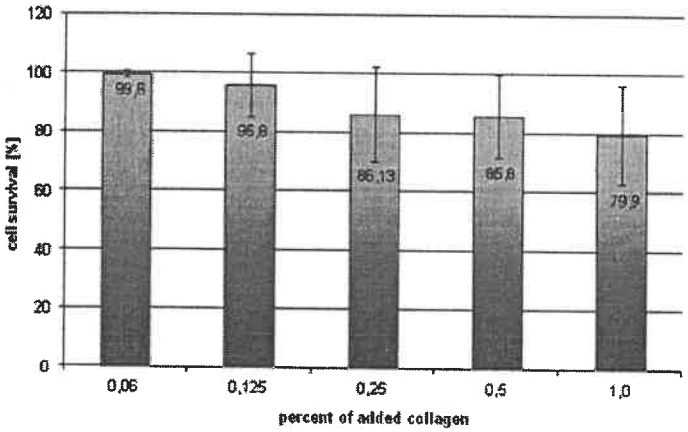

Fig. 1. The effect of fish collagen formulation treatment on the proliferation of fibroblasts was evaluated by the MTT assay. Bars show the mean of four replicates. Error bars indicate standard deviations

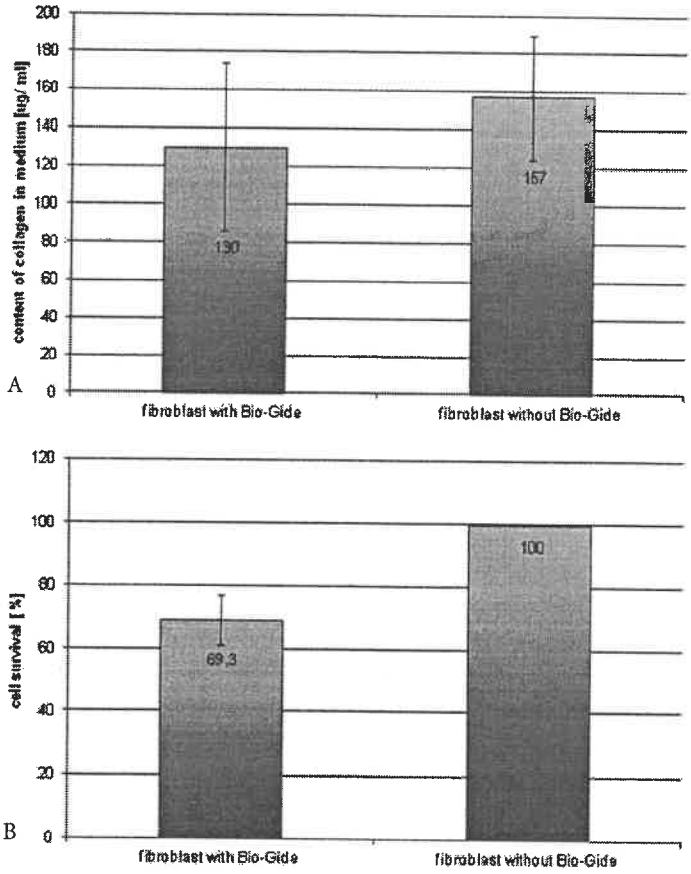

Fig. 2. The biological effects of Bio-Gide® on fibroblasts; A. the induction of production of endogenous collagen; B. the cytotoxicity evaluated by MTT assay

sources of active collagen. In literature there are known studies on comparisons of physical and chemical properties of type I collagen from bird feet, bovine skin, porcine skin, frog skin and shark skin [8]. The study of Song et al. [3] shows that a novel form of acid-soluble collagen was extracted from jellyfish. According to Song et al. [3] jellyfish collagen exhibited higher cell viability than other naturally derived biomaterials like bovine collagen, hyaluronic acid, gelatin and glucan but jellyfish collagen showed a lower Pro (prolin) content compering to calf skin collagen. The jellyfish collagen cytotoxicity in vitro was investigated using different kinds of cells (human fibroblasts, endothelial cells, smooth muscle cells and chondrocytes) by MTT assay. In particular, the viability of fibroblasts in contact with jellyfish collagen was much higher (146 ± 5.4% at the day 10) than of fibroblasts in contact with bovine collagen (112.10 ± 7.8% at day 10). They also found that jellyfish collagen induced an immune response similar to one triggered by gelatin and bovine collagen [3]. Our study investigates cytotoxicity of new collagen formulation derived from silver carp on human fibroblast in vitro. The obtained results show that the cytotoxicity (79.93% ± 16.76%) does not exceed the IC_{50} and is comparable to the cytotoxicity of Bio-Gide® collagen membrane (69%) which was evaluated in the same conditions.

There are known results of stimulation of cells and tissues by fish extract of collagen containing small molecules of active peptides. The experimental formulation probably impacts on different biochemical processes such as proliferation, angiogenesis, apoptosis, wound healing, fibrosis and acts on the hormonal and immune. In our studies it has been validated that fish collagen from the skin of silver carp is a safe material for tissue applications. The research had revealed that 1% of fish collagen effected the proliferation of human fibroblasts and its use has not induced a significant cytotoxic effect on those cells in comparison to control group.

Subsequent laboratory and clinical research of the collagen preparations will focus on the assessment of its efficiency in the process of early healing of soft tissue mouth wounds. Results of initial trials seem to be promising, thus supporting the idea of using collagen gel in dentistry. Additionally, further research of safe fish collagen and FCP also appears to be promising not only in regard to soft tissue healing, but also in bone healing and regeneration [14].

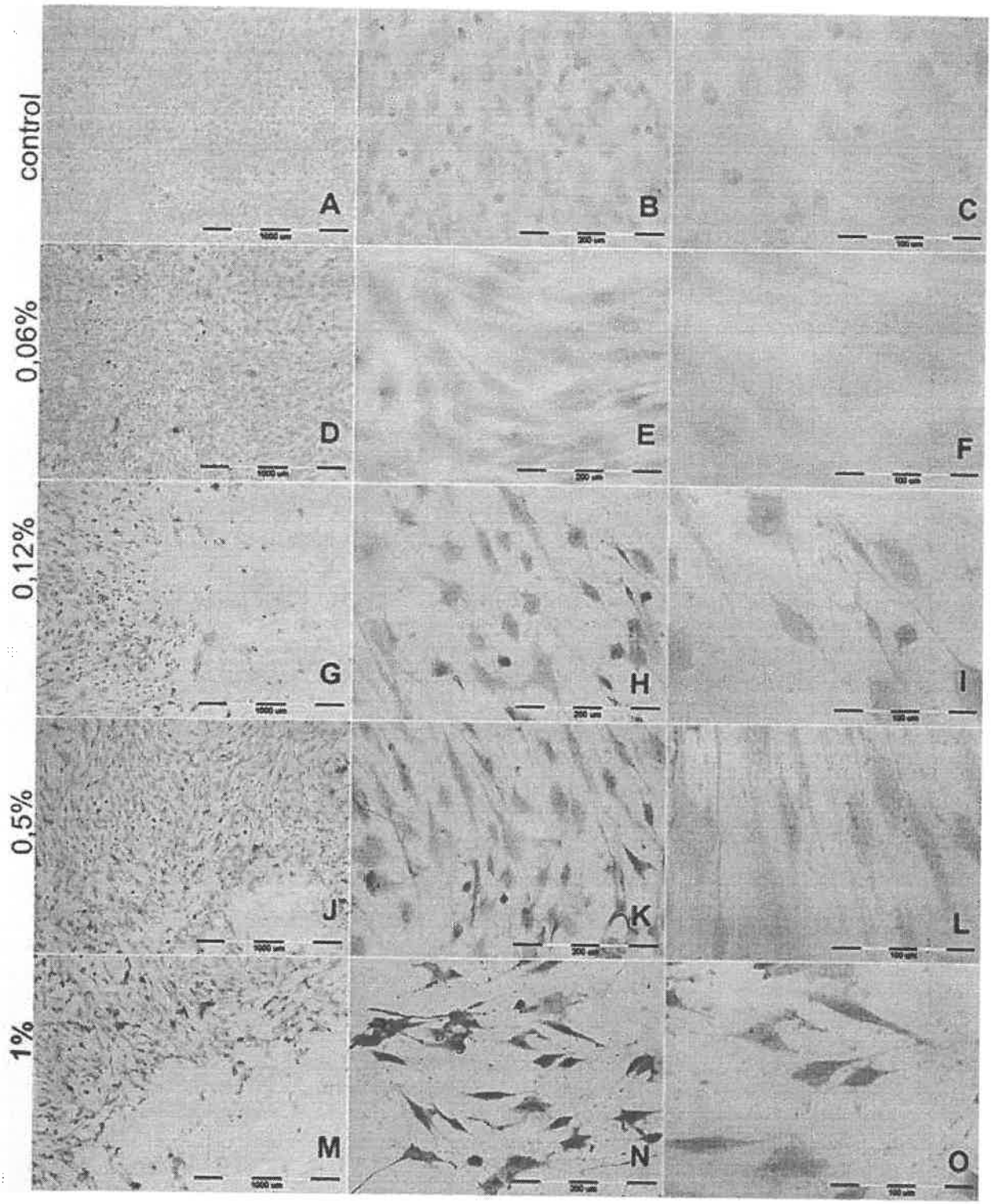

Fig. 3. Photos of fibroblast with immunocytochemically stained collagen type I. On panels A, B, C control cells are shown; on panels D, E, F are the cells cultured in the presence of 0.06% fish collagen; on panels G, H, I 0.12% respectively; in panels J, K, L: 0.5%; in panels M, N, O: 1%. Magnification on panels A, D, G, J, M: 40X; B, E, H, K, N: ×200; C, F, I, L, O: ×400

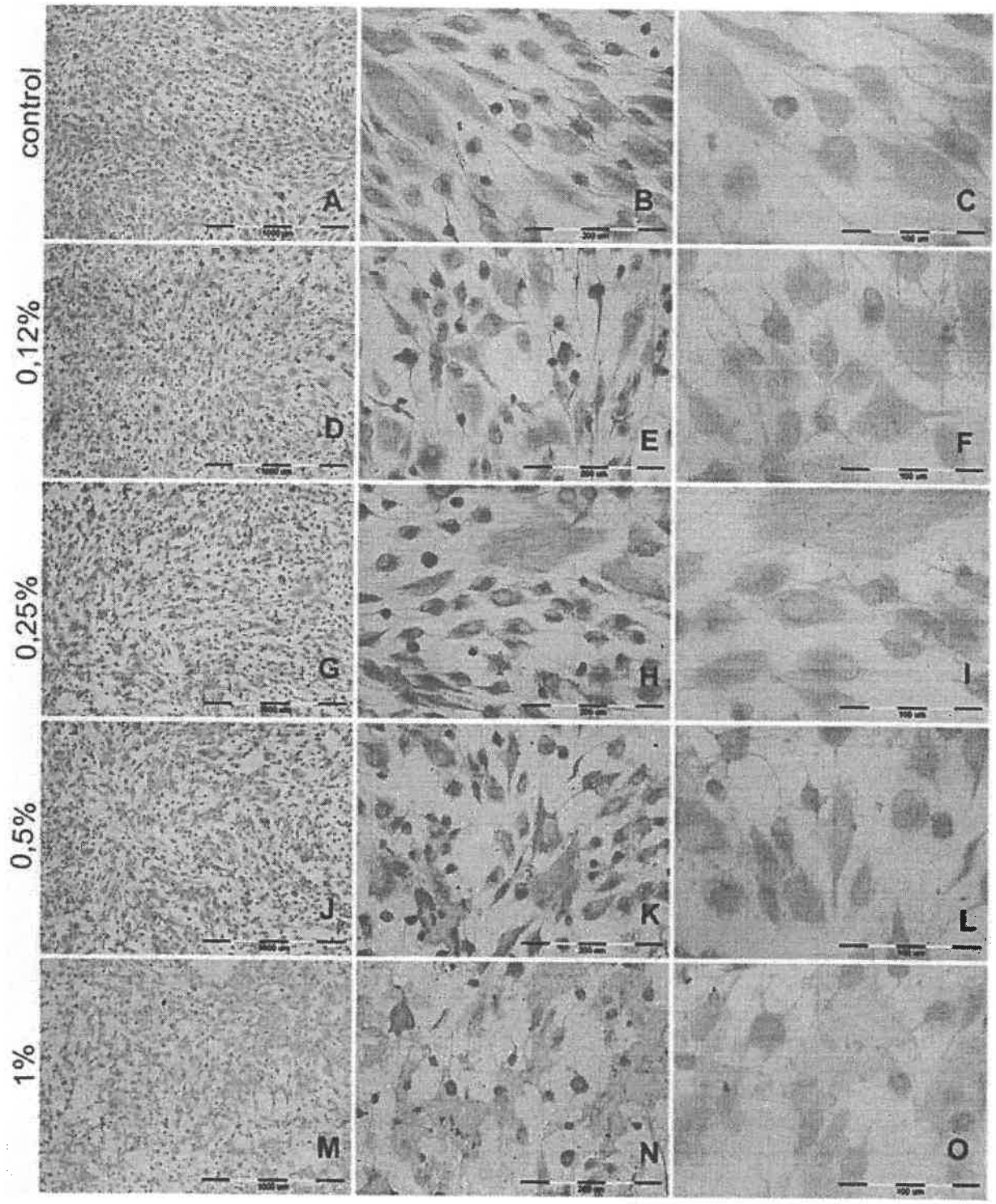

Fig. 4. Photos of fibroblast with immunocytochemically stained collagen type III. On panels A, B, C control cells are shown; on panels D, E, F are the cells cultured in the presence of 0.12% fish collagen; on panels G, H, I 0.25% respectively; in panels J, K, L: 0.5%; in panels M, N, O: 1%. Magnification on panels A, D, G, J, M: 40X; B, E, H, K, N: ×200; C, F, I, L, O: ×400.

References

[1] **Patino MG, Neiders ME, Andreana S, Noble B, Cohen RE:** Collagen as an implantable material in medicine and dentistry. J Oral Implant 2002, 5, 220–225.

[2] **Di Lullo GA, Sweeney SM, Korkko J, Ala-Kokko L, San Antonio JD:** Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 2002, 277, 4223–4231.

[3] **Davidson VL, Sittman DB:** Biochemistry, Urban and Partner, Wrocław 2002, 4th edition, 49–55.

[4] **Kuzan A, Chwiłkowska A:** Heterogeneity and functions of collagen in arteries. Pol Merkur Lekarski 2011, 31, 111–113.

[5] **Song E, Kim SY, Chun T, Byun H-J, Lee YM:** Collagen scaffolds derived from a marine source and their biocompatibility, Biomaterials 2006, 27, 2951–2961.

[6] **Yamada S, Nagaoka H, Terajima M, Tsuuda N, Hayashi Y, Yamauchi M:** Effects of fish collagen peptides on collagen post-translational modifications and mineralization in an osteoblastic cell culture system. Dent Mater J 2013, 32, 88–95.

[7] **Unsal B, Ozcan G, Tuter G, Kurtis B, Yalim M:** Evaluation of initial attachment of human gingival fibroblasts cells to biodegradable membrane *in vitro* by light and scanining electron microscopy. J Oral Sci 1999, 41, 57–60.

[8] **Dominiak M, Łysiak-Drwal K, Saczko J, Kunert-Keil C, Gedrange T:** The clinical efficacy of primary culture of human fibroblasts in gingival augmentation procedures – a preliminary report. Ann Anat 2012, 194, 502–507.

[9] **Simain-Sato F, Lahmouzi J, Heinen E, Defresne MP, De Pauw-Gillet MC, Grisar Th, Legross JJ, Legrand R:** Graft of autologous fibroblasts in gingival tissue *in vivo* after culture in vitro preliminary study on rats. J Periodontol Res 1999, 34, 323–328.

[10] **Lin YK, Liu DCh:** Comparison of physical-chemical properties of type I collagen from different species. Food Chemistry 2006, 99, 244–251.

[11] **Yamada K, Yamaura J, Katoh M, Hata K-I, Okuda K, Yoshie H:** Fabrication of cultured oral gingiva by tissue engineering techniques without materials of animal origin. J Periodontol 2006, 77, 672–677.

[12] **Liu DC, Lin YK, Chen MT:** Optimum condition of extracting collagen from chicken feet and its characteristics. Asian-Australasian J Animal Sciences 2001, 14, 1638–1644.

[13] **Li H, Liu B, Gao L, Chen HL:** Studies on Bullfrog Skin Collagen. Food Chem 2004, 84, 65–69.

[14] **Cheng W, Yan-Hua R, Fang-Gang N, Guo-An Z:** The content and ratio of type I and III collagen in skin differ with age and injury. Afr J Biotechnol 2011, 10, 2524–2529.

[15] **Robinson JJ:** Comparative biochemical analysis of sea urchin and rat tail tendon. Comparative Bioch Physiol 1997, 117, 307–313.

[16] **Saczko J, Dominiak M, Kulbacka J, Chwiłkowska A, Krawczykowska H:** A simple and established method of tissue culture of human gingival fibroblasts for gingival augmentation, Folia Histochem Cytobiol 2008, 46, 117–119.

[17] **Hoyer B, Bernhardt A, Heinemann S, Stachel I, Meyer M, Gelinsky M:** Biomimetically mineralized salmon collagen scaffolds for application in bone tissue engineering. Biomacromolecules 2012, 13, 1059–1066.

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The Effect of Silver Carp Skin-Derived Peptides on the Activities of VEGF and Hsp70.1 Gene Transcription Promoters

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Studies conducted on human cell culture models have demonstrated that collagen-derived peptides can exert a beneficial effect in medicine. However, all these studies were conducted using animal collagen samples, most often originating from bovine or porcine skin. Currently attempts are being made to replace animal collagen with fish collagen.
Objectives. The aim of the study was to compare the effect of silver carp skin-derived peptide extract on the transcriptional activities of human VEGF and hsp70.1 gene promoters inserted into the plasmids with secreted alkaline phosphatase as a reporter gene.
Material and Methods. Changes in the activity of the promoters were investigated using a HEK293FT cell line transfected with pVEGF-SEAP or pHsp70-SEAP. The cells were cultured in dishes containing peptides separated using reverse-phase high performance liquid chromatography.
Results. The study demonstrated that the silver carp skin-derived peptide extract exerts both an inhibitory effect on the VEGF gene promoter and activating effect on the hsp70.1 gene promoter. Higher biological activity was recorded in the case of a freshly prepared peptide extract compared to one stored at 4°C for three months.
Conclusions. The silver carp skin-derived collagen peptides influence VEGF and hsp70.1 gene promoters' transcriptional activity (**Adv Clin Exp Med 2016, 25, 3, 415–423**).

Key words: collagen, fish collagen, skin-derived peptide, gene promoters.

For over 20 years collagen of animal origin has been one of the hopes for elaborating novel therapeutic strategies. Studies conducted on animal models have demonstrated that collagen-derived peptides can be beneficial in fighting cancer, accelerating wound healing, and repairing damaged neurons, among other applications. However, all these studies were conducted using animal collagen samples, most often originating from bovine or porcine skin. Currently, due to reported cases of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD), scientists are trying to replace animal collagen with another type, e.g. fish collagen [1].

Studies on extracellular matrix proteins, including collagen, elastin, laminin and proteoglycans (among others), have revealed that they participate in the regulation of key cellular processes such as cell migration, adhesion and differentiation, organogenesis, mitogenesis and apoptosis [2].

This regulation is mediated by signal peptides, so-called matrikines, produced after a partial proteolysis of the matrix components; this term was first proposed by Maquart to define peptides that

activate specific signaling pathways by binding to specific receptors located on the cell surface [3]. The signals reach numerous cells such as fibroblasts, smooth muscle cells, epithelial cells, macrophages, neutrophils, leukocytes, monocytes and lymphpocytes [4]. They stimulate fibroblasts, both in cell cultures and *in vivo*, to increase their production of collagen [5]. Signal peptides produced as a result of metalloproteinase action can be up to about 20 kDa in size. These peptides are recognized by specific receptors located on the cell surface [6].

The number of collagen applications in medicine has increased thanks to a better understanding of the biological properties of peptides. The small size of peptides allows them to penetrate natural integuments, which results in a better therapeutic effect. They are applied to stimulate wound healing, to stop bleeding [7], and to reconstruct nerve fibers [8]. The use of collagen peptides for drug delivery is under investigation [9]. Collagen peptides demonstrate biological activity both in physiological and pathological processes, which makes them a good tool for therapies for various kinds of pathophysiological diseases, including vascular diseases, rheumatoid arthritis and all types of cancers.

This study focused on two system regulators which were expected to be regulated by skin-derived matrikines, namely vascular endothelial growth factor (VEGF) and heat shock protein hsp70.1. In a previous study the authors used mass spectrometry to demonstrate that fish skin contains small peptides (containing 7–29 amino acid residues) which could be potential matrikines [10]. The most common peptides were derived from decorin, lumican and α1, α2 and α3 chains of type I collagen, among others. One of the purposes of the present study is to investigate the suitability of the peptide extracts obtained to produce preparations that can accelerate wound healing.

VEGF is one of the most important regulators of angiogenesis. It binds to three types of receptors located on the surface of endothelial cells: VEGFR-1 (flt-1), VEGFR-2 (KDR), and neurophilin. Some studies also report VEGFR-3 (FLT-4) [11]. These receptors stimulate cell migration, proliferation, permeability and activity [12]. Expression of the VEGF-A gene is regulated in different tissues by a number of factors, including cytokines (e.g. EGF, TGF-β) [13], growth factors, prostaglandins, nitric oxide [14] and oncogenes (e.g. Ras) [15]. VEGF also plays an important role in the tissue regeneration process, in organ mass adjustment [16] and neurogenesis, including orientation of axonal growth [17]. Currently, angiogenesis inhibitors which could be applied in the treatment of vascular diseases and cancer are under investigation. One of the therapeutic strategies involves collagen-derived matrikines as a factor inhibiting the growth of cancer cells [18]. These matrikines include endostatin, restin and vastatin, as well as non-collagenous domains of collagen IV. Endostatin specifically blocks the development of new blood vessels, causing latency and regression of the tumor [18].

Hsp70.1 is the main representative of the heat shock proteins, and also regulates their expression. These proteins protect the cell against the harmful consequences of environmental and physiological stress, i.e. heat, mechanical damage of tissue, UV radiation, hypoxia, heavy metals, reactive oxygen species and cell differentiation [19]. They prevent protein aggregation and the formation of abnormal connections between them. They have the ability to fold unfolded proteins, and thus they can be applied to slow down such diseases as Parkinson's, Alzheimer's and Huntington's diseases. They also initiate the immune response, including the production of pro-inflammatory cytokines, expression of MHC class II, and the release of nitric acid. They play an important role in antigen presentation and tumor immunity [20]. Heat shock protein activity also occurs during tissue damage and its regeneration, as the damaged skin barrier no longer protects against any adverse impact of the external environment [21]. A high level of hsp70 protein is recorded in the case of well-healing wounds, while in the case of chronic wounds its expression is low or does not occur [22]. Both increased production of heat shock proteins and their active participation in the process of wound healing have been demonstrated over a period of approximately two to ten hours after the damage [23, 24]. It is known that increased production of hsp70 protein may occur in the case of tissue damage that is caused both by internal tissue factors, such as PHSRN peptide derived from fibronectin [25], and bacterial lipopolysaccharides [26], where hsp70 expression inhibits cytokine excretion induced by lipopolysaccharides; this increases the survival rate of (for example) rats injected with lipopolysaccharides [27].

To date, no research concerning the impact of fish collagen peptides on the hsp protein family has been carried out. All the studies of collagen peptides in the available literature have focused on animal-derived collagen. Thus, knowledge of the biological activity and possible applications of such fish peptides in new therapeutic strategies is scarce. One of the advantages of the use of fish collagen is that it does not transmit viruses to humans, which makes fish collagen peptides potentially safer than animal-derived ones.

Therefore, this study aims at verifying whether the peptides of fish collagen exhibit pro- or anti-angiogenic characteristics, and whether they

activate the heat shock response. The study concentrated on the effect of peptides on the key regulator of angiogenesis: VEGF, whose activity contributes, among other things, to the growth and metastasis of cancer cells. Moreover, the biological activity of fish peptides was checked for response to thermal shock, using a plasmid with the hsp70.1 gene promoter sequence. The activation of this gene may also have a therapeutic relevance.

Material and Methods

Plasmids

pVEGF-SEAP is the plasmid containing cDNA of the VEGF human gene promoter with a reporter gene of the secreted alkaline phosphatase based on the pSEAP2-control plasmid [28, 29].

pHsp70-SEAP is the plasmid containing cDNA of the hsp70.1 human gene promoter with a reporter gene of the secreted alkaline phosphatase based on the pSEAP2-control plasmid.

The hsp70.1 gene promoters were cut from the pHBCAT plasmid (received from Prof. R. Morimoto at Northwestern University, USA) using *BamHI* enzyme. They were then cloned, using the blunt-end approach, into the SEAP2 control plasmid (Clontech Laboratories Inc., Mountain View, USA) in place of the SV40 promoter that was cut out using *BglII* and *NruI* enzymes. *BamHI* and *BglII* restriction sites were blunted using mung bean nuclease. The correctness of the construct was confirmed by sequencing.

Cell Line Culture

The HEK293FT cell line was grown on the complete culture medium DMEM/F-12 (Sigma-Aldrich, St. Louis, USA) with the addition of 7% FBS (Sigma-Aldrich). Transfection was performed using TransFast reagent (Promega, Fitchburg, USA).The culture media were supplemented with penicillin and streptomycin, as well as antibiotic-antimycotic solution (Sigma-Aldrich).

Measurement of the Protein Activity of the Secreted Alkaline Phosphatase (SEAP)

The quantity of SEAP protein in the culture medium was investigated using a GREAT EscAPe SEAP Detection Kit (BD Clontech, Takara Bio Inc., Shiga, Japan). In the case of both VEGF and the hsp70 promoter, 24 h after the transfected cells had been seeded into 24-well plates, 50 µL of the culture media was collected into Eppendorf tubes and centrifuged. The supernatant was subjected to the procedure specified by the manufacturer, and finally the level of chemiluminescence was measured with a Victor device (PerkinElmer Inc., Waltham, USA) for 10 s. During each measurement of the activity of the transcription promoter, the survival rate of the cell culture, which was tested with a standard MTT assay, was taken into account. The percentage of activity change was calculated for successive fractions relative to the transfected cells cultured into an empty well.

Preparation of the Collagen Extract

Skins of silver carp, cleaned of muscle tissue and fat, were sanitized with 1% hydrogen peroxide. Then 100 mL of sterile deionized water was added to the 5 g of skin, which was incubated in a water bath at 60°C (or 80°C to examine thermal resistance) for two hours, and stirred frequently. The obtained gel was filtrated on 10 kDa centrifugal filters (Centricon, Millipore Corporation, Billerica, USA) at 20°C. The extract was concentrated *in vacuo* to 10–40-fold and stored at 4°C.

Separation of the Extract of Collagen Peptides on Reverse Phase High Performance Chromatography (RP-HPLC)

A column Ultrasphere C-18 (Hichrom Limited, Reading, UK) was used. In different experiments, from 75 µL to 250 µL of the extract was applied. The peptides were eluted with a gradient of 0.01% acetic acid/acetonitrile, 0–80% acetonitrile, 1 mL/min. To avoid too much dilution of the sample, the separation was performed under conditions of a considerable slope gradient of acetonitrile, so the separation was far from optimal. Eleven successive fractions containing peptides were collected in 24-well plates. The twelfth well was filled with a control sample without peptides. Two separations were performed on each plate. The plates were dried at 37°C, then the HEK293FT cells transfected with a suitable plasmid were seeded on them and grown on the DMEM/F-12 culture medium with 7% FBS and antibiotics.

Results

An example separation of peptides on the RP-HPLC bed (with the absorbance value measured at 280 nm), is shown in Fig. 1. The first hydrophilic peptides that exhibited a weak affinity for the li-

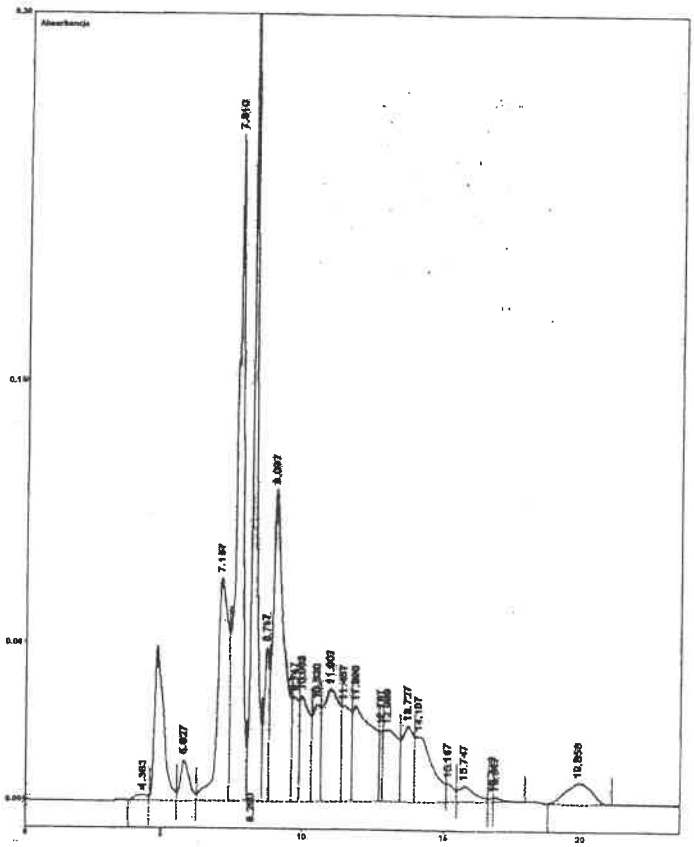

Fig. 1. Example separation chart of silver carp skin-derived collagen peptides on an RP-HPLC, using an ultrasphere C18 column

Gradient elution, flow rate 1mL/min, gradient: 0–1 min; 100%; 0.01% acetic acid; 1–8 min; 0–40% acetonitrile, 8–14 min; 80% acetonitrile and 20%; 0.01% acetic acid; 15–24 min; 0.01% acetic acid. Fractions were collected from the 4th to the 14th min onto a 24-well plate.

gand were eluted. The extracts of collagen peptides from different samples showed significant differences when observed in their chromatograms. Hydrophilic peptides (4–10 min of separation) were especially variable, and therefore they were not included in certain result sets.

The Effect of Collagen Peptides on the VEGF-A Human Gene Promoter

The PVEGF-SEAP plasmid containing cDNA of the VEGF human gene promoter with a reporter gene of the secreted alkaline phosphatase based on the pSEAP2-control plasmid was constructed by one of the authors of this paper (JGW) in the Department of Biotechnology of the Medical University of Gdańsk, Poland. The effectiveness of the measurement method activating the VEGF promoter in HEK293 cells was checked using hydrogen peroxide, which is known to stimulate the VEGF promoter. Stimulation of the promoter was 2.6-fold of the control sample at a concentration of 200 μM of H_2O_2, and a concentration of 500 μM was lethal for HEK293FT cells.

Whether silver carp skin-derived collagen peptides can affect the activity of the VEGF-A promoter and thereby participate in the regulation of angiogenesis was investigated. For this purpose HEK293FT cells transfected with pVEGF-SEAP plasmid were cultured on 24-well plates filled with the separated and evaporated extract of collagen peptides. Samples were collected 24 h after the seeding of the transfected cells on the collagen peptides, and the transcription activity of the VEGF promoter was measured using the aforementioned SEAP detection kit (BD Clontech). Ten samples of silver carp skin-derived collagen peptides were measured. The concentration of peptides before chromatographic separation was 11 mg/mL, and the amount applied to the RP-HPLC was 50–250 μL. The results were divided by the value of the survival rate of the cell culture that was ascertained by the MTT assay. Subsequently, the percentage of change in VEGF promoter activity level relative to the control sample (cells transfected with the pVEGF-SEAP plasmid and not cultured on collagen peptides) was calculated.

The series of experiments showed that the fish-derived collagen peptides generally inhibit the activity of the VEGF promoter (Fig. 2). In some cases (the fractions containing hydrophilic peptides) activation of only the VEGF promoter was observed, which indicated that the overall effect is a result of inhibitory and activating peptides. In hydrophobic fractions, a stronger inhibitory effect on the activity of the VEGF promoter occurred, but generally all the fractions influenced the VEGF promoter.

Due to the fact that commercial collagen extracts contain lactic acid, which is difficult to sepa-

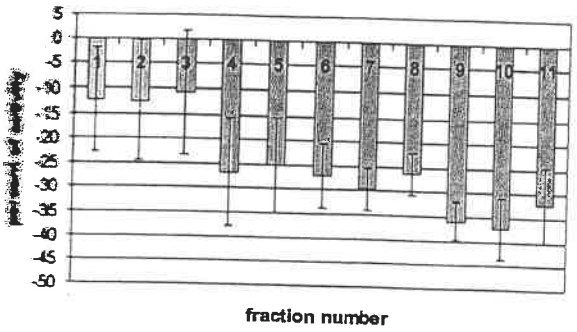

Fig. 2. The level of activation of human VEGF gene promoter induced by silver carp skin-derived collagen peptides

The successive bars represent the mean of 10 experiments for each successive fraction of the collagen peptide extracts separated by RP-HPLC. The fractions correspond to one-minute separation times, with fraction 1 starting from the fourth minute and fraction 11 corresponding to a retention time of 14 min. The vertical axis represents the activity of the VEGF promoter relative to the control, expressed as a percentage. Standard error is indicated in each column. Most of the fractions exhibited inhibition of the VEGF promoter.

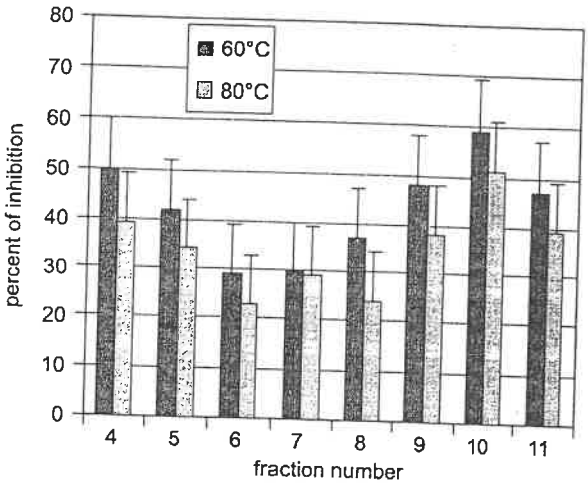

Fig. 3. A comparison of the inhibition level of the VEGF promoter by successive fractions of collagen peptide extracts, separated by RP-HPLC, prepared at two temperatures: 60°C and 80°C

The dark bars represent peptide extraction at 60°C and the light ones represent extraction at 80°C. On the Y axis the inhibition of the VEGF promoter is expressed as a percentage relative to the activity of cells that were not treated with the collagen peptides. Each bar represents the mean of 4 preparations.

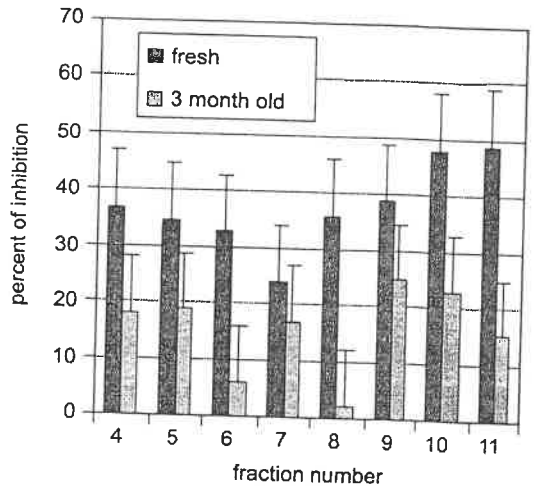

Fig. 4. The influence of the 3-month storage of the concentrated solution of peptides on their activity on VEGF

The dark bars represent the inhibition activity of freshly prepared concentrated peptides; the light bars show the inhibition activity of concentrated peptides stored in a refrigerator for three months at 4°C. Each bar shows the mean results of four experiments. VEGF promoter inhibition activity is given in percentages relative to the activity of HEK293 cells which were not treated with peptides.

rate from biologically active peptides present in the extract, a more drastic method was used: extraction in deionized water at 60°C (and separately at 80°C to check the stability of the peptides in the extraction process), which does not introduce any additional chemical substances. Figure 3 presents the inhibiting impact of the individual fractions produced in the chromatographic separation of the extracts at a concentration of 11.5 mg/mL and at temperatures of 60°C and 80°C, respectively. Four repetitions for each temperature were performed.

The series of experiments showed that the extracts of collagen peptides prepared at 60°C and 80°C differ in their activity on the VEGF promoter by approximately 15% (Fig. 3). Taking into consideration that, according to van't Hoff's rule, for each 10°C rise in reaction temperature the rate of reaction (including decomposition) increases 2–4 fold, the preparations prepared at 60°C can be regarded as fully active.

The activity of collagen peptides in the extract was also tested after three months of storage at 4°C. Four assays were performed on a 3-month-old extract of collagen peptide, and the results were compared with 4 assays made shortly after the preparation of the extract. The results are presented in Fig. 4. The figure shows an approximately two-fold decrease in activity after 3 months of storage at 4°C.

In further tests the effect of the peptide concentration on the level of inhibition of the VEGF gene promoter was examined. Four concentrations of peptides obtained after the HPLC columns were compared (applying 75 µL, 150 µL, 200 µL or 250 µL of the sample to the RP-HPLC at a concentration of 11 mg/mL). Four assays for each quantity were performed; the results were averaged and are presented in Fig. 5.

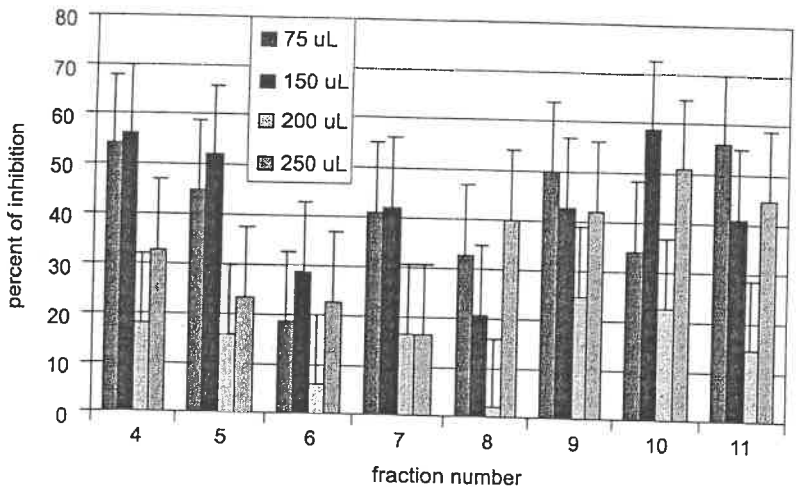

Fig. 5. A comparison of VEGF promoter inhibition levels by fractions of collagen peptides at different concentrations

The different concentrations of peptides resulted from differences in the amount of concentrated extract applied to the RP-HPLC column. The bars in each group correspond to 75 μL, 150 μL, 200 μL and 250 μL, respectively; the average results of 4 tests at each concentration are presented. The inhibition is given in percentages relative to the activity of HEK293 cells that were not treated with peptides.

This experiment showed that an increase in the concentration of the collagen peptides does not increase the inhibition of the VEGF promoter's transcriptional activity. It is probable that at these concentrations the promoter has already been saturated. However, it should be considered that once the amount of the extract applied to the chromatographic column is increased, the resolving power decreases.

The Effect of Collagen Peptides on the hsp70.1 Human Gene Promoter

The effects of collagen peptides on the hsp70.1 human gene promoter were investigated using a pHsp70-SEAP plasmid constructed by one of the authors of this study (DW) in the Department of Biotechnology of the Medical University of Gdańsk, Poland. It contains a gene-encoding secreted alkaline phosphatase under the control of the the 70.1 human gene transcriptional promoter. The transcriptional activity of the hsp70 gene was measured with the same SEAP detection kit mentioned above, using secreted alkaline phosphatase as the reporter gene.

In order to verify whether the function of the hsp70.1 human gene promoter situated on the pHsp70-SEAP plasmid was correct, the HEK-293FT cells transfected with this plasmid were subjected to heat shock in a shaking water bath. Samples of the culture medium were collected every 2 h; each collection was followed by a change of the medium, so that the level of protein production over time was known. The change in transcriptional activity was investigated with the SEAP detection kit previously described. Six hours after the heat shock, the activity of the hsp70.1 promoter in HEK293 cells subjected to a temperature of 43.5°C had increased 4-fold.

In order to investigate the biological activity of the collagen extracts, HEK293FT cells transfected with the pHsp70-SEAP plasmid were cultured on 24-well plates filled with the separated extract of collagen peptides, prepared as described above. Samples of the medium were collected 24 h after the seeding of the transfected cells on the collagen peptides. The hsp70.1 promoter's transcriptional activity was measured in relation to the survival rate of the cell culture. The results were processed as in the case of the VEGF gene. Ten assays were performed using silver carp skin-derived collagen peptides at a concentration of 11 mg/mL before the chromatographic separation. The results are presented in Fig. 6.

This series of experiments showed that collagen peptides activate the hsp70.1 human gene promoter at a 1.5-fold level. In the fractions showing relatively high hsp70.1 promoter activity, the peptides are hydrophobic, and they are eluted last in chromatographic separation.

As in the case of the VEGF promoter, experiments were also conducted to check whether the extraction temperature affects the level of the peptides' biological activity on the hsp70.1 promoter. The activities of collagen peptides extracts whose concentration before the chromatographic separation equaled 11 mg/mL and which were prepared at 60°C and 80°C were compared. For each temperature 4 assays were performed, comparing the fractions of the extract separated on RP-HPLC. The results are shown in Fig. 7.

Contrary to expectations, a comparison of the effect of collagen peptides prepared at 2 different temperatures, 60°C and 80°C, on the activity of the hsp70.1 promoter revealed that in fractions 7, 8 and 10 the peptides prepared at 80°C evoked a considerably stronger activation.

In the next stage of the study a test was performed to find out whether different concentrations of peptides affect the level of activation of the hsp70.1 gene promoter. In this experiment 3 different amounts of peptide extract were ap-

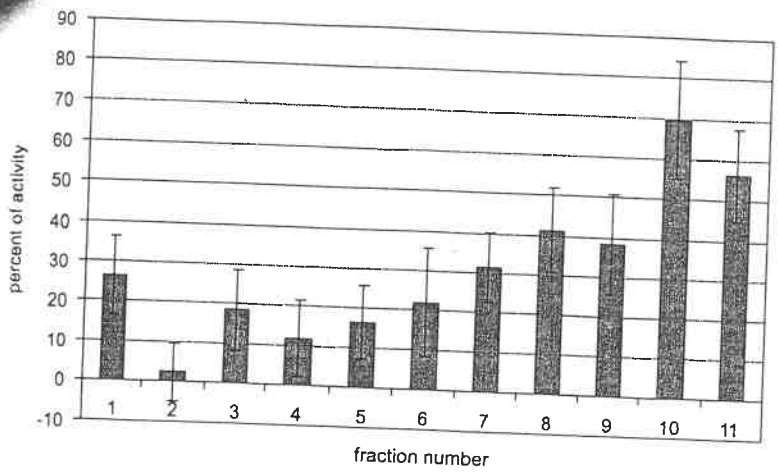

Fig. 6. The level of activation of the hsp70.1 human gene promoter induced by silver carp skin-derived collagen peptides

The successive bars represent the mean of 10 experiments for successive fractions of collagen peptide extracts separated by RP-HPLC. The fractions are analogous to the chromatographic separation shown in Fig. 2. The vertical axis represents the activity of the hsp70.1 promoter relative to the control sample, expressed as percentages. Standard error is indicated in the columns. The majority of fractions showed activation of the hsp70.1 promoter.

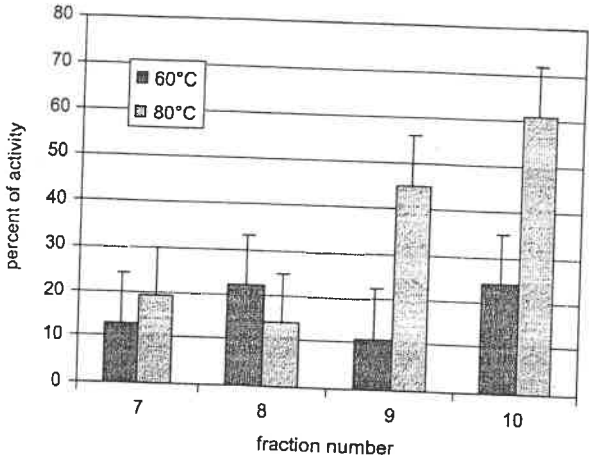

Fig. 7. A comparison of the activation level of the hsp70.1 promoter by fractions of collagen peptide extracts prepared at 60°C and 80°C

The dark bars represent extraction of the peptides at 60°C, and the light ones represent extraction at 80°C. The change in activity of the hsp70.1 promoter is expressed as a percentage relative to the activity of cells which were not treated with collagen peptides. Each bar represents the average of four preparations.

plied to the RP-HPLC column at a concentration of 11.5 mg/mL: 100 µL, 150 µL and 200 µL. Four assays for each amount were performed, and then the results were averaged and are presented in Fig. 8.

The figure shows that there is an increase in hsp70.1 promoter activation between 100 µL and 200 µL.

The study has demonstrated the effect of silver carp skin-derived collagen peptides on the transcriptional activity of the VEGF and hsp70.1 genes promoters. It has been shown that VEGF activity is inhibited on average by 20–30%, while in the case of the hsp70.1 gene, transcriptional activity increased by 30–50%. As a result of extraction of the peptides with water at 60–80°C, a biologically active preparation was obtained without the presence of any organic acids or salts that would contaminate the preparation in the case of acid or alkaline hydrolysis. However, storage of the preparation for 3 months at 4°C reduced the biological activity of the extract to approximately one half in terms of VEGF gene transcription. This probably translates into similar changes in industrial silver carp skin-derived collagen extract obtained with the use of lactic acid.

The results obtained show that fish skin-derived peptides may be applied as an anti-angiogenic factor and as a factor stimulating the repair response by activation of the heat shock protein re-

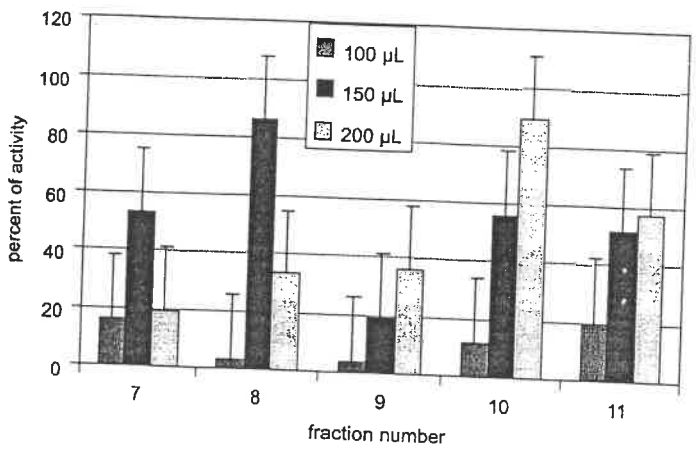

Fig. 8. A comparison of the level of stimulation of the hsp70.1 promoter activity by collagen peptide fractions of different concentrations

The different concentrations of peptides resulted from differences in the amount of concentrated extract applied to the RP-HPLC column. The bars in each column represent 100 µL, 150 µL and 200 µL, respectively. Each bar represents the average of 4 tests at each concentration.

sponse. The study was a preliminary one, and was performed on HEK293 cell line only, and thus it requires confirmation with the use of other cell lines. This includes fibroblasts, especially in terms of wound healing, for which silver carp skin-derived collagen extracts have already shown their effectiveness. However, the inhibitory effect on VEGF observed in this study is ambiguous. As some animal collagen peptides are already being clinically tested for use as angiogenic inhibitors to inhibit tumor cell growth, fish peptides might also be applied in a similar way. Due to the origin of the skin extract, stimulation of the hsp70.1 gene transcription caused by bacterial LPS should be excluded. However, the literature data show that the hsp70.1 protein occurs in well-healing wounds, so stimulation of hsp70.1 expression was expected. The effect of fish-derived collagen peptides on VEGF and heat shock proteins has not yet been examined.

This paper demonstrated that hydrophobic peptides are characterized by the greatest biological activity. This is favorable in the sense that silver carp skin-derived peptide separation using extraction with organic solvents, e.g. chloroform, leads to active peptides with some degree of hydrophobicity [10], which in turn allows the peptides to be concentrated in a simple manner for use in cosmetic and medicinal products. Another important characteristic of small peptides is their effective penetration into the dermis, which has been confirmed for fish peptides obtained by extraction with enzymes [30].

References

[1] **Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, Tanaka M:** Isolation and characterization of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). Food Chemistry 2005, 93, 475–262.

[2] **Tran KT, Griffith L, Wells A:** Extracellular matrix signaling through growth factor receptors during wound healing. Wound Repair Regen 2004, 12, 262–268.

[3] **Maquart FX, Pasco S, Ramont L, Hornebeck W, Monboisse JC:** An introduction to matrikines: Extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. Crit Rev Oncol Hematol 2004, 49, 199–202.

[4] **Desoize B:** Stromal reaction and tumour growth. Crit Rev Oncol Hematol 2004, 49, 173–176.

[5] **Chai HJ, Li JH, Huang HN, Li TL, Chan YL, Shiau CY, Wu CJ:** Effects of sizes and conformations of fish-scale collagen peptides on facial skin qualities and transdermal penetration efficiency. J Biomed Biotechnol 2010, 2010:757301. DOI: 10.1155/2010/757301. Epub 2010 Jun 8.

[6] **Maquart FX, Bellon G, Pasco S, Monboisse JC:** Matrikines in the regulation of extracellular matrix degradation. Biochimie 2005, 87, 353–360.

[7] **Fujioka K, Maeda M, Hojo T, Sano A:** Protein release from collagen matrices. Adv Drug Deliv Rev 1998, 31, 247–266.

[8] **Ahmed MR, Venkateshwarlu U, Jayakumar R:** Multilayered peptide incorporated collagen tubules for peripheral nerve repair. Biomaterials 2004, 25, 2585–2594.

[9] **Olsen D, Yang CL, Bodo M, Chang R, Leigh S, Baez J, Carmichael D, Perala M, Hamalainen ER, Jarvinen M:** Recombinant collagen and gelatin for drug delivery. Adv Drug Deliv Rev 2003, 55, 1547–1567. DOI: 10.1016/j.addr.2003.08.008.

[10] **Wojtkowiak D, Frydrychowski AF, Hadzik J, Dominiak M:** Identification of Small Peptides of Acidic Collagen Extracts from Silver Carp Skin and Their Therapeutic Relevance. Adv Clin Exp Med 2016, 25, 2, 227–235.

[11] **Xie K, Wei D, Shi Q, Huang S:** Constitutive and inducible expression and regulation of vascular endothelial growth factor. Cytokine Growth Factor Rev 2004, 15, 297–324.

[12] **Toi M, Bando H, Weich HA:** Vascular endothelial growth factor and its relationships with endogenous inhibitors in a breast cancer microenvironment manipulated by hormonal therapy: A hypothetical consideration. Biomed Pharmacother 2005, 59, Suppl 2, 344–347.

[13] **Liekens S, De Clercq E, Neyts J:** Angiogenesis: Regulators and clinical applications. Biochem Pharmacol 2001, 61, 253–270.

[14] **Dvorak H:** Angiogenesis: Update 2005. J Thromb Haemost 2005, 3, 1835–1842.

[15] **Loureiro RMB, D'Amore PA:** Transcriptional regulation of vascular endothelial growth factor in cancer. Cytokine Growth Factor Rev 2005, 16, 77–89.

[16] **Luo J, Xiong Y, Han X, Lu Y:** VEGF non-angiogenic functions in adult organ homeostasis: Therapeutic implications. J Mol Med (Berl) 2011, 89, 635–645. DOI: 10.1007/s00109-011-0739-1. Epub 2011 Mar 2.

[17] **Eichmann A, Simons M:** VEGF signaling inside vascular endothelial cells and beyond. Curr Opin Cell Biol 2012, 24, 188–193. DOI: 10.1016/j.ceb.2012.02.002. Epub 2012 Feb 25.

[18] **Cao Y:** Endogenous angiogenesis inhibitors and their therapeutic implications. Int J Biochem Cell Biol 2001, 33, 357–369.

[19] **Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK:** Novel signal transduction pathway utilized by extracellular HSP70: Role of TLR2 and TLR4. J Biol Chem 2002, 277, 15028–15034. Epub 2002 Feb 8.

[20] **Thériault JR, Mambula SS, Sawamura T, Stevenson MA, Calderwood SK:** Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS Lett 2005, 579, 1951–1960.

[21] Laplante AF, Moulin V, Auger FA, Landry J, Li H, Morrow G, Tanguay RM, Germain L: Expression of heat shock proteins in mouse skin during wound healing. J Histochem Cytochem 1998, 46, 1291–1301.
[22] Oberringer M, Baum HP, Jung V, Welter C, Frank J, Kuhlmann M, Mutschler W, Hanselmann RG: Differential expression of heat shock protein 70 in well healing and chronic human wound tissue. Biochem Biophys Res Commun 1995, 214, 1009–1014.
[23] Helbig D, Bodendorf M, Anderegg U, Simon JC, Paasch U: A human skin explant model to study molecular changes in response to fractional photothermolysis: Spatio-temporal expression of HSP70, Medical Laser Application 01/2010; 25, 173–180. DOI: 10.1016/j.mla.2009.12.002.
[24] Mushtaq S, Naqvi ZA, Siddiqui AA, Ahmed N: Albumin precursor and Hsp70 modulate corneal wound healing in an organ culture model. Acta Histochem 2011, 113, 36–42. DOI: 10.1016/j.acthis.2009.07.009. Epub 2009 Sep 9.
[25] Ko JA, Yanai R, Quan WY, Morishige N, Nishida T: Up-regulation of HSP-70 by the fibronectin-derived peptide PHSRN in human corneal epithelial cells. Biochem Biophys Res Commun 2008, 370, 424–428. DOI: 10.1016/j.bbrc.2008.03.093. Epub 2008 Mar 27.
[26] Yao Y, Zhang G, Zhang Y, Li W, Wang C, Yin C, Zhang F: Lipopolysaccharide pretreatment protects against ischemia/reperfusion injury via increase of HS70 and inhibition of NF-κB. Cell Stress Chaperones 2011, 16, 287–296. DOI: 10.1007/s12192-010-0242-6
[27] Dokladny K, Lobb R, Wharton W, Ma TY, Moseley PL: LPS-induced cytokine levels are repressed by elevated expression of HSP70 in rats: Possible role of NF-κB, Cell Stress Chaperones 2010, 15, 153–163. DOI: 10.1007/s12192-009-0129-6. Epub 2009 Jun 24.
[28] Dulak J, Tomala K, Loboda A, Józkowicz A: Nitric oxide-dependent synthesis of vascular endothelial growth factor is impaired by high glucose. Life Sci 2004, 75, 2573–2586.
[29] Grzenkowicz-Wydra J, Cisowski J, Nakonieczna J, Zarebski A, Udilova N, Nohl H, Józkowicz A, Podhajska A, Dulak J: Gene transfer of CuZn superoxide dismutase enhances the synthesis of vascular endothelial growth factor. Mol Cell Biochem 2004, 264, 169–181.

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