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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, 385–392**).

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 application seems to be biomaterial. Using collagen as a biomaterial for tissue regeneration introduces some disadvantages vs. 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 III a1, 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 Wroclaw Medical University , 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 and 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 2 programs were used: the R statistical package for Windows and MedCalc for Windows.

Results

The Effect of Experimental Collagen Formulation Treatment on Fibroblasts

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 (Fig. 3, 4). 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. 3: B, K, N). 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 sources of active collagen. In literature there

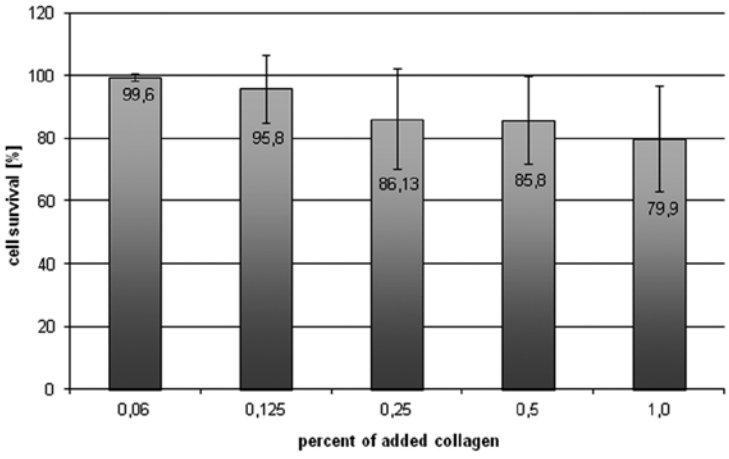

Fig. 1. The effect of fish collagen formulation treatment on the proliferation of fibroblasts was evaluated by the MTT assay. Bars show the mean of four replicates. Error bars indicate standard deviations

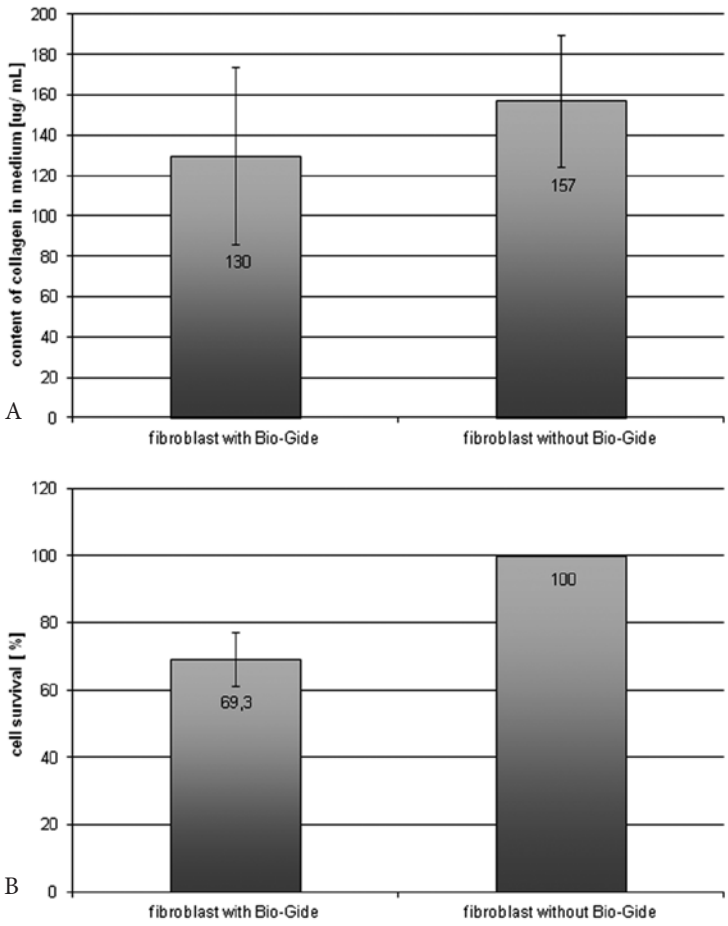

Fig. 2. The biological effects of Bio-Gide® on fibroblasts; A – the induction of production of endogenous collagen; B – the cytotoxicity evaluated by MTT assay

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].

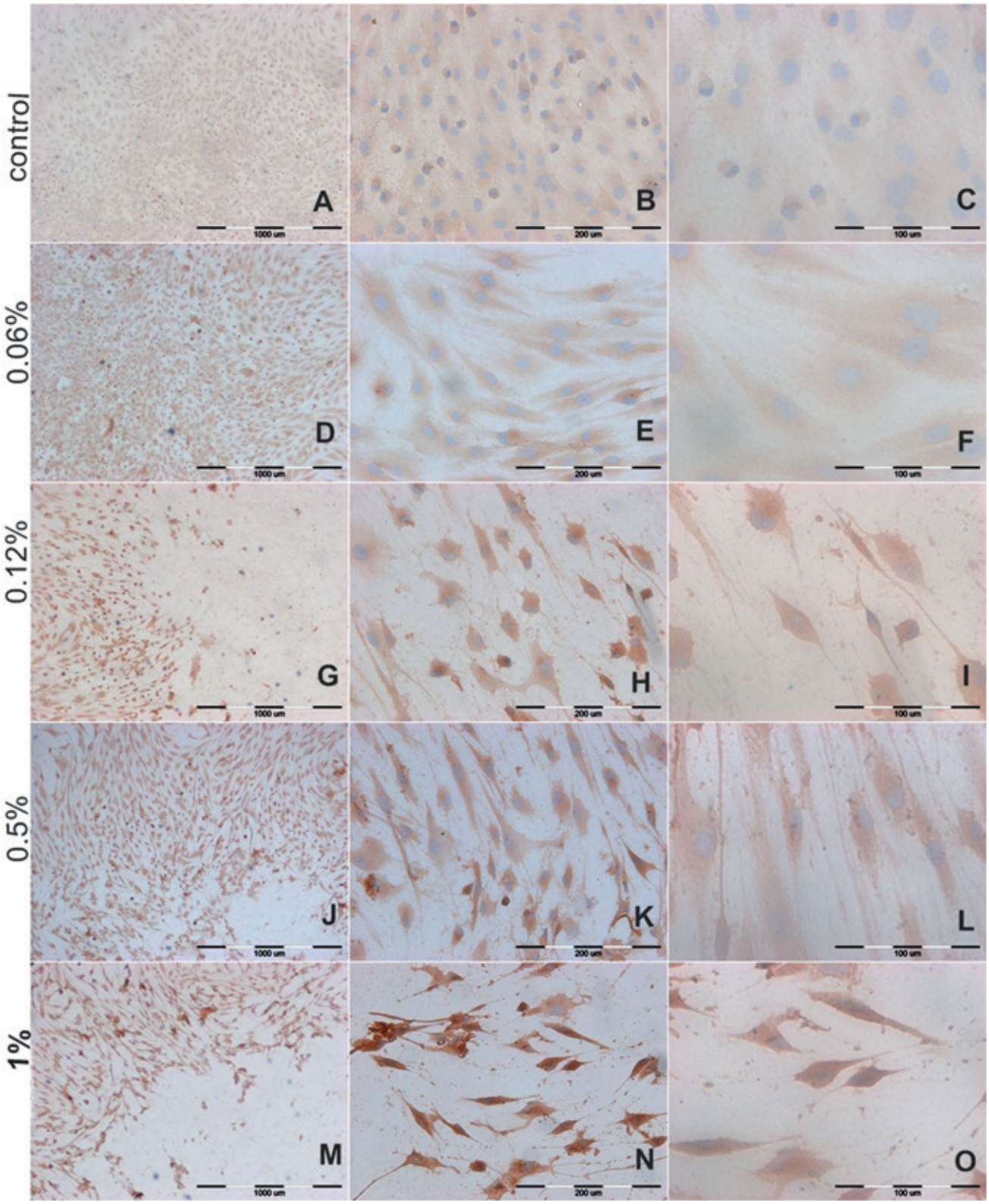

Fig. 3. Photos of fibroblast with immunocytochemically stained collagen type I. On panels A, B, C control cells are shown; on panels D, E, F are the cells cultured in the presence of 0.06% fish collagen; on panels G, H, I 0.12% respectively; in panels J, K, L – 0.5%; in panels M, N, O – 1%. Magnification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

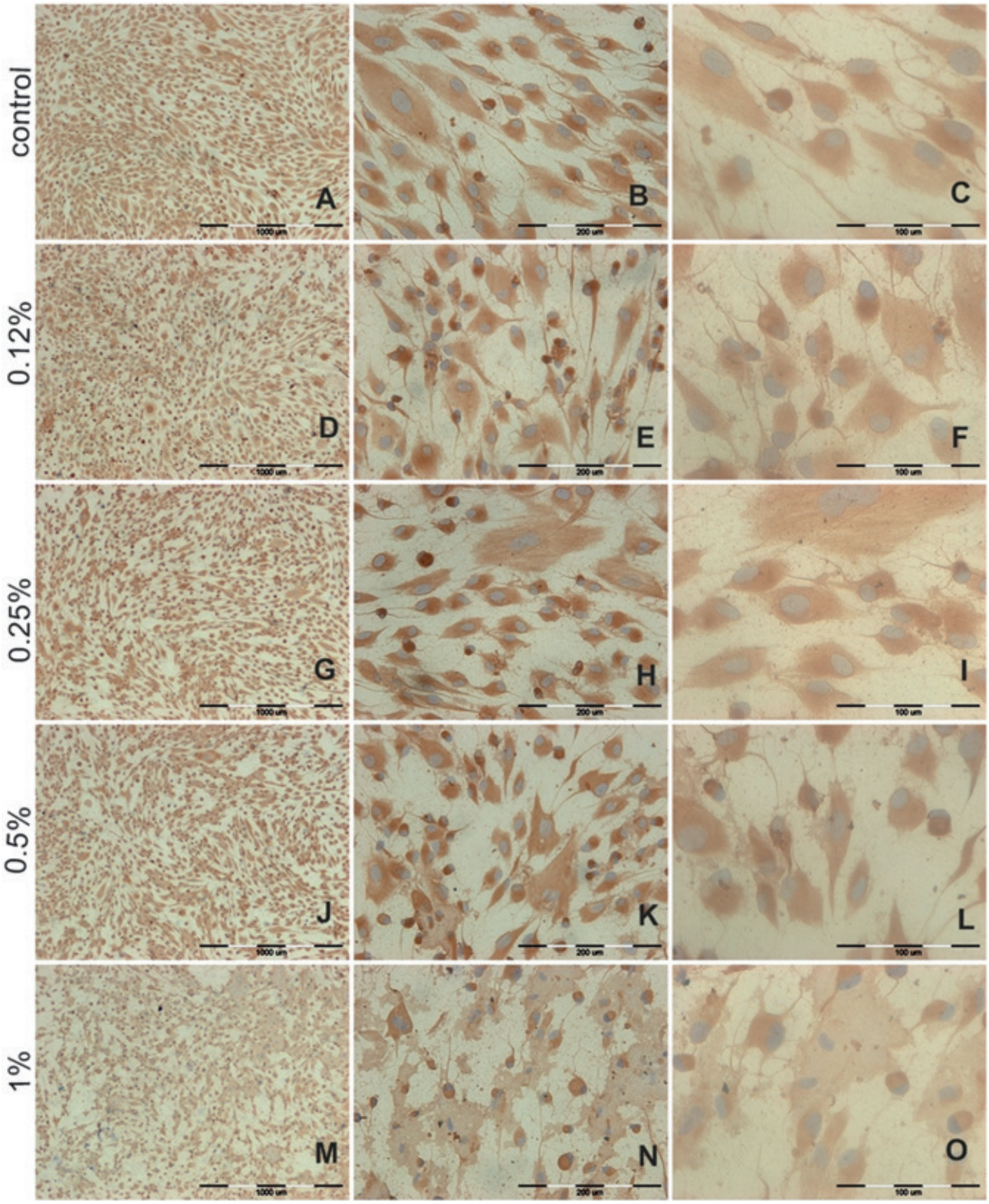

Fig. 4. Photos of fibroblast with immunocytochemically stained collagen type III. On panels A, B, C control cells are shown; on panels D, E, F are the cells cultured in the presence of 0.12% fish collagen; on panels G, H, I 0.25% respectively; in panels J, K, L – 0.5%; in panels M, N, O – 1%. Magnification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

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