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EVALUATION OF GENISTEIN ABILITY TO MODULATE CTGF mRNA/PROTEIN EXPRESSION, GENES EXPRESSION OF TGFβ ISOFORMS AND EXPRESSION OF SELECTED GENES REGULATING CELL CYCLE IN KELOID FIBROBLASTS *IN VITRO*

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Abstract: Keloids are characterized by overgrowth of connective tissue in the skin that arises as a consequence of abnormal wound healing. Normal wound healing is regulated by a complex set of interactions within a network of profibrotic and antifibrotic cytokines that regulate new extracellular matrix (ECM) synthesis and remodeling. These proteins include transforming growth factor β (TGFβ) isoforms and connective tissue growth factor (CTGF). TGFβ1 stimulates fibroblasts to synthesize and contract ECM and acts as a central mediator of profibrotic response. CTGF is induced by TGFβ1 and is considered a downstream mediator of TGFβ1 action in fibroblasts. CTGF plays a crucial role in keloid pathogenesis by promoting prolonged collagen synthesis and deposition and as a consequence sustained fibrotic response. During keloids formation, besides imbalanced ECM synthesis and degradation, fibroblast proliferation and it's resistance to apoptosis is observed. Key genes that may play a role in keloid formation and growth involve: suppressor gene p53, cyclin-dependent kinase inhibitor CDKN1A (p21) and BCL2 family genes: antiapoptotic BCL-2 and proapoptotic BAX. Genistein (4',5,7-trihydroxyisoflavone) exhibits multidirectional biological action. The concentration of genistein is relatively high in soybean. Genistein has been shown as effective antioxidant and chemopreventive agent. Genistein can bind to estrogen receptors (ERs) and modulate estrogen action due to its structure similarity to human estrogens. Genistein also inhibits transcription factors NFκB, Akt and AP-1 signaling pathways, that are important for cytokines expression and cell proliferation, differentiation, survival and apoptosis. The aim of the study was to investigate genistein as a potential inhibitor of CTGF and TGFβ1, β2 and β3 isoforms expression and a potential regulator of p53, CDKN1A (p21), BAX and BCL-2 expression in normal fibroblasts and fibroblasts derived from keloids cultured *in vitro*. Real time RT-QPCR was used to estimate transcription level of selected genes in normal and keloid fibroblasts treated with genistein. Secreted/cell-associated *CTGF* protein was evaluated in cell growth's medium by ELISA. Total protein quantification was evaluated by fluorimetric assay in cells lysates (Quant-iT TM Protein Assay Kit). It was found that TGFβ1, β2 and β3 genes expression are decreased by genistein. Genistein suppresses the expression of CTGF mRNA and CTGF protein in a concentration dependent manner. p53 and p21 genes expression are modulated by genistein in concentration dependent manner. The agent also modulates BAX/BCL-2 ratio in examined cells *in vitro*.

Keywords: keloids, fibroproliferative disorders, genistein, connective tissue growth factor, transforming growth factor, cell cycle

Keloid disease is an abnormal cutaneous fibroproliferative disorder of unknown etiopathogenesis. Keloids present as benign fibroproliferative tumors are considered to be scars that occur when full thickness wounds heal abnormally (1–6). The molecular abnormalities in keloids that correlate with molecular mechanisms in normal wound healing can be categorized into the synthesis and degradation of extracellular matrix (ECM) components, cytokines and growth factors, and apoptotic pathways (6, 7). ECM deposition in any tissue is regulated by a balance between synthesis and degradation of individual components (8). The major component of keloids ECM is mainly either type III collagen (early) or type I collagen (late) because keloid is a type of scar which depends on its maturity (5, 6).

The synthesis of type I collagen is highly regulated by different cytokines at transcriptional level. Especially, isoforms β1 and β2 of transforming growth factor (TGFβ1 and TGFβ2), members of TGFβ family, enhance type I collagen gene expression. Moreover, they prevent collagen degradation

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by stimulating production of protease inhibitors (9). In contrast, TGFβ3, which is predominantly induced in the later stages of wound healing, has been found to reduce connective tissue deposition (10). Connective tissue growth factor (CTGF) is a cysteine-rich protein (CCN2) induced by TGFβ1 in connective tissue cells e.g., keloid fibroblasts which are implicated as mediators of elevated ECM deposition (11, 12). Elevated CTGF expression causes cell activation and tissue fibrosis (13–19). Sustained CTGF *s*ynthesis might be responsible for maintenance of the fibrotic phenotype in keloids (11, 16, 18, 19).

During keloids formation, besides imbalanced ECM synthesis and degradation, fibroblast proliferation and it's resistance to apoptosis is observed (2, 20–23). Key genes that may play a role in keloid formation and growth involve: suppressor gene p53, cyclin-dependent kinase inhibitor p21 (CDKN1A) and BCL2 family genes: antiapoptotic BCL-2 and proapoptotic BAX. The most crucial gene in the network of pathways governing cell's division and apoptosis is p53. The p53 tumor suppressor functions as transcription factor, by inducing or repressing different genes. It can also act as direct inducer of apoptosis by translocation into mitochondria (24). The p21 (also known as $p21^{WAF1/Cip1}$) is a negative regulator of cell divisions. It inhibits the kinase activity of the cyclin-dependent kinases (CDKs) CDK2 and CDK1 leading to growth arrest at specific stages in the cell cycle. In addition, by binding to proliferating cell nuclear antigen (PCNA), p21 interferes with PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication and modulating various PCNA-dependent DNA repair processes (25). BCL2 family genes are involved in intrinsic, mitochondrial pathway of apoptosis. In response to stress activation, BAX forms a homodimer and releases cytochrome c from the mitochondria, which results in caspase-9 activation and apoptosis. BCL-2 homodimers play opposite, protective role by stabilization of mitochondrial membrane (26).

Keloids are important problem in dermatology and esthetic dermatosurgery. In spite of long-lasting studies on keloid formation, there is no one satisfactory method to be successful in keloid prevention and treatment (14, 27, 28). There are some interesting perspectives with the use of genistein, which possesses antiproliferative and proapoptotic properties in many human cancer cell lines (29–36).

Genistein is a natural component of the plant belonging to isoflavones class. Soybean is the most abundant source of naturally occurring genistein. Genistein (4',5,7-trihydroxyisoflavone) exhibits antioxidant properties (37–40) and multidirectional action at the molecular level including i.a., estrogenic and antiestrogenic activity; inhibition of: protein tyrosine kinases (PTKs), topoisomerase II, phosphatidylinositol turnover and proteins involved in multidrug resistance of cancer cells. Genistein also interacts with other potential cellular target proteins (36, 41–50). Multidirectional molecular action of genistein led to its use as a modulator of cell proliferation, apoptosis, differentiation and cell cycle progression, particularly cancer cells. Recent report has shown that genistein is a potent cancer chemopreventive agent (51).

The aim of the study was to investigate genistein as a potential modulator of CTGF gene expression (mRNA and protein expression) and TGFβ1, β2 and β3 genes expression (mRNAs expressions) and was to enquire whether genistein exerts antiproliferative or apoptotic activity through effect on p53, p21 (CDKN1A), BCL-2 and BAX genes expression in keloid fibroblast and normal dermal fibroblasts cultured *in vitro*.

EXPERIMENTAL

Cell culture

Normal human dermal fibroblasts line (NHDF, Adult) was obtained from the tissue culture collection of Clonetics® (Lonza Walkersville Inc., Walkersville, MD, USA). Keloid fibroblasts line (KEL FIB) was obtained from the tissue culture collection of ATCC® (LGC Standards, Teddingtin, UK). Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and fetal bovine serum (FBS) were purchased from ATCC® (LGC Standards, Teddington, UK). Antibiotic (penicillin-streptomycin solution), Dulbecco's phosphate buffered saline without Mg^{2+} and Ca^{2+} and 1x Trypsin with EDTA were purchased from Sigma Aldrich (St. Louis, USA). Nunc EasYFlasks with filter 25 cm^2 culture surface area was purchased from Thermo Scientific Nunc® (Roskilde, Denmark); 96-wells microplates and other plastic materials were purchased from Sarstedt (Nümbrecht, Germany).

Normal human dermal fibroblasts (NHDF) and keloid fibroblasts (KEL FIB) were cultured in DMEM with L-glutamine supplemented with 10% FBS and 1% pen/strep solution (100 U/mL penicillin, 200 μg/mL streptomycin). Cells were maintained at 37°C and 5% CO_2 atmosphere in a humidified incubator. At confluence, cells were routinely passaged. Cells from passages 4 to 6 were used in the study. For the quantitative determination of

examined mRNAs expression, the cells were seeded at the density of 5×10^5 per 5 mL medium in 25 cm^2 surface area culture flasks. For the quantitative determination of CTGF protein expression and total protein quantity, the cells were seeded at the density of 5×10^3/well per 300 µL medium in a 96-wells microplate. After 24 h, when cells adhered to the bottom of wells and started growing, the media were changed to media containing genistein.

Preparation of genistein dilutions

Genistein from *Glycine max* (soybean) used in the study was supplied by Sigma Aldrich® (cat. no. G6776). Genistein was dissolved in dimethyl sulfoxide (DMSO) as 1% stock solution (concentration of genistein 37 mM) and diluted in the growth medium (DMEM) with fetal bovine serum (FBS) and antibiotics for normal dermal fibroblast and keloid fibroblast cultures. Genistein in: 370, 185, 92.5, 37, 18.5, 3.7, 1.85, 0.37 µM and 0 µM (control) concentrations was used in the study to evaluate CTGF mRNA/protein expression and genes expression of TGFβ isoforms. For evaluation of the effect of soybean genistein on the expression of selected genes regulating cell cycle genistein in: 370, 37, 3.7, 0.37 µM and 0 µM (control) concentrations was used.

The expression of selected genes regulating cell cycle (p53, p21, BCL-2, BAX) and CTGF, TGFβ1, β2, β3 isoforms mRNAs expression in NHDF and KEL FIB cells treated with genistein

For the analysis of selected mRNAs expression, NHDF and KEL FIB cells were seeded at the density of 5×10^5 per 5 mL medium in 25 cm^2 surface area culture flasks. After 24 h of incubation under standard conditions (37°C, 5% carbon dioxide and 95% humidity), DMEM with L-glutamine, 10% FBS and 1% pen/strep in fibroblasts cultures were changed to proper mediums supplemented with genistein. Cells were treated with genistein for 72 h under standard conditions. Control groups of cells constituted NHDF and KEL FIB cells cultured 72 h in standard culture media described above.

RNA extraction and real-time RT-QPCR assay

Total RNA was extracted from control cell cultures and cell cultures incubated with genistein with the use of TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration was determined spectrophotometrically, on the basis of absorbance values at the wavelength of 260 nm, using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, USA). All RNA extracts were treated with DNase I (MBI Fermentas) according to the manufacturer's instruction.

Transcriptional activity of p53, p21, BCL-2, BAX, CTGF, TGFβ1, β2, β3 genes was evaluated by the use of real-time RT-QPCR technique with a SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, QIAGEN, Valencia, CA, USA). The analysis was carried out using an Opticon™ DNA Engine Sequence Detector (MJ Research, USA). Real-time RT-PCR assay was performed in triplicate for each sample. The thermal cycling conditions for one-step RT-QPCR assay were as follows: 50°C for 30 min for reverse transcription, 95°C for 15 min followed by 45 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 10 min.

mRNA (RT-QPCR) quantitative detection of CTGF, p53, p21, BCL-2, BAX were conducted on the basis of primer pairs designed for reference sequences (Gene Bank www.ncbi.nlm.nih.gov) with the following sequences:

CTGF mRNA: 5'-GTgACgAgCCCAAggACCAAACCT-3' (forward),
5'-TggACCAggCAgTTggCTCTAATCATAgTTg-3' (reverse);
p53 mRNA: 5'-TAACAgTTCCTgCATgggCggC-3' (forward),
5'-AggACAggCACAAACACg CACC-3' (reverse);
p21 mRNA: 5'-CACTCCAAACgC CggCTgATCTTC-3' (forward),
5'-TgTAgAgCgggCCTTTgAggCCCTC-3' (reverse);
BCL-2 mRNA: 5'-TTgTggCCTTCTTTgAgTTCggTg-3' (forward),
5'-ggTgCCggTTC AggTACTCAgTCA-3' (reverse);
BAX mRNA: 5'- CCTgTgCACCAA ggTgCCggAACT-3' (forward),
5'-CCACCCTggTCT TggATCCAgCCC-3' (reverse).

mRNA (RT-QPCR) quantitative detection of TGFβ1, β2 and β3 isoforms was conducted based on the published sequences (52).

The generally used housekeeping genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a potential, accessible endogenous controls for experimental conditions. The PCR primers for β-actin and GAPDH were purchased from PE Applied Biosystems (PE Applied Biosystems, Inc., Foster, CA, USA).

The mRNA copy numbers of examined genes were obtained by amplifying commercially available standard of β-actin cDNA (TaqMan® DNA Template Reagents Kit; PE Applied Biosystems, Inc., Foster, CA, USA). The control genes were amplified in triplicate under conditions applied for the target genes.

Following RT-PCR, the samples were subjected to temperature ramp from 60 to 95°C at the rate of 0.2°C/s with continuous fluorescence monitoring for melting curve analysis.

Specificity of real-time RT-QPCR reactions for analyzed genes was experimentally confirmed by 8% polyacrylamide gel electrophoresis and determination of the amplimer melting temperatures. No primer-dimers or unspecific by-products were generated during applied RT-QPCR amplification cycles.

Finally, specificity of RT-PCR reaction was confirmed by determining characteristic temperature of melting for each amplimer (p53 = 81.8°C; p21 = 83.3°C, BCL-2 = 82.6°C; BAX = 82.0°C; CTGF = 82.7°C; TGFβ1 = 85.5°C; TGFβ2 = 80.4°C; TGFβ3 = 80.8°C) and by 8% polyacrylamide gel electrophoresis of RT-PCR products with their visualization using silver staining (121 base pair (bp) for p53 amplimer, 101 bp for p21 amplimer, 114 bp for BCL-2 amplimer, 99 bp for BAX amplimer, 109 bp for CTGF amplimer, 152 bp for TGFβ1 amplimer, 201 bp for TGFβ2 amplimer and 121 bp for TGFβ3 amplimer).

Enzyme-linked immunosorbent assay (ELISA) quantitative measurement of CTGF protein

CTGF protein expression was measured by ELISA in cell culture media (NHDF and KEL FIB) according to the manufacturer's protocol (USCN Life Science Inc., Wuhan, P.R. China). The kit is a sandwich enzyme immunoassay for *in vitro* quantitative measurement of CTGF protein in human serum, plasma, cell culture medium and other biological fluids. ELISA was performed in triplicate for each sample and in duplicate for each standard and control. Optical density readings of each well (standards and samples) were taken at 450 ± 10 nm on the Asys UVM 340 Microplate Reader (Biogenet). The concentration of CTGF protein in the samples was determined by comparing the O.D. of the samples to the standard curve. The standard curve concentrations used for the ELISA's were: 30, 15, 7.5, 3.75, 1.88, 0.94 and 0.47 ng/mL. The minimum detectable concentration of human CTGF protein is typically less than 0.21 ng/mL.

The total protein concentration measurement in cell cultures

Total protein was extracted from control cells and cells treated with genistein (NHDF and KEL FIB) with the use of CellLytic™M kit (Sigma Aldrich®, Saint Louis, MO, USA) following the protocol of manufacturer. Total protein concentration (μg/mL) was determined in triplicate using a Qubit protein assay kit (no. Q3321; Invitrogen) on a Qubit 2.0 fluorometer (Invitrogen).

Statistical analysis

Statistical analysis was carried out using Statistica PL 8.0 software. The Shapiro-Wilk test for data normality was applied. Statistical analysis of the data showed their normality, therefore all the results are represented as the means ± SD. The one-way ANOVA followed by *post hoc* Dunnett's test was used to determine the significant differences between a control group (cells untreated with genistein) mean and the remaining genistein treatment group means in an analysis of variance setting. The difference between control cells (NHDF *vs*. KEL FIB) was analyzed using the Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase GAPDH as potential candidates for endogenous control

The generally used housekeeping genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as potential, accessible endogenous controls for experimental conditions. The transcriptional activity of β-actin (*ACTB*) and GAPDH genes were detected in both cell lines (NHDF and KEL FIB) untreated genistein.

The expression of β-actin gene exhibited significant differences between normal and keloid fibroblasts ($p = 0.004$, Student's *t*-test) and was lower in NHDF cells ($3.44 \times 10^7 \pm 4.81 \times 10^6$ copy number of mRNA per 1 μg of total RNA) compared to KEL FIB cells ($4.12 \times 10^7 \pm 2.03 \times 10^6$ copy number of mRNA per 1 μg of total RNA). Statistical analysis of the experimental data with the use of ANOVA revealed statistically significant differences ($p < 0.05$) in β-actin gene expression between control cells and cells treated with genistein, similarly as described previously (53). Moreover, expression of β-actin mRNAs changed, depending on genistein concentration, which excludes its application as endogenous control that might serve to normalize expression results of examined genes (54).

The expression of GAPDH mRNA exhibited no significant differences between normal and keloid fibroblasts ($p = 0.272087$, Student's *t*-test) and was lower in KEL FIB cells ($4.16 \times 10^4 \pm 3.5 \times 10^3$ copy number of mRNA per 1 μg of total RNA) compared to NHDF cells ($1.94 \times 10^5 \pm 2.04 \times 10^4$

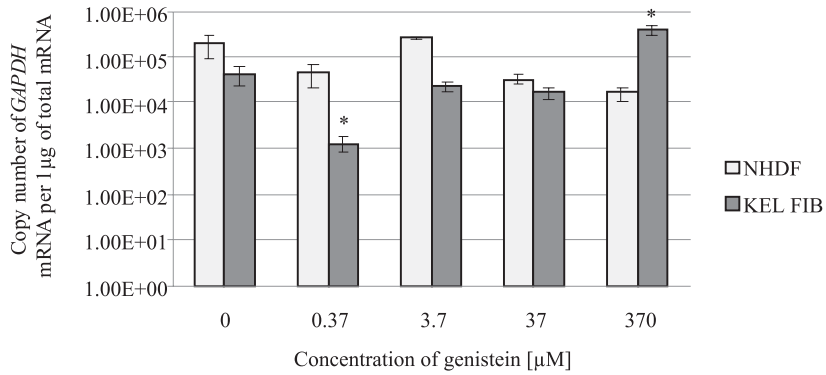

Figure 1. Comparison of the copy number of GAPDH mRNAs per 1 µg of total RNA in NHDF/KEL FIB cells treated with various genistein concentration *vs*. respective control NHDF/KEL FIB cells. * $p < 0.01$

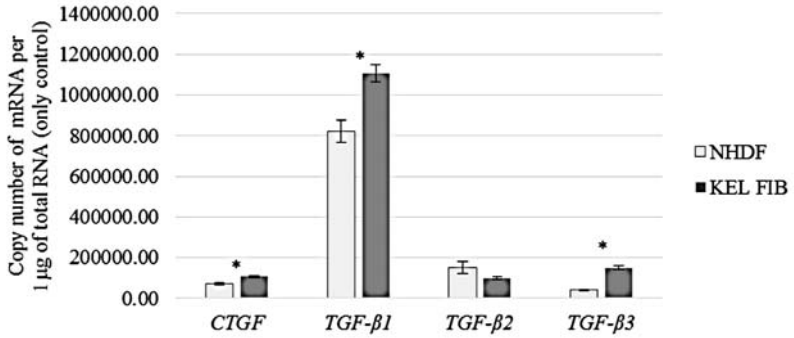

Figure 2. The native expression of *CTGF*, *TGFβ1*, *β2* and *β3* isoforms in NHDF control cells and KEL FIB control cells. * $p < 0.05$ (comparison between control NHDF cells and KEL FIB cells)

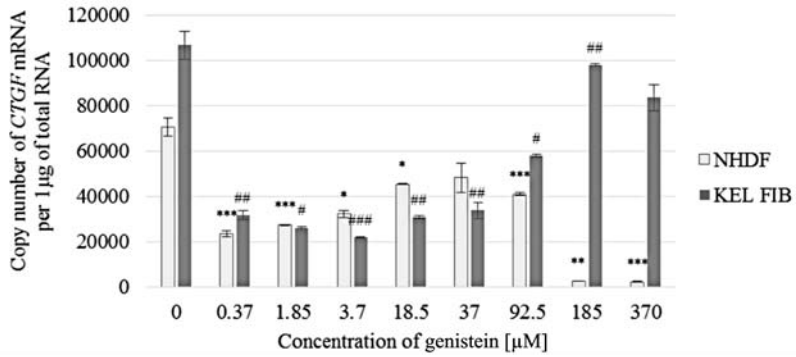

Figure 3. Expression of *CTGF* mRNA in NHDF cells and KEL FIB cells determined by real-time PCR. Comparison of the copy number of *CTGF* mRNAs per 1 µg of total RNA in NHDF cells treated with various genistein concentration *vs*. control NHDF cells (*p) and in KEL FIB cells treated with various genistein concentration *vs*. control KEL FIB cells (#p). */# $p < 0.01$, **/## $p < 0.001$, ***/### $p < 0.0001$ (*post hoc* Dunnett's test)

copy number of mRNA per 1 µg of total RNA) (Fig. 1). Statistical analysis of the experimental data revealed no statistically significant differences in GAPDH mRNA expression between NHDF control cells and NDHF cells treated with genistein ($p = 0.39094$, ANOVA). Statistical analysis of the experimental data with the use of ANOVA revealed statistically significant differences in GAPDH gene expression between KEL FIB control cells and KEL FIB cells treated with genistein ($p = 0.000022$, ANOVA). Expression of GAPDH mRNA in KEL FIB cells exposed to genistein in concentration 0.37 and 370 µM compared to control KEL FIB cells showed statistical significance ($p = 0.00059$ and $p =$

0.005634, respectively; *post hoc* Dunnett's test) (Fig.1).

The results of the statistical analysis showed no possibility of using *GAPDH* as endogenous control to normalize the results of examined genes expression.

The native mRNA expression of CTGF, TGFβ1, β2 and β3 isoforms in NHDF control cells and KEL FIB control cells

The transcriptional activity of all examined genes was detected in unstimulated (control) NDHF cells and KEL FIB cells. The expression of CTGF mRNA exhibited significant differences between normal and keloid fibroblasts ($p = 0.0154$, Student's *t*-test) and was lower in NHDF cells ($7.06 \times 10^4 \pm 7.92 \times 10^3$ copy number of mRNA per 1 μg of total RNA) compared to KEL FIB cells ($1.07 \times 10^5 \pm 1.26 \times 10^4$ copy number of mRNA per 1 μg of total RNA). Likewise, statistical analysis revealed significant differences in *TGFβ1* mRNA ($p = 0.0106$, Student's *t*-test) and *TGFβ3* mRNA ($p < 0.0001$, Student's *t*-test) between normal and keloid fibroblasts. The expression of *TGFβ1* mRNA and *TGFβ3* mRNA was lower in NHDF cells ($8.21 \times 10^5 \pm 8.67 \times 10^4$ and $4.0 \times 10^4 \pm 7.04 \times 10^3$ copy number of mRNA per 1 μg of total RNA, respectively), compared to KEL FIB cells ($1.11 \times 10^6 \pm 1.03 \times 10^5$ and $1.49 \times 10^5 \pm 1.87 \times 10^4$ copy number of mRNA per 1 μg of total RNA, respectively). No marked changes in *TGFβ2* mRNA were demonstrated between control NHDF cells ($1.50 \times 10^5 \pm 5.99 \times 10^4$ copy number of mRNA per 1 μg of total RNA) and control KEL FIB cells ($9.96 \times 10^4 \pm 1.36 \times 10^4$ copy

Table 1. The results of the statistical analysis (*post hoc* Dunnett's test) used to determine the differences between TGFβ1, β2, β3 mRNAs expression between control cells (cells untreated genistein) and cells treated with genistein in NHDF cells.

Genistein concentration [μM]	The results of *post hoc* Dunnett's test presented as *p* value		
	TGFβ1	**TGFβ2**	**TGFβ3**
0.37	0.000007	0.000005	0.000007
1.85	0.000005	0.012149	0.000416
3.7	0.000014	0.005199	0.007349
18.5	0.000023	0.000423	0.000246
37	0.000006	0.000006	0.000005
92.5	0.000044	0.000237	0.000124
185	0.000007	0.000042	0.000336
370	0.000005	0.000005	0.000006

Table 2. The results of the statistical analysis (*post hoc* Dunnett's test) used to determine the differences between TGFβ1, β2, β3 mRNAs expression between control cells (cells untreated with genistein) and cells treated with genistein in KEL FIB cell cultures.

Genistein concentration [μM]	The results of *post hoc* Dunnett's test presented as *p* value		
	TGFβ1	**TGFβ2**	**TGFβ3**
0.37	0.000006	0.000005	0.000005
1.85	0.000005	0.000005	0.000005
3.7	0.000006	0.000005	0.000005
18.5	0.000005	0.000005	0.000005
37	0.000005	0.000005	0.000005
92.5	0.000005	0.000005	0.000005
185	0.000005	0.000005	0.000005
370	0.000009	0.000005	0.000005

number of mRNA per 1 µg of total RNA) ($p > 0.05$, Student's *t*-test) (Fig. 2).

CTGF mRNA expression in NHDF and KEL FIB exposed to genistein

Treatment of NHDF cells and KEL FIB cells with genistein in each applied concentration resulted in downregulation of CTGF mRNA expression as compared with untreated cells in both lines ($p = 0.000004$, $p = 0.001009$, respectively; ANOVA) (Fig. 3).

Statistical analysis revealed no significant decrease in CTGF mRNA expression in NHDF cells exposed to genistein in concentration of 37 µM ($p = 0.064446$, *post hoc* Dunnett's test) (Fig. 3) and in KEL FIB cells exposed to genistein in concentration 370 µM ($p = 0.992769$, *post hoc* Dunnett's test) compared to the respective control cells, only. The other concentration of genistein showed significant differences compared to control NHDF cells and control KEL FIB cells, respectively (Fig. 3).

CTGF protein expression in NHDF and KEL FIB exposed to genistein

Statistical analysis revealed no significance differences between obtained CTGF protein concentration (ng/mL) (Fig. 4a) and CTGF protein concentration recalculated *per* total amount of protein in the cell lysates (Fig. 4b) ($p > 0.05$, Student's *t*-test), in both cells lines (NHDF and KEL FIB).

The expression of CTGF protein exhibited significant differences between normal and keloid fibroblasts ($p = 0.00001$, Student's *t*-test) and was about 5 times lower in NHDF cells (3.511 ± 1.046 ng/µL) compared to KEL FIB cells (26.133 ± 2.202 ng/µL).

Exposure of NHDF cells to genistein in each applied concentration resulted in downregulation of CTGF protein expression as compared with untreated NHDF cells ($p = 0.0261$, ANOVA). Statistical analysis revealed significant decrease in the CTGF protein expression in NHDF cells exposed to genistein in concentration 1.85 µM ($p = 0.024373$, *post*

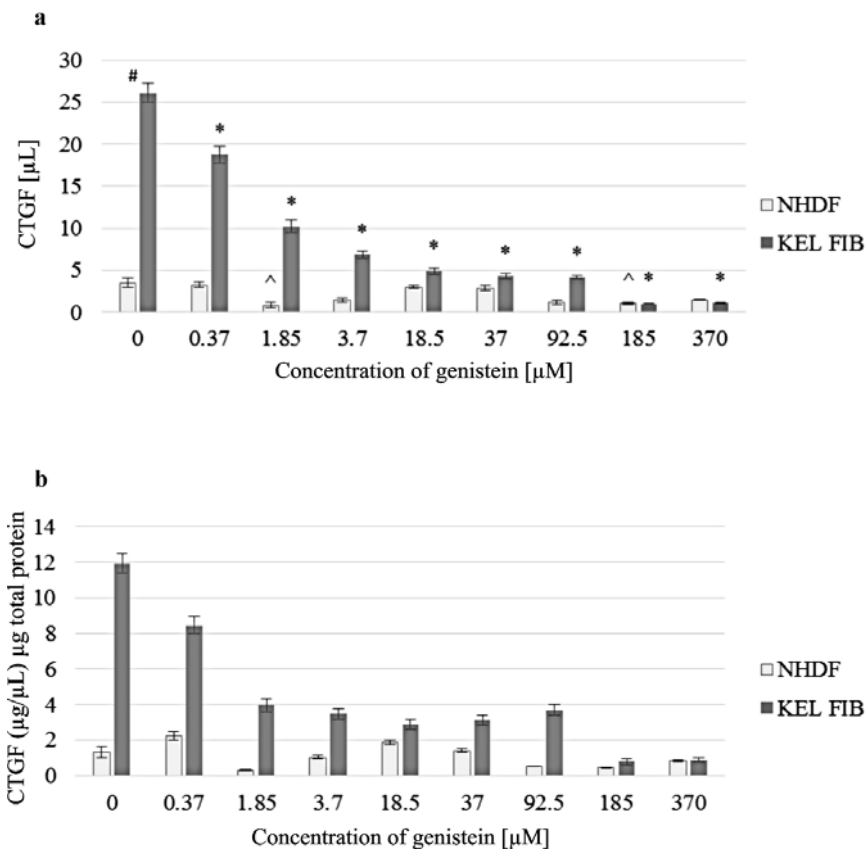

Figure 4. Expression of CTGF protein in NHDF cells and KEL FIB cells determined by ELISA. **a** Comparison of CTGF protein (µg/µL) between: control normal dermal fibroblasts (NHDF) and control keloid fibroblasts (KEL FIB) (# $p < 0.05$, Student's *t*-test), NHDF control cells and genistein treated NHDF cells (^ $p < 0.05$, Dunnett's test) and KEL FIB control cells and genistein treated KEL FIB cells (* $p < 0.00001$, Dunnett's test). **b** Comparison of CTGF protein (µg/µL) and CTGF protein (µg/µL) recalculated per µg of total protein (in each concentration $p > 0.05$, Student *t*-test)

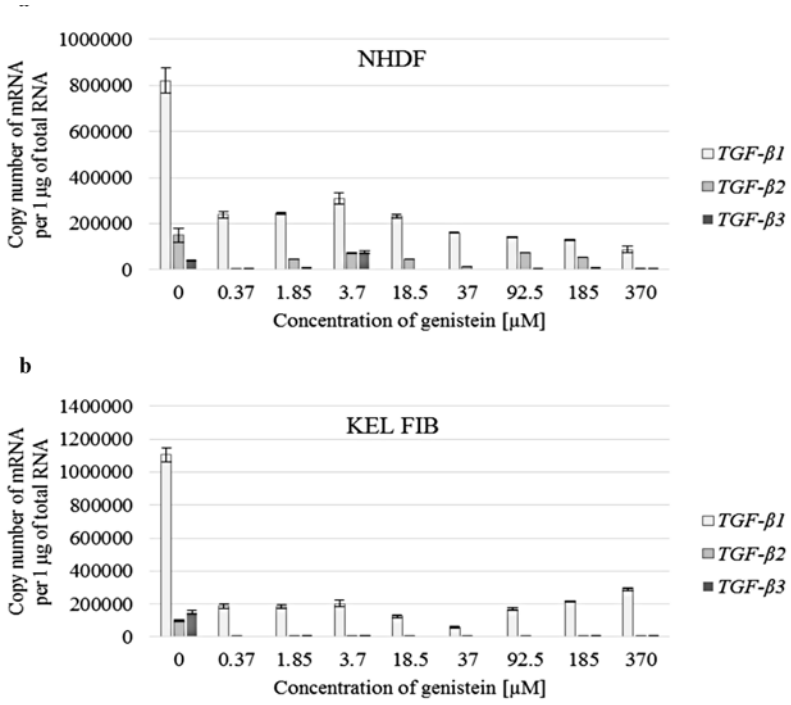

Figure 5. Expression of TGFβ1, β2, β3 mRNAs in cells treated with genistein determined by real-time PCR **a** – NHDF cells, **b** – KEL FIB cells

hoc Dunnett's test) and 185 µM ($p = 0.040428$, *post hoc* Dunnett's test), compared to NHDF control cells (Fig. 4a).

Similarly, genistein treatment of KEL FIB cells resulted in downregulation of CTGF protein expression as compared with untreated, control cells ($p < 0.0001$, ANOVA). Statistical analysis revealed significant decrease in the CTGF protein expression in KEL FIB cells exposed to genistein in each applied concentration, compared to control cells (Fig. 4a).

TGFβ1, β2, β3 mRNA isoforms expression in NHDF cells and KEL FIB exposed to genistein

Treatment of NHDF cells with genistein in each applied concentration resulted in downregulation of: TGFβ1 mRNA expression ($p < 0.000001$, ANOVA) and TGFβ2 mRNA expression ($p = 0.041047$, ANOVA), as compared with untreated control cells. Genistein downregulated TGFβ3 mRNA expression in NHDF cells ($p < 0.000001$, ANOVA), except for cells treated with 3.7 µM genistein ($7.5 \times 10^4 \pm 1.31 \times 10^4$ copy number of mRNA per 1 µg of total RNA) where an increase of expression was noted, as compared with untreated control cells ($4.00 \times 10^4 \pm 7.04 \times 10^3$ copy number of mRNA per 1 µg of total RNA) (Fig. 5a).

Statistical analysis (*post hoc* Dunnett's test) revealed significant differences in TGFβ1, β2, β3 mRNAs expression in NHDF cells exposed to various concentration of genistein compared to the untreated control cells (Tab. 1).

Exposure of KEL FIB cells to genistein in each applied concentration resulted in downregulation of TGFβ1 mRNA expression ($p < 0.000001$, ANOVA), TGFβ2 mRNA expression ($p < 0.000001$, ANOVA) and TGFβ3 mRNA expression ($p < 0.000001$, ANOVA), as compared with untreated control cells (Fig. 5b).

Statistical analysis (*post hoc* Dunnett's test) revealed significant differences in TGFβ1, β2, β3 mRNA expression in KEL FIB cells exposed to various concentrations of genistein compared to the untreated control cells (Tab. 2).

The effect of soybean genistein on the expression of selected genes regulating cell cycle in fibroblasts derived from keloids

The basal expression of p53, p21, BAX and BCL-2 mRNAs in NHDF and KEL FIB cells

The transcriptional activity of all examined genes was detected in unstimulated (control) NDHF cells and KEL FIB cells. No marked changes in p53

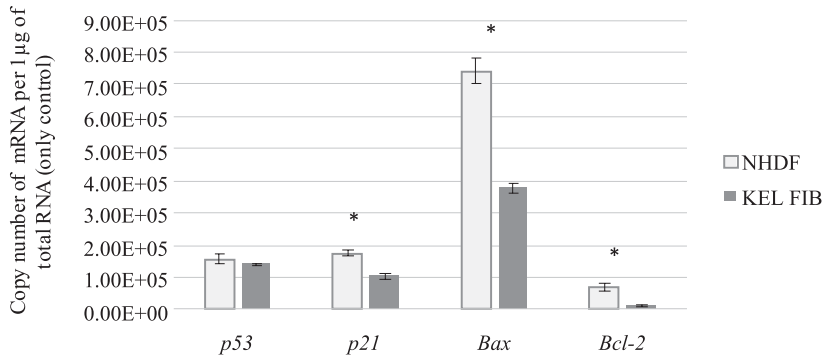

Figure 6. The native expression of p53, p21, BAX and BCL-2 in NHDF control cells and KEL FIB control cells. * $p < 0.05$ (comparison between control NHDF cells and KEL FIB cells)

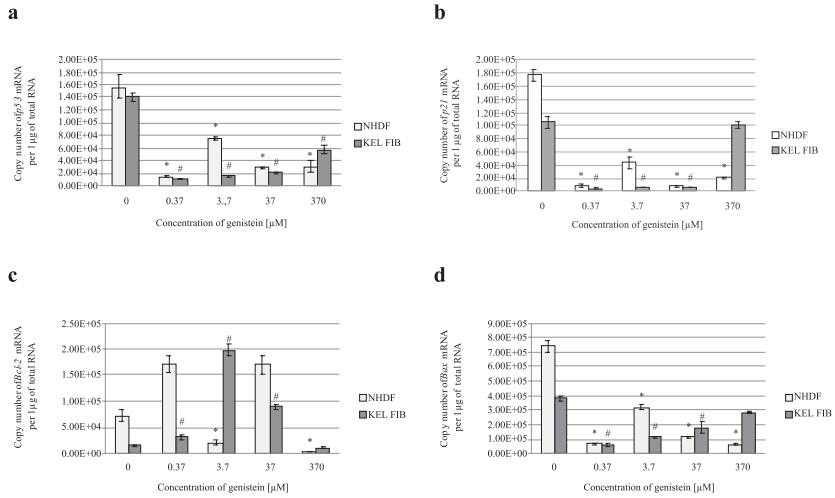

Figure 7. Expression of p53, p21, BCL-2 and BAX mRNAs in NHDF cells and KEL FIB cells determined by real-time PCR. Comparison of the copy number of mRNAs per 1 µg of total RNA: **a** p53, **b** p21, **c** BCL-2, **d** BAX (*$p < 0.05$ NHDF cells treated with various genistein concentration *vs*. control NHDF cells; #$p < 0.05$ KEL FIB cells treated with various genistein concentration *vs*. control KEL FIB cells)

gene activity were demonstrated in normal fibroblast ($1.56 \times 10^5 \pm 3.71 \times 10^3$ copy number of mRNA per 1 µg of total RNA) and keloid fibroblasts ($1.41 \times 10^5 \pm 1.11 \times 10^3$ copy number of mRNA per 1 µg of total RNA). ($p = 0.516160$, Student's *t*-test). Statistical analysis revealed significant reduction in p21 gene expression ($1.05 \times 10^5 \pm 1.78 \times 10^4$ copy number of mRNA per 1 µg of total RNA) ($p = 0.006554$, Student's *t*-test), BCL-2 gene expression ($1.42 \times 10^4 \pm 3.97 \times 10^3$ copy number of mRNA per 1 µg of total RNA) ($p = 0.012359$, Student's *t*-test) and BAX gene expression ($3.80 \times 10^4 \pm 2.53 \times 10^4$ copy number of mRNA per 1 µg of total RNA) ($p = 0.002177$, Student's *t*-test) in keloid fibroblasts as compared with normal fibroblast: p21 (CDKN1A) ($1.77 \times 10^5 \pm 1.61 \times 10^4$ copy number of mRNA per 1 µg of total RNA), BCL-2 ($7.19 \times 10^5 \pm 2.28 \times 10^4$ copy number of mRNA per 1 µg of total RNA), BAX ($7.42 \times 10^5 \pm 8.56 \times 10^4$ copy number of mRNA per 1 µg of total RNA) (Fig. 6).

P53 gene expression in NHDF and KEL FIB exposed to genistein

Treatment of NHDF cells and KEL FIB cells with genistein in each applied concentration resulted in downregulation of p53 mRNA expression as compared with untreated cells, both lines ($p = 0.000014$, $p = 0.000001$, respectively; ANOVA). Statistical analysis (*post hoc* Dunnett test) revealed significant decrease in the p53 mRNAs expression in NHDF cells exposed to genistein in concentration: 0.37 µM ($p = 0.000011$), 3.7 µM ($p = 0.001276$), 37 µM ($p = 0.000036$) and 370 µM ($p = 0.000042$) compared to NHDF control cells.

Genistein in concentrations: 0.37 μM (p = 000005, Dunnett's test), 3.7 μM (p = 000005, Dunnett's test), 37 μM (p = 000005, Dunnett's test) and 370 μM (p = 0.000019, Dunnett's test) downregulated constitutive transcriptional activity of p53 gene in KEL FIB cells (Fig. 7a).

P21 gene expression in NHDF and KEL FIB exposed to genistein

Exposure of NHDF cells and KEL FIB cells to genistein in each applied concentration resulted in downregulation of p21 mRNA expression as compared with untreated cells, both lines (p = 0.000002, p = 0.000001, respectively; ANOVA). Statistical analysis (*post hoc* Dunnett test) revealed significant decrease in the p21 mRNAs expression both in NHDF cells exposed to genistein in concentration: 0.37 μM (p = 0.000006), 3.7 μM (p = 0.000014), 37 μM (p = 0.000006) and KEL FIB cell exposed to genistein at the same concentration (p = 0.000005), (p = 0.000005), (p = 0.000006), respectively. Genistein in 370 μM concentration decreased expression of p21 mRNA in NHDF cells (p = 0.000069) compared to the NHDF control cells. Genistein in 370 μM concentration induced opposite effect in keloid fibroblasts cultured *in vitro,* where was noted increase of p21 expression ($1.01 \times 10^5 \pm 1.22 \times 10^4$ copy number of mRNA per 1 μg of total RNA) in comparison to control KEL FIB cells ($1.05 \times 10^5 \pm 1.78 \times 10^4$ copy number of mRNA per 1 μg of total RNA). Nevertheless, no statistically significant differences were revealed (p > 0.05, Dunnett's test) (Fig. 7b).

BCL-2 gene expression in NHDF and KEL FIB exposed to genistein

Exposure of NHDF cells to genistein in the concentration: 0.37, 3.7, 37 and 370 μM changed expression of BCL-2 gene in normal dermal fibroblast (p = 0.000003, ANOVA) and in keloid fibroblasts (p = 0.000002, ANOVA). The expression of BCL-2 revealed similar quantity in cells treated with 0.37 μM ($1.72 \times 10^5 \pm 3.32 \times 10^4$) and 37 μM genistein ($1.71 \times 10^5 \pm 3.41 \times 10^4$) to control cells ($7.19 \times 10^5 \pm 2.28 \times 10^4$) (p > 0.05, Dunnett's test). The expression of BCL-2 was significantly lower in cells treated with 3.7 μM genistein ($2.06 \times 10^4 \pm 8.28 \times 10^3$) and 370 μM genistein ($2.43 \times 10^3 \pm 3.75 \times 10^2$ copy number of mRNA per 1 μg of total RNA) compared to control NHDF cells (p = 0.025015, p = 0.00024, respectively; *post hoc* Dunnett's test). Expression of BCL-2 revealed similar quantity in cells treated with 370 μM genistein ($1.05 \times 10^4 \pm 1.20 \times 10^3$) compared to control KEL FIB cells (p > 0.05, Dunnett's test). Treatment of KEL FIB cells with 0.37, 3.7 and 370 μM genistein resulted in significant decrease (*post hoc* Dunnett's test) in BCL-2 expression in comparison to control KEL FIB cells (p = 0.042235, p = 0.000012, p = 0.00007, respectively) (Fig. 7c).

BAX gene expression in NHDF and KEL FIB exposed to genistein

Treatment of NHDF cells and KEL FIB cells with genistein in each applied concentration resulted in downregulation of BAX mRNA expression as compared with untreated cells, both lines (p = 0.000001, p = 0.000006, respectively; ANOVA). The expression of BAX in NHDF cells was significantly lower in cells treated with 0.37 μM genistein ($6.60 \times 10^4 \pm 1.94 \times 10^4$), 3.7 μM genistein ($3.19 \times 10^5 \pm 4.64 \times 10^4$), 37 μM genistein ($6.60 \times 10^4 \pm 1.94 \times 10^4$) and 370 μM genistein ($1.13 \times 10^5 \pm 1.22 \times 10^4$ copy number of mRNA per 1 μg of total RNA) compared to control NHDF cells (p = 0.000005, p = 0.000482, p = 0.000006, p = 0.000005, respectively;

Table 3. BAX/BCL-2 ratio for mRNA levels in normal fibroblasts (NHDF line) and fibroblast derived from keloid (KEL FIB) treated with genistein *p < 0.05 NHDF cells treated with various genistein concentration *vs*. control NHDF cells; #p < 0.05 KEL FIB cells treated with various genistein concentration *vs*. control KEL FIB cells.

Genistein concentration [μM]	BAX/BCL-2 mRNA ratio	
	NHDF	KEL FIB
0	10.31 ± 3.76	26.67 ± 6.40
0.37	0.38 ± 0.07 *	2.00 ± 0.22 #
3.7	15.48 ± 5.60	0.58 ± 0.07 #
37	0.66 ± 0.04 *	2.00 ± 0.09 #
370	25.76 ± 2.15 *	27.04 ± 1.89

post hoc Dunnett's test).The expression of BAX in KEL FIB cells was significantly lower in cells treated with 0.37 μM genistein ($6.04 \times 10^4 \pm 1.26 \times 10^4$), 3.7 μM genistein ($1.15 \times 10^5 \pm 8.15 \times 10^3$) and 37 μM genistein ($1.79 \times 10^5 \pm 7.73 \times 10^4$) compared to control KEL FIB cells ($p = 0.000008$, $p = 0.000145$, $p = 0.002822$, respectively; *post hoc* Dunnett's test). Although, expression of BAX gene was lower in cells treated with 370 μM genistein ($6.26 \times 10^4 \pm 8.07 \times 10^3$) compared to control KEL FIB cells, statistical analysis revealed no significant differences ($p > 0.05$, Dunnett's test) (Fig. 7d).

Proapoptotic ratio BAX/BCL-2 in normal and keloid fibroblasts

The BAX/BCL-2 ratio determines cell susceptibility to apoptosis. The BAX/BCL-2 mRNA ratio was determined based on real time RT-PCR results (Tab. 3).

Statistical analysis revealed significant increase in BAX/BCL-2 mRNA ratio in control fibroblasts derived from keloid ($p = 0.03621$, Student's *t*-test) compared to control normal fibroblasts. Treatment of NHDF cells with genistein resulted in statistically significant decrease of BAX/BCL-2 ratio in cells treated with 0.37, 37 and 370 μM genistein ($p = 0.003651$, $p = 0.004211$, $p = 0.002644$, respectively; Dunnett's test). No marked changes in BAX/BCL-2 mRNA ratio were demonstrated with 3.7 μM genistein stimulated cells ($p > 0.05$; Dunnett's test).

Keloid fibroblasts exposed to 0.37, 3.7 and 37 μM genistein revealed significant decrease of BAX/BCL-2 mRNA ratio compared to control KEL FIB cells ($p = 0.000211$, $p = 0.000055$, $p = 0.000200$, respectively; Dunnett's test). Keloid fibroblast exposed to 370 μM genistein revealed no differences in BAX/BCL-2 mRNA ratio in comparison to control fibroblast derived from keloids ($p > 0.05$; Dunnett's test).

DISCUSSION

Keloids are abnormal wound responses in predisposed individuals and represent a connective tissue response to skin injury. Treatment of keloids includes surgical, pharmacological and physical methods. Each of these methods can be applied as monotherapy, but combining them gives better results. Moreover, various modalities of therapy may be applied, but none of them has been proved entirely successful (55, 56). Keloids are abnormal cutaneous fibroproliferative disorders which grow beyond the confines of the original wound, invading the normal surrounding skin and they rarely regress over time (1, 6, 55). Cutaneous fibroproliferative disorders other than keloids include systemic sclerosis and hypertrophic scars (56, 57) and CTGF is their effective marker (57). Moreover, expression of CTGF in keloid fibroblast is dependent on fibroblast location and is more concentrated in keloid fibroblasts at the expanding invasive border of the keloid scar (6, 58). In the study, the keloid fibroblast culture revealed an increase of CTGF mRNA and an increase of CTGF protein expression compared to normal fibroblast confirming contribution of CTGF in keloid fibroblast pathology.

CTGF is up-regulated at the transcriptional level by a number of specific factors such as TGFβ and endothelin-1 (ET-1) or nonspecific factor (mechanicals stress, hypoxia) (16–19, 37, 57). Several studies describe the role of TGFβ in keloids and hypertrophic scars. CTGF expression increased more than 100-fold after stimulation with TGFβ1 and more than 75-fold after the addition of TGFβ2 and TGF-β3 (10).

TGFβ1 stimulation induces collagen synthesis in keloid and hypertrophic scar fibroblasts, but TGFβ1 upregulation alone seems not to be sufficient for excessive scarring (59, 60). In animal models, TGFβ2 induces cell proliferation and collagen production in scars (61, 62). The role of TGFβ3 in wound healing and pathological scarring is not fully understood, but TGF-β3 is both scar inducing and scar reducing in animals (63).

Our data revealed increased expression of TGFβ1 mRNA in keloid fibroblasts compared to normal dermal fibroblast, confirming results from other studies (64, 65). Lee et al. (64) describe increased TGF-β2 protein expression in keloid derived fibroblasts compared to normal skin fibroblasts. They did not define the biopsy site from which the fibroblasts were harvested and the time points for analysis of mRNA and protein expression may diverge. Our data reveal a decrease (no statistical significance) of TGFβ2 mRNA expression in keloids fibroblast compared to normal dermal fibroblasts, confirming Seiferts results (66). The study revealed increased expression of TGFβ3 mRNA in keloid fibroblasts compared to normal dermal fibroblast, in contrast to Lee's et al. study which revealed no difference between keloid derived fibroblasts and normal human skin fibroblasts. Seifert results revealed significantly decreased expression of TGFβ3 mRNA in keloids compared to hypertrophic scars to control skin (66).

Our study seems to confirm the thesis that the increased expression of CTGF by keloid fibroblasts

could be a response to TGFβ1 stimulation. The TGFβ signal is mediated by SMADs intracellular proteins. Changes in this signaling pathway regulate TGFβ expression and induce gene expression in the cell. The SMAD signaling pathway is crucial for simultaneous activation of several collagen genes by TGFβ and other ECM-related genes are identified as gene targets downstream of TGFβ (67). Moreover, functional Smad element resides within the CTGF promoter (68). However, the ability of TGFβ to fully induce the CTGF promoter and protein also requires protein kinase C and the ras/MEK/ERK MAP kinase cascade (69, 70).

Inhibitors of CTGF expression may be used as anti-fibrotic therapies (71) *via* TGFβ-dependent or TGFβ-independent mechanism. Inhibitors of TGFβ or endothelin receptors may be used to reduce CTGF expression in skin fibroproliferative disease such as scleroderma (72, 73). Ilprost, synthetic prostacyclin and 9-cis-retinoic acid reduced CTGF expression in scleroderma fibroblasts (74). One of the mechanisms of ilprost's action is antagonizing MEK/ERK signaling (75). U0126 is a highly selective inhibitor of both MEK1 and MEK2, a type of MAPK/ERK kinase, it reduces CTGF (CCN2) expression in response to TGFβ and ET-1 in scleroderma fibroblasts as well as constitutive over-expression of CTGF in the pancreatic cancer cell line (76). TNFα suppresses TGFβ-induced gene expression in fibroblasts but has no appreciable effect on the constitutive CTGF expression in scleroderma fibroblasts (77). Caffeine also reduces TGFβ-induced CTGF expression in hepatocytes by blocking Smad activation (78). Many *in vitro*, epidemiological and animal model studies proved application of genistein in the prophylaxis and treatment of i.a., menopause, cancer, cardiovascular disease and cystic fibrosis (79). Recent studies are focused on application of this natural isoflavone in fibroproliferative diseases. The results of the study revealed that genistein decreased mRNA and protein expression of CTGF in keloid fibroblast in a concentration-dependent manner. Moreover, genistein decreases TGFβ1, β2 and β3 genes expression (mRNA level) in keloid fibroblast. Han et al. (80) showed that genistein can effectively inhibit TGFβ1-induced invasion and metastasis in pancreatic cancer cell line Panc-1 and could partly suppress the expression of CTGF gene and its protein stimulated TGFβ1 in rat renal mesangial cells and probably decreases the accumulation of extracellular matrix (ECM) and has the potential ability of anti-fibrosis (81).

Keloid derived fibroblasts have a greater proliferative capacity than normal dermal fibroblasts (21, 82). Furthermore, the proliferation profile of fibroblasts in keloid *in vivo* has been properly documented (83) and clearly demonstrated differences in the proliferation of cells between the center and the edge of the lesion. Besides, proliferation, apoptosis, and necrosis occur simultaneously in keloids and these processes are distinctly compartmentalized, too. When keloid matures, apoptosis and necrosis result in selective removal of certain cellular populations resulting in the characteristic avascular fibrotic collagenous lesion, whereas proliferation of fibroblasts in the keloid dermis propagates the fibrosis (21). Moreover, keloid fibroblasts fail to undergo apoptosis and thus, continue to produce and secrete extracellular matrix components (20).

The induction of apoptosis is partly mediated, intracellularly, by several genes, such as p53, BCL-2, BAX, and p21. The p53 tumor suppressor gene is a cell cycle regulator able to induce cell cycle arrest to allow DNA repair or apoptosis. p21 is activated by the p53 protein, and an increased level of p21 is associated in cyclin-containing complexes with decreasing cyclin-dependent activity in damaged cells destined to apoptosis. BCL-2 functions as a suppressor of apoptotic death triggered by a variety of signals, and is negatively regulated by wild type p53. BCL-2 overexpression has been shown to inhibit apoptosis induced by a variety of stimuli, whereas, a predominance of BAX over BCL-2 accelerates apoptosis upon apoptotic stimuli (84). The aim of the study was to evaluate the influence of genistein on transcriptional activity of genes encoding p53, p21, BCL-2 and BAX in keloid fibroblasts. Results of the study reveal no marked changes in p53 gene expression between normal dermal fibroblast and fibroblast derived from keloid and marked decrease of p21 gene expression in keloid fibroblast compared to normal fibroblasts. De Felice et al. found that in keloid fibroblasts a p53 (TP53) under expression, due to the sequence mutations, in concert with ΔNp63 (an isoform of the p63 gene) activation, is central in the mechanism involved in keloid proliferation (85).

In the study, exposure of NHDF cells and KEL FIB cells to genistein in each applied concentration resulted in downregulation of p53 mRNA and p21 expression as compared with untreated cells, both lines. The effects of genistein may depend on the levels of endogenous p53 in the cells. p53 is known to induce DNA repair enzymes, and cells containing wildtype p53 may have repair of the DNA damage caused by genistein treatment. Tumor cells, either deficient in p53 or with very low doses of it, replicate through the damage and are more susceptible to genistein effects (34). The obtained results may indi-

cate that fibroblast derived from keloid compartment where cells proliferation and apoptosis were balanced, and keloid fibroblast not existed as malignant cells. Besides, in normal cells p53 is found at very low levels (86). The increase in the level of active p53 protein leads to an inhibition of entry into S-phase or the induction of apoptosis. Genistein has been identified as a protein tyrosine kinases (PTKs) inhibitor, which play a key role in oncogenesis, control of cell growth and apoptosis, therefore genistein is a potent inhibitor of cell proliferation, oncogenesis and clonogenic ability in animal and human cells (84).

P21 expression is usually controlled at the transcriptional level by both p53-dependent and p533-independent mechanisms. Cells lacking p21 appear to undergo apoptosis normally. The results of the study (decrease of p21 gene expression in cells KEL FIB cells and NHDF cells treated with genistein) are contrary to Upadhyay et al. results (87), which revealed that p21 is up-regulated by genistein treatment, and greatly induced at RNA and protein levels in normal breast epithelial cells, whereas its level was only slightly induced in malignant MDA-MB-231 breast epithelial cells and not detectable in malignant MCF10CA1a breast epithelial cells. Therefore, p21 is responsible for differential sensitivity of genistein among these cell lines (87). Li et al. revealed up-regulation of p21 and BAX expressions and down- regulation of p53 and BCL-2 expression in genistein-treated breast cancer cells MDA-MB-231 (84).

BAX and BCL-2 have been reported to play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. Increased expression of BAX can induce apoptosis, while BCL-2 protects cells from apoptosis. Ratio of BAX/BCL-2 is important for the survival of drug-induced apoptosis in leukemia cell lines (84, 88). The study revealed significant reduction in BCL-2 and BAX genes expression in keloid fibroblasts as compared with normal fibroblast. Moreover, treatment of NHDF cells and KEL FIB cells with genistein in each applied concentration resulted in downregulation of BAX mRNA expression as compared with untreated cells, both lines. Expression of BCL-2 was dependent of genistein concentration. Genistein decreases BAX/BCL-2 ratio both, in normal fibroblast and keloid fibroblast, which may indicate its structural similarity to 17β-estradiol.

Genistein and 17β-estradiol may act by increasing the expression of BCL-2 and decreasing the expression of BAX, resulting in a protective effect (89). This protective effect may also come from the stimulation of estrogen receptor β, which activates the estrogen response element of the BCL-2 gene, and then increases transcription and translation to upregulate the expression of BCL-2 (90).

CONCLUSION

Summarizing the results of the study, genistein *in vitro* suppresses the expression of CTGF mRNA and CTGF protein probably in TGFβ-dependent mechanism in keloid fibroblasts but its potential application as a antifibrotic factor in keloids treatment requires further research.

Genistein does not induce the p53 and p21 genes expression, therefore it seems that it does not induce apoptosis in monoculture of keloids fibroblast. Genistein revealed cytoprotective effect stimulating BCL-2 gene expression in fibroblasts derived from keloids.

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