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CYCLOSPORIN A AFFECTS THE PROLIFERATION PROCESS IN NORMAL HUMAN DERMAL FIBROBLASTS

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Abstract: Cyclosporin A is an immunosuppressant drug that is used not only in solid transplant rejection, but also in moderate and severe forms of psoriasis, pyoderma, lupus or arthritis. Serious side effects of the drug such as skin cancer or gingival hyperplasia probably start with the latent proliferation process. Little is known about the influence of cyclosporin A on molecular signaling in epidermal tissue. Thus, the aim of this study was to estimate the influence of cyclosporin A on the process of proliferation in normal human dermal fibroblasts. Fibroblasts were cultured in a liquid growth medium in standard conditions. Cyclosporin A was added to the culture after the confluence state. Survival and proliferation tests on human dermal fibroblast cells were performed. Total RNA was extracted from fibroblasts, based on which cDNA and cRNA were synthesized. The obtained cRNA was hybridized with the expression microarray HGU-133A_2.0. Statistical analysis of 2734 mRNAs was performed by the use of GeneSpring 13.0 software and only results with $p < 0.05$ were accepted. Analysis of variance with Tukey *post hoc* test with Benjamini-Hochberg correction for all three (8, 24, 48 h) culture stages (with and without cyclosporin A) was performed to lower the number of statistically significant results from 679 to 66, and less. Between statistically and biologically significant mRNAs down-regulated were *EGR1*, *BUB1B*, *MKI67*, *CDK1*, *TTK*, *E2F8*, *TPX2*, however, the *INSIG1*, *FOSL1*, *HMOX1* were up-regulated. The experiment data revealed that cyclosporin A up-regulated *FOSL1* in the first 24 h, afterwards down-regulating its expression. The *HMOX1* gene was up-regulated in the first stage of the experiment (CsA 8 h), however, after the next 16 h of culture time its expression was down-regulated (CsA 24 h), to finally increased in the later time period. The results indicate that cyclosporin A had a significant effect on proliferation in normal human dermal fibroblasts through the changes in the expression of genes related to the cell cycle and transcription regulation process.

Keywords: cyclosporin A, fibroblasts, proliferation, microarray

Abbreviations: ANOVA – analysis of variance, FGM – fibroblasts growth medium, NHDF – normal human dermal fibroblasts, SRB – sulforhodamine test, XTT – tetrazolium test

Cyclosporin A is an efficient immunosuppressive drug, which is used mostly for long-term therapy in patients after various solid organ transplantations (1), though its clinical use is also extended to rheumatological and dermatological disorders (2, 3). Cyclosporin A acts on dermal structures showing a visible therapeutic effect in psoriasis, pyoderma, arthritis or other skin lesions after oral use (3). The drug influences various biological processes in mammalian cells (4) and is a well-known inhibitor of calcineurin, having an impact on the NFAT (nuclear factor of activated T-cells) signaling pathway. Such function can result in the inhibition of the pathway in turn having a crucial impact on cell homeostasis (5). Despite wide use, knowledge about the effects of the drug on cellular processes is limited. New technologies such as oligonucleotide microarrays allow investigating the reaction of cell signaling pathways to various drugs, thus providing accurate data that can have both positive and negative therapeutic relevance.

Cyclosporin A has many adverse side effects such as gingival hyperplasia (6) and cancer (7), which are connected with excessive cell proliferation. Due to the increasing use of the drug and long-term therapy in new diseases, the reasons for these

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side effects are being intensively investigated (8, 9). A well-known side effect in long-term therapy with cyclosporin A in transplant recipients is skin cancer. In cases of dermatological use in small doses the drug has not observed tumor symptom (10).

Understanding the effect of cyclosporin A on the process of proliferation of human dermal fibroblasts can contribute to a better understanding of its biological activity. It may also indirectly help to recognize the cells' mechanism of action, when exposed to cyclosporin A. Thus, the aim of this study was to evaluate the influence of cyclosporin A on the proliferation process in normal human dermal fibroblasts.

EXPERIMENTAL

Reagents

Cyclosporin A (3*S*, 6*S*, 9*S*, 12*R*, 15*S*, 18*S*, 21*S*, 24*S*, 30*S*, 33*S*)-30-ethyl-33-[(1*R*, 2*R*, 4*E*)-1-hydroxyl-2-methyl-4-hexen-1-yl]-6,9,18,24-tetraisobutyl-3,21-diisopropyl-1,4,7,10,12, 15,19,25,28-nonamethyl-1,4,7,10,13,16,19,22,25,28,31-undecaazacyclotritriacontane-2,5,8,11,14,17,20,23,26,29,32-undecone) was obtained from Novartis Pharma GmbH as Sandimmun Neoral oral solution 50 mg/mL in 0.9% NaCl; agarose, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), sulforhodamine B (SRB), phosphate-buffered saline (PBS), trichloric acid (TCA) and ethidium bromide were purchased from Sigma Aldrich. TRIZOL and SuperScript Choice reagents were from Invitrogen Inc. RNeasy Mini Kit columns and Sample Cleanup Module were from Qiagen and DNase I from Fermentas Inc. BioArray HighYield RNA Transcript Labeling Kit was purchased from Enzo Life Science. GeneChip Expression 3'-Amplification Reagents Hybridization Control Kit and microarrays were purchased from Affymetrix Co. CC-2511 cell strain and FGM™-2 bulletkit were purchased from Lonza Group Ltd.

Cell culture

Normal human dermal fibroblasts (NHDFs) were cultured in a fibroblasts growth medium (FGM) in plates with antibacterial filters (25 cm^2, Nunclon™), at constant conditions: temperature 37°C, 5% CO_2 and humidified atmosphere, according to the Lonza protocol.

Cell survival test

NHDF cells survival was performed by using *in vitro* tetrazolium test (XTT), which indicates the number of cells based on mitochondrial activity of living cells (*In Vitro* Toxicology Assay Kit XTT based). The cultured fibroblasts when in the log phase of growth and at a final cell number of 10^6 cells/cm^2 were used for the experiment. Each procedure had included also a blank sample with only fresh FGM medium without cells. The cells were seeded in 96-well plates to which cyclosporin A was added at a concentration range from 0.1 to 100 000 ng / 1 mL of the medium and cultured 24 h (in eight replicates). All procedures and reagents were used according to the producer protocol. Absorbance of the XTT formazan was measured at a wavelength of 450 and 690 nm using the MRX Revelation plate reader (DYNEX Technologies).

Cell proliferation

NHDF cells proliferation test was performed using *in vitro* sulforhodamine B, which indicates the cell answer to cyclosporin A (*In Vitro* Toxicology Assay Kit Sulforhodamine B based). After the cells reached the confluent phase, cyclosporin A was added to the same amount of cells as previously at a concentration range from 0.1 to 100 000 ng / 1 mL in a 96-well plates for 24 h (in eight replicates). After the incubation time the cells were washed with PBS, fixed in 10% TCA and stained with sulforhodamine B according to producer protocol as previously. Absorbance of incorporated dye has been measured at a wavelength of 565 and 690 nm using the MRX Revelation plate reader (DYNEX Technologies).

RNA isolation

The NHDFs with cyclosporin A at a concentration of 100 ng / 1 mL of the medium were cultured for 8, 24 and 48 h in standard condition as described previously. Total RNA was isolated from the harvested fibroblasts without and with cyclosporin A using TRIZOL reagent according to the producer's protocol. Isolated RNA has been purified with DNase I and RNeasy Mini Kit columns. The purity of the RNA was evaluated spectrophotometrically (GeneQuant II, Pharmacia Biotech) and by 1% agarose gel electrophoresis (Mini, Kucharczyk) stained with ethidium bromide.

HGU-133A_2.0 microarray performance

Obtained RNA was used for the expressions microarray performance, which is starting from the synthesis of cDNA by using the SuperScript Choice reagent. Biotinylated cRNA synthesis was carried out using BioArray HighYield RNA Transcript Labeling Kit and its fragmentation done using the Sample Cleanup Module. The preparation of the

hybridization mixture was performed using the GeneChip Expression 3'-Amplification Reagents Hybridization Control Kit, and subjected to hybridization with HG-U133A_2.0 microarrays. All the mentioned steps were performed according to the producer protocol. Fluorescence intensity signal was measured with Affymetrix GeneArray Scanner 3000 7G (Affymetrix). The quality of cDNA and cRNA were controlled using electrophoresis and spectrophotometer, as previously.

All results from twelve microarrays were normalized by the RMA (Robust Microarray Analysis) method transforming data to logarithmic values (log_2). Normalized values were grouped accordingly to the time in which they were cultured with cyclosporin A, namely CsA 8 h, CsA 24 h, CsA 48 h and non-exposed control group (all in triplicate).

Statistical analysis

The obtained results were subjected to the statistical analysis using Statistica 11.0 (StatSoft) and GeneSpring 13.0 (Agilent) software. Apart performed repetitions of the microarray experiment, for each mRNA was eleven probe sets for which fluorescence signals were measured by the scanner readings, and were analyzed with Wilcoxon's test. Additionally, mined mRNAs related to the proliferation process have been compared using ANOVA and Tukey *post hoc* test with Benjamini-Hochberg correction. The differences in gene expression between fibroblast samples with cyclosporin A and non-exposed cells were calculated as a fold change (FC = 1.0). Additionally, the individual comparisons were performed using Student's *t*-test. All results were statistically significant at $p \leq 0.05$.

RESULTS

Cell survival and proliferation

The measured changes in the amount of formed formazan (mitochondrial dehydrogenases of viable cells reduce the tetrazolium ring of XTT, yielding an orange formazan derivative) indicate the degree of cyclosporin A cytotoxicity (a decrease in differences in absorbance of viable cells relative to non-treated), which has been shown on Figure 1. The greatest statistically significant viability of the tested fibroblasts was shown for a concentration of 100 ng/mL compared to others ($p = 0.01$). This amount of cyclosporin A has not shown toxic effects and resulted in significant increase in growth of fibroblasts.

The SRB test results indicate that cyclosporin A has no effect on the proliferation process in the investigated human dermal fibroblasts. The comparison between the control and cyclosporin A exposed fibroblasts at the concentration range of 0.1 to 100 000 ng/mL indicated significant differences only for the two samples shown in Figure 2. The highest tested concentration demonstrated strong cytotoxicity.

Proliferation-linked gene expression

The number of proliferation-linked mRNA was obtained based on the NetAffx Analysis Center database (www.affymetrix.com). Descriptive statistics were calculated for the 2734 probe sets of

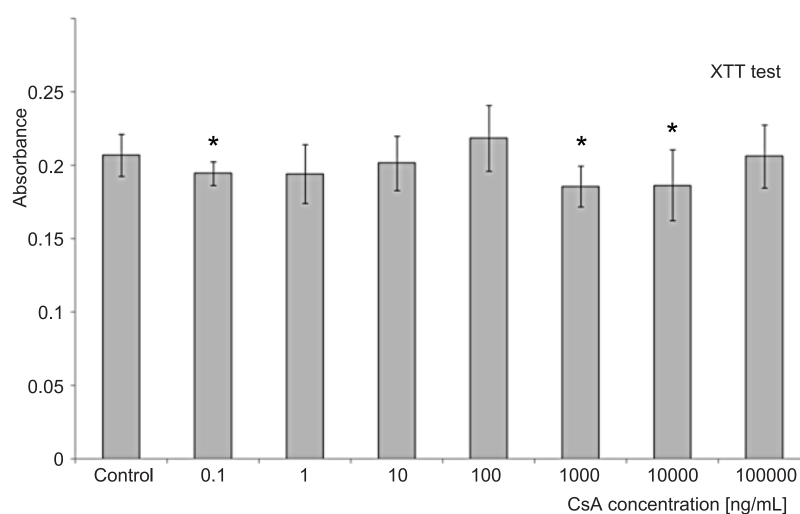

Figure 1. Influence of different concentrations of cyclosporin A on survival of human dermal fibroblasts Each point represents the mean of eight experiments and their standard deviation; * shows $p \geq 0.05$ comparing to control (for 0.1 ng/mL $p = 0.03$; 1000 ng/mL $p = 0.01$; 10 000 ng/mL $p = 0.02$).

Table 1. Fold change of proliferation-linked gene in human dermal fibroblasts with cyclosporin A exposed 8, 24 and 48 h.

Probe set ID	Gene symbol	Access. no.	p-value	FC CsA 8 h	Regulation	FC CsA 24 h	Regulation	FC CsA 48 h	Regulation
201626_at	*INSIG1*	U96876	0.0050	1.40	up	1.23	up	1.24	up
204420_at	*FOSL1*	NM_005438	0.0005	1.36	up	-1.27	down	-1.61	down
203665_at	*HMOX1*	AY460337	0.0003	1.29	up	-1.05	down	1.41	up
210052_s_at	*TPX2*	BC020207	0.0030	-3.07	down	-1.62	down	-1.48	down
219990_at	*E2F8*	NM_024680	0.0040	-3.10	down	-1.64	down	-2.71	down
204822_at	*TTK*	NM_003318	0.0068	-3.16	down	-1.64	down	-1.89	down
203213_at	*CDK1*	NM_001786	0.0001	-4.21	down	-1.35	down	-2.33	down
212022_s_at	*MKI67*	NP_002408	0.0010	-4.32	down	-1.85	down	-1.99	down
203755_at	*BUB1B*	NM_001211	0.0022	-4.72	down	-1.77	down	-1.67	down
201693_s_at	*EGR1*	NC_000005	0.00003	-17.8	down	-2.12	down	-2.23	down

ID – identity number of Affymetrix database, access. no. – accession number to Gene Bank, FC – fold change of CsA *vs.* control, up – up-regulated, down – down-regulated.

mRNAs (9th of November 2014). The obtained results were visualized as a whisker box plot in Figure 3. Differences between the normalized fluorescence signals for fibroblasts exposed to cyclosporin A and the control ranged from a minimum of -3.0 for CsA 8 h to a maximum value of over 1.0 for CsA 48 h (deviated values in Fig. 3 named as differentiating mRNAs). More differentiating genes in positive values were observed for the control fibroblasts. The use of ANOVA and Tukey *post hoc* test with Benjamini-Hochberg correction in the comparison of the CsA samples to the control limited the number of differentially expressed mRNAs to 679, at a statistical significance of $p \leq 0.05$ (Fig. 4). Taking into comparison the results from the previous analysis, only 66 common mRNAs were revealed for all three study groups. There were also 25 mRNAs specifically differentiating the CsA 8 h, as well as 111 the CsA 24 h and 65 in the long-term group of CsA 48 h.

Taking into consideration the highest fold change of differentially expressed gene for CsA 8 h, the statistically and biologically significant mRNAs not less than 1.2 and no greater than -3.0 were selected (Table 1). The *EGR1* (Early Growth Response 1), *BUB1B* (Budding Uninhibited by Benzimidazoles 1 b), *MKI67* (Marker of proliferation Ki-67), *CDK1* (Cyclin-dependent kinase 1), *TTK* (tramtrack), *E2F8* (E2F transcription factor 8) and *TPX2* (Microtubule-associated Targeting Protein) were down-regulated in all times of culture with cyclosporin A. The experiment data also revealed that exposure to cyclosporin A up-regulated *INSIG1* (Insulin Induced Gene 1). Genes such as *FOSL1* (Fos-related antigen 1) and *HMOX1* (Heme Oxygenase 1) up-regulated expression in the first 8 h and showed significant down-regulation after 24 h. In the case of *HMOX1*, the expression was raised once more in the final 48 h. All mentioned genes had a significant influence on the progress of the fibroblast cells' proliferation.

DISCUSSION AND CONCLUSIONS

According to the survival and proliferation tests, only a cyclosporin concentration of 100 ng/mL showed an increase in absorbance, but without statistical significance, (Fig. 1 and Fig. 2; $p > 0.05$). In relation to the SRB test there was no significant difference of cell growth with the cyclosporin A compared to the non-exposed culture at the investigated range. Only at the highest concentration (100 000 ng/mL) were toxic effects on the tested cells observed (Fig. 2). Zhou et al. (11) had stated that

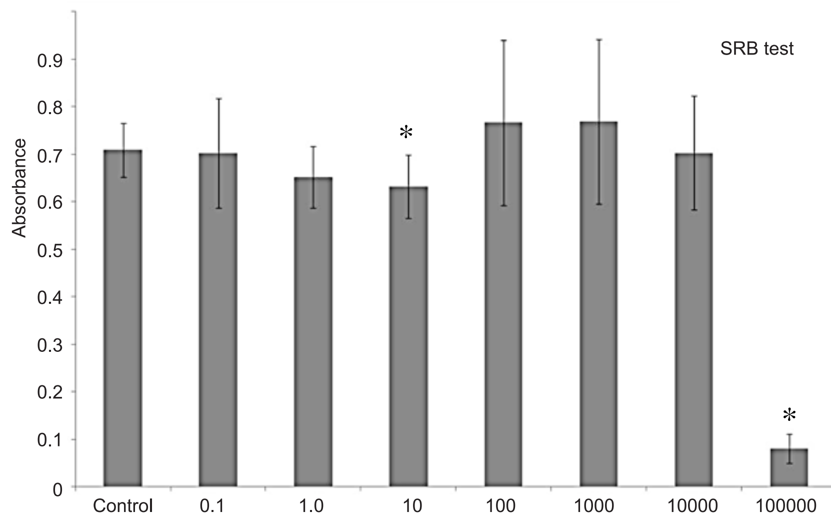

Figure 2. Influence of different concentrations of cyclosporin A on proliferation of human dermal fibroblasts. Each point represents the mean of eight experiments and their standard deviation; * shows $p \geq 0.05$ comparing to control (for concentration 10 ng/mL $p = 0.04$ and 100 000 ng/mL $p = 0.0000$).

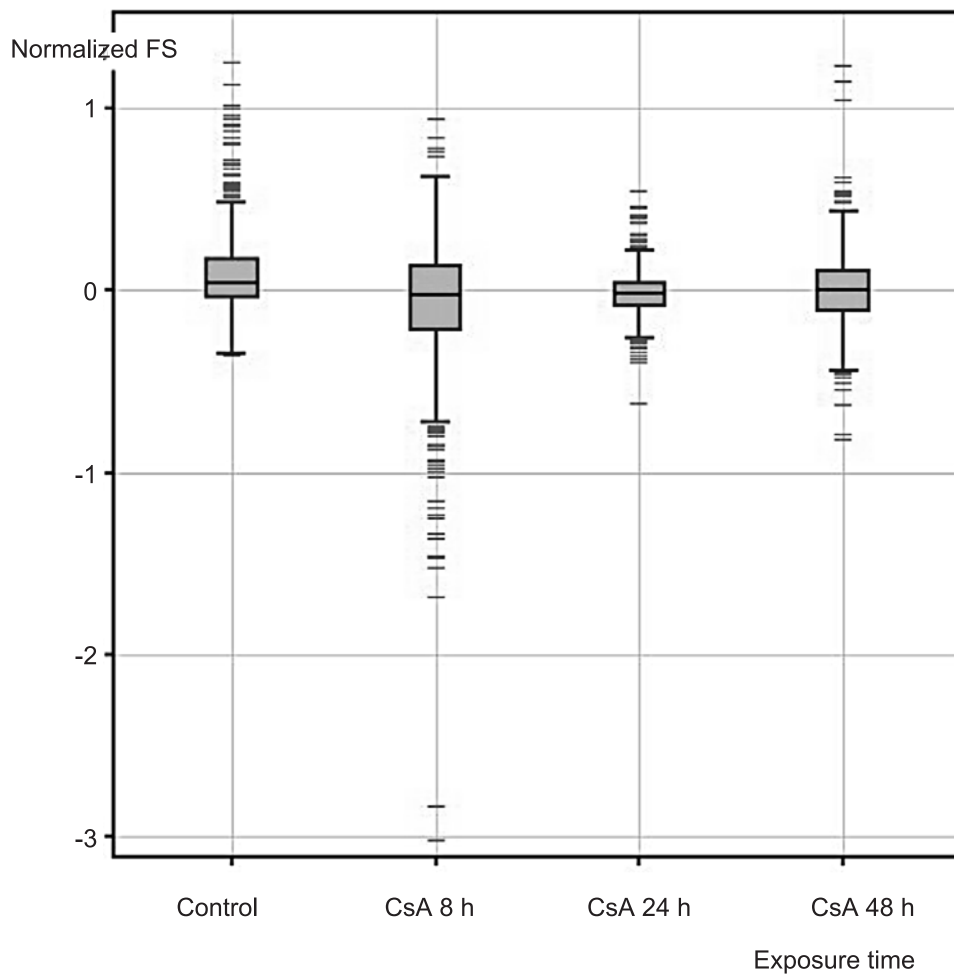

Figure 3. Comparison of proliferation-linked mRNAs (n = 2734) in NHDFs exposed to cyclosporin A in different time of culture and control

cyclosporin A in a concentration above 1000 ng/mL inhibited mesangial cell proliferation and cell cycle progression. Abe et al. (12), studying the effect of cyclosporin on fibroblast proliferation, had shown its cytotoxicity at a concentration of 10 and 100 ng/mL (13). However, the results of cell viability were comparable to those described above. It is well known that cyclosporin suppresses human dermal fibroblast proliferation (13, 14).

The usual therapeutic dose of cyclosporin in skin diseases such as psoriasis is 3 mg per kg of body weight, which gives a blood concentration from 80 to 200 ng/mL (15, 16). Other studies stated that the concentration of cyclosporin A in the blood of psoriasis patients was in a range from 100 to 1000 ng/mL and has been considered as clinically relevant (17, 18). The concentration of cyclosporin A used in our experiment was similar. The applied concentration may exert moderate proliferative effect on dermal fibroblasts as has been shown in the results (but without statistical significance). Based on the performed survival and proliferation tests, the non-toxic concentration of cyclosporin A, which also has clinical significance in skin diseases (15-18) was chosen for NHDFs analysis. Thus, to assess the expression of genes related to the proliferation process the optimal cyclosporin A concentration was 100 ng/mL.

Our findings showed that cyclosporin A in this concentration changed the expression of proliferation-linked genes in NHDFs culture. This is the first study on the effect of cyclosporin A on human dermal fibroblasts proliferation based on microarray analysis. The obtained results can be a valuable indicator showing the tendency of the cell and its reaction to a drug in large-scale usage. A strong effect of cyclosporin A on proliferation-linked genes is visible in the obtained 679 significant mRNAs (selected from all 2734). The biggest change was observed in CsA 8 h, when its concentration was still at a high level and the number of differentiating genes was the highest (Fig. 3). Although the fibroblast reaction to cyclosporin A was tested only in one of the most commonly used concentrations in the studies on cultured cells and clinically significant (12, 15-18), its effectiveness was tested in three stages of culture (CsA 8 h, CsA 24 h and CsA 48 h). It was found that the number of differentially expressed genes was reduced in time, which may arise from the effect of its biotransformation. From this point of view, checking the differences in expression of key signal transmitters linked to proliferation gave an inside view to the cell functioning under stress caused by the drug. Comparison of the results with and without cyclosporin A, as well as between them at various culture times (Fig. 4), and the highest FC and specificity of probe sets had ten statistically significant genes changing expression regardless to the culture time (Table 1). Cyclosporin A changed the expression of proliferation-linked genes in NHDFs, partic-

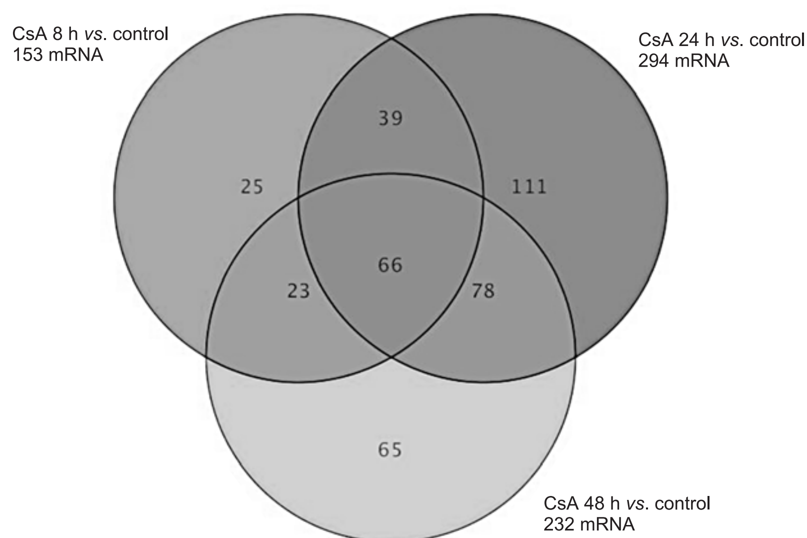

Figure 4. Number of common and differentially expressed proliferation-linked mRNAs exposed to cyclosporin A in various time of culture, compared to control (n = 679).

ularly those, which have a critical function in the cell cycle and transcription regulation, as well as in apoptosis and differentiation of cells.

The insulin induced gene 1 (*INSIG1*) is responsible for coding an endoplasmic reticulum membrane protein that regulates cholesterol concentration, lipogenesis, and glucose homeostasis in the cell (19). It has been demonstrated that *INSIG1* is expressed predominantly in liver tissue, but in preadipocyte it inhibits adipogenesis (20, 21), as well as in cultured animal cells (19, 22). Similarly, in this experiment the *INSIG* gene expression was up-regulated as in primary mouse embryonic fibroblasts (3T3-L cells) exposed to silibinin (23) and glucose (24). Thus, cyclosporin A has the hardest effect on *INSIG1* expression in CsA 8 h, when there is a great demand for cholesterol. Hence, the drug can have indirect influence on proliferation by the regulation of sterols and glucose homeostasis in fibroblasts.

Fos-related antigen 1 (*FOSL1* or c-Fos) encodes the leucine zipper protein, which is required for the creation of the AP-1 transcription factor complex. In turn, c-Fos also has the ability to act as a transcription factor and regulates proliferation, differentiation, and transformation of the cells (25). The increase of *FOSL1* in CsA 8 h (Table 1) has influence on the cell growth. Decrease of its expression in the next culture time indicates the suppression of this gene. Expression of *FOSL* is regulated during the cell cycle and probably has an essential impact on cell proliferation and survival (26), but its function is not clear. The proliferative role of c-Fos as an inducer was only shown in exponentially growing cells regardless of the cell cycle phase (27). Up-regulation of *FOSL1* was stated in most tumor tissues, however, its over-expression can result in fibroblasts transformation (28).

The heme oxygenase 1 (*HMOX1*) encodes an enzyme that is participating in heme catabolism by cleaving heme to biliverdin. It was stated in knockout mice that up-regulation of the *HMOX1* gene can indicate an adaptive and protective mechanism against oxidative damage (29). In human skin fibroblasts, transcriptional activity of *HMOX1* was increased under the influence of different chemical and physical agents (30), which was also noticed after 8 and 48 h of exposure to cyclosporin A (CsA 8 h and CsA 48 h; Table 1). Transient reduction in the expression of this gene was observed in CsA 24 h, which can probably be connected to cyclosporin biotransformation and response to its metabolites. It has been observed that over-expression of the *HMOX1* gene in fibroblast cells exposed to chromium was increased in the early hours of incubation, but after 24 h it was significantly decreased (31), similarly to the results presented here. Studying the influence of other agents on *HMOX1* gene expression has exhibited a significant dose-dependent decrease (32) and incubation time-dependent or concentration-dependent increase or decrease (33).

Cyclosporin A suppressed transcriptional activity of *EGR1*, *BUB1B*, *MKI67*, *CDK1*, *TTK*, *E2F8* and *TPX2* genes regardless to the culture time of NHDFs (Table 1). All these genes have a role in cell cycle regulation, thus influencing the cell proliferation (34, 35). *EGR1* and *E2F8* are responsible for encoding a transcription factor (35, 36). The products of the *BUB1B*, *CDK1* and *TTK* are related to protein kinases (34, 37, 38), and the *MKI67* gene is a well-known proliferation marker, but little is known about the microtubule-associated *TPX2* gene. Decreased expression of those genes in fibroblasts exposed to cyclosporin A can indicate diminished cell proliferation, despite that the used concentration was not toxic (Fig.1-2). Cyclosporin A strongly reduced gene expression of *EGR1*, *CDK1* and *MKI67* in NHDFs, which was also seen in the performed tests through high standard deviation and lack of significant increase in absorbance (Fig. 2). *BUB1* inhibition decreases the cell proliferation (37), but over-expression of *TTK* blocks photoreceptor cell differentiation (38). It was also confirmed that the level of *E2F8* was decreased in each treated cell line with nobiletin (39), and cyclosporin A similarly acted in the tested NHDFs.

Furthermore, it was observed that many chemical carcinogens added to the cell culture also decrease the expression of genes involved in proliferation (40), as these results show. Tiu et al. (41), in studying the short-time (24 h) cyclosporin (5 mg/mL) effect on the squamous cell line (SCC-015), stated that ”cyclosporin activates and inhibits a wide array of genes important for life processes and should be used cautiously for treatment of psoriasis and atopic eczema, especially in children”. Our results indicate that cyclosporin affects proliferation of human dermal fibroblasts on the molecular level, however, the drug does not have an immediate effect on cell survival. Inhibition of proliferation (down-regulated genes) and occurrence of adaptive and protective mechanisms against the oxidative stress (upregulated *HMOX1*) as well as disruption in lipogenesis (up-regulated *INSIG1*) are well-known symptoms of carcinogenesis in the initial stage (42). The observed changes in the proliferation of linked genes leading to metabolic disruption and possibly to further stages of carcinogenesis in the cells indicate that cyclosporin

behaves like a typical carcinogen. From the clinical point of view to reduce this side effect, the patients should necessarily take antioxidants.

Based on the results of the proliferation assay (Fig. 2), a statistically significant increase of cell growth was not observed in the investigated range of concentrations, only toxic effects (10 and 100 000 ng/mL), but the expression of genes important to proliferation underwent silencing. Cyclosporin A did not allow an increase in the expression of genes playing an important role in the cell cycle and transcription process, which could mean that in human skin fibroblasts at this (therapeutic) dose, an intense process of carcinogenesis probably will not start. An alternate explanation of the obtained results may be that the task of cyclosporin A in various therapies is to inhibit the immune response, which involves cytokines that are linked through signaling pathways to genes directly affecting the process of proliferation and hence the observed effect. Could it also indicate that immunosuppression is the beginning of the long process of carcinogenesis?

In summary, the presented results indicate that cyclosporin is able to affect NHDFs proliferation. Significant changes were also observed in the expression of genes related to the regulation of the cell cycle and transcription in the cultured fibroblasts exposed to cyclosporin.

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