

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**Discovering new complex mechanisms for regulating
hair growth - Focus on outer root sheath
keratinocytes**

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Investigation of new, complex mechanism in hair cycle regulation – focus on the outer root sheath keratinocytes

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The Examination takes place at Department of Physiology Faculty of Medicine, University of Debrecen

April 25, 2018, 10 am

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

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INTRODUCTION

Nowadays, when there is increasing emphasis on attaining and maintaining an aesthetic appearance; millions of men and women struggle to achieve what they perceive to be the perfect image, posing new challenges for doctors and researchers alike. Hair growth disorders present a significant quality-of-life impairment for many people worldwide. It is unsurprising therefore, that in the interest of finding new and effective treatments for these disorders, the investigation of „hair biology” is a common goal for many researchers around the globe. Our own workgroup– partly through collaborations with cosmetic companies – has been a one of the pioneers of this field, and has shown that both adenosine and caffeine plays an important role in the regulation of the hair cycle.

Adenosine is a locally produced essential mediator, with widespread physiological functions (e.g. angiogenesis, vasodilation), as well as an important role in energy transfer and neurotransmission. It’s beneficial effect on hair growth through increasing hair shaft elongation and thickening has been described previously. It has also been shown that adenosine increases the production of certain hair cycle modulators (vascular endothelial growth factor [VEGF], fibroblast growth factor-7 [FGF-7]) from cultured HF-derived dermal papilla (DP) cells through adenosine receptor (ADOR) signaling. There were nevertheless many open questions as to how adenosine influences other cells of the HF, and whether other HF compartments also contribute to hair cycle regulation upon adenosine challenge.

Caffeine is one of the most well-known alkaloid compounds of our age, due to the culinary delights its natural source, the coffee plant (*Coffea arabica*, *Coffea canephora*) imparts. It is also one of the most widely-used natural, pharmacologically active compounds in the world. Thanks to its hydrophobic nature it is capable of penetrating through the skin barrier, partly through the hair canal and specifically the HFs. Its subsequent complex biological activity has

resulted in increased popularity in certain cosmetic products, specifically in the treatment of male pattern hair loss, where HFs are „stuck” in the telogen phase because of high testosterone (TST) levels. Caffeine has a positive effect in these cases by reintroducing these hair follicles (HF) into the anagen phase. In our investigations we wished to gain a deeper mechanistic insight into how caffeine regulates the human hair cycle.

We performed our experiments partly on human HF organ cultures isolated from skin samples acquired from plastic surgery and partly from outer root sheath (ORS) keratinocytes isolated from HFs via enzymatic digestion.

AIMS

The role of adenosine and caffeine in hair growth is not fully elucidated, so in our experiments we aimed to answer the following questions:

What potential role does adenosine play in regulating the biological processes of human HFs and HF-derived ORS keratinocytes in vitro, and which of its receptors mediate its putative effects?

Can caffeine, as natural compound that potentially induces hair growth, elicit its hair cycle regulatory effect in female donors; how do these possible effects influence the biological processes of human HFs and HF-derived ORS keratinocytes in both men and women, and what signaling pathways underlie these effects?

MATERIALS AND METHODS

Isolation and maintenance of HFs:

Human anagen VI HFs were isolated from human skin samples obtained from healthy individuals undergoing neurosurgery with written informed consent, adhering to Helsinki guidelines, and after obtaining the permission of the Institutional Research Ethics Committee and the Government Office for Hajdú-Bihar County (protocol No.: DE OEC RKEB/IKEB 3724-2012; document IDs: IX-R-052/01396-2/2012, IF-12817/2015, IF-1647/2016, IF-778-5/2017). Our collaborator (Dr. T. Fischer) took biopsies from women undergoing face-lift surgery, and male HFs were taken from electively taken biopsies (0.5 x 1.5 cm) from the balding vertex region in the border area of the dense to the shedding area (androgen-sensitive) of men affected with androgenetic alopecia (AGA) in moderate stage (Norwood-Hamilton stage III vertex and IV (5)). The study was approved by the Ethics Committee of the University of Lübeck (reference 06-109) and written informed consent was obtained from the patients in accordance with the “Helsinki Declaration”. After isolation HFs were maintained in Williams’ E medium supplemented with 2 mM L-glutamine, 10 ng/ml hydrocortisone, 10 mg/ml insulin, and antibiotics. Culture medium was changed every other day, whereas treatment with various compounds was performed daily. For immunofluorescent staining and histomorphometry, follicles were frozen at -80 °C and further processed after 6 days in culture. Length measurements on individual cultured HFs were performed using a light microscope with an eyepiece measuring graticule. Elongation was calculated for each hair follicle separately, by subtracting the length measured on day 0 from the value of the relevant day.

Culturing of human HF-derived ORS keratinocytes

Human plucked eyebrow or scalp HFs of several male donors were obtained after written informed consent from healthy individuals adhering to Helsinki

guidelines and after Institutional Research Ethics Committee permission. Plucked HFs were digested using trypsin to obtain ORS keratinocytes. Similarly, human dermal fibroblasts (FBs) were obtained from de-epidermized dermis of human skin samples using enzymatic digestion. ORS keratinocyte cultures were kept on feeder layer of non-proliferating FBs treated with mitomycin C in a 1:3 mixture of supplemented Ham's F12 and Dulbecco's modified Eagle's medium supplemented with 10% Fetal Clone II, 0.1 nM cholera toxin, 5 µg/ml insulin, 0.4 µg/ml hydrocortisone, 2.43 µg/ml adenine, 2 nM triiodothyronine, 10 ng/ml epidermal growth factor, 1 mM ascorbyl-2-phosphate and antibiotics.

Determination of cellular proliferation

The degree of cellular growth (reflecting number of viable cells) was determined by measuring the DNA content of cells using CyQUANT Cell Proliferation Assay Kit. ORS keratinocytes (10000 cells/well) were cultured on mitomycin treated FBs feeder cells (900 cells/well) in 96-well black-well/clear-bottom plates in quadruplicates. Supernatants were removed and the plates were subsequently frozen at -70°C . The plates were then thawed at room temperature, and 200 µl of CyQUANT dye/cell lysis buffer mixture was added to each well. Fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using a FlexStation II 384 Fluorescence Imaging Plate Reader.

Determination of apoptosis in ORS keratinocytes

Mitochondrial membrane potential of ORS keratinocytes as early marker of apoptosis was determined using a MitoProbe DiIC₁(5) Assay Kit using previously optimized protocols. Cells (10000 cells/well) were cultured on mitomycin treated FBs feeder cells (900 cells per well) in 96-well black-well/clear-bottom plates in quadruplicates. After removal of supernatants, cells were incubated for 30 minutes with DiIC₁(5) working solution and the fluorescence of DiIC₁(5) was measured at 630 nm excitation and 670 nm emission wavelengths using a FlexStation II 384 Fluorescence Imaging Plate Reader.

Determination of necrosis in ORS keratinocytes

Necrotic cell death was determined by SYTOX Green nucleic acid staining, in parallel with the above assessment of apoptosis. SYTOX Green is able to penetrate only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible staining. Cells were cultured and treated as described above. Supernatants were then discarded and the cells were incubated with 1 μ M SYTOX Green solution. Fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths using the above mentioned FlexStation II 384.

Quantitative “Real-Time” PCR (RT-qPCR)

Total RNA was isolated using TRIzol reagent and digested with recombinant RNase-free DNase-1 according to the manufacturer’s protocol. After isolation, 1 μ g of total RNA was reverse-transcribed into cDNA using the High Capacity cDNA kit following the manufacturer’s instructions.

Quantitative real-time PCR was performed on ABI Prism 7000 or Stratagene Mx3005p sequence detection system by using 5’ nuclease assay using specific TaqMan primers and probes. The amount of the transcripts was normalized to the expression of the internal control gene, using the Δ Ct method. All experiments were performed in triplicates.

Determination of protein secretion (ELISA)

Supernatants of ORS keratinocytes after treatments were taken at 24, 72 and 120 h for quantitative determination of transforming growth factor- β 2 (TGF- β 2) and insulin-like growth factor-1 (IGF-1) protein levels using specific ELISA kits, which were pre-coated with the specific antibodies, following the manufacturer instructions.

Fluorescent labeling

Immunolabeling of adenosine receptors (ARs)

To detect the four type of ARs on isolated HFs and ORS keratinocytes, we performed indirect fluorescent immunolabeling. Cryosections of isolated HFs fixed with ethanol:acetic acid or acetone-fixed ORS keratinocytes grown on coverslips were first incubated with different primary rabbit antibodies (diluted in specific antibody diluent [DCS]). Then, followed by incubation with Alexa Fluor 488 dye-conjugated goat anti-rabbit secondary IgG according to standard procedures nuclei were counterstained with 4',-6-diamidino-2-phenylindole (DAPI) and sections were mounted with Fluoromount-G aqueous medium. Images were acquired using an Eclipse E600 fluorescent microscope.

Immunolabeling of Ki67 proliferation marker

Cellular proliferation was also assessed by immunocytochemical labeling of the proliferation nuclear marker Ki67. In brief, cells were fixed in acetone and then incubated with a mouse anti-Ki67 antiserum. Ki67 immuno-reactivity was visualized by a rhodamide-conjugated secondary antibody. Nuclei were counterstained by DAPI and sections were mounted with Fluoromount-G aqueous medium.

Ki-67/TUNEL Double Labeling

To simultaneously detect proliferating and apoptotic cells in the HFs, Ki-67 immunolabeling and terminal dUTP nick end labeling (TUNEL) were performed in a double-staining protocol. Cryosections were fixed in formalin/ethanol/acetic acid and labeled with a digoxigenin-deoxyUTP (ApopTag Fluorescein In Situ Apoptosis detection kit) in the presence of terminal deoxynucleotidyl transferase according to the manufacturers protocol, followed by overnight incubation with a mouse anti-Ki-67 antiserum. TUNEL+ cells were visualized by an anti-digoxigenin FITC-conjugated antibody (ApopTag kit), whereas Ki-67 was

detected by an Alexa Fluor 568 dye-conjugated secondary antibody. Cells positive for Ki-67 or TUNEL were counted per hair bulb and were normalized to the number of nuclei (DAPI+).

HF immuno-fluorescence of TGF- β 2 and IGF-1

To detect the key-catagen inductor TGF- β 2 and the major growth factor IGF-1, immunofluorescence with tyramide signal amplification (TSA) procedure was used. Frozen sections were fixed with acetone and endogenous peroxidases were quenched using 3% H₂O₂. Blocking was performed by the incubation with 2% normal goat serum for TGF- β 2 or 2% normal rabbit serum for IGF-1. Sections were incubated with polyclonal rabbit anti-TGF- β 2 IgG or with polyclonal goat anti-IGF-1 IgG. After overnight incubation, the slides were treated with the secondary biotin-labeled goat anti-rabbit IgG for TGF- β 2 or with the secondary biotin-labeled rabbit anti-goat IgG for IGF-1. Staining was developed using TSA Fluorescein System for TGF- β 2 or TSA Tetramethylrhodamine System for IGF-1 in the following way: slides were incubated with horseradish-peroxidase-conjugated streptavidin, and labeled using fluorescein tyramide reagent or rhodamine tyramide reagent for TGF- β 2 or IGF-1, respectively. Sections were counterstained with DAPI and mounted in Fluoromount-G mounting medium. All fluorescent images were then obtained using the Keyence BZ-8000 inverted fluorescence microscope.

„DP Stalk” analysis

Quantitative method to analyze the migrating FBs cell number, from the DP to the DP stalk, in a well-defined reference area. In catagen stage, more DAPI+ cells migrate to the DP stalk than in anagen VI HF. Number of DAPI+ cells in a standardized area of the dermal papilla (DP) stalk was counted on Ki-67/TUNEL double labeled sections to further characterize the hair cycle quantitatively.

Histology and quantitative histomorphometry

Cryosections (6 μm) of cultured HFs were fixed in acetone, air-dried, and processed for routine histology. Hematoxylin and eosin (HE) staining was used for studying HF morphology and hair cycle stage (anagen and different stages of catagen) of each HF was assessed according to defined morphological criteria.

For statistical analysis (hair cycle score [HCS]), anagen VI HFs were arbitrarily attributed a score of 100, HFs in early catagen a score of 200, in mid-catagen of 300, and in late catagen of 400. The sum of scores per group was then divided by the number of investigated HFs. The mean value of these scores therefore is a reliable quantitative indicator of the mean HF stage that had been reached on average by a larger population of HFs after culture.

Statistical Analysis

Values are presented as mean \pm SEM in every group. To compare the mean values of multiple groups, statistical analysis was subsequently performed by One-way ANOVA and Dunnett or Bonferroni post hoc tests, as appropriate. A value of $p < 0.05$ was used to determine statistical significance. Differences in distribution of HFs among different hair cycle stages was compared pairwise by Fisher's exact test. Origin 9.0 and IBM SPSS Statistics 23.0 were used to plot the data and perform statistical analysis, respectively.

RESULTS

1. Overview of the effects of adenosine on the hair cycle

Adenosine stimulates HF growth, matrix keratinocyte (MK) proliferation and prolongs the anagen phase

In our experiments we followed the elongation of HFs treated with 50 and 100 μM adenosine. According to our results both concentrations were capable of significantly stimulating the elongation of HFs.

To learn more about the underlying mechanisms behind these effects HFs were frozen after the completion of elongation studies and sections were prepared for immunohistofluorescent experiments. These showed that adenosine could significantly and dose-dependently increase the number of Ki67 positive, proliferating cells in the HF bulb. We next performed histomorphometric analysis of HFs following HE staining. In good accordance with these results we found that adenosine applied at 100 μM inhibited the transition of HFs into the catagen phase, since a larger portion of HFs remained in the anagen phase. Following this observation, we next investigated the putative catagen-blocking and proliferation-inducing effect of adenosine in the presence of TGF- β 2, which has been shown to induce the catagen transition in HFs.

Adenosine blocks the catagen-inducing effect of TGF- β 2

In the first step of our experiments we examined the elongation of HFs. TGF- β 2 applied alone significantly decreased HF-elongation, which effect could be significantly blocked by the coapplication of 100 μM adenosine. In order to confirm the specificity of the effect of adenosine, the experiment was also performed in the presence of the generic ADOR antagonist CGS15943, which completely abrogated the anagen-protective effect of adenosine.

We further analyzed the ratio of proliferating and apoptotic cells using Ki67-TUNEL immunostaining. Supporting our HF elongation results we found that the

proportion of apoptotic cells increased significantly in the TGF- β 2 treated group compared to the control sections, and in parallel, the number of Ki67 positive, i.e. proliferating cells, decreased. Adenosine was also effective in blocking this effect: in the HFs treated with both TGF- β 2 and adenosine we saw no significant difference compared to the control group in the ratio of Ki67 positive cells, however the percentage of TUNEL positive cells was significantly decreased. These effects were adenosine-dependent, since the pan-ADOR antagonist CGS15943 completely blocked the protective effect of adenosine upon TGF- β 2 treatment.

Histomorphometric hair cycle analysis showed that, as expected, TGF- β 2 applied alone induced significant catagen induction. Adenosine was able to block this effect on HFs both in the mid- and late-catagen phase, while simultaneously increasing the ratio of anagen and early-catagen HFs. CGS15943 once again abrogated the effect of adenosine, since the percentage distribution of this group is almost identical to the TGF- β 2 treated group only. Our results show therefore that adenosine, and in a broader context signalization through the adenosine receptors regulates the hair cycle by inhibiting the induction of the catagen phase and prolonging the anagen phase.

To further support our results, we performed DP stalk analysis of the treated HFs, and in good accordance with the above detailed experiments we found that adenosine could effectively (and ADOR-dependently) counteract the catagen-inducing effect of TGF- β 2.

HFs and ORS keratinocytes both express ADOR isoforms

We investigated the expression of certain ADOR subtypes in human HFs to further elucidate the signaling processes behind the effects observed above. After reverse transcription of the complete mRNA from anagenic follicles, cDNA was generated from three different donors. Subsequently, it was demonstrated by RT-

qPCR that all four ADOR subtypes (A_1 , A_{2A} , A_{2B} and A_3) are expressed on HF, and of these (at least at the mRNA level) A_{2B} is the most highly expressed isoform.

In order to obtain more accurate information regarding the presence and localization of ADORs at the protein level, immunohistochemical detection of frozen sections of human anagen HFs was performed. We found that, although different receptor subtypes have different expression patterns, all four receptors are expressed at the protein level in human HFs.

Since the ORS keratinocyte layer plays an important role in hair cycle regulation, and these cells showed positive expression of all ADOR isotypes we continued our studies on primary ORS keratinocyte cultures isolated from HFs.

In full agreement with our previous experiments on HFs, we found that expression of all four ADOR isoforms was detected on primary human ORS keratinocytes at both the mRNA (RT-qPCR), and protein (immunofluorescent labeling) levels, with A_{2B} receptor once again showing the highest expression of all isoforms.

Investigation of the possible mode of action of adenosine

Our experiments to date have convincingly demonstrated the effect of adenosine on the hair cycle, and as such we found it important to gain insight into the underlying mechanism of action. ORS cells were treated with adenosine in combination with CGS15943 (ADOR pan-antagonist) or MRS1754 (selective A_{2B} antagonist). We next investigated the effect the above-mentioned treatments had on the expression of certain negative and positive hair cycle regulator genes using RT-qPCR technique. According to our results adenosine decreased the expression of TGF- β 2 and epidermal growth factor (EGF) – both known to be catagen-inducing mediators – while increasing the expression of the anagen and pigmentation promoting stem cell factor (SCF) and insulin-like growth factor 1 receptor (IGF1R).

These results are in good accordance with our prior experiments and provide insights into the complex processes leading to the effects of adenosine treatment on the hair cycle. It is important to highlight that coapplication of the pan-ADOR antagonist CGS15943 could effectively block these effects, which means that adenosine-induced expression changes occurred in an adenosine receptor-dependent manner. Given that A2B shows the highest mRNA expression among adenosine receptors in our cells, we also performed these experiments in the presence of the selective A2B receptor antagonist MRS1754. We found that the use of the specific antagonist resulted in similar expression changes as those elicited by the pan-antagonist, which suggests that in our case adenosine acts through the A2B receptor.

2. Examination of the effects of caffeine on the hair cycle

Male and female human HFs respond differently to caffeine stimulation

According to our results, in HFs from male donors, the co-administration of caffeine with TST was able to halt the effects of TST, i.e. the inhibition of hair shaft elongation and the anti-proliferative effect on matrix keratinocytes (MKs). In light of the fact that the problems associated with hair growth disorders are not limited to men, we also added female donors to our experiments. According to our studies, TST treatment reduced the elongation of HFs, but treatment with different concentrations of caffeine was able to compensate for this in a statistically significant way.

Caffeine stimulates the proliferation of MKs from HFs from female and male donors

As a continuation of our experiments, we wanted to gain a deeper insight into the cellular effects of caffeine on HFs. As a first step, we examined how it influences the proliferation of MKs. We used TST treatment alone or in combination with caffeine on HF organ cultures, and our samples were labeled with Ki67-TUNEL combined staining. Caffeine, when used at a concentration of 0.001%, was able to significantly increase the number of Ki67 positive cells in both the control and TST treated male donors. HFs from female donors, were much more responsive to TST treatment as it greatly reduced the number of proliferating MKs. This effect was partially offset by the addition of 0.0005% caffeine, which increased the ratio of Ki67 positive cells to around 30%, which was no longer significantly different from the control group.

Caffeine enhances proliferation of cultured ORS keratinocytes

After determining that caffeine enhances the proliferation of MKs in HF organ culture, we wanted to further investigate its cellular effects, so we continued our studies using primary ORS keratinocyte cultures from male donors.

Fluorescent CyQUANT assay was used for ORS keratinocyte proliferation assay. According to our results, caffeine significantly increased cell proliferation. This effect was most pronounced in the 24-hour studies, with a 160% increase compared to the control group.

Ki67-specific immunocytochemical staining was used to independently confirm the results of our proliferation assays. Following quantitative analysis of the data, we found that 24-hour caffeine treatment significantly and dose-dependently increased the proportion of ORS keratinocytes with positive Ki67 immunostaining.

Caffeine reduces the cell death-inducing effect of TGF- β 2 on ORS keratinocytes

It has been described that TGF- β 2 induces the transition into the catagen phase by initiating cell death processes in several cell populations of HFs (including ORS keratinocytes). Based on this fact, we planned our next experiments to determine whether caffeine, in addition to its already known proliferation inducing effect, can counteract the onset / progression of cell death processes in ORS keratinocytes. Combined DilC1 (5) -SYTOX Green labeling showed no significant difference when using caffeine alone, whereas TGF- β 2 significantly reduced DilC1 (5), which correlates with mitochondrial membrane potential, and increased SYTOX Green staining which marks cell membrane disintegration, in other words TGF- β 2 induced early cell death pathways. However, with caffeine treatment these changes proved to be preventable. In addition to TGF- β 2, our experiments were repeated with the known catagenic inducer endocannabinoid anandamide (AEA) and were in good agreement with the above. Again, caffeine exhibited a dose-dependent protective effect, as did the positive controls (IGF-1 and keratinocyte growth factor [KGF]) used.

Caffeine completely suspended the catagen-inducing effect of TST in men and was partially effective in women

After examining the cellular effects of caffeine, its effects on the hair cycle were monitored. After TST and caffeine treatment of HFs, HE staining and histomorphometric analysis were performed. We found that TST reduced the proportion of anagen phase VI HFs in men by 20% compared to the control group. In contrast, co-administration with caffeine showed a marked increase in the proportion of HFs in the anagen phase, up to 70%. Similar, albeit less pronounced, changes were observed in female donors, i.e., the catagen-inducing effect of TST was partially reversible at different concentrations of caffeine. These results were clearly confirmed in the case of male donors by examining the hair cycle score (HCP): TST treatment increased the HCP value, while in the case of male donors the concomitant use of caffeine resulted in normalization, whereas in female donors caffeine was ineffective.

Caffeine treatment differentially influences the intracellular expression of the hair cycle modulator TGF- β 2 and IGF-1 in HFs from male and female donors

After demonstrating the ability of caffeine to reverse the catagenic phase-inducing effect of TST on male HFs, we focused our attention on critical hair cycle regulatory pro-anagen (IGF-1) and pro-catagen (TGF- β 2) factors. As a first step, immunohistochemical detection of hair cycle regulators was performed on sections from TST and caffeine treated and subsequently frozen HFs. In addition to the ORS layer, TGF- β 2 expression was also observed in the cells of the Henle layer (IRS). As expected TST treatment significantly increased the expression of catagen-inducing TGF- β 2 in HFs from male donors, and consistently with our previous results co-treatment with caffeine resulted in normalization of intrafollicular TGF- β 2 protein expression. In other words, caffeine was able to counteract the TGF- β 2 expression inducing effect of TST. The results obtained from HFs from female donors showed slightly different results, as TST treatment

did not increase the expression of TGF- β 2, but co-treatment with caffeine also significantly reduced the protein expression compared to the control group.

In the next step, changes in IGF-1 expression were monitored in the experimental system described above. According to our results, expression of IGF-1 protein was observed in both the ORS and the inner root sheath (IRS) layer. In male donors, the decrease in intrafollicular expression caused by TST treatment was not only offset by the use of caffeine, but also showed a significant increase compared to the control group. In contrast, in female donors, the effects of TST treatment showed significant donor dependence, whereas caffeine alone resulted in a significant increase in IGF-1 expression.

Caffeine affects TGF- β 2 and IGF-1 expression and release on ORS keratinocytes

Primary ORS keratinocyte cultures were once again used to examine the cellular expression of TGF- β 2 and IGF-1. RT-qPCR was used to investigate the expression of specific mRNA, while ELISA was used to investigate the release of molecules at the protein level. Isotretinoin, which was used as a positive control as pro-catagen inducer significantly increased the detectable amount of TGF- β 2 in the supernatant of the cells, whereas caffeine caused a significant decrease in TGF- β 2 release after 120 hours of treatment.

In the gene expression study of IGF-1, mRNA transcripts in three of the six male donors were below the detection threshold, while in three donors we were able to detect expression, albeit at low levels. In two of the latter three donors, caffeine treatment (0.001%) was able to significantly increase IGF-1 expression, whereas in the third donor no significant change was seen. Analysis of the changes at the protein level by ELISA revealed that the treatment with 0.001% caffeine significantly increased the release of IGF-1 at each of the three time points examined.

DISCUSSION

Hair and hair growth disorders negatively impact the quality of life of millions of people worldwide. Androgenetic alopecia (AGA) is responsible for a significant percentage of the population's hair loss, affecting both men and women. The molecular background of this process is the increased sensitivity of pathogenetically predisposed HFs to dihydrotestosterone (DHT). In addition to the commonly used 5-alpha-reductase (5-AR) inhibitor Finasteride or Dutasteride, and topical Minoxidil, low-frequency laser interventions, mesotherapy and hair transplantation are the most commonly used therapeutic interventions, all used with varying (often disappointing) degrees of effectiveness. The development of new pharmacological therapeutic agents is slow. The unclear pathophysiology, which results in inadequate therapy, and ever-widening social demands, has led many research teams around the world to better understand the biology of hair growth. Based on these facts we investigated the direct effect of adenosine and caffeine on HF and ORS keratinocytes and also their indirect effect on modulating the hair cycle.

Adenosine as a potential hair cycle regulatory molecule

The beneficial systemic effects of adenosine are widely known, so it is not surprising that its use is gaining traction in HF biology as well. Previous clinical studies have reported the beneficial effects of adenosine on hair growth in both male pattern hair loss (AGA) and female hair loss: topical adenosine treatment increased both hair diameter and hair growth rates in Japanese and Caucasian populations. Although these results describe adenosine as a positive hair cycle regulatory molecule, the underlying processes behind these effects were not investigated, so the exact mechanism of action of adenosine remains to be elucidated.

In good agreement with previous clinical studies, we found that adenosine treatment increased hair growth *in vitro*. In order to better understand the

molecular pathways underlying these effects, using immunofluorescent and histological labeling, we found that adenosine treatment increased the proliferation of intrafollicular MKs, and decreased the number of HFs in the regressive, catagen phase while simultaneously increasing the ratio of anagen HFs. All these results suggest that adenosine is a potential regulator of the hair cycle, which hypothesis we wished to test from another angle as well. To this end, we used TGF- β 2 to induce the catagenic phase in human HF organ culture, followed by adenosine treatment. From these experiments we came to the following conclusions: adenosine could partially block the elongation-inhibiting effect of TGF- β 2, it increased the proliferation of MKs and decreased the number of apoptotic cells, thereby preventing the effects of the catagen inducer, as well as blocking the entry of HFs into the catagen phase, and preserving the morphological characteristics of anagen HFs. To confirm our results, we performed a DP stalk analysis which also supported our findings that adenosine is able to counteract the catagenic phase induction effect of TGF- β 2. In order to identify potential signaling targets for hair growth, we used the ADOR pan-antagonist CGS15943, the presence of which completely abrogated the adenosine HF-longening and MK proliferative and anagenic phase-prolonging effects.

Based on data from the literature on HF organ cultures established from murine whiskers adenosine plays a role in maintaining the anagen phase and enhances the cysteine uptake of intrafollicular cells, which is a characteristic marker of proliferating cells. Based on our results so far, the effect of adenosine on human and mouse HF was essentially similar.

The molecules involved in hair cycle regulation and their receptors have been extensively documented in the literature in several mesenchymal and epithelial compartments of HF, covering an extensive paracrine regulatory system for hair cycle regulation. Modulation of the paracrine regulatory loops may provide effective therapeutic solutions for hair growth problems, so in the next

step of our experiments we investigated how adenosine treatment influences the expression of potential hair cycle regulating molecules on ORS keratinocytes.

Previous studies in mice have shown that adenosine treatment was able to enhance the expression of certain positive hair cycle regulatory factors (FGF2, FGF7, IGF-1 and VEGF) in murine whisker-derive DP cell cultures. Experiments on human DP cell cultures have shown that the FGF7 expression-enhancing effect of adenosine was abolished in the presence of the specific A_{2B} receptor blocker alloxazine, and the expression of the A_{2B} receptor on DP and ORS cells was detected by immunohistochemistry.

In good agreement with previous data from the literature, we found that all four ADOR subtypes are expressed on the HF, the expression pattern of the A_{2B} receptor is as described above, and in fact, its expression level is the highest at the mRNA level of ADORs. In our work, we investigated the expression of ADORs in primary ORS keratinocyte cultures and showed similar results to those seen in the HF: we detected the expression of all four ADOR subtypes at both protein and mRNA levels and found that the A_{2B} receptor had the highest expression of specific transcripts.

Next, adenosine treatment was performed on ORS keratinocyte cell cultures and we found that adenosine enhanced the expression of both IGF-1R, which is involved in the initialization of the anagen phase, and SCF, which is responsible for the pigmentation associated with said phase. It also reduced the expression of the well-known catagen inducers TGF- β 2 and EGF. All these results suggest that ORS keratinocytes play an important role in mediating the anagen supporting and hair growth-promoting effects of adenosine through mesenchymal-epithelial paracrine communication between certain HF cell groups.

Although the exact characterization of the HF adenosine system is still to come, our results provide a deeper insight into the effect of adenosine on hair growth and intrafollicular proliferation and bring us closer to understanding the potential signaling pathways and also highlight the role of ORS keratinocytes in

adenosine signaling. Based on all these results, the role of adenosine in hair growth and its potential as a therapeutic target in the treatment of hair growth disorders is further highlighted.

Caffeine as a potential hair cycle regulatory molecule

The effect of caffeine on hair growth is known from the literature reported previously by our co-authors: caffeine plays a role in inducing the re-entry of HFs, which are "trapped" in the telogen phase by elevated TST levels in AGA. In addition, there is a growing number of clinical studies describing the efficacy of using caffeine alone or in combination with other agents against hair loss in AGA.

As the pathologies associated with hair loss affect more and more people, including women, we explored the potential role of caffeine in regulating the hair cycle by extending our current experiments to women donors as well.

We first demonstrated in microdissected HF organ cultures from female donors that caffeine can counteract the inhibitory effect of TST on longitudinal hair growth, however it is important to note that HFs from women donors responded more sensitively to caffeine, so we had to decrease the applied dose compared to what we found effective in men in previous experiments.

To further understand the underlying intrafollicular processes, we performed immunofluorescent labeling and found that caffeine treatment was able to significantly increase the number of proliferating MKs, thereby counteracting the effect of TST on both female and male donors. Following histomorphometric analysis, it was found that, in addition to its effect on the proliferation of MKs, caffeine was able to counteract the catagen-inducing effect of TST, since the proportion of HFs in the anagenic hair cycle phase was not reduced in the HFs treated with both TST and caffeine compared to the control group.

Expanding our experiments to include primary ORS keratinocyte cell culture we demonstrated through diverse methodological approaches that caffeine treatment resulted in increased cell proliferation and reduced apoptotic rate, even

in the presence of catagen inducers TGF- β 2 and AEA. These findings are consistent with previous studies in mice, where caffeine applied to vascular smooth muscle cells increased cell proliferation, decreased apoptosis, and the release of reactive oxygen species. It was also able to halt the antiproliferative effect of TST on human epidermal keratinocytes and to restore TST-damaged skin barrier functions in *in vivo* human studies.

According to our results from microdissected HF organ culture and primary ORS keratinocyte cells caffeine extends the anagen phase, enhances proliferation and reduces apoptosis thereby counteracting the catagen-inducing effects of TST and TGF- β 2. In the next phase of our experiments, we investigated the effect of caffeine on the expression of well-known hair cycle regulators, the aforementioned anagen-inducer IGF-1, and the catagen inducer, TGF- β 2.

Using immunofluorescent labeling on microdissected HF organ culture, we found that caffeine effectively counteracted the increase in TST-induced TGF- β 2 protein expression in men, whereas in women, although the TST-inducing effect was absent, the expression level of TGF- β 2 was still significantly decreased compared to the control. According to our results, TST had a catagen inducing effect in both sexes, but increased the expression of TGF- β 2 only in males, while this effect was absent in female donors. Caffeine also increased the expression of the anagen-inducing IGF-1 protein, thereby counteracting the effect of TST on IGF-1 expression in males, whereas in female donors caffeine alone resulted in a significant increase in IGF-1.

Our results are also supported by our studies on primary ORS keratinocyte cell cultures – which cells play a key role in the regulation of hair cycle regulation – where we recorded similar effects as described above: caffeine significantly reduced the expression of TGF- β 2 at the protein level and increased the expression of IGF-1 on both the mRNA and protein levels according to our RT-qPCR and ELISA studies respectively.

In conclusion, our *in vitro* experiments on ORS keratinocytes and microdissected HF organ cultures demonstrate the complex role of caffeine in hair biology. The overall beneficial effects raise the potential of caffeine as a putative therapeutic agent in the treatment of hair growth disorders. Our experiments shed light on the effects of caffeine on hair growth, regardless of the differences observed between the sexes, and this is the first study on the effects of caffeine on microdissected HF organ culture that investigates HFs from female donors. The underlying mechanisms behind this difference in caffeine sensitivity between the sexes has not been elucidated as of yet.

A possible hypothetic mechanism, or where the strands intertwine

Caffeine achieves most of its biological effects by interrupting adenosine tone by inhibiting ADORs. This begs the question: how can two molecules with a high degree of structural analogy, which can cause opposing molecular effects, influence the hair cycle in the same way? Of course, to answer this question, experimental studies are needed to deepen the underlying signaling processes, so our assumption remains purely hypothetical. A potential milestone of adenosine A_{2B} receptor signaling may be adenylate cyclase (AC) stimulation, resulting in increased cyclic adenosine monophosphate (cAMP) levels. In contrast, caffeine (in addition to inhibiting ADORs) can also inhibit the degradation of cAMP through inhibition of phosphodiesterase (PDE), which can (like adenosine) lead to increased levels of cAMP.

Gender differences in hair growth may be due to differences in the effect of sex hormones on the level of intrafollicular secondary messengers. According to data in the literature, estrone enhances AC and DHT inhibits it - thus the former increases the level of cAMP, while the latter decreases it. Elevated DHT levels in AGA combined with HFs that are genetically sensitive to these high levels results in the increased conversion of terminal hair to vellus hair, and subsequent partial or complete baldness, which may be due to DHT inhibition of AC function and

concomitant decreased cAMP levels. In our experiments with caffeine, we found that HFs of female donors showed increased sensitivity to this compound. It is possible that the increased activity of AC due to estrogen may also play a role in higher intracellular cAMP levels. Thus caffeine, which inhibits PDE activity to increase cAMP levels, results in a greater effect in women than in men, who have less basal AC activity.

Elevated cAMP levels may influence diverse paracrine signaling pathways, and thus may play a role in the increased production of anagen-promoting factors IGF-1 and SCF as well as the decreased production of catagen-inducers such as TGF- β 2 and EGF. According to our results the expression level of these hair cycle regulatory molecules showed significant changes upon adenosine and caffeine treatments. It is likely that elevated cAMP levels also improve microcirculation in the HF's environment thereby increasing nutrient and oxygen delivery, which are essential for HF growth and development. Although these ideas are purely theoretical, they show good accordance with data in the literature and we can safely posit that the use of caffeine and/or adenosine-based therapies holds promise to be an extremely effective new tool for the treatment of AGA, without significant side effects.

APPENDIX



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List of publications related to the dissertation

1. **Herczeg-Lisztes, E.**, Tóth, I. B., Bertolini, M., Szabó, I. L., Zákány, N., Oláh, A., Szöllősi, A. G., Paus, R., Bíró, T.: Adenosine promotes human hair growth and inhibits catagen transition in vitro: role of the outer root sheath keratinocytes.
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DOI: <http://dx.doi.org/10.1111/bjd.13114>
IF: 4.275





List of other publications

3. Szabó, I. L., **Herczeg-Lisztes, E.**, Béke, G., Tóth, K. F., Paus, R., Oláh, A., Bíró, T.: The phytocannabinoid (-)-cannabidiol (CBD) operates as a complex, differential modulator of human hair growth: anti-inflammatory submicromolar versus hair growth inhibitory micromolar effects.
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