Report

Effect of caffeine and testosterone on the proliferation of human hair follicles *in vitro*

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Abstract

Background Androgenetic alopecia (AGA) is a common problem in men of all ages, affecting approximately 50% at 50 years of age. The underlying cause is an androgen-dependent miniaturization of genetically predetermined hair follicles. Here, the hair organ culture model was used to investigate the effects of testosterone and caffeine; the latter being a promising candidate for hair growth stimulation.

Methods Hair follicles from 14 biopsies, taken from the vertex areas from male AGA patients, were cultivated for 120–192 h *in vitro* with normal William's E medium (control) or William's E medium containing different concentrations of testosterone and/or caffeine. Hair shaft elongation was measured daily and at the end of cultivation, cryosections of follicles were stained with Ki-67 to evaluate the degree and localization of keratinocyte proliferation.

Results Significant growth suppression was found in hair follicles treated with 5 μg/ml testosterone. This was counteracted by caffeine in concentrations of 0.001% and 0.005%. Moreover, caffeine alone led to a significant stimulation of hair follicle growth. These results were confirmed immunohistochemically by Ki-67 staining.

Conclusions Androgen-dependent growth inhibition of *ex vivo* hair follicles from patients suffering from AGA was present in the human hair organ culture model, a constellation which may serve for future studies to screen new substances against androgen-dependent hair loss. Caffeine was identified as a stimulator of human hair growth *in vitro*; a fact which may have important clinical impact in the management of AGA.

Introduction

Androgenetic alopecia (AGA) is a common problem in men of all ages, commonly beginning at 20 years of age with a prevalence of approximately 50% at the age of 50 years.[1,2] The development of AGA is predominantly androgen-dependent and modulated via the testosterone metabolite dihydrotestosterone (DHT) and the expression of hair follicle-related androgen receptor (AR).[3,4] DHT, a metabolite of circulating testosterone, is produced systemically and by intrafollicular conversion of testosterone to DHT by 5alpha-reductase within the hair follicle of genetically predisposed men.[5–7] The DHT is the effector hormone causing a continuous shortening of hair growth (anagen) cycles in favor of longer telogen phases, followed by miniaturization of the hair follicle and finally visible cessation of hair growth in genetically predetermined areas in the frontal-temporal and vertex regions.[2,5,8,9]

To date, only two FDA-approved drugs (oral finasteride and topical minoxidil) are available for treatment of AGA.[10,11] However, 20–30% of AGA patients receiving these drugs are nonresponders – a fact that encourages a search for alternative substances for the treatment of AGA. Such substances may either exert a complementary effect by targeting alternative nonandrogen related mechanisms of AGA or act synergistically with the established antiandrogenic drugs.

Caffeine is a well-known substance, yet little is known about its effect on human hair follicle growth. As a phosphodiesterase-inhibitor, caffeine increases cAMP levels in cells and therefore promotes proliferation by stimulating cell metabolism; a mechanism which would counteract testosterone/DHT-induced miniaturization of the hair follicle.[12] In a male skin organ culture model (MSOCM), caffeine was shown to reverse the inhibiting effect of testosterone on keratinocyte proliferation.[13] The hair organ culture model (HOCM) is a suitable model for investigating the inhibitory and/or stimulatory effect of various substances on human hair follicles which are extracted *in toto* and cultivated for up to 10 days.

Whether the results from the MSOCM would be transferable to the hair follicle model was the aim of the present study. Hence, we cultivated *ex vivo* human hair follicles derived from scalp biopsies from affected vertex areas from male AGA

Table 1 Indicated concentrations of testosterone and caffeine alone and in combination were used for the experiments

William's E medium		Additional combination of active ingredients used
Testosterone	5 ng/ml*	Testosterone 5, 10, and 50 ng/ml + caffeine 0.15%*
Testosterone	10 ng/ml*	Testosterone 50 ng/ml + caffeine 0.01%*
Testosterone	50 ng/ml*	Testosterone 50 ng/ml + caffeine 0.005%*
Testosterone	0.5 μg/ml*	Testosterone 50 ng/ml + caffeine 0.001%*
Testosterone	1 μg/ml*	Testosterone 5 μg/ml + caffeine 0.01%*
Testosterone	5 μg/ml*	Testosterone 5 μg/ml + caffeine 0.005%*
Caffeine	0.15%*	Testosterone 5 μg/ml + caffeine 0.001%*
Caffeine	0.05%*	
Caffeine	0.01%*	
Caffeine	0.005%*	
Caffeine	0.001%*	

*In William's E medium.

patients *in vitro* and aimed to identify the maximal inhibitory concentration of testosterone and at what concentration this inhibitory effect would be reversed by caffeine.

Materials and Methods

Scalp biopsies

Human hair follicles were taken from scalp biopsies (0.5 × 1.5 cm) under local anesthesia from the vertex region in the border area of the dense to the shedding area of 14 male patients (aged 20–45 years) suffering from androgenetic alopecia in middle-stage showing slight to moderate shedding of the vertex area (stage III vertex and IV Norwood-Hamilton classification[1]). The study was approved by the Ethics Committee of the Friedrich-Schiller-University Jena and written informed consent was obtained from the patients in accordance with the "Helsinki declaration".

Hair follicle extraction and cultivation

Scalp skin biopsies were transferred directly after excision into physiologic 0.9% sodium-chloride solution in which they were kept at maximum 1 h until further processing. Single hair follicles were prepared by first horizontally cutting the biopsy with a scalpel at the dermo–subcutaneous fat interface and transferring the subcutaneous part containing intact anagen hair follicles to Petri dishes containing Earl's balanced salt solution (1/3) and Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (2/3) (Sigma, St. Louis, MO). Then, intact anagen hair follicles were carefully micro-dissected with their root sheaths from subcutaneous fat by softly grasping the follicle by the outer root sheath with watchmaker's forceps and pulling with slight traction; the extracting procedure was carried out under the sight of a binocular and cold-light source. After extraction, single hair follicles were transferred to 24-well-plates, one follicle per well containing 500 μl William's E culture medium (PromoCell, Heidelberg, Germany). The culture medium was supplemented with L-glutamin (200 mM), insulin from lyophilized and irradiated bovine pancreas (10 μg/ml), hydrocortison (10 ng/ml) and antibiotic solution (penicilline/streptomycine 100 IE/ml). Cultivation was carried out at 37 °C and 5% CO_2 over 120–192 h and hair shaft elongation was measured each day by using a scaled microscopic eyepiece. The William's E medium with the above mentioned supplements was used as normal cell medium (control) and treatment medium was defined as supplemented William's E medium containing different concentrations of caffeine and/or testosterone (see Table 1). Control and treatment media were changed every other day.

Ki-67 immunohistochemistry

At the end of the cultivation period, hair follicles were carefully removed from the wells with micro-tweezers and transferred to micro-boxes made of aluminum foil. The boxes, 1 × 1 × 1 cm in size, had a longitudinal hollow on the bottom in which the hair follicle was exactly placed to ensure stable horizontal position during the freezing process. This process was induced by pouring freezing-gel (Tissue-Tek, O.T.C.; Miles Diagnostics, Elkhart, IN) onto the hair follicle, followed by immediate deep freezing in liquid nitrogen. Frozen sections with 3–5-μm thickness were performed with a kryotom (Frigocut 2800E; Leica, Nussloch, Germany) and 6–12 cryosections from each follicle were placed on mounted slides (SuperFrost Plus; Menzel, Braunschweig, Germany) and dried overnight at 37 °C. After fixation, sections were incubated with Ki-67 antibody (DAKO, Glostrup, Denmark), and detection-kit AEC was used in conjunction with the staining device Nexes (both of Ventana Instruments, Illkirch, France). Evaluation of Ki-67-positive cells was performed after assessment of staining intensity for each follicle and quantification of Ki-67 positive keratinocytes. For this purpose, a semiquantitative score with values from 0–3 was developed. This score separately quantified the number and distribution of positive matrix keratinocytes in different layers above the hair papilla and positive keratinocytes in the basal, middle and upper outer root sheath. Score values were subdivided by ranking numbers from 5–40 for sub-specification, which is described in Table 2.

Table 2 Scores with ranking-values for the assessment of the number and distribution of Ki-67 positive keratinocytes indicating cell proliferation in cryosections from hair follicles

Score-value	Definition	Staining grade	Ranking-value
Dermal papilla			
0	0–3 cells Ki-67 positive		5
1	Ki-67-positive cells in basal layer of supra-papillary matrix keratinocytes	a) Single cells	10
		b) 50% cells stained	15
		c) 100% cells stained	20
2	Ki-67-positive cells in basal and 2–4 higher layers	a) Single areas < 50%	25
		b) Almost full area with 50–75% staining	30
		c) continuous staining (maximum 2–3 gaps)	35
3	Ki-67-positive cells in > 5 layers		40
Outer root sheath			
0	0–3 cells Ki-67 positive		10
1	> 3 cells, but single		20
2	Chain-like stringing together of cells (single)		30
3	Continuous chain-like stringing of cells in 2–3 rows		40

Statistical analysis

The mean values of hair shaft elongation measurements were calculated from all follicles ($n = 6–8$) for each treatment condition per biopsy and the minimum of three biopsies were taken for each experimental setting showing effect of testosterone alone, caffeine alone and a combination of both substances. Repeated measures ANOVA with appropriate post hoc testing was performed with GraphPad Prism ver.4.0 (GraphPad Software Inc., San Diego, CA). Differences were considered significant when $P < 0.05$. For statistical analysis of Ki-67 staining, the mean values of ranking numbers were collected from 6–8 follicles per single treatment condition and Student's *t*-test checked for unpaired observation.

Results

Hair shaft length measurements

Inter-individual variability of number of extracted hair follicles per biopsy caused a situation that not all substances could be included in every experiment, resulting in differences in the number of used concentrations of testosterone and caffeine per experiment.

First, a suppressive effect of testosterone was shown in 11 of 14 experiments after 120 h of hair follicle growth in culture: in follicles of the first two scalp biopsies, testosterone concentrations of 5, 10 and 50 ng/ml were used; 50 ng/ml being the most suppressive. The mean hair shaft length under 50 ng/ml testosterone, applied in six experiments, was 94.3% compared with control. Higher concentrations of 0.5 μg/ml and 5 μg/ml showed hair shaft length of 78.6% and 78.9% vs. control, respectively (Fig. 1). The suppression was significant at a concentration of 5 μg/ml, which was therefore chosen for further experiments.

Caffeine was tested in parallel with testosterone, either in combination or alone. In the first three experiments, caffeine

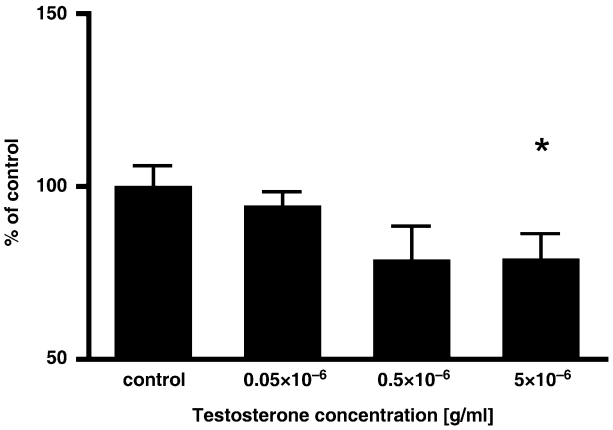

Figure 1 Testosterone at concentrations of 50 ng/ml, 0.5 μg/ml and 5 μg/ml suppressed hair follicle growth assessed at 120-h cultivation time to 94.3%, 78.6% and 78.9% vs. control, respectively. Significant suppression was observed under 5 μg/ml. Data from six independent experiments are shown with mean ± SEM, $*P = 0.0234$

at the concentration of 0.15% was used in combination with testosterone which resulted in stronger suppression than testosterone alone. Also, the lower concentration of 0.05% showed a suppression (data not shown). As a consequence of the observed inhibitory effects in high concentrations of caffeine, lower concentrations of 0.005% and 0.001% were used in further experiments. In the majority of the experiments with these low concentrations, a stimulatory effect was noted, but some experiments showed neutral or slightly inhibitory effects. However, statistically significant differences compared with control were only observed in experiments in which caffeine showed stimulation of hair follicle growth. The cumulative significant effect in these experiments after

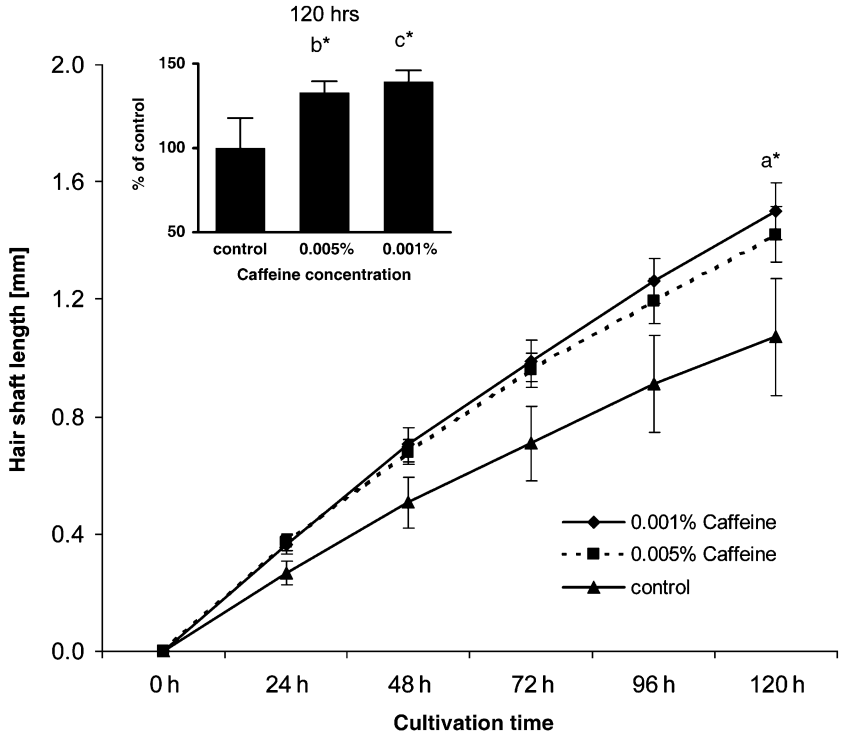

Figure 2 Caffeine led to significantly higher hair shaft elongation at 120 h cultivation time in concentrations of 0.005% and 0.001% compared with caffeine-free control. The effect at this time-point was 33% and 39.4% increase, respectively. Values represent means ± SEM from two representative experiments; a* = caffeine vs control, $P = 0.038$; b* = caffeine 0.005%, $P = 0.044$; and c* = caffeine 0.001%, $P = 0.0275$.

120 h in culture was an increase of 33% and 39.4% for incubation with caffeine at concentrations of 0.005% and 0.001%, respectively (Fig. 2).

To evaluate the effects of caffeine on prolongation of significant follicle growth in culture compared with caffeine-free control, the study investigated hair follicle growth during an extended cultivation time of 192 h. The growth-rate per 24 h observed in the follicles treated with William's E medium displayed a significance level of only $P < 0.05$ after a 96-h cultivation period and no significant hair growth per 24 h after 144 h. In contrast, hair follicles treated with caffeine 0.001% and 0.005% showed a highly significant ($P < 0.001$) 24-h growth-rate until 144 h, and still further significant hair growth (significance level of $P < 0.01$) until the end of the cultivation period of 192 h. Potent stimulation was also observed in absolute hair shaft length measurements. The differences in growth under caffeine at the concentration of 0.005% compared with the control reached a level of significance of $P < 0.05$ after 72 h, of $P < 0.01$ after 96 h and of $P < 0.001$ between 120–192 h. Even more potent stimulation was observed in hair follicles treated with 0.001% caffeine. The difference compared with the control reached a significance level of $P < 0.01$ after only 72 h and of $P < 0.001$ between 96–192 h (Fig. 3).

After evaluation of the biologic effects of the substances alone, showing suppression of hair follicle growth by testosterone and stimulation by caffeine, combinations of both substances were used. The suppressive effect of testosterone (5 μg/ml) was counteracted by caffeine at the concentration of 0.005% showing a normal growth curve as under William's E medium alone, and caffeine at 0.001% led to a slight stimulation compared with control (Fig. 4).

Ki-67 immunohistochemistry

The immunohistological staining with Ki-67 confirmed the results obtained by hair shaft elongation measurements. Higher ranking values indicated stronger keratinocyte proliferation which was reached in the dermal papilla under treatment with caffeine 0.001% and 0.005% (Fig. 5a,b). The ranking-value was significantly different versus the control, exclusively under treatment with 0.005% caffeine, but there was also a clear tendency of stronger Ki-67 staining at 0.001% concentration. Also suppression of Ki-67 was observed in parallel to inhibition of hair shaft elongation by the treatment of testosterone (5 μg/ml) which was counteracted by caffeine 0.005% and 0.001% concentrations (Fig. 6a,b). In the outer root sheath, Ki-67 staining was also positive and more strongly expressed under caffeine than under control (Fig. 5b).

Discussion

The objective of the present study was to investigate if the suppressive effect of testosterone on keratinocyte proliferation, found in the MSOCM,[13] also applies to the HOCM using whole human androgen sensitive hair follicles[14,15] from male patients with AGA. These follicles were taken *ex vivo* and cultivated *in vitro* for a period of 120 h and 192 h. The

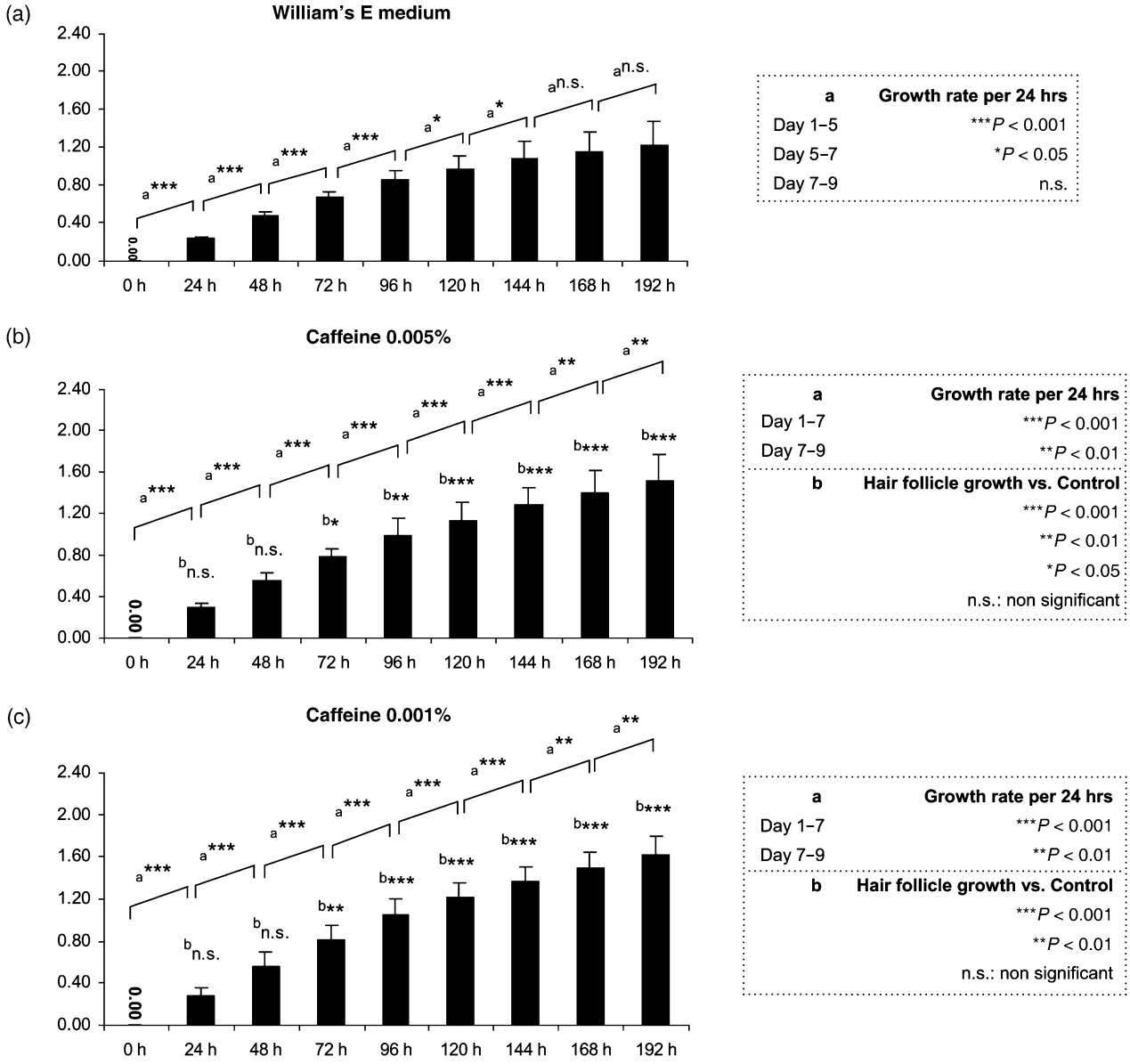

Figure 3 Hair follicle growth per 24 h under William's E medium was significant until 144 h of cultivation (a), whereas caffeine at concentrations of 0.005% (b) and 0.001% (c) showed significant 24 h growth-rate to 192 h. Absolute hair shaft length was significantly different under both concentrations of caffeine compared with control at 72 h of cultivation

experiments with testosterone alone in the HOCM confirmed its suppressive effects observed in the MSOCM. The most suppressive effect of testosterone was found at concentrations of 0.5 μg/ml and 5 μg/ml, which were higher than physiological ones found in human plasma (9 ng/ml) and the ones used in the above-mentioned skin model (5 ng/ml).[13] Men with AGA usually show normal levels of testosterone, and the androgenetic hair loss is caused by increased levels and/or activity of the converting enzyme 5alpha-reductase in the predisposed hair follicle[3,16]. Higher levels of testosterone were necessary in the hair organ culture model owing to the fact that vascular nutrition was missing and therefore testosterone had to reach the papilla by diffusion through outer and inner hair root sheath requiring higher concentrations. It may be criticized that testosterone is not the inducer of AGA. Testosterone, however, is the "prerequisite" of AGA and absence of testosterone leads to absence of male-pattern baldness as shown in castrated men.[17] The DHT is the effector substance in AGA, and local DHT levels in the scalp/hair follicle result from systemic and local conversion of testosterone to

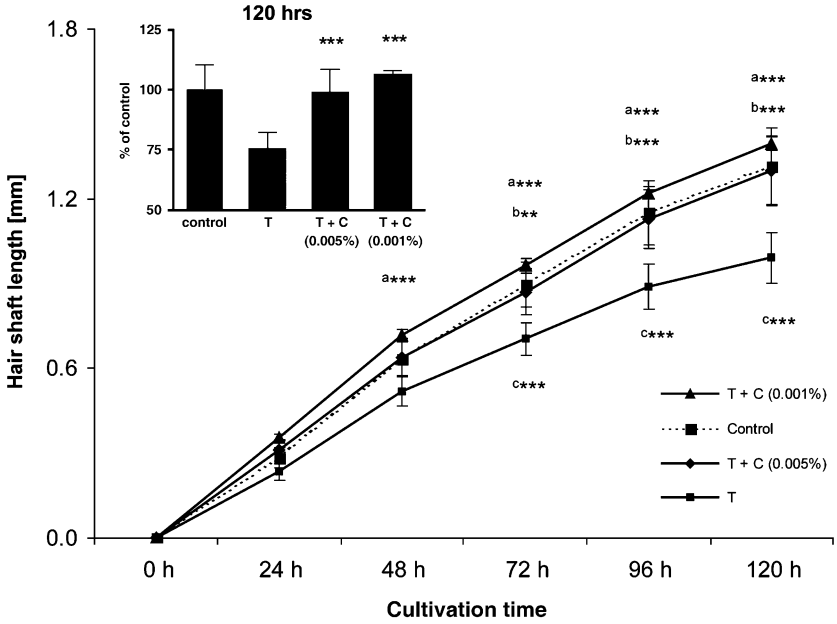

Figure 4 Significant suppression of hair follicle growth by testosterone (T) alone (5 μg/ml) was observed after 72 h of cultivation in comparison with the hair follicles cultivated with pure William's E medium (control). Addition of caffeine at the concentration of 0.005% to the culture medium containing testosterone reverses the testosterone-induced suppression of follicle growth. The addition of caffeine 0.001% to testosterone containing medium even stimulated hair growth to levels above the normal growth level under standard culture conditions (William's E medium without active substances except standard supplements). Where, T is 5 μg/ml testosterone and C is caffeine: (a) $***P < 0.001$, caffeine 0.001% + testosterone 5 μg/ml vs. testosterone 5 μg/ml alone; (b) $***/**P < 0.001/0.01$, caffeine 0.005% + testosterone 5 μg/ml vs. testosterone 5 μg/ml alone; and (c) $***P < 0.001$: Testosterone 5 μg/m vs. control (William's E medium). Asterisks in inset represent significance between T + C compared with T alone

DHT.[18] In extracted hair follicles from patients with AGA, local testosterone metabolism with production of DHT was significantly higher than in hair follicles isolated from occipital (nonbalding) scalp regions owing to increased 5alpha-reductase activity.[7,19] In the present *in vitro* study, the situation with "systemic" testosterone delivered to the hair follicle within the media fluid is imitated and in both systems (*in vivo* and *in vitro*) testosterone is then converted to DHT by the follicle itself via the intrafollicular enzyme 5alpha-reductase.[4,5,7]

For caffeine, dose-response experiments showed that a concentration of 0.15% was stimulatory in the MSOCM,[13] but suppressive in the hair follicle model. Also concentrations of 0.05% and 0.01% showed suppression. These high concentrations were regarded as nonphysiologic or toxic, an effect which can be observed in many substances. High concentrations may cause an over-stimulation of hair follicle metabolism resulting in extensive consumption of energy reserves, exhaustion of the proliferation capability and finally lack of hair shaft elongation. To date, it is not known which mechanism is relevant for the stimulatory effects of caffeine on hair follicle growth. Different mechanisms of caffeine observed in other models might be responsible: In alveolar macrophages caffeine exerted an antiapoptotic effect at low concentrations while showing proapoptotic effects at high concentrations. The antiapoptotic effect was accompanied by reduced production of super-oxide dismutase.[20,21] Other mechanisms are the arrest of cell growth (G0/1-arrest),[22] increase of prostaglandin levels or suppression of Cdc2 kinase and MAP kinases.[12] The stimulatory effect of caffeine in lower concentrations (0.001% and 0.005%) may be explained by the classic phosphodiesterase inhibition[12] and by a direct effect against apoptosis, which is induced in AGA.[23]

Ki-67 is a marker to detect positive nuclei in matrix keratinocytes indicative for proliferation of anagen hair follicles.[24–27] Although Ki-67 ranking-values were only significant for 0.005% caffeine, there was a clear tendency for high Ki-67 ranking-values also for 0.001%. This slight discrepancy to hair shaft elongation may be explained by variances of Ki-67 staining caused by factors of cryosection processing, staining procedure and affinity of Ki-67 antibody. Similar to other studies, the authors also found Ki-67 staining in outer root sheath cells which may contribute to proliferation and nutrition of the hair follicle.[28]

Variability in the response to caffeine as well as to testosterone were most probably owing to inter-individual differences in the sensitivity of hair follicles from different individuals and even in the variability of follicles from the same biopsy. Dermal papillae *in vitro* express considerable activity of

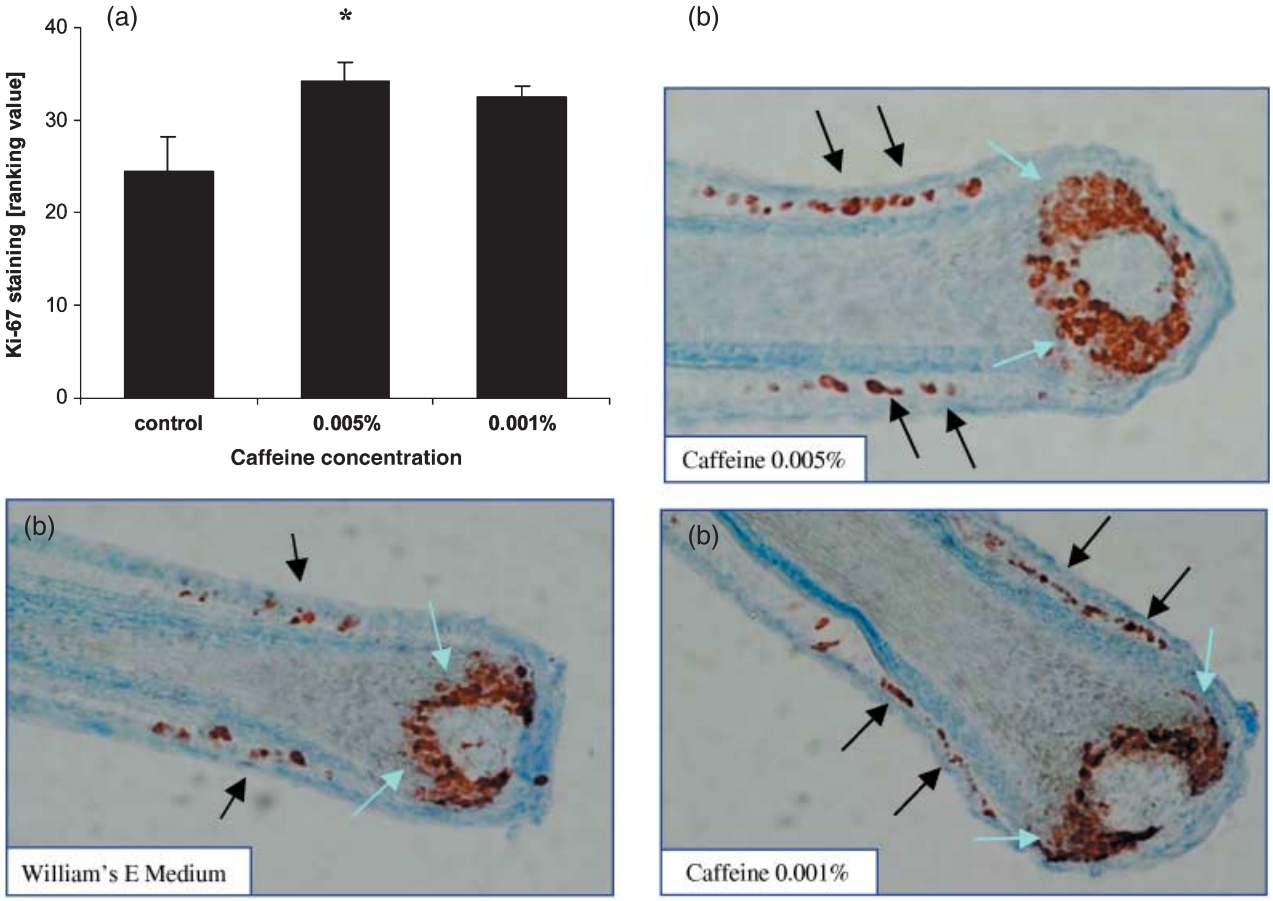

Figure 5 (a,b) Histologic ranking-values of Ki-67 positivity in dermal papilla matrix keratinocytes under caffeine 0.005% (n = 6) and 0.001% (n = 6) showed increase of proliferation compared with control (n = 8) which was significant for 0.005% (a). Immunohistochemistry of follicles treated with caffeine showed Ki-67 positive matrix keratinocytes in 2–4 layers above the dermal papilla with only a few gaps (blue arrows) while control follicles revealed only positivity in the angles of the basal region and bigger gaps in the latero-apical region (blue arrows). The outer root sheath showed chain-like staining in caffeine treated follicles compared with single positive cells in controls (black arrows). Ki-67 staining with AEC detection-kit and magnification ×20 (b)

5alpha-reductase, although with great interfollicular variability.[29] Different responsiveness from different patients may be explained by varying stages of disease activity, duration and clinical expression of AGA. The general variability of follicles to grow *in vitro* is another factor, as growth conditions in culture differ generally from natural hair growth *in vivo* or in skin equivalents:[30] Blood circulation with supply of oxygen, vitamins and minerals is missing as well as important regulatory structures (pilo-sebaceous bulb-unit, perifollicular connective tissue sheath, and epidermis). However, the advantages of the HOCM are the cultivation of human hair follicles as an entire organ to screen for stimulatory and/or inhibitory effects of substances which has successfully been shown for TGF-beta, EGF, IGF-1, minoxidil and 17alpha-estradiol.[14,15,31] The novel concept of this study is to take androgen-sensitive follicles selectively from patients with specific diagnosis of AGA and directly from the location where hair shedding is clinically observed, a procedure which would guarantee a high transferability from laboratory findings to the clinical situation of men with AGA *in vivo*.

In conclusion, male androgenetic alopecia is characterized by shortening of the anagen phase of genetically predisposed hair follicles induced by extra-follicular testosterone which is metabolized to DHT by intrafollicular 5alpha-reductase. This constellation was reproduced in the HOCM and the suppressive effect of testosterone was reversed by 0.001% and 0.005% caffeine. The results obtained *in vitro* constitute the basis for possible clinical effects of topically applied caffeine in the management of androgenetic alopecia.

Acknowledgment

This study was kindly supported by Dr Kurt Wolff GmbH, Bielefeld, Germany.

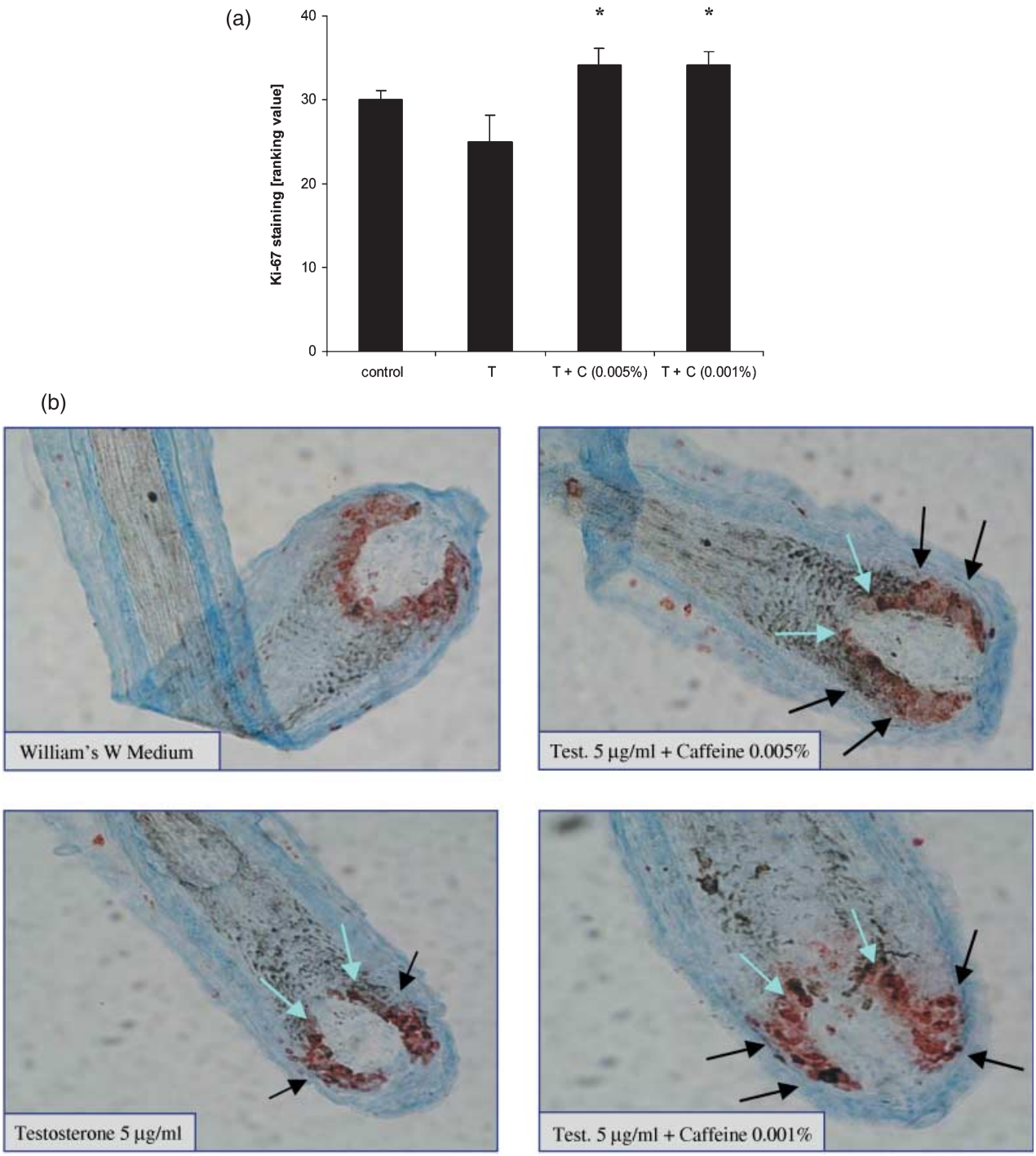

Figure 6 (a,b) Testosterone treated follicles (n = 6) showed a lower Ki-67 score-value than control (n = 8), whereas follicles incubated with testosterone and caffeine (n = 6) showed significantly higher values than testosterone alone (a). The immunohistochemical staining under testosterone showed positivity in 2–3 layers above the dermal papilla (black arrows) with lack of staining in the apical pole (blue arrows), whereas treatment with caffeine led to positivity in 2–4 layers (black arrows) and a tendency of closing the gap on the apical pole (blue arrows) compared with the testosterone treated follicles (b). T is 5 µg/ml testosterone, C is caffeine; Ki-67 staining with AEC detection-kit and magnification ×20

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