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An Analysis of the Safety and Efficacy of Topical Zinc-Thymulin to treat Androgenetic Alopecia

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Abstract

Objective: To assess the safety and efficacy of the metallopeptide zinc-thymulin (ZT) for treating androgenetic alopecia (AGA). Previous *in vitro* studies have described that different thymic peptides can both increase and decrease anagen (thymulin and thymosin beta-4, respectively). Zinc is an essential element and serum zinc deficiency can cause hair loss.

Methods: Eighteen consecutive adult subjects were recruited, 17 males and 1 female, age range 35-90 years (mean 55.4, SD 13.3) with a diagnosis of AGA, Norwood classification 2-7, and hair loss duration range of 3-40 years (mean 15.8, SD 9.6). The trial duration for each subject ranged from 4-10 months. The test compound ZT was synthesized by standard Fmoc peptide protocols and administered in water based topical spray to the scalp. Baseline and after treatment images for hair growth were graded by two blinded assessors using two validated scales: 1. numerical visual analog scale (VAS) for global assessment 2. hair growth index (HGI) of images under higher magnification for percentage changes of vellus, intermediate and terminal hair.

Results: ZT demonstrated no adverse systemic effects or local side effects of redness or scalp irritation in any subject over a total of 3,300 treatment days. Three subjects who were concurrently using minoxidil (N=2) and minoxidil / finasteride (N=1) did not report any drug interaction with ZT. VAS hair assessment improvement was significant in subjects who completed 6 months of treatment (P=0.045, t-test). HGI assessment showed a significant increase in the number of newly observed intermediate hairs in previous "absent hair" regions (P<0.0001) with an average increase of vellus type (32%) and intermediate type (23%) hairs at 6 months. Melanogenesis was observed in several subjects.

Conclusion: Topical applications of ZT demonstrated safety and established efficacy for initiating and maintaining anagen to treat male pattern baldness when applied for>6 months.

Keywords: Zinc-thymulin; Thymulin; Zinc; Androgenetic alopecia; Hair growth; Anagen; Melanogenesis; Male pattern baldness; Hair loss; Topical treatment

Introduction

Androgenetic alopecia (AGA) is a global problem and has an increasing prevalence with age, for example in Australia (author's country) the rate increases in men from 31% (age 40-55 years) to 53% (age 65-69 years) [1]. The condition is common in women [2] and can cause moderate psychological stress [3] through to high levels of anxiety and depression [4].

Most published research on the treatment of AGA has investigated surgical hair transplantation procedures and medication to control hair loss that includes finasteride, dutasteride and minoxidil. In contrast, there are very few studies evaluating the potential of endogenous and natural compounds to induce hair growth in humans. The purpose of this research study was to assess thymulin, a native human immune system peptide that was chemically combined with zinc, an essential element.

Thymulin is a nonapeptide derived from the thymus gland. Early reports of thymus extract stimulating hair growth was in 1986 [5] and 2000 [6]. Conclusive work for thymulin's activity on the hair follicle was recently published by Meier et al. [7] where this *in vitro* study investigated several thymic peptides and showed thymulin could extend anagen. However, the biological activities of both the thymus gland and serum thymulin concentrations are critically dependent on the presence of zinc [8]. The consequence of zinc deficiency in human's results in a lack of T cell differentiation [9] and the element is important for synaptic integration and plasticity [10].

The clinical symptoms of zinc deficiency include hair loss, diarrhea, impotence, weight loss and impaired wound healing [11]. Despite an adequate intake of zinc in the diet its absorption and bioavailability can be impaired by inhibitors such as phytic acid [12] that is found in cereals, legumes and nuts [13]. Other studies [14] showed significantly less zinc and copper in the hair of AGA subjects and a review of topical formulations reported that zinc administration could improve AGA [15].

In the scientific literature to date, there has been no previous *in vivo* human study investigating the biological activity of the combined zinc-thymulin complex for hair loss. The purpose of this investigation was to evaluate a synthesized topical form of the metallopeptide zinc-thymulin (ZT) for safety in humans and determine its preliminary efficacy in an open label trial design to treat male pattern baldness.

Methods

Subjects

Male and female adult subjects with a diagnosis of AGA were invited to participate in the study. Each subject provided written informed consent except the 90 year old subject who gave verbal consent as he was unable to provide written consent due to arthritis of the hand. The study was conducted in compliance with the Declaration of Helsinki, International Conference on Harmonization Good Clinical Practice guidelines. Eighteen consecutive adult subjects were recruited (17 males and 1 female) with an age range 35-90 years (mean 55.4, SD 13.3) with a diagnosis of AGA Norwood-Hamilton classification 2-7, and with a hair loss history ranging from 3-40 years (mean 15.8, SD 9.6). Entry time points of the volunteers occurred over 6 months with the trial completed at the same endpoint resulting in duration of treatment for subjects in the trial ranging from 4-10 months.

Materials

Thymulin has an amino acid sequence pyruvate-Glu-Ala-Lys-Ser-GIn-Gly-Gly-Ser-Asn (one letter sequence pEAKSQGGSN) and was synthesized using standard Fmoc protocols on a peptide synthesizer (AAPPTEC Corp. Louisville, Kentucky, USA). Amino acids, resin, reagents and resin cleavage chemicals were analytical grade (AAPPTEC; Labsupply, Sydney, Australia). Confirmation and purity of thymulin was performed by liquid chromatography coupled to an electrospray mass spectrometer (LCMS). Chemical methodology was adapted from previous LCMS peptide and steroid hormone methods published by the author [16,17]. The LCMS was a Shimadzu 2010AD system.

Purity of the peptide was established at >95% (Figure 1) with LC conditions employing reverse phase chromatography with mobile phase A 0.1% acetic acid in MilliQ water and phase B of analytical grade methanol, gradient 0-50% B over 30 minutes, using a 2.1 mm x 150 mm C8 column (Grace Vydac). The operating conditions of the MS were 1.5 kV detector voltage in positive ion mode, heat block and CDL temperature at 200C, 1.5 L/min nitrogen nebulizing gas, m/z scan range 200-1000.

The mass spectra of the eluting peak showed two m/z values of 430 and 375. At the stated MS operating conditions m/z 430 represents the thymulin peptide (mass 858.85) that displayed the $[M+2H]^{2+}$ state i.e. 429 x 2 (double electron charge) +1 proton from acetic acid=observed mass 859 (Figure 1). The m/z 375 is an expected but variable phase between pyruvate and the amino acids glutamic acid and glutamine that occurs uniquely under electrospray kV detector conditions [18].

After peptide synthesis the residual trifluoroacetic acid and resin cleavage chemicals were removed by lyophilization with 10% acetic acid, repeated three times. Attachment of the zinc ion was performed by mixing thymulin with zinc oxide (1:3 w/w) in 10% acetic acid and lyophilized to yield ZT. The compound was then dissolved in distilled water with preservatives benzoic acid, sodium benzoate and potassium sorbate.

Citric acid and sodium bicarbonate were used as buffers that resulted in a colorless and odorless solution at pH 5.4. The product given to subjects was a 100 ml spray bottle containing 0.0005% ZT as the active test agent. Subjects were instructed to spray 1-2 ml of solution, twice daily, and rub into the scalp. The spray bottle solution was to be kept away from direct sunlight and heat. Subjects were resupplied with fresh solutions every 6-8 weeks during the trial.

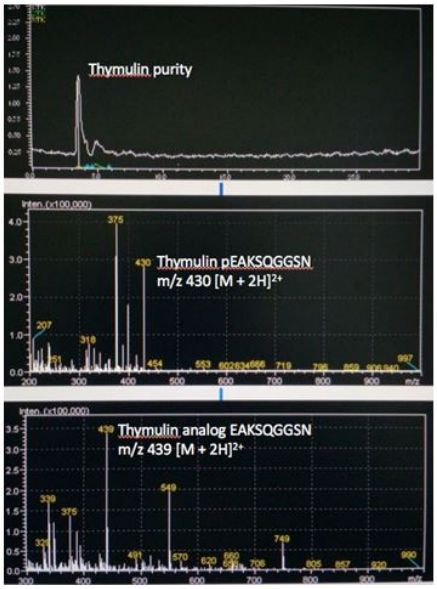


Figure 1: Purity and confirmation of thymulin. Top image shows purity of the thymulin peak (retention time 3.7 minutes) on the mass spectrometer in full scan mode m/z 100-1000 using gradient chromatography. Middle image of thymulin (pEAKSQGGSN, molecular weight 858.85) confirms identity of the peptide peak with m/z at the double charged state 430 $[M+2H]^{2+}$ and the 375 peak of pyruvate-glutamic acid (pE) interaction in the mass spectrometer electrospray conditions. Bottom image shows tentative confirmation of the pE using a thymulin analog containing non-pyruvate glutamic acid (EAKSQGGSN, molecular weight 876.9) showing spectra at m/z 439 $[M+2H]^{2+}$ and 375. Additional m/z 749 is the double charged state of 375 $[M+2H]^{2+}$. A further m/z 549 is an unknown post-translational modification of the analog peptide.

Hair growth assessment

Images were captured on a Samsung Galaxy Note 4 phone in high resolution (16 MB image) with flash, F 2.2 and focal length 31 mm. For assessment, baseline and after treatment images were de-identified, paired and randomly allocated with respect to left/right sides. Images were presented for analysis to assessors in Powerpoint format files on a 55 inch high resolution television screen. All images and original assessor scorecards were archived in PDF format for external reviewer confirmation.

Two validated assessment methods were used to grade the changes: 1. Numbered visual analog scale (VAS) where the assessor gives a value between 0-10 of the image (0=total absence of hair, 10=full abundant hair) [19]. 2. Hair Growth Index (HGI) that is a more in-depth scale of analysis where higher magnification of images can identify the four types of hair regrowth that are designated as absent, vellus, intermediate or terminal [20]. For each image these four growth stages are given percentage values that added together give a raw score total of 100%. Each percentage value for absent, vellus, intermediate, and terminal are multiplied by 0,1,2,3 respectively to yield a final adjusted score ranging from a possible minimum score of 0 (total absence of

hair) to a maximum score of 300 (full terminal hair). Scores were pooled for paired t-test, and P<0.05 was established for significance. Assessment was graded by two independent assessors in a single blind manner. Assessors underwent three training sessions over two weeks prior to the final evaluation. Reliability and validation of the assessors using the scoring methods was conducted by a test/retest at two weeks with Pearson's R. Results showed good intrasubject reliability at two weeks (assessor #1 R=0.69, assessor #2 R=0.71, respectively); and similarly, intersubject reliability had improved to a high level at two weeks (first training session R=0.54, third training session at two weeks R=0.76).

Results

Demographic data of the 18 subjects and their individual trial duration of ZT use are shown in Table 1. The VAS analysis showed no significant growth in the total group (P=0.07) at the end of the trial but significant growth was observed in the 11 subjects who had completed at least 6 months of treatment (P=0.045) (Table 2) (Figure 2).

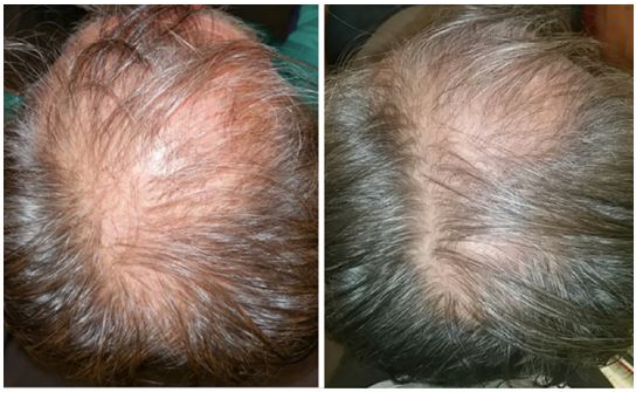


Figure 2: Subject #18 VAS (0-10) assessment.

Subject	Gender	Age (years)	Hair loss (years)	Norwood scale	Other current treatments	Zinc-thymulin use (months)
1	male	35	10	2		9
2	female	49	3	2		8
3	male	64	5	4	minoxidil	7
4	male	61	11	3 vertex		4
5	male	53	30	5A		6
6	male	69	15	5A		6
7	male	55	15	6		9
8	male	44	15	2A	finasteride and minoxidil	6
9	male	35	15	5A		4
10	male	49	10	7	minoxidil	5
11	male	64	5	3 vertex		5
12	male	68	5	6		6
13	male	90	40	7		5
14	male	55	20	7		4
15	male	50	10	2A		5
16	male	44	20	7		6
17	male	50	30	4		10
18	male	60	15	5A		6

Table 1: Demographic data of subjects and trial duration.

	Subject	VAS baseline	VAS after treatment
Assessor #1	1	7	8
	2	7	8
	3	8	6

	4	6	8
	5	9	8
	6	7	8
	7	8	6
	8	8	9
	9	9	8
	10	6	7
	11	9	8
	12	7	8
	13	5	8
	14	7	6
	15	7	8
	16	6	7
	17	8	9
	18	8	9
Assessor #2	1	7	6
	2	7	8
	3	7	6
	4	6	8
	5	6	7
	6	7	6
	7	5	4
	8	8	9
	9	6	7
	10	4	5
	11	8	7
	12	6	8
	13	5	8
	14	7	6
	15	7	6
	16	4	5
	17	7	8
	18	8	9

Table 2: VAS (0-10) of each subject by the two assessors (#1 and #2). [P=0.07 for pooled results, P=0.045* (t-test) for subjects using zinc-thymulin for >6 months].

In 10 subjects the images could be magnified with acceptable clarity and definition to perform the HGI analysis. In the raw score analysis (Table 3) there was significantly less "absent hair" after treatment (P=0.008) and a significant increase of intermediate hair in the baseline/after treatment category (P=0.02). In the HGI adjusted scores

(Table 4) there was confirmation of the increase of intermediate hair when compared for the baseline/after treatment category (P=0.03).

Only one side effect was reported in the entire study from a non-active constituent. Subject #1 used the topical spray each day for nine months and reported a single transient episode of one hour of redness to the forehead after exposure to the sun for several hours, followed by a facial forehead exfoliative scrub cream and then application of the spray.

The investigator (ERV, a maxillofacial surgeon) considered that the redness was due to the action of the citric acid buffer on the combined sun-irradiated and abraded skin where it mimicked a low grade cosmetic facial chemical peel effect.

The subject continued to use the spray with no further recurrence.

	subject	%A before	%A after	%V before	%V after	%I before	%I after	%T before	%T after
A#1	2	50	20	10	25	10	25	30	30
	3	30	20	5	10	5	10	60	60
	4	50	40	10	25	20	25	20	10
	7	50	50	20	10	20	20	10	20
	9	30	40	40	20	10	20	20	30
	10	50	50	30	20	10	15	10	15
	14	50	50	20	10	10	10	20	30
	16	50	40	10	25	10	25	30	10
	17	50	40	0	15	20	25	30	20
	18	30	15	10	5	20	25	40	55
A#2	2	40	20	10	30	30	30	20	20
	3	40	30	0	20	10	30	50	20
	4	40	10	10	30	30	30	20	30
	7	20	30	10	0	40	30	30	40
	9	10	10	50	40	20	20	20	30
	10	50	40	10	20	20	30	20	10
	14	40	50	20	10	20	20	20	20
	16	50	40	0	30	20	20	30	10
	17	30	30	10	20	40	30	20	20
	18	30	10	10	10	10	20	50	60
P=			0.008*		0.17		0.02*		0.86

Table 3: Hair Growth Index analysis of raw percentage scores (N=10 subjects) by each assessor (A#1 and A#2). The four hair types graded as: A (absent), V (vellus), I (intermediate), T (terminal) before and after trial completion. P values are shown at the bottom of the table.

	subject	A before	A after	V before	V after	I before	I after	T before	T after
Assessor#1	2	0	0	10	25	20	50	90	90
	3	0	0	5	10	10	20	180	180
	4	0	0	10	25	40	50	60	30
	7	0	0	20	10	40	40	30	60
	9	0	0	40	20	20	20	60	90
	10	0	0	30	20	10	30	30	45

	14	0	0	20	10	20	20	60	90
	16	0	0	10	25	20	50	90	30
	17	0	0	0	15	40	50	90	60
	18	0	0	10	15	40	50	90	60
Assessor#2	2	0	0	10	30	60	60	60	60
	3	0	0	0	20	20	60	150	60
	4	0	0	10	30	60	60	60	90
	7	0	0	10	0	80	60	90	120
	9	0	0	50	40	40	40	60	90
	10	0	0	10	20	40	60	60	30
	14	0	0	20	10	40	40	60	60
	16	0	0	0	30	40	40	90	30
	17	0	0	10	20	80	60	60	60
	18	0	0	10	10	20	40	150	180
P=					0.18		0.03*		0.52

Table 4: Hair Growth Index analysis of adjusted scores (N=10 subjects) by each assessor (#1 and #2). The four types graded as: A (absent), V (vellus), I (intermediate), T (terminal) before and after trial completion. P values are shown at the bottom of the table.

Discussion

Safety

The ZT topical formulation was shown to be very safe with only one side effect from a non-active constituent reported in 18 test subjects over a total of 111 months (~3,300 days). Specifically, there was no redness, scalp irritation or deterioration of hair color, quality or quantity. In addition, three subjects were using minoxidil and/or finasteride during the trial with no observable drug interaction suggesting concurrent use of these approved FDA drugs with ZT is possible. The excellent safety profile of ZT demonstrated in this study offers an opportunity for treating people with alopecia where side effects of finasteride and minoxidil may be problematic; depression, suicidal thoughts and persistent sexual problems from finasteride [21], pruritis and local irritation from minoxidil [22].

Anagen initiation

In the transition to observed hair development there was a highly significant increased number of intermediate hairs that had progressed from the absent hair stage (P<0.0001) indicating widespread hair follicle activation (Figures 3 and 4). To confirm this effect subject #17 (30 year hair loss) underwent a fluorescence spectroscopy procedure of the scalp. The images showed 6 pre-existing terminal hairs under visible white LED light but spectroscopy revealed an additional 59 developing vellus hairs (Figure 5). Further spectroscopic research is required to confirm this effect but this potentially indicates that extended use of ZT may treat people with Norwood classification 6-7. Supporting evidence was seen when comparing the assessor pooled responses of 'total absent hair' where 65% (13/20) of subjects improved.

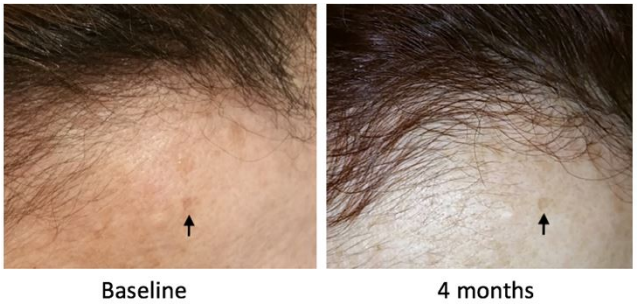


Figure 3: Subject #2 comparison photos with HGI assessment and melanogenesis. Arrow shows scar used for location.

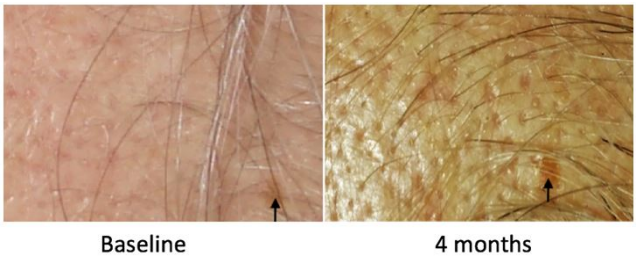
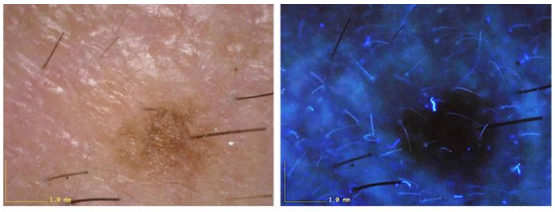


Figure 4: Subject #4 comparison photos with HGI assessment and melanogenesis. Arrow shows pigmented lesion used for location.



Figures 5: Subject #17 of images taken after 10 months ZT treatment of a bald patch comparing white LED image of 6 pre-existing visible terminal hairs to fluorescence spectroscopy with an additional 59 hair follicles developing vellus hair.

At the anatomical level the neuroendocrine regulation of hair would occur through the sensory divisions of the trigeminal nerve for the anterior two-thirds of the scalp, greater occipital nerve for the posterior third of the scalp and sympathetic innervation associated with the blood supply. At the molecular and cellular level Paus et al. [23] in a detailed review of the neuroendocrinology of the hair follicle reported on several endogenous peptides and hormones involved in stimulating the anagen phase, and ZT could be considered as an additional synthetic compound based on endogenous (thymulin) and natural (zinc) constituents.

The delivery of ZT used in this study was from a twice daily topical spray. To further improve the efficacy of ZT for the initiation and maintenance of anagen there are now available new sophisticated transdermal drug delivery systems. Technology such as biodegradable microspheres composed of polylactic acid, polyglycolic acid and alginate have a high benchmark of safety. Potentially, biospheres containing ZT and delivered by local injections or through adhesive patches would be a safe and practical method to reduce the time to obtain visible cosmetic improvement.

Melanogenesis

Melanogenesis was clearly observed with the higher magnification images in several older subjects who developed numerous intermediate hairs that were dark and resembled the color of hair when they were younger (Figures 3 and 4). The work by Meier et al. [7] investigated thymulin's role on the anagen phase but this current ZT study identifies further positive cosmetic effects of the test agent by inducing melanogenesis.

Conclusion

This *in vivo* study of a topical ZT application showed it to be a safe and efficacious compound to stimulate anagen in humans. Thymulin is an endogenous peptide and zinc oxide is a widely used medical and dental chemical compound, both with established safety profiles. In addition they are naturally occurring with prior published work on their individual mechanisms of action at the molecular and cellular level. Combining these two compounds into a single synthetic form also showed excellent human biological safety.

The study was conducted in 18 consecutive subjects to confirm its likely widespread therapeutic benefit for treating hair loss in the general population. This study examined ZT at the anatomical level utilizing scoring methods for the purpose of determining if meaningful cosmetic improvement was occurring in a realistic timeframe. Global visual improvement was statistically significant when ZT was used for>6 months. The study also showed that ZT initiated anagen in AGA subjects with Norwood-Hamilton classification 5-7. Moreover, several subjects were concurrently using finasteride and minoxidil with no evidence of adverse drug interactions with ZT. The compound was seen to induce melanogenesis and suggests further cosmetic applications in addition to its anagen stimulating properties.

Acknowledgements

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Conflict of Interest

The author discloses a financial interest in this study and has filed IP on zinc-thymulin (Australian Patent Application number 2016902192 filed June 2016).

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