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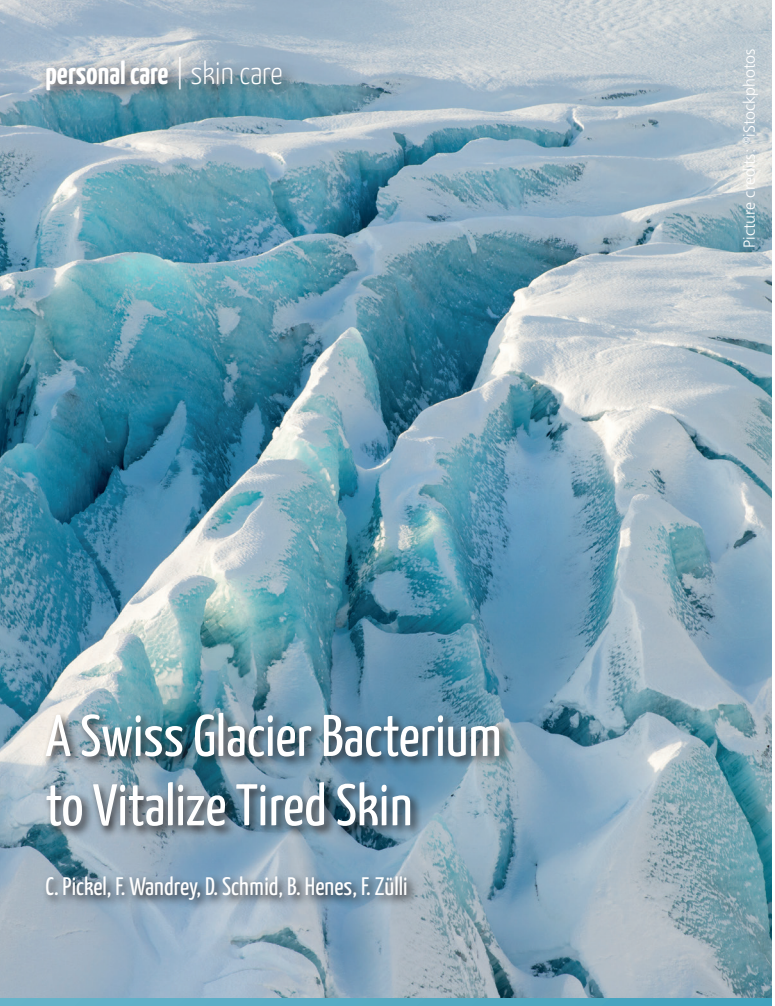

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A Swiss Glacier Bacterium to Vitalize Tired Skin

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Today's society is characterized by demanding jobs, long working hours and an increasingly hectic lifestyle, which frequently results in a lack of sleep. This is often markedly reflected on the skin by a tired appearance of the face. Recent research showed that also on the molecular level, lack of sleep causes stress and leads to premature aging. A major stress mechanism, the unfolded protein response (UPR) of the endoplasmic reticulum (ER), gets compromised by sleep deprivation, leading to the accumulation of misfolded proteins which damage the cell. To target this novel cellular aging mechanism, an extract of the psychrotolerant Swiss glacier bacterium *Iodobacter ssp*. was developed and analyzed for its efficacy to reduce ER stress and visible signs of tiredness. Treatment of aged fibroblasts with the extract led to an increase in the expression of ER chaperones which mediate the UPR and energized cells through an increase of ATP levels in a cellular model of sleep deprivation. Placebo-controlled randomized clinical studies conducted with sleep-deprived and overworked volunteers demonstrated that treatment with the Iodobacter-derived active ingredient IceAwake™ improved several skin parameters associated with skin aging, leading to a vitalized and rejuvenated appearance.

Introduction

Lack of sleep impacts on the cellular stress response

A long day at work, then you get stuck in commuter traffic, just quickly prepare dinner – time flies and suddenly it's past bedtime… most people can relate too well! In today's hectic society, lack of sleep is a common phenomenon which often results in a tired appearance marked by a loss of radiance, an increase in wrinkles such as crow's feet and dark circles around the eyes. But besides causing a tired appearance on the macroscopic level, sleep deprivation is also an aging factor on the molecular level.

It affects a cellular process termed the unfolded protein response (UPR), which is initiated upon accumulation of misfolded proteins and required for the clearance of these potentially toxic proteins [1]. The UPR takes place in the endoplasmic reticulum (ER), an intermediate station during the life cycle of the vast majority of cellular proteins. Following their synthesis from mRNA through ribosomes, proteins are transported into the ER, where a class of helper proteins, termed chaperones, assists in the correct folding and assembly of proteins and protein complexes. Only then, they can continue their journey in the correct shape and fulfill their intended functions in the cell. Cellular stress was shown to upregulate BiP (binding immunoglobulin protein), one of the major chaperones of the ER which is also involved in the UPR [2, 3]. Interestingly, recent research showed that expression of BiP is upregulated at night prior to increased expression of collagen, thereby supporting cellular regeneration processes [4]. It has further been shown that sleep deprivation affects protein folding and causes ER stress, leading to the activation of the UPR and an increase in the expression of BiP [5, 6]. This likely helps the cells to cope with the stress and aids regeneration and repair of the cell. However, it was shown that in aged cells, not only the basal expression of chaperones is decreased, but also their ability to activate the UPR upon sleep deprivation is reduced [3, 7]. The resulting accumulation of wrongly folded proteins causes further ER stress and leads to subsequent damage of the cell. Besides impairing the functions of the ER itself, ER stress also results in the formation of mitochondria-associated membrane (MAM) contact points, through which stress signals are transferred to the mitochondria [8]. In line with this, sleep deprivation also causes a reduction of mitochondrial activity, leading to reduced energy levels as reflected by a drop in the levels of ATP, the cellular energy currency [9]. This further prevents the activity of ATP-dependent chaperones upon lack of sleep, deteriorating cellular stress and causing cell damage.

Taken together, inadequate sleep, similar to UV irradiation or oxidative stress, is correlated with reduced skin health, as it weakens the skin's ability to repair itself at night and consequently accelerates skin aging. Therefore, active ingredients for cosmetic use which support the skin in coping with cellular stresses due to lack of sleep are desirable.

Harnessing the potential of extremophile organisms

Most organisms are viable only under (close to) optimal conditions, such as mild temperatures, atmospheric pressure, neutral pH and salt concentrations close to the organism's own. However, there are various groups of organisms termed "extremophiles" that have evolved to being able to survive and even thrive under extreme conditions. Among these, there are prokaryotes, e.g. archaea and bacteria, but also eukaryotes and even metazoans which grow despite very high or low temperatures, pH values, salt concentrations or pressures, and even under permanent ionizing or UV radiation. These organisms can be regarded as true masters of survival, and understanding their survival strategies has been the subject of numerous scientific studies. It has, for example, been shown that bacteria that are able to proliferate at temperatures as low as 5°C (so-called psychrotolerants) express enzymes which are active even at low temperatures and contain a large number of secondary metabolites with various functions [10, 11]. Transferring the capabilities of these extraordinary organisms into skin care by harnessing their biological potential and using them as a source for interesting and novel secondary metabolites for use in cosmetic application is of big interest for cosmetic scientists.

A freshness kick for tired skin with a Swiss glacier bacterium

In order to discover and harvest novel extremophile microorganisms for use in cosmetics, an expedition to a glacier in Valais, Switzerland, was undertaken. Due to the continuous shrinking of glaciers in the past decades, more and more microbes that have been hidden below permanent ice for centuries have become accessible. A sample of the soil exposed underneath the retreating glacier was taken and analyzed for its microbial content. This led to the identification of *Iodobacter ssp.*, a rod-shaped bacterium which belongs to the group of cold-tolerant organisms. After many years below the glacier ice layer, it has been reawakened and harnessed for the development of a novel active ingredient for skin care. Large-scale cultivation under optimized conditions and extraction of the *Iodobacter ssp.* strain followed by spray-granulation of the extract on a maltodextrin carrier yields the active ingredient IceAwake™ [INCI: Succinic Acid (and) Maltodextrin (and) Aqua/Water]. The efficacy of this novel active to reduce ER stress as a cause of prematurely aged and tired skin was investigated *in vitro* and *in vivo*.

Methods and Results

Iodobacter ssp. extract re-activates chaperone expression in aged fibroblasts

To assess the effect of *Iodobacter ssp.* extract on the process of protein folding, the expression of helper proteins required for flawless protein production was analyzed in aged cells. For this, normal human dermal fibroblasts were cultured for 17 passages prior to the experiment in order to mimic the aging process. Following treatment of these aged fibroblasts with 1 % *Iodobacter ssp.* extract for 24 h, cells were harvested, and total RNA was extracted. An untreated control was performed in parallel. Complementary DNA (cDNA) was synthesized from total RNA and the expression of target genes was assessed by RT-qPCR.

Among others, the expression of several chaperones involved in protein folding in the ER was analyzed in aged fibroblasts treated with an extract of *Iodobacter ssp.* and compared to an untreated control. The expression levels of key chaperones involved in the UPR, namely BiP, endoplasmin, calnexin and calreticulin, were increased by up to 100 % in aged fibroblasts when treated with 1 % *Iodobacter ssp.* extract (**Fig. 1**).

Previous research has shown that cellular stress, caused by factors such as sleep deprivation, leads to an upregulation of various ER chaperones [5, 6]. This is an important mechanism that helps the cells to prevent the accumulation of misfolded proteins. However, according to recent studies, aged cells lose the capacity to activate the UPR and therefore can no longer efficiently prevent protein misfolding and aggregation [3, 7]. Additionally, the expression of BiP, the main chaperone responsible for assisting correct protein folding and preventing of protein aggregation, declines in aged cells [7]. Together, this leads to an amplification of the ER stress and initiates a vicious cycle in which aged cells can only insufficiently recover from stress during the night and therefore get damaged even further. The results of this study show that treatment with an extract of *Iodobacter ssp.* reverses these aging effects by supporting protein folding in the ER via upregulation of chaperone expression.

ER stress is reduced by *Iodobacter ssp.* extract in a cellular model of sleep deprivation

Cellular stress caused by sleep deprivation not only impairs ER function and protein folding, but also affects mitochondria,

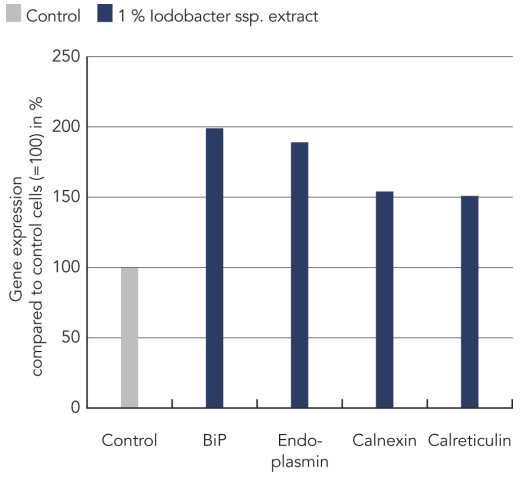

Fig. 1 Gene expression of chaperone genes in aged fibroblasts treated with *Iodobacter ssp.* extract relative to untreated cells.

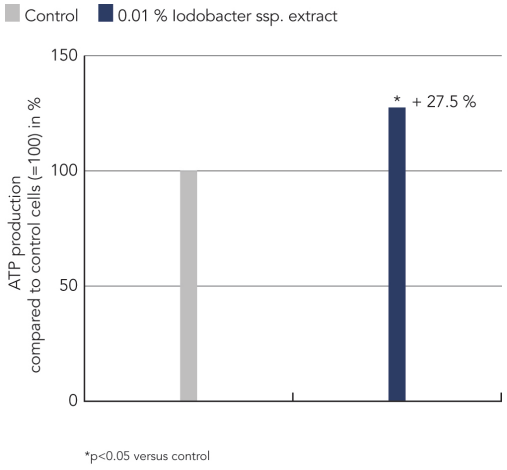

Fig. 2 ATP levels in AD fibroblasts upon treatment with *Iodobacter ssp.* extract relative to untreated cells.

the cells' powerhouses required for production of the energy equivalent ATP. A decrease in ATP levels is therefore a sign of impaired mitochondrial function [9]. Moreover, the formation of so-called mitochondria-associated membrane (MAM) contact points between mitochondria and the ER was shown to be an indicator of these impairments [8]. The efficacy of *Iodobacter ssp.* extract to alleviate these signs of cellular stress was tested in fibroblasts isolated from a patient with Alzheimer's disease (AD), which combine impairment of mitochondrial function and increased ER-stress and can thus be used as a cellular model for sleep deprivation.

In a first experiment, AD fibroblasts were treated with 0.01 % *Iodobacter ssp.* extract for 120 minutes or left untreated prior to measurement of ATP levels by chemiluminescence. Compared to the untreated control, *Iodobacter ssp.* extract increased ATP production by 27.5 % after 120 minutes of treatment (**Fig. 2**).

In a second experiment, the formation of MAM contact points resulting from interactions between the mitochondrial protein GPR75 and the ER protein SERCA2 was assessed in AD fibroblasts. As before, these cells were treated with 0.01 % *Iodobacter ssp.* extract for 120 minutes or left untreated, and healthy cells were cultured in parallel. Subsequently, MAM contact points were stained and the signal as well as nuclear counterstaining was visualized by fluorescent microscopy. Quantification showed that the number of MAM contact points was increased in the sleep deprived cell model, while treatment with the *Iodobacter ssp.* extract led to a significant and visible reduction of MAM contact points in these cells (**Fig. 3** and **4**).

The results of these experiments confirm that an extract of *Iodobacter ssp.* supports cells in dealing with mitochondrial and ER stress. The extract alleviates known signs of cellular stress and supports the function of ER as well as mitochondria. Moreover, an increase in ATP levels boosts chaperone function, as the activity of these helper proteins is energy dependent. Therefore, treatment with *Iodobacter ssp.* extract facilitates correct protein folding through an additional mechanism and despite aging and sleep deprivation.

IceAwake™ ameliorates visible signs of tiredness in a mixed study population

The efficacy of the active ingredient IceAwake™ to reduce visible signs of tiredness was further evaluated in two independent randomized, double-blind, placebo-controlled clinical studies. For this, twenty-one Caucasian men and women (44-66 years, mean age: 53.7 years) and twenty-three Asian women (41-57 years, mean age: 50.7 years) with chronic lack of sleep and/or a tired appearance with dark circles around the eyes and medium-deep crow's feet wrinkles were enrolled. Having completed a washout phase of three to seven days, the volunteers were asked to apply a cream containing 2 % IceAwake™ on one half of the face while treating the other half of the face with a placebo cream without an active ingredient. At the beginning of the study and after two weeks of twice daily treatment, the wrinkle depth of crow's feet was determined using a PRIMOS Premium (GF Messtechnik, Germany) or a PRIMOSlite (Canfield, Germany). Moreover, the skin radiance and visible tiredness of the volunteers was evaluated by clinical grading performed by experts.

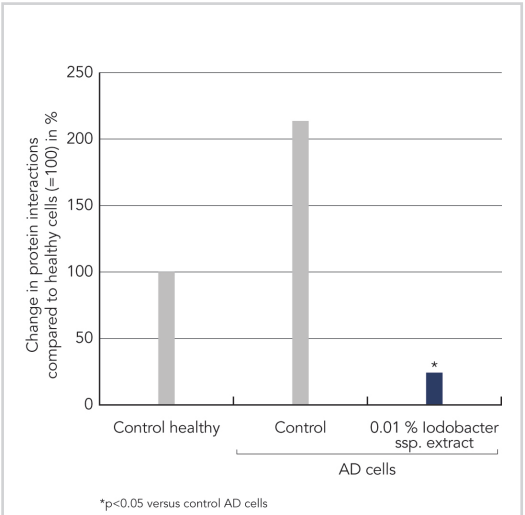

Fig. 3 Quantification of MAM contact points in healthy cells or AD fibroblasts treated with the Iodobacter active or left untreated.

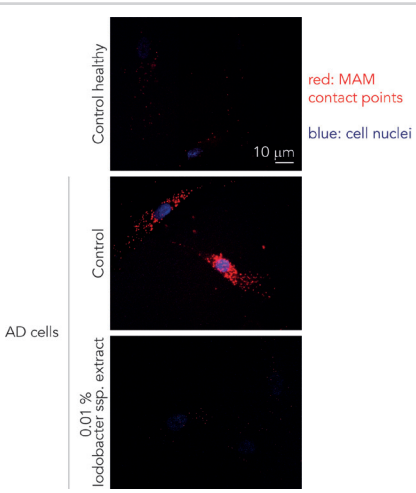

Fig. 4 Immunofluorescence staining of MAM contact points in healthy cells or AD fibroblasts treated with the Iodobacter active or left untreated.

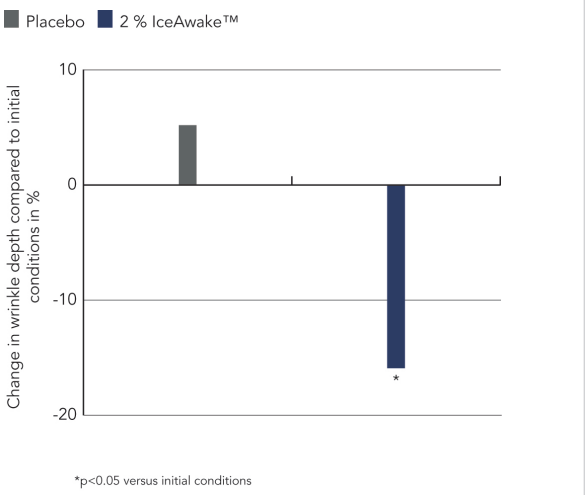

Fig. 5 Decrease of wrinkle depth upon treatment with IceAwake™.

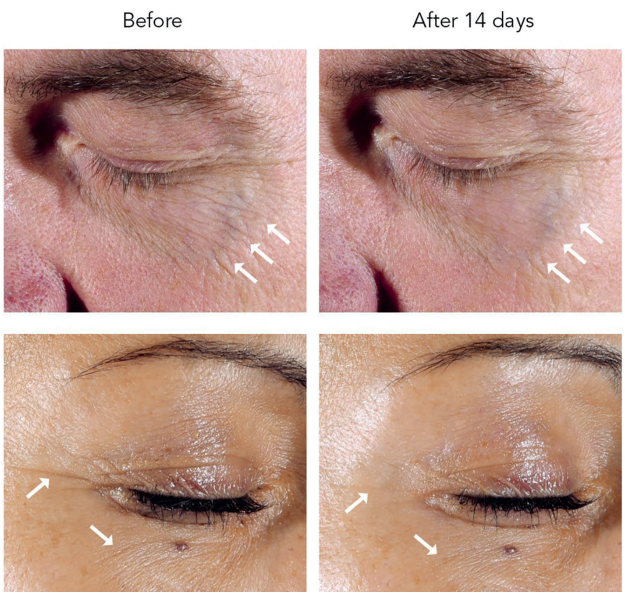

Fig. 7 Visible improvement of wrinkle depth and dark circles with IceAwake™.

After only 14 days of application of a cream containing 2 % IceAwake™ on Caucasian skin, the wrinkle depth of crow's feet was significantly reduced by 15.9 % compared to initial conditions (**Fig. 5**). Moreover, clinical-grade evaluation confirmed a significant reduction of visible facial tiredness compared to initial conditions, which was observed in 71 % of the volunteers (**Fig. 6**). The reduction in wrinkle depth as well as the improvement of tiredness were also visible in photographs of male and female volunteers (**Fig. 7**).

In a questionnaire about their lifestyle, the Asian volunteers enrolled in the second clinical study indicated to have late bedtime (56.5 % go to bed after midnight), sleep only very little hours (34.8 % sleep less than five hours per night) and with bad to very bad quality (95.6 %), while being faced with night overtimes or heavy workload at least once per week (60.8%). In this study population, the significant reduction of wrinkle depth following 14 days of treatment with a cream containing 2 % IceAwake™ was confirmed (data not shown). In addition, skin radiance significantly increased by 9.2 % compared to initial conditions and the placebo treatment (**Fig. 8**). Overall, these data demonstrate that after just 14 days of application, IceAwake™ rejuvenates tired skin by reducing wrinkle depth and visible signs of tiredness as well as increasing skin radiance.

Conclusion

The active ingredient IceAwake™ was developed using an extract of *Iodobacter ssp.*, a microorganism discovered in the soil under a retreating Swiss glacier. This extremophile bacterium has likely survived under permanent ice for centuries and is therefore an interesting source for an active ingredient to vitalize end energize tired skin. Mechanistic studies showed

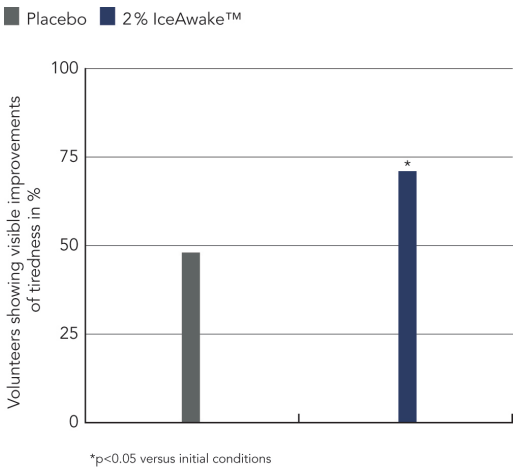

Fig. 6 Reduction of tired appearance upon treatment with IceAwake™.

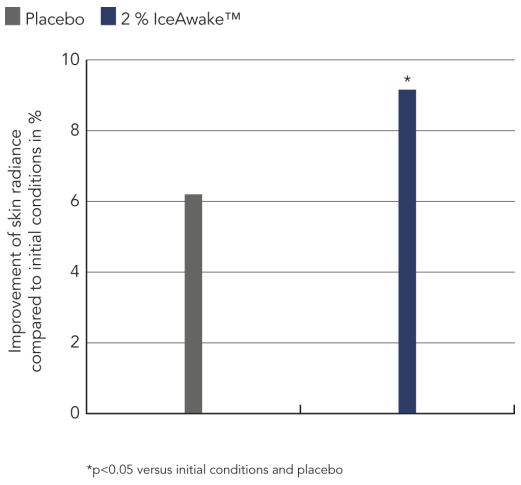

Fig. 8 Increase in radiance with IceAwake™.

that treatment of cells with *Iodobacter ssp.* extract leads to an upregulation of key chaperones involved in protein folding and thereby supports the removal of accumulated, misfolded proteins. The increased ATP levels following treatment with the *Iodobacter ssp.* extract additionally ensure the availability of the energy required for chaperone activity. Both mitochondrial and ER function are known to be impaired upon aging and sleep deprivation [3, 7, 8]. The results show that these impairments were improved in stressed cells upon treatment with *Iodobacter ssp.* extract. These findings translate to clinical studies, where it was found that application of a cream containing IceAwake™ leads to a significant and visible improvement of the wrinkle depth of crow's feet compared to a placebo cream. Moreover, the positive effect on tired and stressed skin was confirmed through clinical grading by experts who attested a significant improvement of skin tiredness and radiance after just two weeks of treatment with the cream containing IceAwake™. Regular application therefore leads to a visible skin rejuvenation and an increase in radiance despite a hectic lifestyle.

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