Fighting ageing in tired skin with a glacier bacterium

Christina Pickel, Franziska Wandrey, Daniel Schmid, Bernhard Henes, Fred Zülli – Mibelle Biochemistry, Switzerland

Abstract

Long working hours and a hectic lifestyle are common phenomena in today's society. This often results in a lack of sleep, which is markedly reflected on the skin by a tired appearance of the face. Also on the molecular level, lack of sleep causes stress and leads to premature ageing. 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. Moreover, mitochondrial ATP production is impaired upon lack of sleep due to the link between ER and mitochondrial stress. To target this novel cellular ageing mechanism, an extract of the psychrotolerant Swiss glacier bacterium *Iodobacter ssp.* was developed and analysed 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. Moreover, *Iodobacter spp.* increased ATP levels in a cellular model of sleep deprivation. Placebo-controlled randomised clinical studies conducted with sleep-deprived and overworked volunteers demonstrated that treatment with the *Iodobacter*-derived active ingredient improved several skin parameters associated with skin ageing, leading to an energised and rejuvenated appearance.

The life cycle of proteins in our cells involves various processes, starting with the transcription from DNA and ending with their degradation. An intermediate station for the vast majority of proteins is the endoplasmic reticulum (ER), where they are correctly folded with the help of chaperone proteins in order to then continue their journey in the correct shape to fulfil their function. The ER also represents an important checkpoint for quality control, and upon accumulation of incorrectly folded proteins, a process termed unfolded protein response (UPR) is initiated to facilitate clearance of these potentially toxic proteins.[1] One of the major chaperones of the ER also involved in the UPR and upregulated by cellular stress is BiP (binding immunoglobulin protein).[2,3] Recent research showed that expression of BiP is upregulated prior to increased expression of collagen at night when it supports 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.[3,5,6] This likely helps the cell to cope with the stress and aids regeneration and repair of the cell. However, basal chaperone expression as well as the ability to activate the UPR upon sleep deprivation were shown to decrease in aged cells.[3,7] This leads to the accumulation of wrongly folded proteins, which cause further ER stress and subsequently damage the cell. Another consequence of ER stress is the formation of mitochondria-associated membrane (MAM) contact points, an interaction platform with mitochondria.[8] In line, 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, sleep deprivation does not only cause a tired appearance on the macroscopic level but is also an ageing factor on the molecular level. Moreover, it has been shown that, similar to UV-irradiation or oxidative stress, inadequate sleep is correlated with reduced skin health, weakens the skin's ability to repair itself at night and can accelerate skin ageing. Therefore, active ingredients for cosmetic use which support the skin in coping with cellular stresses due to lack of sleep are desirable.

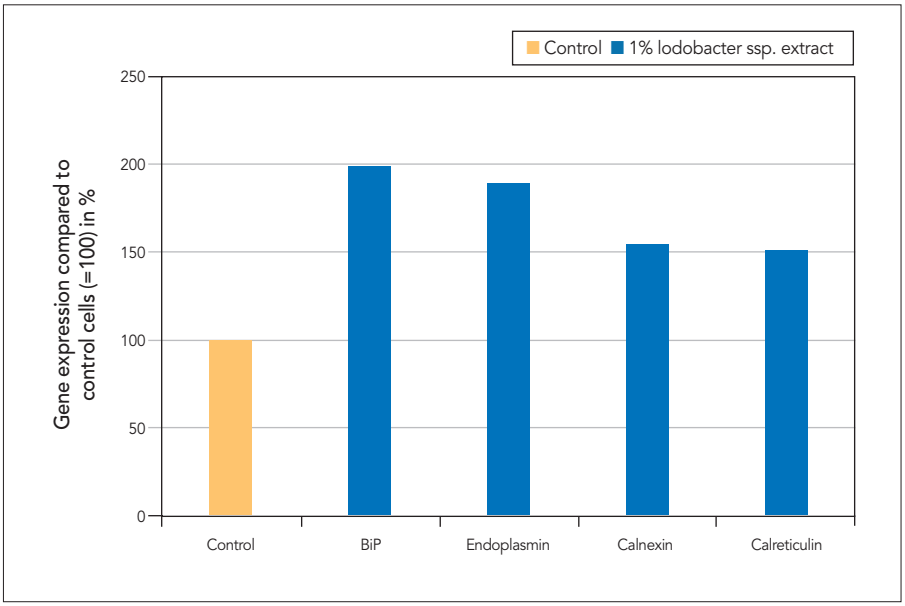

Figure 1: Gene expression of chaperone genes in aged fibroblasts treated with *Iodobacter ssp.* extract relative to untreated cells.

Extremophile bacteria – Masters of survival in a hostile environment

Extremophile organisms are masters of

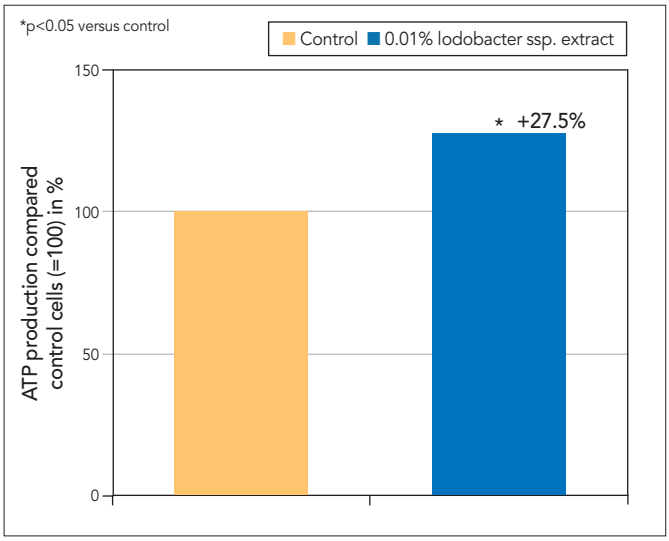

Figure 2: ATP levels in AD-fibroblasts upon treatment with Iodobacter ssp. extract relative to untreated cells.

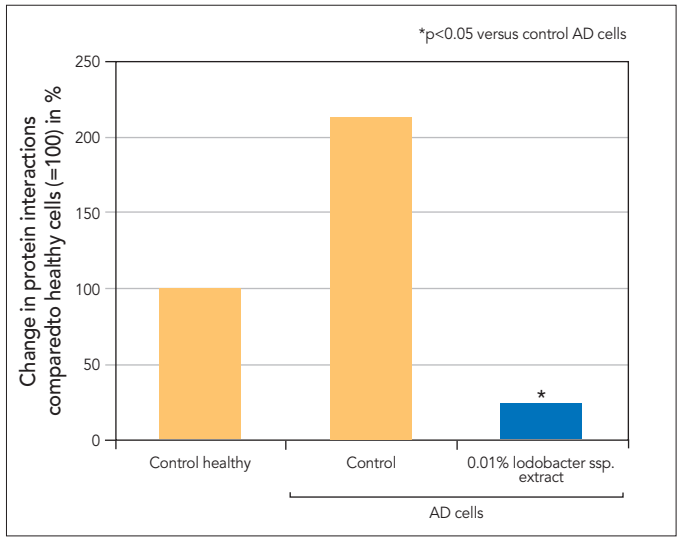

Figure 3: Quantification of MAM contact points in healthy cells or AD-fibroblasts treated with the Iodobacter active or left untreated.

survival as they are able to adapt to conditions generally considered as hostile to life. There are prokaryotes, e.g. archaea and bacteria, but also eukaryotes and even metazoans which survive in extreme conditions such as very high or low temperatures, pH values, salt concentrations or pressures, and even ionizing or UV radiation. In order to adapt to such conditions, extremophiles developed various strategies which help them to cope with these stresses. For psychrotolerant bacteria, which are able to proliferate at temperature as low as 5°C, survival is amongst others ensured by the following strategies: 1) incorporation of polyunsaturated fatty acids into the cell membrane in order to maintain membrane fluidity, 2) reduction of the freezing point of the intracellular fluid by addition of solutes such as trehalose, 3) expression of enzymes that are active even at low temperatures, and 4) production of a large number of secondary metabolites with various functions.[10,11] These strategies could on the one hand be harnessed by the modern industry due to their biotechnological potential, and on the other hand psychrotolerant bacteria themselves represent interesting novel sources for secondary metabolites for cosmetic or pharmaceutical application.

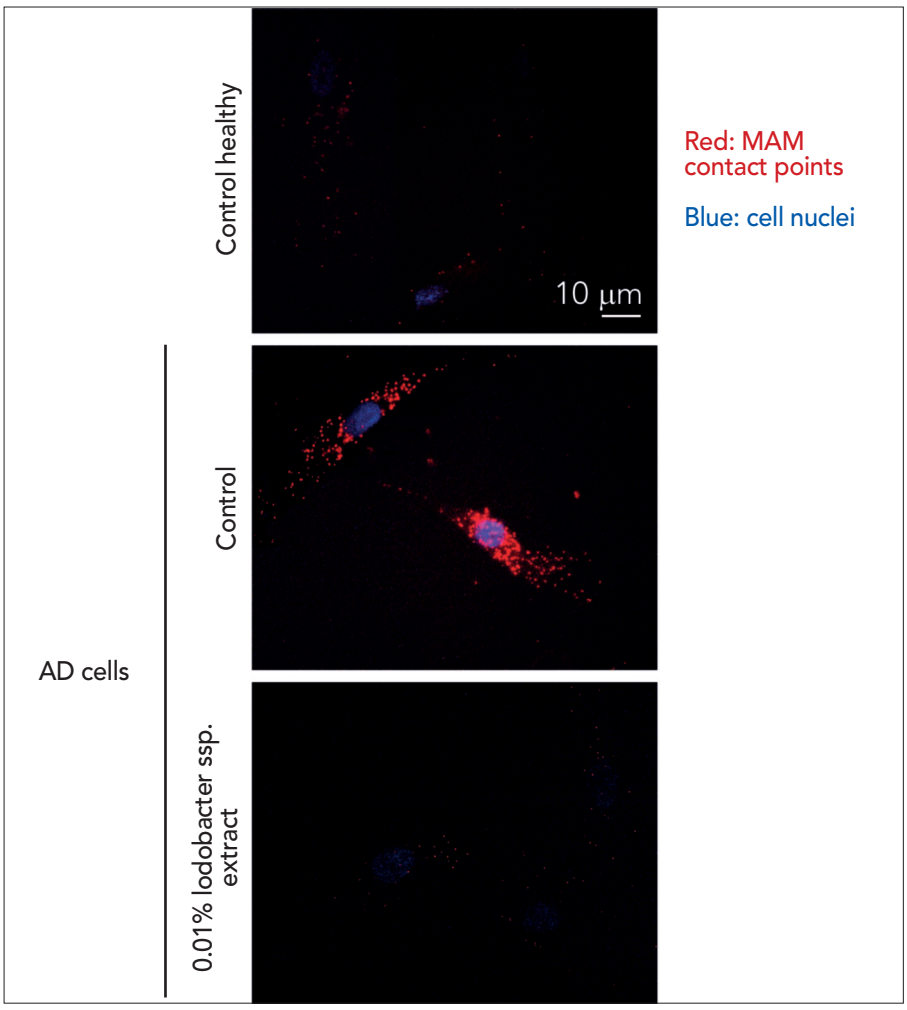

Figure 4: Immunofluorescence staining of MAM contact points in healthy cells or AD-fibroblasts treated with the Iodobacter active or left untreated.

A Swiss glacier bacterium for refreshing tired skin

The continuous shrinking of glaciers in the past decades made more and more microbes accessible that have been hidden below permanent ice for centuries. In order to discover and harvest novel extremophile microorganisms for use in cosmetics, an expedition to a glacier in Valais, Switzerland, was undertaken. A sample of the soil exposed underneath the glacier was analysed for its microbial content, leading to the identification of *Iodobacter ssp.* After many years below the glacier ice layer, this cold-tolerant, rod-shaped bacterium has been reawakened and harnessed for the development of a novel active ingredient for skin care. Large-scale cultivation under optimised conditions and extraction of the *Iodobacter ssp.* strain followed by spray-drying 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*.

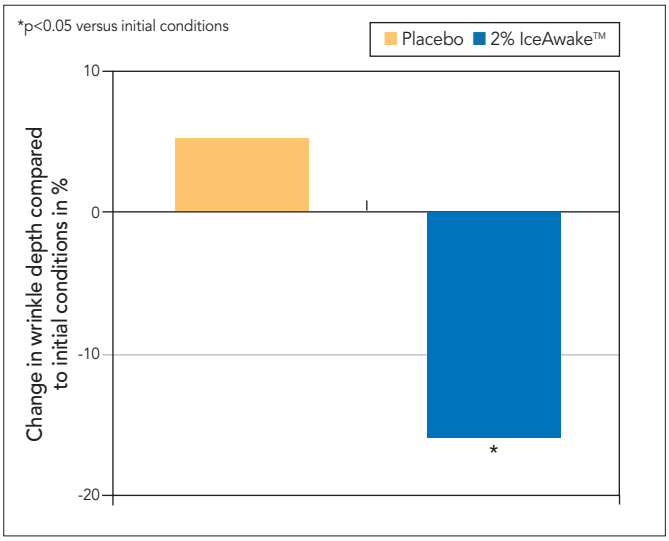

Figure 5: Decrease of wrinkle depth upon treatment with IceAwake™.

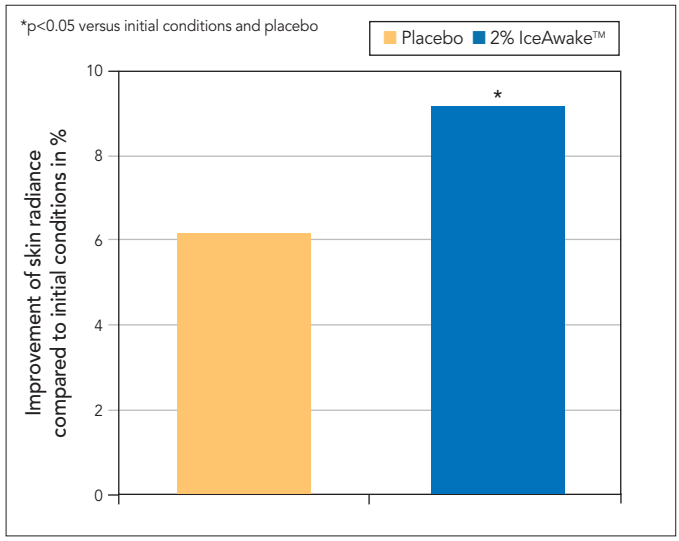

Figure 6: Reduction of tired appearance upon treatment with IceAwake™.

Materials and methods

Gene expression in aged fibroblasts

To mimic the ageing process, normal human dermal fibroblasts were cultured for 17 passages prior to the experiment. Aged fibroblasts were subsequently treated or not (control) with 1 % *Iodobacter ssp.* extract for 24 h. All experimental conditions were performed in n=3. Following treatment, cells were harvested, and total RNA was extracted from the pooled sample of each condition using TriPure Isolation Reagent® (Roche, Mannheim) according to the supplier's instructions. Complementary DNA (cDNA) was synthesised from total RNA in the presence of oligo(dT) primer and Transcriptor Reverse Transcriptase (Roche). RT-qPCR for the target genes was performed in n=2 using the LightCycler® system (Roche).

Determination of ATP production in a cellular model of sleep deprivation

Fibroblasts isolated from a patient with Alzheimer's disease (AD) combine impairment of mitochondrial function and increased ER-stress and were used as a cellular model for sleep deprivation. AD-fibroblasts were grown in assay medium which inhibited TCA cycle activity, thereby inducing additional mitochondrial stress. Following a wash, cells were incubated with 0.01% *Iodobacter ssp.* extract for 120 minutes or left untreated prior to measurement of ATP levels by chemiluminescence. All measurements were performed in quadruplicates.

Fluorescent staining of mitochondria-associated membrane (MAM) contact points

AD-fibroblasts were treated with 0.01% *Iodobacter ssp.* extract or left untreated for 120 minutes, healthy cells were grown in parallel and left untreated. Cells were subsequently fixed and permeabilised, and MAM contact points were stained using the DuoLink® II *in situ* red proximity ligation assay (Sigma-Aldrich, USA) according to the manufacturer's instructions. Cell nuclei were counterstained with 4',6'-diamin-2-phenylindol (DAPI). Fluorescent signals resulting from protein interactions (<40 nm) between GPR75 and SERCA2, as well as nuclear stain were visualised by immunofluorescence microscopy (Zeiss, Germany) and quantified. All measurements were performed in quadruplicates.

Skin adaptation in clinical studies with overworked volunteers

The anti-tiredness effect of *Iodobacter ssp. extract* was evaluated in two randomised, double-blind, placebo-controlled clinical studies involving twenty-three Asian women (41-57 years, mean age: 50.7 years) and twenty-one Caucasian men and women (44-66 years, mean age: 53.7 years), respectively. For inclusion into the study populations, volunteers were required to present with a lack of sleep and/or tired appearance with dark circles around the eyes and medium-deep crow's feet wrinkles. After a wash-out phase of three to seven days, volunteers applied a cream containing 2% *Iodobacter*-derived active ingredient on one half of the face and a corresponding placebo cream on the other half of the face twice daily for 14 days. The wrinkle depth of crow's feet was determined using a PRIMOS Premium (GF Messtechnik, Germany) or PRIMOSlite® (Canfield, Germany). Additionally, a clinical expert grading of skin radiance and visible tiredness was performed on photographs taken of the volunteers using a Visia® CR camera (Canfield, USA) or a ColorFace® camera (Newtone Technologies, France).

Results and discussion

Iodobacter ssp. extract induces chaperone expression in aged fibroblasts

The effect of *Iodobacter ssp.* on the expression of several chaperones involved in protein folding in the ER was analysed in aged fibroblasts treated with an extract of *Iodobacter ssp.* and compared to an untreated control. Upon treatment with 1% *Iodobacter ssp. extract* for 24 h, 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 compared to untreated cells (Fig 1).

It has previously been shown that various ER chaperones are upregulated upon cellular stress caused for example by sleep deprivation.[5,6] However, recent research revealed that aged cells lose the capacity to activate the UPR, and the cells consequently fail to prevent protein misfolding and aggregation.[3,7] Moreover, the basal expression of BiP, the main ER chaperone responsible for assistance in protein folding and prevention of protein aggregation, has been shown to decline in aged cells.[7] This causes further stress to the ER, initiating a vicious cycle which leads to insufficient recovery of the cells during sleep. Treatment with an extract of *Iodobacter ssp.* reverses these ageing effects and supports protein folding in the ER by upregulating chaperone expression.

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

Sleep deprivation causes cellular stress which results in impaired mitochondrial and ER function. Both the decrease in ATP levels, as well as the formation of mitochondria-associated membrane (MAM) contact points, were shown to be indicators of these impairments.[8,9] In order to assess the efficacy of *Iodobacter ssp.* extract to alleviate these signs of stress, cells of an

Alzheimer's disease (AD) patient were used as a cellular model for sleep deprivation. Treatment of AD-fibroblasts with 0.01 % *Iodobacter ssp.* extract significantly increased ATP levels by 27.5 % after 120 minutes compared to untreated cells (Fig 2). Moreover, the number of MAM contact points, which was increased in AD fibroblasts compared to healthy control cells, significantly decreased upon treatment with an extract of *Iodobacter ssp.* (Fig 3 and 4).

Together, these results show that an extract of *Iodobacter ssp.* alleviates signs of cellular stress and supports the functions of both the mitochondria and the ER for more efficient protein folding despite ageing and lack of sleep.

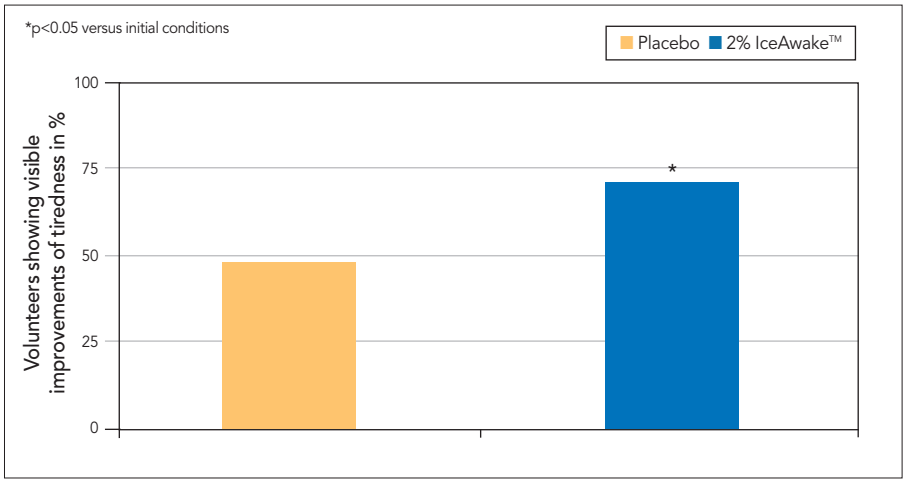

Figure 7: Visible improvement of wrinkle depth and dark circles with IceAwake™.

Amelioration of visible signs of tiredness skin in a mixed study population

The efficacy of the Iodobacter ssp. extract was further assessed in a placebo-controlled clinical study including female and male Caucasian volunteers with a tired appearance marked by dark circles around the eyes and medium-deep crow's feet. Following application of a cream containing 2% *Iodobacter* active for 14 days, 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).

A second, placebo-controlled clinical study with overworked Asian volunteers with little and bad quality sleep confirmed the significant reduction of wrinkle depth following 14 days of treatment with a cream containing 2% *Iodobacter* active (data not shown). Additionally, skin radiance significantly increased by 9.2% compared to initial conditions (Fig 8).

Overall, these data demonstrate that after just 14 days of application, *Iodobacter ssp.* extract rejuvenates tired skin by reducing wrinkle depth and visible signs of tiredness as well as increasing skin radiance.

Conclusion

Using the extract of *Iodobacter ssp.*, the novel active ingredient IceAwake™ was developed. Targeting a recently discovered aging mechanism based on protein folding and chaperone activity, treatment of stressed cells with the extract improved the ER stress response *in vitro*. Clinical studies proved a positive effect on tired and stressed skin. Regular application leads to a visible skin rejuvenation and an increase in radiance despite a hectic lifestyle. PC

Figure 8: Increase in radiance with IceAwake™.

References

1 Walter P, Ron D. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. *Science*. 2011; **334**(6059):1081-1086

2 Gething MJ. Role and regulation of the ER chaperone BiP. *Semin Cell Dev Biol*. 1999; **10**(5): 465-472

3 N Naidoo et al. Aging Impairs the Unfolded Protein Response to Sleep Deprivation and Leads to Proapoptotic Signaling. *J Neurosci. 2008;* **28**(26):6539-6548

4 Pickard A et al., Preservation of circadian rhythms by the protein folding chaperone, BiP. FASEB J. 2019; 33(6): 7479-7489

5 Naidoo N et al. Sleep deprivation induces the unfolded protein response in mouse cerebral cortex. *J Neurochem*. 2005; **92**(5):1150-1157

6 Naidoo N et al. Aging and sleep deprivation induce the unfolded protein response in the pancreas: implications for metabolism. *Aging Cell*. 2014; **13**(1): 131-141

7 RC Taylor, Aging and the UPR(ER). *Brain Res*. 2016; **1648**(Pt B): 588-593

8 van Vliet AR, Agostinis P. Mitochondria-Associated Membranes and ER Stress. *Curr Top Microbiol Immunol*. 2018; 414: 73-102

9 Chikahisa S, Séi H. The role of ATP in sleep regulation. *Front Neurol.*, 2011; 2: 87

10 Russell NJ. Psychrophily and resistance to low temperature. In: C Gerday and N Glansdorff (eds) Extremophiles (Life under extreme environmental Condition), Encyclopedia of Life Support Systems (EOLSS) Developed under the Auspices of the UNESCO. Eolss Publishers, Oxford, UK.

11 Zakhia F et al. Cyanobacteria in Cold Ecosystems. In: R Margesin et al., Psychrophiles: from Biodiversity to Biotechnology. Springer-Verlag Berlin Heidelberg, 2008; Chapter 8, 121-135

12 Naidoo N. Cellular stress/the unfolded protein response: Relevance to sleep and sleep disorders.