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# Skin-protecting dinucleotide examined

It has become evident that stress is one of the major characteristics of our modern life, and shown in much data is that stress has a direct effect on skin health and ageing.

Stress can come from pollution, weather conditions, cigarette smoke, radiation, heavy metals, and ultraviolet (UV) rays.

Among these stresses, UV is the primary concern in the fight against photoageing, since it damages collagen, elastin, melanocytes, and the skin's barrier function, resulting in wrinkles, sagging, uneven skin tone, dark spots, and dry skin.

Consequently, developing active ingredients to help the skin fight ageing and stress has become a common point of interest in the cosmetics sector.

The ISP Global Skin Research Center, located in Sophia Antipolis, France, has developed a cosmetic active ingredient obtained by biotechnology from *Artemia*, a plankton* that lives in hypermineral lakes. When environmental conditions turn critical (stress), *Artemia* enters a state of dormancy, where it encapsulates and accumulates a vital molecule, Diaguanosine Tetraphosphate (GP4G), a principal source of phosphate bonds with high energetic potential used for DNA protection as well as energy storage.

When environmental conditions turn favourable again, *Artemia* "awakens" and re-hydrates, and different metabolic activities take place again. Ultimately, GP4G is transformed into adenosine triphosphate (ATP). GP4G provides *Artemia* with energy and protection for its survival and development.

The objective of this study was to demonstrate that GP4G applied to the skin provides tremendous effects by supplying energy, protecting DNA, protecting against UVB and ageing, and ensuring a general overall better condition of the skin.

*Technical Editor's comment:
Common brine shrimps (*Artemia*) are in the phylum *Arthropoda*, class *Crustacea*. *Artemia* are (strictly speaking) zooplankton like *Copepods* and *Daphnia*.

*UV is the primary concern in the fight against photoageing.*

## Materials and methods

**Primary human fibroblasts** were obtained from clonetics. Skin explant proceeded from plastic surgery. Thereafter, an established skin organ culture method was used.[1] An HaCaT cell line was obtained from American Type Culture Collection (ATCC). Human fibroblasts were treated with *Artemia* extract for 24 hours, then exposed to UVB irradiation. A Di Methyl Thiazolyl blue Tetrazolium (MTT) bromide colorimetric assay was carried out 24 hours later.[2]

**Cultured skin samples** were studied by routine direct immunofluorescence (DIF) microscopy following standard methods.[3] For heat shock protein studies, rabbit polyclonal antibody anti-Hsp70 was used. Mouse anti-CD1a antibody was used for Langerhans cell staining.

**Immunoblotting studies** were performed by means of previously described methods[4] using NuPAGE 12% gel. Polyclonal rabbit anti-collagen antibody, monoclonal mouse anti-pan keratin antibody, and polyclonal rabbit anti-fibronectin were purchased by TEBU.

**The cellular content** in HSP70 mRNA was evaluated using a probe stained by digoxigenine in standard Northern blot experiments.[5]

**Comet assay (or single cell gel electrophoresis)** is a short and sensitive micro-electrophoretocal technique that enables the detection of single- and double-strand DNA breaks in individual cells.[6] Briefly, after treatment, cells were confined in an agarose gel and lysed in a highly saline buffer detergent. The DNA was thereafter denatured by an alkaline bath followed by a short electrophoresis, then revealed by propidium iodide. The DNA of damaged cells stretched out towards the anode in proportion to the number of breaks and formed a "comet." Lesions were evaluated using a program that helped determine the percentage of DNA damage.

Cultured human keratinocytes received *Artemia* extract 24 hours before and after their irradiation with 100 mJ/cm$^2$ of UVB. A TUNEL assay[7] was performed 24 hours later.

A standard Enzyme Linked Sorbent Assay (ELISA) method[8,9] was carried out on human HaCaT cells. Cells received 3% *Artemia* extract for 24 hours, followed by UVB irradiation. Total cytokine level was assessed for IL-1 alpha, IL-8 and TNF-alpha. The level of the cytokines was expressed in pg/mg protein.

## Results

In order to demonstrate cell metabolism activation, ATP content in fibroblast treated cells was measured. Based on a cAMP competitive immunoassay system from Amersham Biosciences, an increased level that reached 40% was measured compared with the basal level.

GP4G was shown to act as an ATP precursor and to demonstrate energising properties. In its biological condition, when *Artemia* was awakened by favourable conditions,[10] GP4G content declined rapidly

while an increase in ATP was observed. GP4G is considered an adaptive mechanism that allows *Artemia salina* to survive in severe environmental conditions. When environmental conditions are critical, *Artemia* accumulates a vital molecule, GP4G, typical for its energy-rich phosphate bonds. It is interesting to note that in this state, GP4G consists of a form of a supply of energy that can be hydrolysed in ATP when environmental conditions become favourable again. Thus, GP4G, either in a biological condition or in the human cell, boosts cAMP-dependent metabolic pathways.

## Epidermal and dermal regeneration

In both immunostaining and immunoblotting experiments, an increase of filaggrin (a major marker of terminal differentiation of the epidermis) and keratin in HaCaT treated cells was observed. This study showed that the *Artemia* extract stimulates filaggrin synthesis and therefore epidermal differentiation. Likewise, a large increase of dermal protein such as fibronectin, and collagen in fibroblast-treated cells was demonstrated. GP4G thus improves the skin barrier function by inducing filaggrin and keratin protein, but, more than this effect, GP4G acts also on an extra cellular matrix by inducing collagen and fibronectin proteins. GP4G offers both epidermal and dermal protection.

## DNA protection

In order to demonstrate DNA protection, experiments were conducted in UVB irradiated keratinocytes. TUNEL technology was used for detection and quantification of apoptosis (programmed cell death). *In situ* detection of DNA breaks by the TUNEL method revealed that the addition of *Artemia* extract into the cell after UVB exposure decreased UVB-induced apoptosis by 35%. In addition, GP4G protection reached 45% when *Artemia* extract was added before and after UVB irradiation. These results are very interesting, and could be compared to the biological role of GP4G in *Artemia salina*. Indeed, *Artemia* in accumulating GP4G is therefore capable of withstanding various aggressions while preserving the integrity of its DNA and proteins.

## Protection against UVB

Based on the previous result showing the effect of *Artemia* extract on DNA protection against UVB rays, the effects of GP4G on a cell exposed to UVB were researched. Experiments were conducted on human fibroblasts and on human skin. First, cell viability was demonstrated to improve by 20% in *Artemia* extract treated cells. In a second step, Comet assay was carried out on cultured fibroblast, and demonstrated that in low dose (30 mJ) and high dose of UVB (60 mJ and 100 mJ), the DNA of cells treated was less damaged than the DNA of untreated cells (Fig. 1).

This experiment was ultimately confirmed by the study on *ex vivo* skin exposed to high doses of UVB (Fig. 2). Indeed, treated skin better preserves its structure and shows few signs of UV damage (e.g. rare presence of sunburn cells). Moreover, the CD1a immunostaining of Langherans cells reveals that skin treated does not display a decrease in the number and distribution of Langherans cells, whereas the placebo-treated skin shows a large decrease in Langherans cells as well as a significant migration of these cells toward the dermis, a sign of immunosuppression induced by UV rays.

All these experiments successfully demonstrated that GP4G offers cells a tremendous protection against UVB effects.

## Improving stress resistance

The aim of this study was to assess the effect of GP4G on different stresses such as heat tolerance both in cell and *ex vivo* skin. Human fibroblasts were cultured with the dinucleotide-phosphate in the medium. Then, cells were submitted to a temperature of 47°C for 1 hour. Living cells were thereafter measured using the MTT technique. In another experiment, human skin explants were treated with either an *Artemia* cream or a placebo cream, then submitted to stress temperature followed by a recovery phase of 24 hours at 37°C.

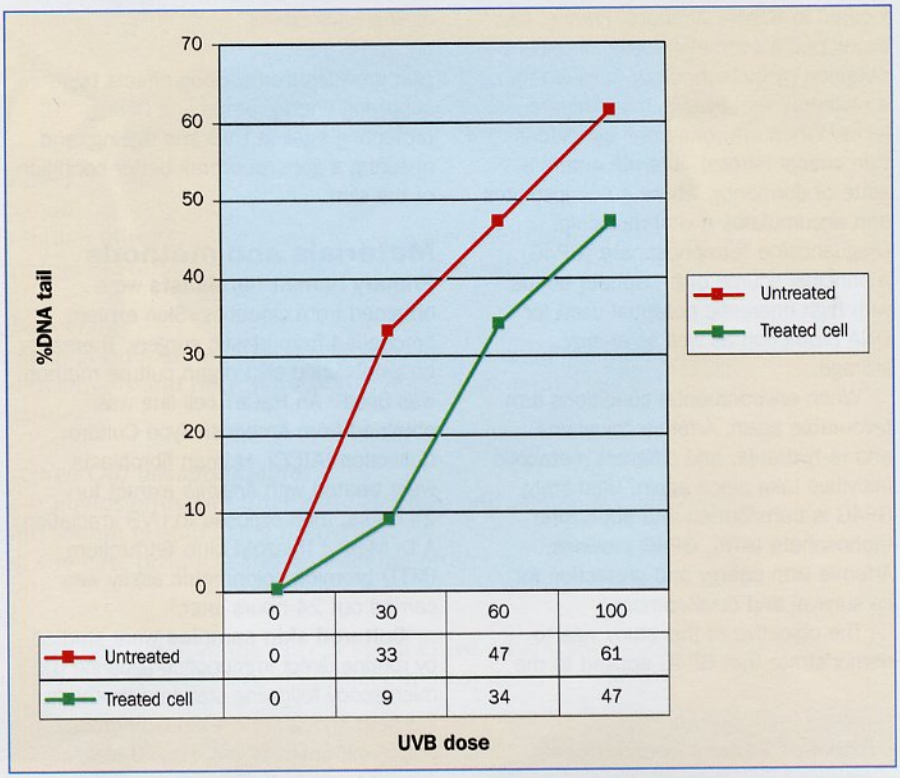
Study results demonstrated that the viability of treated cells submitted to heat shock increased in comparison with untreated cells. In hematoxylin-eosin staining, the treated cells were less affected and more resistant to the effects of heat compared with the control cells.

An *Artemia* extract-treated skin sample exposed to heat stress presented a well-preserved structure and minimal sign of heat injury compared with the placebo-treated skin.

## Additional studies

If the beneficial effect of GP4G itself on the cyste of *Artemia salina* can be explained, how can its effect on skin be explained? GP4G was demonstrated to stimulate proteins such as keratin or filaggrin. The question is: can GP4G stimulate other proteins with a protective role?

Focus was placed on heat shock proteins (HSPs), molecular chaperones




| | 0 | 30 | 60 | 100 |
|---|---|---|---|---|
| Untreated | 0 | 33 | 47 | 61 |
| Treated cell | 0 | 9 | 34 | 47 |

**Figure 1:** *DNA degradation by Comet assay, in cells irradiated with 30, 60 and 100 mJ/cm² of UVB. Mean percentage of DNA tail in a population of cells exposed to a range of UVB doses: x-axis shows the dose in joules per square centimeter; y-axis represents mean percentage of DNA in the tail.* Artemia *extract-treated cells exhibit very little UVB-induced DNA degradation, demonstrated by a low percentage of DNA in the tail compared to the untreated cells.*

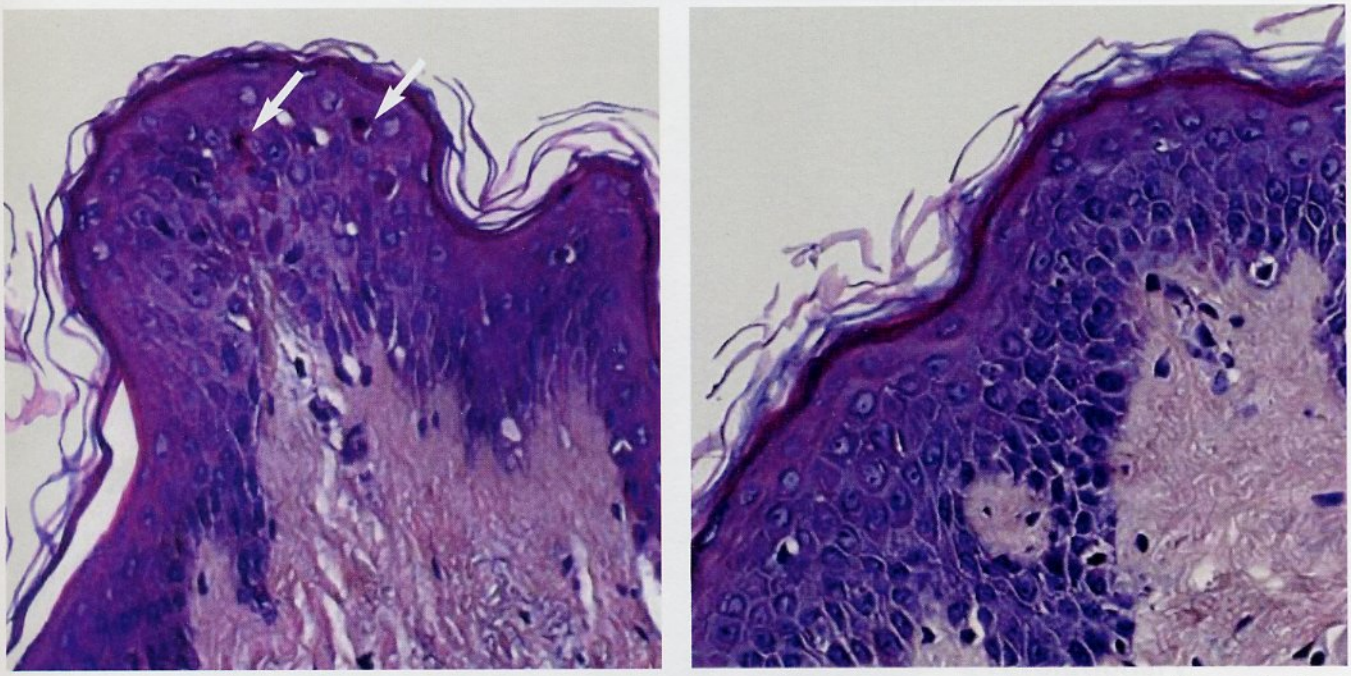


**Figure 2:** *H&E staining of human skin after UV stress. Left: Control UVB-untreated. Right: UVB-*Artemia *extract treated skin. In the control, skin exhibits many signs of UV damage such as numerous sunburn cells (arrows), disturbance and vacuolisation of the dermal-epidermal junction. Right:* Artemia *extract significantly protects the skin from UV damage. Skin structure is well preserved and rare signs of UVB insult are seen.*

that play an important role in protecting the cell from different types of stress. Briefly, HSPs have been shown to possess a variety of physiological functions in both stress and no-stress conditions, emphasising the important role of these proteins in this mechanism. HSPs include two types of molecules belonging to HSP70.[11] In biological conditions, HSPs are important for cell development and differentiation, and also help protein folding and assembly, whereas in stress conditions, HSP70 binds to unfolded and misfolded proteins, acting as a "molecular chaperone."

The objective of the study was to evaluate HSP70 expression by immunostaining in human skin cells and on skin samples. HSP70 messenger RNA content was also assessed. Application of GP4G significantly induced HSP70 synthesis in both keratinocytes and fibroblasts. Similar studies on human skin showed that the application of *Artemia* extract in a cream formula significantly induced and increased HSP70 protein expression compared with placebo-treated skin (Fig. 3). In another blot experiment, a strong increase in HSP70 mRNA level was observed. Indeed, HSP70 mRNA content reached its maximum at a 70% increase, three hours after the beginning of cell treatment by the dinucleotide phosphate.

In addition, different studies strongly suggest that *Artemia* extract induction of HSP70 is stress-free. Such studies included cell viability using MTT colorimetric assay, which showed no loss of cell viability. Likewise, studies on IL-1 and IL-8 synthesis showed no induction of these cytokines, indicating the absence of cellular stress.[12] Thus, it is very interesting to note that *Artemia* extract provides substantial protection by a remarkable mechanism: it induces HSP70 expression in human skin and human cells without stressing cells.

## Cytokine regulation

Following up on these observations of inducing HSP70 expression in a stress-free manner, the anti-inflammatory effect of *Artemia* extract was investigated on IL-1, IL-8 and TNF synthesis. Other experiments showed that HSP70 production under heat, chemical or radiation stress inhibits cytokine production in human stress in a concomitant fashion.[13] In order to understand a possible role of *Artemia* extract in the same way, ELISA tests were performed on human HaCaT keratinocytes treated with GP4G for 24 hours before UVB irradiation. Interestingly, a significant decrease in IL-1 level was found in the *Artemia* extract-induced cells at different time points. Similarly, a decrease in IL-8 level in treated cells was found at all time points. Likewise, cells treated with *Artemia* extract showed a decrease in the powerful mediator of inflammation TNF content. All the decrease is statistically significant with the Student T test. These studies indicate *Artemia* extract or, more precisely, HSP70 induction, without stress, by *Artemia* extract in human cells provides a new and interesting means of treatment of some kinds of inflammatory states of the skin by reducing the cytokine response.

## Effect of *Artemia* extract on retinoid-treated skin

According to the previous studies of the role of *Artemia* extract in leading to the decrease of cytokine molecule in various stress conditions, interest turned to the effect of GP4G on cells and skin treated with "all trans retinoic acid" (ATRA) for a prolonged period. It should be remembered that a topical treatment of retinoic acid can be used for different skin diseases. The results of the studies discussed in this article showed that the administration of ATRA in cells decreased the level of HSP70. This effect was observed within the first week of application and became increasingly clear with time. The administration of *Artemia* extract on ATRA-treated cells and on ATRA-treated skin samples clearly increased HSP70 levels in both cells and skin. Moreover, several previous studies have established that ATRA topical application increases inflammatory cytokine.[14] The effect of *Artemia* extract on IL-1 levels in ATRA and GP4G treated cells was researched. The results confirmed both the pro-inflammatory effect of ATRA, in particular on the IL-1 synthesis, and the effect of *Artemia* extract on the decrease in inflammatory cytokines. This study substantiated the role of GP4G in the improvement of skin comfort in association with retinoids.

## Conclusion

An overall increased awareness of the risks linked to sun exposure has uncovered a renewed interest in protecting skin from

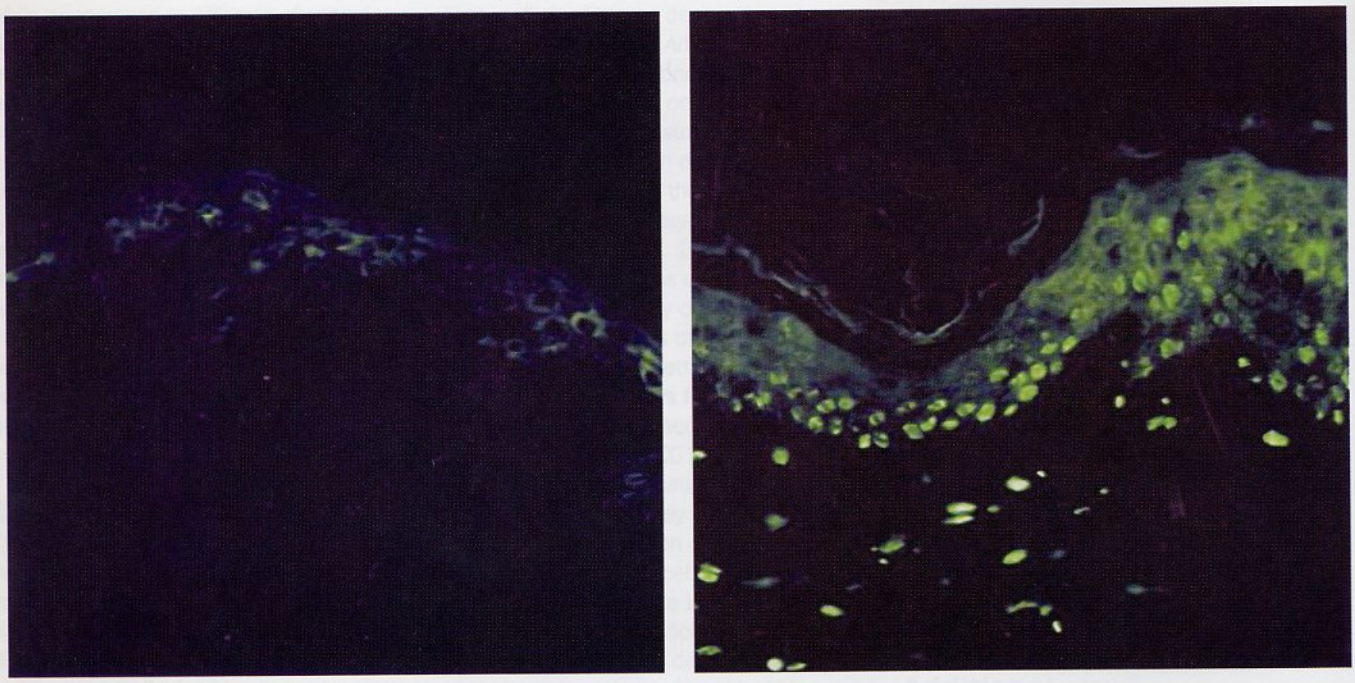


**Figure 3:** *Left: Control untreated skin. Right:* Artemia *extract treated skin. Application of* Artemia *extract significantly induced HSP70 in the skin's keratinocytes and fibroblasts in comparison to untreated skin.*

the harmful effects of UV rays. Today, UV rays are known to induce cellular damage reaching DNA and contributing to photoageing and carcinogenesis.[15] Exposure to sunlight and other factors may hasten the skin-ageing process – the factors include cigarette smoke, air pollution, harsh climatic conditions, and the stresses of modern life.

*Artemia* extract, through both GP4G and HSP, offers great skin protection. Indeed, *Artemia* extract possesses multiple protective effects, some of which are mediated by the induction of HSP70, the first fighter of cellular stress. In addition to the protective HSP effect, *Artemia* extract acts as a "cyto-nutritive" energiser of the cell through GP4G. GP4G, through GTP, binds G-proteins and enhances cell metabolism. GP4G is also a rich source of phosphorous and guanine, constitutive elements of essential molecules (phospholipids, DNA, RNA, etc). Phosphorous is a trace element present in numerous cell structures such as membrane phospholipids. Guanine is a nucleotide essential for RNA and DNA synthesis as well as for the regeneration of cutaneous cells.

GP4G acts as a genuine biological booster on skin cells, both improving the skin barrier and promoting the regeneration of the dermis. Furthermore, study data demonstrated that *Artemia* extract protects and decreases DNA damage. GP4G can be used in reinforcing the protection of sunscreens.

Moreover, *Artemia* extract offers an anti-inflammatory action by diminishing the level of inflammatory cytokines. Thus, *Artemia* extract offers great interest for therapeutical and cosmetic purposes.

GP4G offers a more comprehensive means to increase cellular defences against stress, and therefore is of great interest for anti-ageing and sun care products as well as for the care of sensitive and irritated skin. PC

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