

A phosphatidylcholine hyaluronic acid chitin–nanofibrils complex for a fast skin remodeling and a rejuvenating look

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Background: The reduction of mortality worldwide has led older individuals to seek intervention modalities to improve their appearance and reverse signs of aging.

Objective: We formulated a medical device as innovative block-polymer nanoparticles based on phosphatidylcholine, hyaluronan, and chitin nanofibrils entrapping amino acids, vitamins, and melatonin.

Methods: Viability and collagen synthesis were controlled on fibroblasts *ex vivo* culture while adenosine triphosphate production was evaluated on keratinocytes culture. Subjective and objective evaluations were performed *in vivo* on selected volunteer patients.

Results: In accordance with our previous studies, both the *in vitro* and *in vivo* obtained results demonstrate the efficacy of the injected block-polymer nanoparticles in reducing skin wrinkling and ameliorating the signs of aging.

Keywords: antiaging agent, scar correction, stretch marks, signaling molecules, photoaging, biostimulation

Introduction

Major advances in the medical field have led to a significant increase in life expectancy, with attendant greater efforts being directed towards healthier living and growing concerns about general face and body appearance.¹ The increasing proportion of women and men interested in skin rejuvenation, influenced in part by the media, has created an enormous demand for so-called antiaging remedies to rejuvenate photodamaged skin in the shortest possible time. Signs of relative skin damage associated with age, such as wrinkling, slackening, and irregular pigmentation, are in fact strongly influenced by environmental factors, particularly lifetime sun exposure.² It has been shown for the normal aging process as well as for photoaging that the induction of reactive oxygen species generates mitochondrial DNA mutations, leading to a defective respiratory chain, especially at the level of the fibroblast cell, and higher production of reactive oxygen species is induced, which, in turn and in a vicious cycle, allows further mitochondrial DNA mutation and mutagenesis, independently of the inducing agent.^{2,3} In addition, the induction of common deletion in human skin fibroblasts is paralleled by a measurable decrease in oxygen consumption as well as by an increase in metalloprotein-1. Many studies have shown, in fact, how the induction of matrix metalloproteinases plays a major role in the pathogenesis of photoaging.^{4,5} Moreover, generation of phosphocreatine, and consequently adenosine triphosphate (ATP), is facilitated when creatine is abundant in cells. Indeed, experimental supplementation of normal human fibroblasts with creatine normalizes mitochondrial mutagenesis, as

well as the functional parameters of oxygen consumption and metalloprotein-1 production.⁶

As a consequence of all these cellular disturbances, aged skin displays an array of fine, superficial facial lines that leads to the formation of deep creases over the forehead and between the eyebrows, periorbitally, and in the nasolabial folds. This has been shown to be due to an altered skin antioxidant network and a decreased epidermal turnover, with a decline in synthesis and secretion of lipids, signaling-peptide molecules, and their receptors.^{7–10}

During the past few years, many different techniques for rejuvenation have been proposed. Among them, injections with fillers and biostimulating agents are used for wrinkle treatment, correction of scars, and soft-tissue augmentation. Increased demand from plastic surgeons for new active medical devices and procedures is accompanied by the increased use of cosmeceuticals and nutricosmetics to obtain beauty and wellness on the inside and outside.¹¹

Project

Drawing on the experience of our group in the use of topical cosmetics^{12–14} and noninvasive procedures adopted to ameliorate the appearance of aging skin,¹⁵ we developed and studied a new medical device to treat facial lines and body contour by balancing skin cell turnover and metabolism.

This product was formulated to increase, accelerate, and ameliorate the activity of both antiaging cosmetics and injected temporary or permanent fillers, which are predominantly based on the use of collagen and hyaluronic acid.^{16,17} Naturally, the product had to be safe, biocompatible, and stable at the implantation site, with minimal complications and no risk of migration.¹⁸ We formulated block-polymer nanoparticles (BPN) of phosphatidylcholine linoleic acid-rich nanocomplexed with hyaluronan and chitin nanofibrils (PHHYCN) encapsulating cholesterol, creatine, caffeine, melatonin, vitamins E and C, and the amino acids glycine and arginine. The high content in linoleic acid of the phosphatidylcholine used allowed the active BPN to quickly reestablish skin-barrier function. Thus, while the phosphatidylcholine-fatty acids of the BPN composition contribute to balancing the disturbed composition and organization of lipids at the level of epidermal keratinocytes and consequently of corneocyte lamellae, the high level of linoleic acid should contribute to reintegrate the reduced level of ceramide 1, structural and stabilizing component of the stratum corneum.^{19,20}

Ceramides are considered essential for barrier function, not only because of their quantitative significance but also because of their amphiphilic structure and long-chain

constituent *N*-acyl fatty acids. They may improve and upregulate the synthesis of flaggrin and consequently the production of normal moisturizing factors.^{21,22}

Moreover, the encapsulated cholesterol, regulating the hydration state of the skin together with the metabolites of phosphatidylcholine, plays an important role in lipid phase packing and in regulation of skin scaling, binding, and keeping water at the level of the epidermis's cells.^{23–31} All these activities are modulated and increased by the chitin nanofibrils (CN) encapsulation methodology.^{32–35}

Finally, the presence in the formulation of creatine is of primary importance in normalizing mitochondrial activity. In addition, melatonin and vitamins C and E are useful components to reintegrate the skin antioxidant network, while glycine and arginine support collagen and elastine synthesis. Also, caffeine has a role in ameliorating cellular tone, its metabolic process, and intercellular signals, according to the recently proposed Nervous Immune Cutaneous Endocrine (NICE) systems concept.^{36–41}

Aim

The aim of the study was to evaluate the rejuvenation effects of injectable active BPN, entrapping the selected active ingredients, on subjects affected by photoaging. In vitro, the activity of this BPN was observed on cultures of stressed keratinocytes and fibroblasts, as well as their ATP and collagen synthesis verified in presence of our product. In vivo, the safety and efficacy of the product to reduce the global appearance of fine, deep lines was established. This innovative nanovehicle based on PHHYCN traps all the active ingredients, allowing their slow release at cell level.^{32–34}

Material and methods

Formulation

Each milliliter contained: hyaluronan salt 1 mg, phosphatidylcholine 3 mg, creatine 0.1 mg, caffeine 0.1 mg, ascorbyl tetraisopalmitate 0.5 mg, vitamin E 10 mg, chitin nanofibrils 1 mg, melatonin 0.1 mg, glucosamine 0.1 mg, glycine 0.1 mg, arginine 0.1 mg, sodium phosphate dibasic 2 mg, potassium dihydrogen phosphate 0.2 mg, sodium chloride 9 mg, sterile water for injection to 1 ml.

Patient enrollment

Before the study, each subject was informed about the purpose of the study, and their written informed consent was obtained according to the ethics of medical-device experimentation. No drugs or cosmetic procedures affecting the course of the antiaging treatment were allowed 2 weeks prior to the study period.

All the 36 volunteer women (age range 30–55 years) between February and April 2011 attended the dermatology outpatient office, as per Lever et al.⁴² The only criterion for entry in the study was the presence of one or more signs of photoaging affecting the face, such as fine wrinkling around the eyes, crease lines around the mouth and cheeks, telangiectasia, wrinkling and spots on the back of the hands, etc, corresponding to degrees 3–5 of the photodigital scale described by Larnier et al⁴³ and previously used by our group.^{44,45}

In vitro studies

Skin aging causes a general reduction of the synthesis of collagen fibres, slowing also keratinocyte turnover and ATP activity. One month before the in vivo intradermal injections the formulation was applied on control ex vivo cultures of fibroblasts and keratinocytes obtained by explants (8 mm diameter) of the abdominal skin of the volunteers. Thus the collagen production of the fibroblasts as well as the ATP produced from the keratinocytes' cultures were controlled for, in accordance with the methodologies used in our previous study.³⁵

Fibroblast activity

According to our previous study³⁵, viability and proliferation of fibroblasts, isolated from the patients' explant were controlled. They were cultured in 9BM medium (Cambrex MD, USA) at 37°C and 5% CO₂ at 50% relative humidity (RH).

For four cultures of fibroblasts, 10 µg/mL of the BPN was added; the other four cultures served as controls. Results are reported in Figure 1, illustrating the percentage media of cell proliferation with respect to control values.

Collagen synthesis

As in our previous study,³⁵ the rate of collagen type-1 synthesis was measured by the use of specific antibodies (enzyme-linked immunosorbent assay) on eight cell cultures, four of which were enriched with 10 µg/mL of the product directly introduced in the culture medium. Four served as nontreated controls. Measurements were done after 6 days of incubation. Results are reported in Figure 2, illustrating the percentage media of collagen increase with respect to the control value.

ATP activity of keratinocytes

As in our previous study,³⁵ the ATP activity was controlled on keratinocyte cultures irradiated by 4 J/cm² UVA + 0.4 J/cm² UVB. Irradiation causes a strong reduction of ATP present and it is dose dependent. Of the twelve dish cultures used, four received 10 µg/mL of the product 24 hours before UV irradiation, whereas eight served as controls (four nonirradiated and four irradiated). The ATP level was detected using ATP Lite-M (Chemiluminescent kit; Packard Bioscience, Groningen, Netherlands). The obtained results are reported in Figure 3, illustrating the percentage media

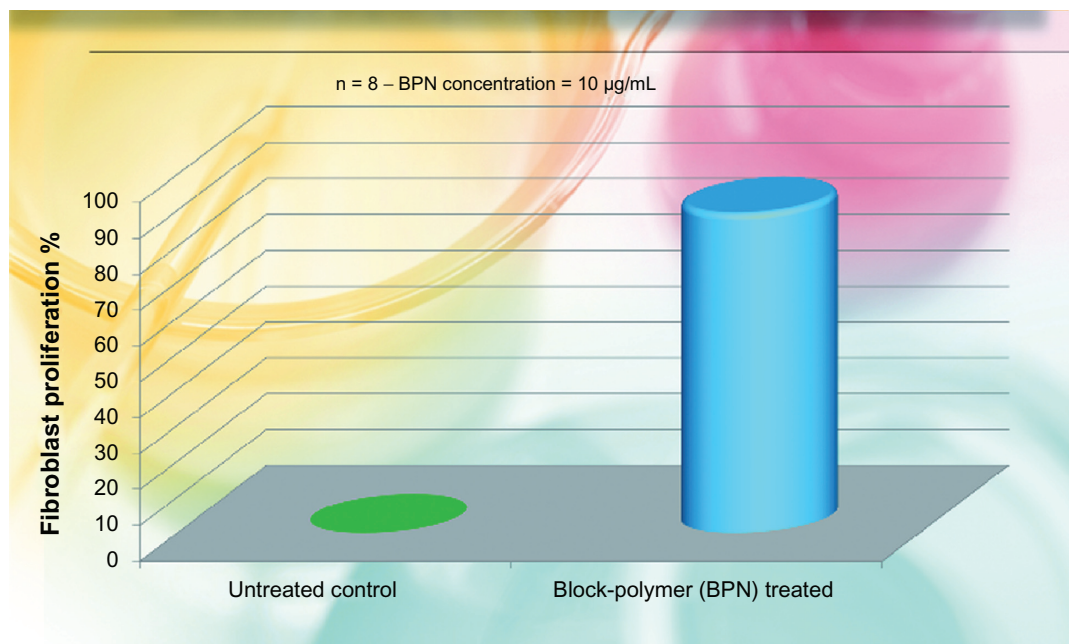


Figure 1 Fibroblast proliferation recovered by the use of the liposomal complex of phosphatidylcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (BPN) vs the untreated control.

Note: BPN values vs control highly significant ($P < 0.001$).

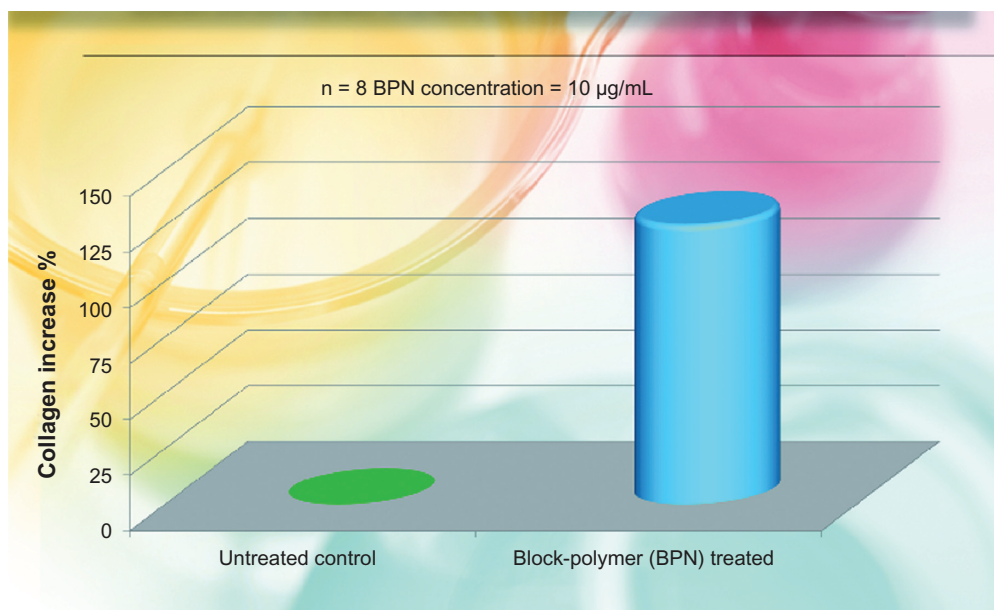


Figure 2 Percent increase of collagen produced by fibroblast cultures added with the liposomal complex of phosphatidilcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (BPN) vs the untreated control.

Note: BPN values vs control highly significant ($P < 0.001$).

of ATP increase with respect to the baseline nonirradiated untreated values.

In vivo studies

Patient enrollment

The possibility of having a unique product composed of different kinds of active ingredients with different pharmacological

activities at different cell levels would help the operator to obtain the best rejuvenation results from the skin biostimulation.

Injection technique

The active BPN was injected directly in different skin areas of the 36 women subjects, mean age 51.3 years. The puncture technique used was based on a single injection every 7 days

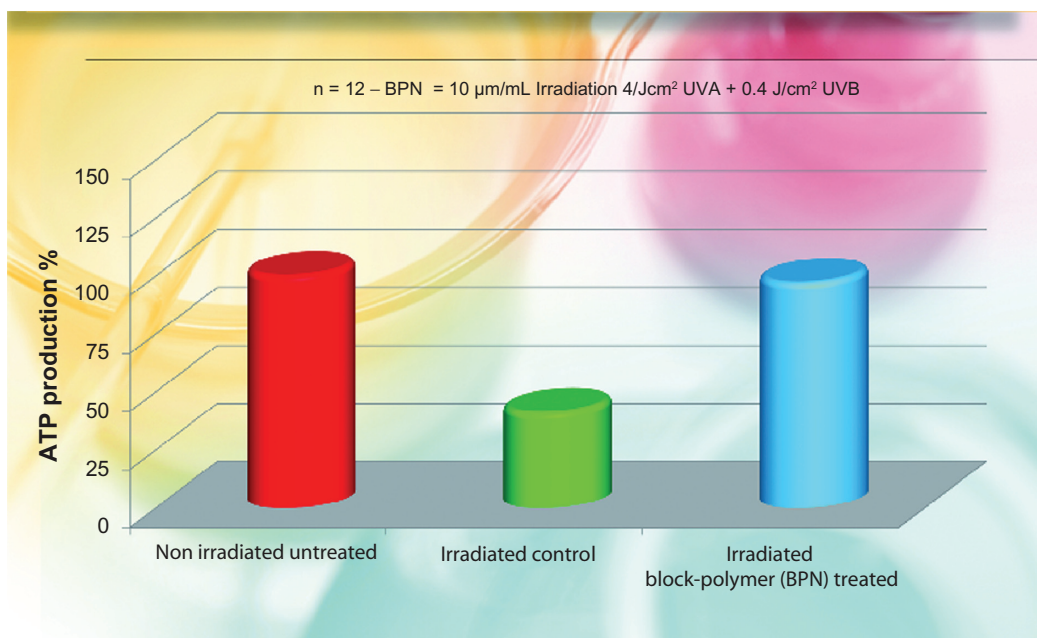


Figure 3 Percent increase of ATP produced by irradiated keratinocyte cultures added with the liposomal complex of phosphatidilcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (BPN) vs the untreated control.

Note: BPN vs irradiated and non irradiated control highly significant ($P < 0.005$).

Abbreviations: ATP, adenosine triphosphate; UVA, ultraviolet A.

for 10 weeks, followed by other injections for a further 2 weeks for a global personalized treatment of 90 days with a final control at day 120 (regression period).

The treatment used was based on the mesotherapy technique, using 1 mL solution and a 30 g needle positioned at 45° to the skin surface. The needle was advanced until the middle to deep subcutis, aspiration was performed to ensure that the tip was not within a blood vessel, and injection was then commenced slowly as the needle was withdrawn. Injection rate was at all times less than 0.3 mL/min. Firm massage, with index finger inside the mouth and thumb outside, was then used to remove any unevenness. The 1-mL quantity is sufficient to treat the entire face. For each skin area, 1–2 mL of the product was used, as per our previous studies.^{44,45}

Control assessment and dermatological evaluation

Control visits and evaluations were undertaken on the first day (D1, baseline) and after 15 (D15), 30 (D30), 45 (D45), 60 (D60), 75 (D75), and 90 (D90) days of treatment, with a follow-up visit at D120 (regression period). The individual signs of photoaging symptoms of skin irritation and the degree of the obtained correction for each treatment and each area were evaluated objectively by an expert dermatologist using a 0–10 visual analog scale with separate scores for each site of the face (0 = no correction; 5 = satisfactory correction; 10 = total correction). The degree of satisfaction with the efficacy of the product was also obtained subjectively by

asking the patients if there was any itching, stinging, or burning sensation. The different mean evaluations are reported in Figure 4, with some photographic examples in Figure 5.

Subjects' evaluation

After the first, second, and third month of treatment, with a follow-up at the fourth month (regression period), the subjects evaluated their satisfaction or dissatisfaction by giving scores on firmness, softness, hydration, and wrinkle appearance, using a scale of 0–4 for each criterion (0 = unsatisfactory; 4 = satisfactory), as per Berardesca et al.⁴⁶ The obtained results are reported in Figure 6.

Results and discussion

The obtained results seem to be in line with our expectations for both the in vitro and in vivo evaluations on the efficacy of the treatment. Practically all the subjects treated during the 90-day period reported that they were satisfied with the general aspect of their skin, which appeared softer and more hydrated since the first month of treatment, as shown in Figure 6. In line with their self-evaluation, the appearance of the fine wrinkling was notably reduced and the consequent skin softness and firmness enhanced during the entire treatment period. It is interesting to note that this general amelioration remained during the regression period also, 30 days after the interruption of the treatment. The same results were clinically observed by the dermatologists involved in the study.

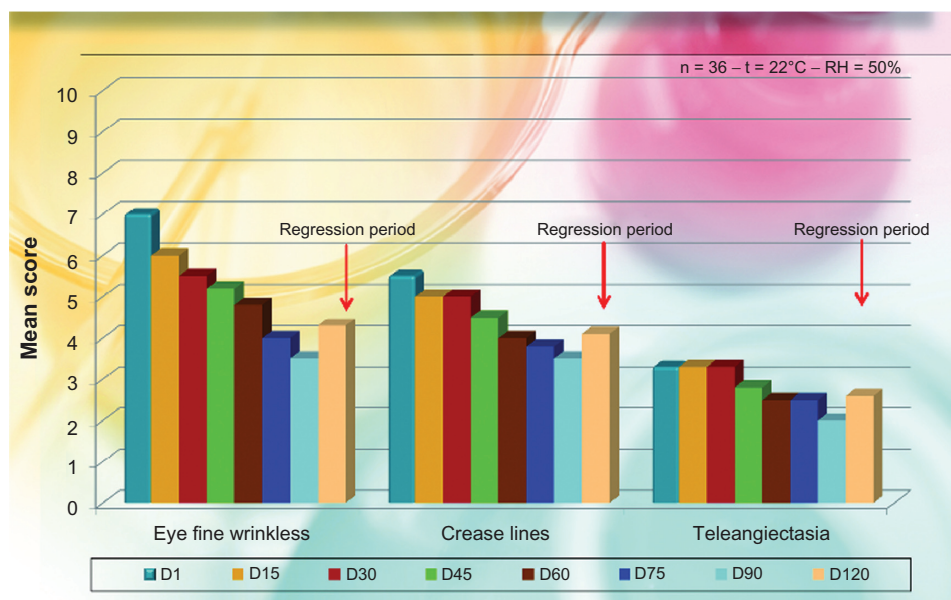


Figure 4 Dermatological mean evaluation on signs of photoaging after injective treatment with phosphatidylcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (BPN).

Note: All *P* values are highly significant as to baseline ($P < 0.005$).

Abbreviations: BPN, block-polymer nanoparticles; RH, relative humidity.

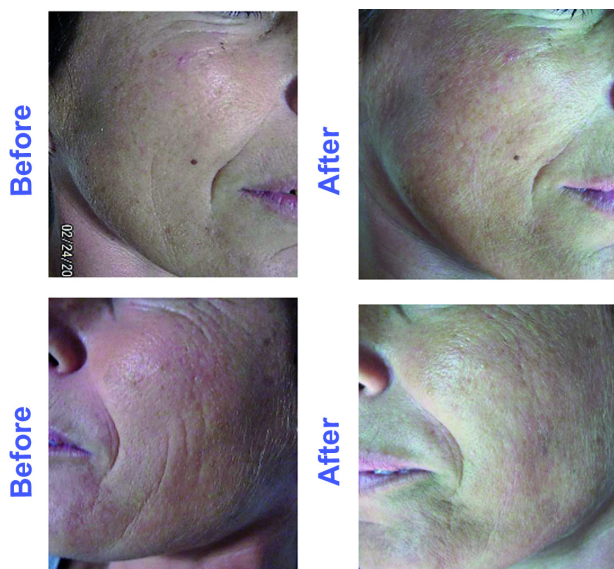


Figure 5 View of nasolabial folds and midcheek before (A and C) and after (B and D) treatment by the active block-polymer nanoparticles at 30 days.

As shown in Figure 4, both fine wrinkling and crease lines were reduced soon after the first 15 days of treatment, as well as the presence of telangiectasia, so that the general appearance of the face was notably ameliorated during the regression period also. This interesting general amelioration is shown in Figure 5, where a reduction of fine lines is evident.

The in vivo results confirmed the in vitro (ex vivo) studies. As evident from Figure 1, the BPN increased fibroblast proliferation by about 80% ($P < 0.001$), along with an increase in collagen production, as shown in Figure 2. Moreover, the increased ATP production of irradiated keratinocytes, has underlined an increased vitality of all the cells involved in daily skin turnover. As shown in Figure 3, the irradiated keratinocytes treated by the use of BPN showed about the same quantity of ATP in comparison with the nonirradiated cultures. From these first results, it is possible to claim that the injectable use of this special formula seems able to stimulate the normal life of all the skin cells of both the epidermis and dermis, improving the general appearance of the face (Figure 5). As previously shown (Figures 1 and 2), both keratinocytes and fibroblasts appear to be able to produce in vitro more ATP and collagen, respectively, so that the final appearance of the skin appears softer and more elastic with reduced numbers of fine wrinkles (Figure 5).

Based on our findings, the BPN encapsulating the active ingredient used, seems to be useful in improving the activity of permanent fillers, rendering it useful as an antiaging remedy for the plastic surgery armamentarium. In conclusion, this innovative biostimulating medical device should be used for wrinkle treatment and rejuvenating looks, as well as an adjuvant in soft-tissue augmentation and stretch-mark corrections.

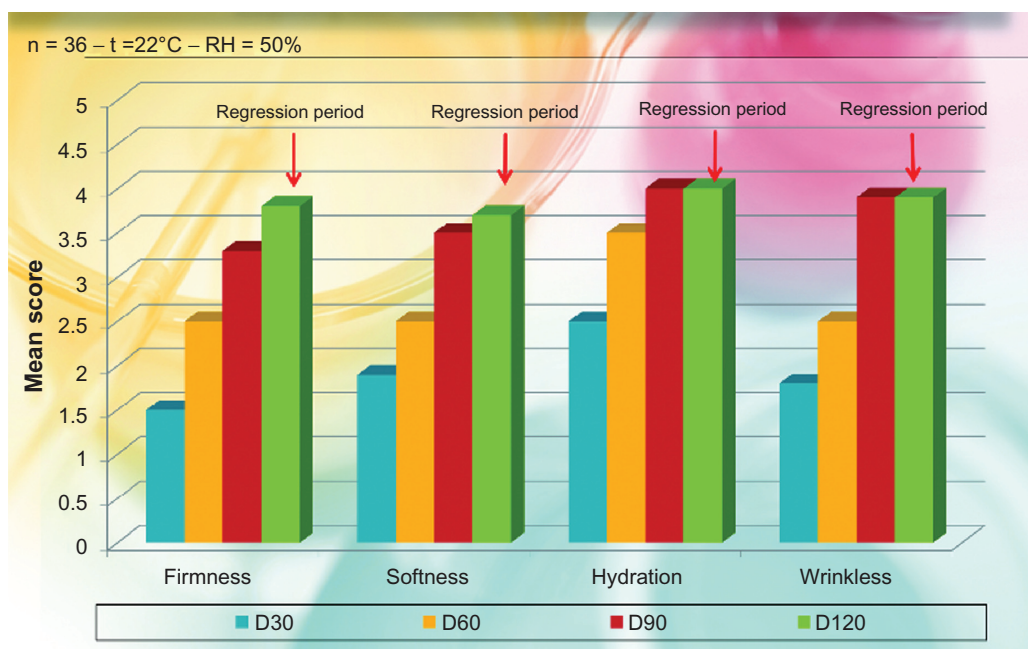


Figure 6 Self evaluation and satisfaction of the subjects treated with phosphatidylcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (BPN) (general degree of satisfaction).

Note: All P values are highly significant as to baseline ($P < 0.001$).

Abbreviations: BPN, block-polymer nanoparticles; RH, relative humidity.

We are continuing with other research for better understanding the mechanism of action of this medical device, and to improve our knowledge on the intimate activity of CN and CN complexes as probable signaling molecules at the level of the skin cells.

Disclosure

The authors report no conflicts of interest in this work.

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