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# Enhanced skin regeneration by nanoegg$^{TM}$ formulation of all-trans retinoic acid

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All-trans retinoic acid (atRA) which could smooth wrinkles and produce less pigmented skin after a few months of treatment has been studied in research into topical treatments for a potent inhibitor of new melanin production. However, the clinical responses of commercial atRA cream predominantly comprise severe inflammation. We report a novel nanotechnology "nanoegg$^{TM}$" system giving improved effects of atRA self-assembly which were coated by $CaCO_3$. Dorsal areas of hairless mouse and porcine skin were employed for administration of nanoegg$^{TM}$ ointment and commercial products. The mRNA for heparin-binding epidermal growth factor-like growth factor (HB-EGF) from tissues was measured by a real-time PCR method. All tissues were stained for detection of hyaluronate and the thickness of the epidermis. A clinical trial in humans was carried out at St.Marianna University in Japan. As a result, the irritation and inflammation associated with atRA molecules were substantially reduced. The physicochemical instability of atRA was also dramatically improved. Furthermore, nanoegg$^{TM}$ enhanced marked expression of mRNA for HB-EGF from keratinocytes, which is known as one of the markers of keratinocyte turnover. Also, production of hyaluronate was surprisingly in the intercellular spaces of the basal and spinous cell layers 2 days after treatment. Even at the low concentration of atRA in the nanoegg$^{TM}$ system, the proliferation and differentiation of keratinocyte was somewhat enhanced. A nanoegg$^{TM}$ may thus not only prevent adverse effects, but also markedly enhance the main effect.

## 1. Introduction

Hyperpigmentation on the face is a symptom that produces great anxiety, especially women from the point of view of beauty. For several years, all-trans retinoic acid (atRA) and other synthetic retinoids have been used widely in the treatment of a variety of skin diseases associated with hyperproliferative processes. Retinoids induce a characteristic series of biochemical and histological modifications when applied to skin. Their effects on keratinocytes are still incompletely understood in spite of the discovery of nuclear atRA receptors, which are highly expressed in human adult skin (Krust et al. 1989).

The physicol-chemical properties of atRA and the characteristics of its interactions with the various environments in which it is distributed *in vivo* affect its biological functions. As a consequence of the presence of a large hydrophobic moiety in atRA, it is poorly soluble in water. However, self-aggregation of atRA can have significant implications for the biological function of this compound. At an alkaline or physiological pH, atRA molecules self-associate, and form micelles in aqueous solution (Yamaguchi et al. 2005). Micelle formation may allow a higher than expected aqueous concentration of atRA to be maintained, and this concentration may be high enough to support diffusional flux of free atRA that is sufficient for short-distance transport in cells or to allow metabolism of free atRA. Consideration of the chemical structure of atRA suggests that its amphipathic nature results in detergent-like characteristics, and several studies have shown that the presence of atRA affects various aspects of membrane structure and function (Dingle and Lucy 1965; Stillwell et al. 1982; Wassall et al. 1988).

The atRA may carry an actual net charge. This feature may result in a stronger surfactant – like characteristic and provide an explanation for its critical micellar concentration (CMC). The CMC of atRA is lower than 1mM (Yamaguchi et al. 2005), but more than three orders of magnitude lower than reported CMC values for bile acids (Small 1971) and tenfold lower than the CMC of the long-chain fatty acid palmitate (Custola et al. 1988). This observation indicates that atRA self-associates into micelles at a substantially lower concentration than other small amphipathic compounds.

The conjugated double-bonds of the isoprenoid chain render atRA susceptible to photodegradation, isomerization, and oxidation. Consequently, naturally occurring atRA is extremely labile. In practice, the structural integrity of

atRA must be maintained *in vivo*. The answer lies in part with the observation that most retinoids *in vivo* are associated with retinoid-binding proteins. Many of these proteins bind their ligands in hydrophobic pockets, thereby effectively shielding them from the aqueous environment. The micelle form is thus expected to protect atRA from nonspecific oxidation and may also stabilize its isomerization state.

atRA is widely used for topical therapy of several skin diseases; it also improves chronic solar damage (Weiss et al. 1988). Topical atRA induces irritation of the skin, which precludes its use in some skin diseases that respond to systemic retinoids (Weiss et al. 1988). Irritation might be explained in part by an overload of atRA dependent pathways with non-physiological amounts of exogenous atRA in the skin. It might thus be expected that if atRA anionic micelles are formulated in a drug delivery system (DDS) with controlled release properties, irritation of the skin might be decreased.

The substance has been approved by the FDA in the U.S., and has been marketed as Renova™ and Retin-A™. Melanin loss and smoother, less-wrinkled skin, however, are frequently accompanied by excess skin irritation after treatment with tretinoin creams (Klingman et al, 1984, 1986; Olsen et al. 1992). Clinical responses predominantly comprise severe irritant dermatitis in the early stages of application of Renova™ and Retin-A™ (around 3 or 4 days). Such damage with a high level of inflammatory events after tretinoin treatment can induce chronic skin pigmentation of the area being treated, potentially resulting in imperfect repair of skin displaying a pigmented appearance.

We have previously reported a novel DDS for atRA, which comprises inorganic-coated atRA micelles (nanoegg™) (Yamaguchi et al. 2005). The conceptual framework of this study is that nanoegg™ plays a crucial role in resolving the low permeability of atRA across the skin, improving lability, and decreasing the strong symptoms of irritation.

## 2. Investigations, results and discussion

### *2.1. Improved lability of atRA in nanoegg™ system*

Fig. 1 shows the high potential of nanoegg™ for improved stability. As atRA has a specific maximum absorbance at 340 ~ 345 nm, the decrease of peak height and another isomeric peak in HPLC are available to investigate loss of activity. It can be seen that the peak height of nanoegg™ scarcely changed during approximately 70 days in an accelerated environment at 40 degrees, even with no antioxidant. It can thus be seen that the lability of the atRA molecule was somewhat improved with respect to temperature, as might be expected. As a result, long-term storage of atRA ointment became possible.

### *2.2. Improved irritation of atRA in nanoegg™ system*

Both Retin-A™, a 0.1% atRA cream, and Renova™, a 0.05% atRA cream, are already marketed commercially by Johnson & Johnson Co. in the U.S. An *in vivo* experiment on the porcine dorsal area was performed for comparison with these commercial products (Fig. 2). With nanoegg™, irritation of porcine skin, which is known to be similar to human skin, showed the lowest level compared with other commercial products. In a tissue experiment on mice and rats, inflammation tests also showed the same result that

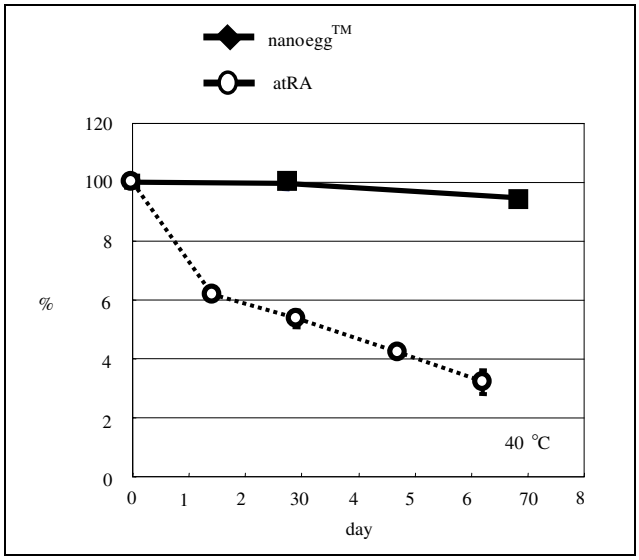


Fig. 1: Stability of atRA in 40 degree environment. The atRA dissolved in ethanol as negative control. Each sample was packed in tube protected from light

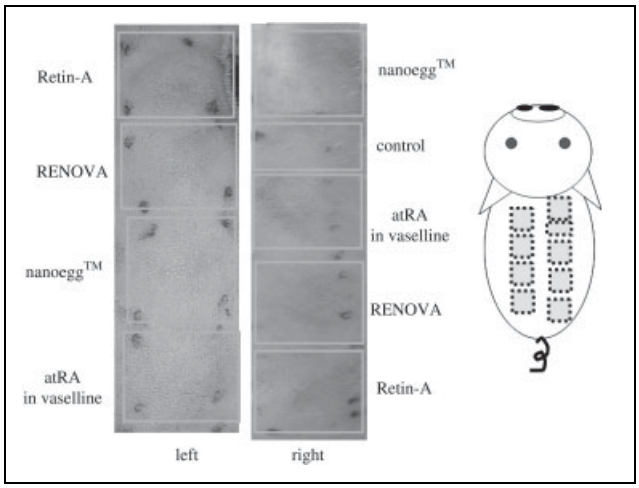


Fig. 2: Irritation test of nanoegg™ 0.1% ointment. Porcine (pot bellied, male, 3 months) dorsal area was utilized for topical administration. Negative control was atRA 0.1% in vaseline. Each compound was administered 30 mg daily on 2 × 2 cm square for 15 days

nanoegg™ seemed to suppress of irritation after daily topical treatment (Yamaguchi et al. 2005). One distinguishing feature of human skin response to atRA is the generation of clinical erythema. Since the erythema response of human skin to atRA is normally dose-dependent (Griffiths CEM et al. 1993), the reduced erythemogenic potential of nanoegg™ would be expected to result in a lack of irritation with decreasing dose of atRA, compared to normal atRA therapy.

### *2.3. Pharmacological effects of nanoegg™*

#### *2.3.1. Expression of mRNA HB-EGF on mouse skin*

Stoll et al. reported that mRNA expression of heparin-binding epidermal growth factor (HB-EGF) is induced by atRA in human keratinocytes and skin (Stoll and Elder 1998). Recently, production of HB-EGF has been identified as one of the markers of turnover of keratinocytes (Stoll and Elder 1998). Fig. 3 shows mRNA HB-EGF expression in hairless mouse dorsal tissues treated with the

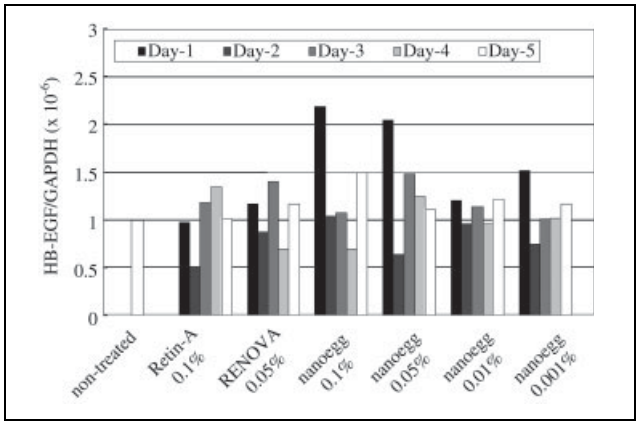


Fig. 3: Expression of mRNA HB-EGF in hairless mice (HR-1, male 5-week) dorsal areas after one-day is treatment at 5-days with no-treatment, nanoegg™ (0.1%, 0.05%, 0.01% and 0.001% respectively), Renova™, Retin-A™. The tissues of dorsal areas were frozen by liquid nitrogen, and RNA extracted by the Isogen method

commercial products Retin-A™ and Renova™ and nanoegg™. One-day's application was performed, and tissue was sacrificed and frozen in liquid nitrogen daily until 5-days. Especially on the first day, excess expression of mRNA HB-EGF was apparent in the nanoegg™-treated group compared with the commercial products, Renova™, Retin-A™. Furthermore even at the most dilute atRA dose, the elevated expression of HB-EGF mRNA observed at day-1 would be expected to be associated accelerated proliferation and differentiation of keratinocytes.

*2.3.2. Proliferation and differentiation of keratinocytes*

It is known that atRA affects multiple biological functions including cell growth and differentiation through regulation of specific genes. Histologically, improvements in appearance of photo-damaged skin following atRA usage are associated with thickening of the epidermis accompanying the reduction in melanin content (Klingman et al. 1984; Klingman AM et al. 1986).

The nanoegg™-treated hairless mice underwent more progressive epidermal hyperplasia than those treated with the commercial products at the same dose, respectively (Fig. 4). Even at the lower dose of atRA (0.001%), histological evaluations suggested effective skin regeneration in mice. The significant increases in epidermal thickness for nanoegg™ treatment seen at all doses were compared with treatment using commercial product creams. The hyperplasia of the epidermis in Fig. 4 almost corresponded to the expression of HB-EGF mRNA in Fig. 3, but it would be seem that a one or two-day period is needed due to the thickened epidermal layer after the production of HB-EGF.

Not only the increase in thickness of viable epidermis but also the expression of hyaluronan from keratinocytes was observed simultaneously with the in nanoegg™ treatment system. Interestingly, wrinkling and skin texture may also be influenced by other epidermal factors apart from HB-EGF expression. The influence of atRA on epidermal differentiation is associated with the metabolism of hyaluronan (HA) by keratinocytes (Tammi et al. 1989). Tammi et al. reported tentative results showing that HA represents atRA-exposed keratinocytes (Tammi et al. 1989). The atRA molecule leads to an accumulation of HA in the superficial layers of the epidermis by stimulating HA synthesis in keratinocytes. In the nanoegg™ system, rapid regulation of HA in the epidermis might effectively help keratinocyte growth. Production of hyaluronan in the epidermis would accelerate turnover of keratinocytes, as the lubricant between cells, eventually contributing to a reduction in macular hyperpigmentation and less apparent fine wrinkles (Yamaguchi et al. 2005).

### *2.4. Clinical trial of nanoegg™ on human skin*

In general, a period of at least a few months is required for clinical effectiveness in treating mottled pigmentation using atRA cream. Given the major increase in thickness of the epidermis in our animal trials (Figs. 3 and 4), a reduction of melanin pigment should rapidly be produced by nanoegg™ 0.05% treatment in humans. As a preliminary clinical trial of *in vivo* treatment of wrinkles and pigmented spots, nanoegg™ therapy was carried out for one month on human faces. The institutional review board of St. Marianna University permitted the clinical trial of nanoegg™ ointment in human faces over 40 years old. The trial was carried out for one month in 30 wrinkles and

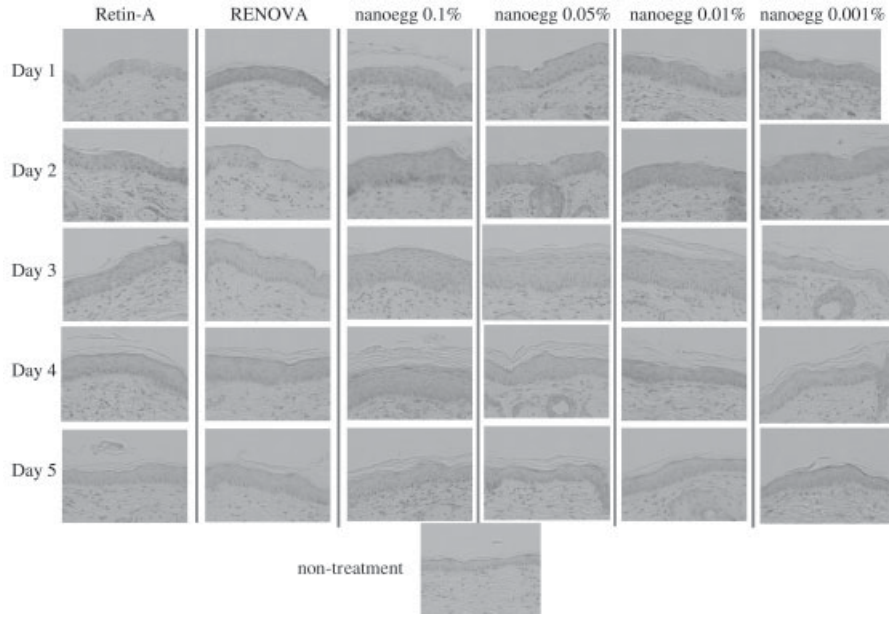


Fig. 4:
Histological evaluations of commercial products and nanoegg™ 0.1%, 0.05%, 0.01% and 0.001% at each day after topical administration until 5-days. All tissues were stained colloidal iron stain method to detect hyaluronan. Blue domain corresponds to the presence of hyaluronan, red domain nuclear in cells. The external treatments were carried out once and the tissues were sacrificed each day until 5-days. 30 mg of compounds was administered to 1 $cm^2$ of dorsal area of hairless mice(HR-1, male, 5-week) respectively

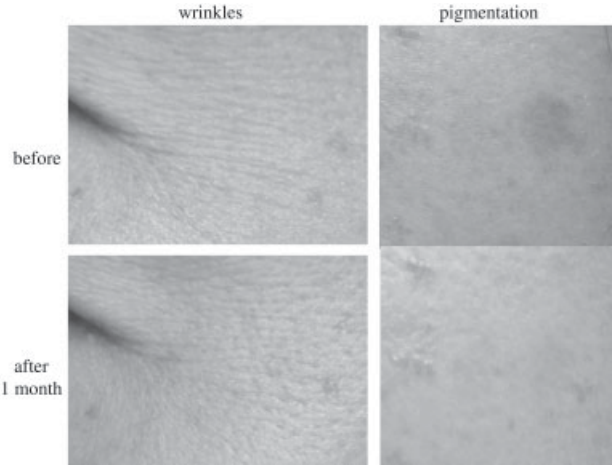


Fig. 5: Pictures of wrinkle and photoaged hyperpigmentation on human faces (female, 48 years old). Before treatment of nanoegg™ 0.05%, and after treatment for one month. The external daily administration was carried out one time at night, contineously

30 brown spots induced by aging and/or UV irradiation. The brown spots were noticeably decreased (Fig. 5). Further, coarse wrinkling at the side of the eye was consistently reduced after nanoegg™ 0.05% treatment without strong irritation and inflammation (Fig. 5). This would certainly be due to the abundant hyaluronan accumulation in the epidermal layer at an early stage. It is usually found that commercial ointment treatment cannot achieve a decrease of hyperpigmentation in only one month, while the nanoegg™ system could readily reduce it without serious irritation, as is obvious in Fig. 5. In fact, after treatment with nanoegg™ for several days, mild inflammation occurred in several subjects. Compared with those of commercial products, these levels were quite mild. Accordingly, we believe that the risks due to continuous treatment with atRA therapy, such as erythema and irritation, can be avoided completely, because the same effect would be obtained by short-term treatment with the nanoegg™ system. At the same time, the water content of the stratum corneum also showed a statistically significant increase with one month's treatment with nanoegg™ (Fig. 6).

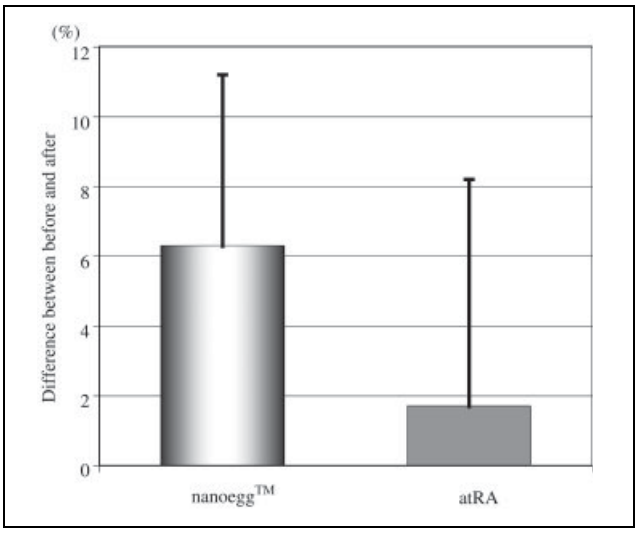


Fig. 6: Change of water content of stratum corneum of human skin. Measurements carried out in people over 40 years old before and after treatment with nanoegg™ and atRA

Up to now, in practice clinical responses have predominantly comprised severe irritant dermatitis in the early stages of application (around 3 or 4 days) of atRA ointments. This problem is generally managed by modifications to dosage and schedule. We describe here the development of a novel DDS using nanotechnology. The conceptual framework of this study is that the nanoegg™ system plays a crucial role in solving the poor lability of atRA and its strong symptoms of irritation. Surprisingly, the efficacy of nanoegg™ suggested not only improved lability and irritation but also a simultaneous enhanced turnover of epidermis. Drastically increased epidermal thickness could be expected to result in a relative decrease in melanin content, and excess expression of HA aids induction of hyperplasia, resulting in less wrinkled skin. This study clearly shows that our newly developed novel DDS technology for nanoegg™ provides great potential for skin regeneration.

## 3. Experimetal

### *3.1. Materials*

All-trans retinoic acid (tretinoin, atRA) was obtained commercially from Wako Pure Chemical Industries Ltd. Polyoxyethylene (20) sorbitan monooleate (Tween 80), sodium hydroxide, ethanol, calcium chloride dehydrate and sodium carbonate were also purchased from Wako Pure Chemical Industries Ltd. All chemical materials were used with no further purification and were of reagent grades.

### *3.2. Nanoegg™ preparation*

The nanoegg™ was prepared using boundary-organized nano-scale reaction droplets. The detailed preparation method is given in the literature (Yamaguchi Y et al. 2005). The samples obtained were stored at 4 °C until use.

### *3.3. Measurement of mRNA HB-EGF in mouse skin tissue*

To detect mRNA HB-EGF, a real-time PCR system was utilized. The samples used for extraction of RNA were hairless mouse (5-week, male) skin tissue in liquid nitrogen. After grinding the mouse skin well, RNA was extracted by the Isogen method. The measurements of mRNA HB-EGF for each sample were carried out using a LightCycler® 2.0 Instrument (Roche Diagnostics GmbH, Germany) with six detection channels and 20 μL capillaries. LightCycler Software 4.0 was installed as a quantitative method to automate grouping, melting curves and analysis.

Biopsy specimens were obtained after the treatment with each material of the dorsal area of hairless mice (HR-1, male, 5-week). All biopsy specimens were taken from the same area in all subjects, i.e., the lateral region on the back of the hairless mouse, to ensure uniformity of sample sites and collection of specimens from the external adhesion area. All biopsy specimens included both epidermal and dermal tissue. All formalin-fixed specimens of dorsal area of hairless mouse (5-week, male) were stained by colloidal iron for the detection of hyarulonan (HA). After the samples were dehydrated, 1-μm sections were cut from the paraffin-embedded specimens, and stained by a suitable method.

### *3.4. Nanoegg™ and USA commercial product treatments of normal mouse and porcine skin*

The nanoegg™ particles, single atRA as a control, and Retin-A™ and Renova™, the commercial products in the USA, were applied externally to dorsal areas ($1.0 \times 1.0$ cm$^2$) of hairless mouse (HR-1, male, 5-week) and porcine (pot bellied, 3 months, male) skin. Each ointment was applied once, and each day tissues were sacrificed until 5-days. The irritation level was observed visually, and recorded photographically.

### *3.5. Clinical trial of nanoegg™ on human skin*

The institutional review board of St. Marianna University permitted the clinical trial of nanoegg™ ointment on human faces of subjects over 40 years old. The trial was carried out for one month on 30 wrinkles and 30 brown spots induced by aging and/or UV irradiation. Each wrinkle and brown spot was selected as one case on one-face, respectively. The concentration of atRA was fixed as 0.05%, and white vaseline was used the ointment base. The treatment on each wrinkle and brown spot was carried out one before sleeping each day. The water content of the stratum corneum was measured by a method using conductivity change to automatically estimate the amount of water. Moisture measurement in skin used an instrument produced Scalar Corporation and made in Japan.

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