

CELLULAR RESPONSES TO EGG-OIL (CHARISMON®)

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Summary: Egg-oil (Charismon®) is known for its beneficial action in wound healing and other skin irritancies and its antibacterial activity. The physiological basis for these actions has been investigated using cells in culture: HaCaT-cells (immortalized human keratinocytes), human endothelial cells in culture (HUVEC), peripheral blood mononuclear lymphocytes (PBML) and a full thickness human skin model (FTSM). Emphasis was on the influence of egg-oil on cell migration and IL-8 production in HaCaT cells, respiration, mitochondrial membrane potential, reactive oxygen (ROS) production and proliferation in HUVEC and HaCaT cells, cytokine and interleukin production in PBML and UV-light induced damage of FTSM. IL-8 production by HaCaT cells is stimulated by egg-oil whilst in phythemagglutinin-activated PBMLs production of the interleukins IL-2, IL-6, IL-10 and IFN- γ and TFN- α is reduced. ROS-production after H₂O₂ stimulation first is enhanced but later on reduced. Respiration becomes activated due to partial uncoupling of the mitochondrial respiratory chain and proliferation of HaCaT and HUVEC is reduced. Recovery of human epidermis cells in FTSM after UV-irradiation is strongly supported by egg-oil. These results support the view that egg-oil acts through reduction of inflammatory processes and ROS production. Both these processes are equally important in cellular aging as in healing of chronic wounds.

Key words: Charismon®; Inflammation; Wound healing; Respiration; Reactive oxygen species; Interleukins; PBML; HaCaT; HUVEC; Human skin model

Introduction

Egg-oil (Charismon®) is a spagyric preparation of egg-oil from chicken egg yolk (1) with a broad action spectrum in patients. Egg-oil contents are listed in table 1. It has been reported to prevent atherosclerosis by improving membrane elasticity (2), relief of gum inflammation and bleeding when applied by using Egg-oil-containing tooth paste, Egg-oil containing ointment improves burn wound healing as well as healing of ulcers with low scar formation only (3). It acts against acne and Herpes zoster, and it exerts antimicrobial (against *Staphylococcus* and *E. coli*) and anti-inflammatory actions (1, 2, 4).

Tab. 1: Chemical composition of egg-oil. Mass spectrometric determination of the contents of egg-oil (Chemisches Untersuchungs-Labor Dr. Lörcher, Heilbronn, Germany). Compositions of different batches are almost identical.

water content	0.09%
total fat	84.7%
triglycerides 56.1%	
phospholipids 36.8%	
partial glycerides 6.7%	
free fatty acids	0.7%

partial glycerides	6.7%
sterols	6.0%
cholesterol (enzymatic determination) 2,2%	
carbohydrates	< 0.5%
fatty acids (percentage distribution)	
C12:0 laurinic acid methylester	0.02%
C14:0 myristinic acid methylester	0.61%
C16:0 palmitinic acid methylester	26.4%
C18:0 stearinic acid methylester	6.18%
C18:1 oleic acid methylester	47.5%
C18:2 linolic acid methylester	18.5%
C18:3 linoleic acid methylester	0.73%
C20:0 arachidonic acid methylester	0.03%
C22:0 behenic acid methylester	0.04%
phosphatidylamine	detectable
retinoic acid derivatives	detectable
tocopherol	11.1 mg/kg
β , γ -tocopherol	31.3 mg/kg
other carotinoids	33.8 mg/kg

Interpretation of this broad spectrum of action is very difficult for two reasons, first egg-oil as a product of biological origin is not a single substance rather a mixture of a variety of fatty acids and retinoids. Strict scientific explanation of the mechanism of action may be impossible for a naturopathic drug of complex composition which is supposed to act only in concert of its constituents. Second, the processes which have been reported to be affected by this compound preparation themselves result from complex interactions of a large number of processes and their regulation. Nevertheless an objective proof of action, not influenced by subjective feelings, is required to establish a preparation as a medical drug, or even of cosmetic value or a valuable food supplement.

Therefore we studied the effects of egg-oil on a variety of physiological reactions in cells in culture. While being aware of the principle limitations cell cultures impose on the deduction of modes of action in the whole human body, the relative clearness and reproducibility of results and the simplicity in handling justifies this reductive approach. Cell cultures can be used as model systems for a large variety of physiological activities if the model character of this approach is taken into account appropriately (5). Following the clinical findings listed above, key events influenced by egg-oil are wound-healing and inflammation. In particular inflammation shows a strong relationship to aging, which may be considered to represent a pro-inflammatory stage (i.e. arthrosis and rheumatic diseases, atherosclerotic reactions). Therefore some significant events important for wound-healing and aging have been investigated in cells in culture: In healing wounds epithelia have to cope with dead cell material (debris) in their environment, with infectious bacteria, and the epithelial cells migrate towards the wound centre during primary wound closure. Infections, probably in concert with the immune reaction against debris may evoke the production of inflammatory cytokines and production of reactive oxygen species (ROS). Increase of ROS also occurs during aging of tissues (6, 7). All these events which represent extraordinary situations within a cell's life will require increased energy metabolism which has been monitored by measuring mitochondrial membrane potential.

The next level of complexity is reached using organ reconstructions in vitro. However, such cultures mimicking the architecture and differentiation of the organ in situ still can be handled reproducibly and analysed as it is possible for cell cultures. Therefore also this type of cultures was included in our study investigating stress response modulation by egg-oil.

Results and Discussion

One of the outstanding clinical achievements of egg-oil is its support in healing chronic wounds when applied as an ointment preparation (3). Model investigations using cells

in culture require the experimental separation of different processes involved in wound healing, but keeping in mind their mutual interaction. We treat this problem by investigating the influences of egg-oil on epithelial cell migration (involved in primary wound closure), inflammatory responses based on lymphocyte activation and resulting in release of inflammatory cytokines from lymphocytes as well as from epithelial (epidermal) cells because both these cell types interact in a complex network (8).

1. Primary wound closure by cell migration and proliferation

For these experiments HaCaT cells (9) were used, a line of human epidermis cells which maintained the ability for differentiation. They were seeded in multiwell plates and cultured in carbonate buffered Hank's medium with 5% fetal calf serum and 1% penicillin-streptomycin at 37 °C in a 5% CO₂ atmosphere. Each single field of cells was separated into two areas by a Teflon block which prevented adhesion of cells to the area covered by the block. 3 days after cell seeding the Teflon blocks were removed and the coverage of the cell free areas by immigrating cells was measured for 4d under control conditions and with egg-oil suspended in the culture medium by sonication at concentrations of 1 : 1000, 1 : 2000, 1 : 5000 and 1 : 10,000. Only after 24 h did the egg-oil treated cultures close the gap a bit faster than controls. After 2d and more no improvement of gap closure occurred.

Proliferation of cell types relevant for wound healing (human umbilical vein endothelial cells (HUVEC) and HaCaT) is reduced in presence of egg-oil which was dissolved in the culture medium by sonication. HUVEC cultures reach much faster the post-proliferative phase (table 2), corresponding to premature senescence as is revealed by concomitant increase of the senescence marker β -galactosidase activity and by apoptotic death of the cultures. On the other hand, proliferation of fibroblasts was not influenced by egg-oil. Because gap or wound coverage result from cell migration and proliferation, acceleration of early gap closure by stimulated migration may be followed by retardation due to the reduction of proliferation.

Tab. 2: Population doublings (PDL) and time required for HUVEC to reach post-proliferative state under control conditions and in presence of different concentrations of egg-oil (dissolved by sonification in culture medium; 3 parallel cultures for each concentration).

	PDL	days
control	22	60
0.067‰	12	50
0.1‰	8	35
0.2‰	5	21

2. Inflammatory responses

2.1 Interleukin production by human epidermal cells (HaCaT)

Interleukins are small molecules which control cell proliferation, cell death, antibody production and gene expression (10). They act by binding to specific membrane receptors either on the cell type by which they are produced or on other cell types. One of the main fields of interleukin action is the regulation of cellular immunity. White blood cells are the main source of cytokines, but other cells like the epidermal cells (HaCaT) used in this study, will also produce interleukins and by this modulate differentiation and immune responses of the different types of white blood cells.

HaCaT cells can be stimulated to differentiate by increasing the Ca^{2+} -concentration in the culture medium. This process goes with some increase in IL-8 production by the cells as was shown by IL-8-ELISA (table 3). IL-8 production can be suppressed with the anti-inflammatory glucocorticoid beta-methasone-17-valerat [20 μM ; BM-17-V]. In undifferentiated HaCaT cultures in Hanks medium overnight exposure to egg-oil significantly stimulates IL-8 production in a concentration dependent manner. In differentiating cells (high Ca^{2+} -culture medium) the concentration dependence is lost. In Hepes buffered medium almost no IL-8 production occurs either in the absence or presence of egg-oil (table 3). The reasons for Hepes-dependence are unknown, but CO_2 buffering corresponds better to the in situ situation.

Tab. 3: IL-8 production by HaCaT-cells. Egg-oil significantly enhances IL-8 production in HaCaT-cell cultures. Differentiating HaCaT (in high Ca -medium) produce more IL-8 under control conditions than those in the proliferative state. Replacing CO_2 buffering by HEPES abolishes IL-8 production and its inducibility by egg-oil. The corticosteroid BM-17-V was used as a control to demonstrate IL-8 inhibition by this general anti-inflammatory drug. Egg-oil was dissolved by sonication ($n = 4$).

condition	IL-8 concentration in medium (pg/mL)
proliferating HaCaT cells	
control	237 \pm 9
BM-17-V	133 \pm 21
egg-oil 1 : 5000	363 \pm 19
egg-oil 1 : 2000	443 \pm 31
egg-oil 1 : 1000	499 \pm 47
differentiating HaCaT cells	
control	333 \pm 13
BM-17-V	7 \pm 4
egg-oil 1 : 5000	553 \pm 30
egg-oil 1 : 2000	437 \pm 43
egg-oil 1 : 1000	507 \pm 49

HaCaT cells in Hepes medium	
control	37 \pm 12
BM-17-V	37 \pm 6
egg-oil 1 : 5000	37 \pm 3
egg-oil 1 : 2000	50 \pm 4
egg-oil 1 : 1000	50 \pm 7

IL-8 stimulates degradation of extracellular matrix material by lymphocytes and thus supports remodelling of extracellular matrix in the wound area and reduces the resistance of ECM against migrating cells (increasing migration).

2.2 Influence on cytokine production by PBMLs

A main factor preventing healing in chronic wounds is inflammation. Therefore the influence of egg-oil (dissolved in ethanol; stock solution: 300 μL ethanol plus 240 μL egg-oil) on inflammatory cytokine release has been investigated. For this purpose peripheral blood mononuclear lymphocytes (PBML) from 3 healthy volunteers have been isolated via gradient centrifugation, seeded in AIM V-medium (Gibco) and stimulated using phythemagglutinin (PHA; 1 $\mu\text{g}/\text{mL}$). After 48h of stimulation, the amount of cytokines released into the medium was determined with ELISA.

Egg-oil itself does not stimulate cytokine production (IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF- α), but PHA-stimulated release of the following cytokines becomes reduced depending on the dilutions of the egg-oil stock solution (Fig. 1):

- IFN- γ (40% at 1 : 2500; no effect \leq 1 : 10,000)
- IL-2 (40% at 1 : 2500; no effect \leq 1 : 10,000)
- IL-10 (70% at 1 : 2500; no effect \leq 1 : 10,000)
- IL-6 (90% at 1 : 2500; no effect \leq 1 : 15,000)
- TNF- α (50% at 1 : 2500; no effect \leq 1 : 7500)

Because PHA does not evoke an IL-4 response, the action of egg-oil on this cytokine could not be determined.

Cytokines exert their action via binding to receptors on the plasma membrane. Therefore the influence on the

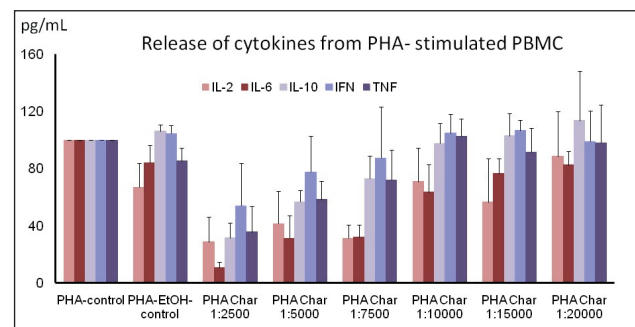


Fig. 1: Influence of egg-oil on interleukin secretion by PHA-stimulated PBML. Interleukins were determined 48h after stimulation with 1 $\mu\text{g}/\text{mL}$ PHA.

presence of the surface receptors CD25, CD54 and CD69 in PHA-stimulated and unstimulated T-lymphocytes was measured using flow cytometry of staining with the corresponding fluorescent antibodies. Only the expression of CD25 is reduced by egg-oil in a concentration dependent manner in stimulated T-cells as well as B-cells.

2.3 Egg-oil-evoked cell death

Observations of cells losing adherence from the cover glass at higher concentrations of egg-oil indicated the need to study noxious effects on cells. Two approaches have been used, the release of LDH and staining of cells with propidium iodide. Both these methods reveal permeabilization of the plasma membrane. However, the LDH release is a graded process whilst propidium iodide uptake is an all or non-event for the decision whether a cell is dead or alive. LDH was determined using the ELISA cytotoxicity test of Boehringer (no. 16447930001) according to the manufacturer's instruction. LDH activity in a supernatant of cells treated with 1% Triton-X-100 served as a marker for maximum release. At the highest egg-oil concentrations (1 : 1250 and 1 : 2500) full LDH release takes place from PBMLs within 24h of action. Lower concentrations increase LDH release from 40–50% (in controls without egg-oil) to about 60% total LDH in PHA-stimulated as well as unstimulated PBML. Propidium iodide uptake remains unaffected by egg-oil in unstimulated PBML, whilst PHA stimulation renders the cells sensitive (base value: 10–12% propidium iodide – positive cells) with an increase by approximately 5% more dead cells (15–17% propidium iodide-positive cells) in presence of egg-oil, independent of its concentration.

This is not a strong effect, but in concert with the suppression of inflammatory cytokine release and reduction of the IL-2 receptor CD25, egg-oil may reduce inflammation considerably without abolishing these reactions.

3. Egg-oil modulated photosensitivity

Sunburn is a special case of skin inflammation. Sunburn not only causes DNA damage by direct interaction between light and DNA but also cell death via necrosis and apoptosis. In addition to DNA strand breaks (which may be repaired), the main chemical reason for cell death is a burst of free reactive oxygen species (ROS) production. This process has been considered to represent a factor also involved in causing aging (11; for critical evaluation of this hypothesis of aging see (7)). We aimed to use artificial sunburn (photo-toxicity of UVB) as a model for inflammation to investigate whether the inflammation suppressive action of egg-oil can be established for this situation as well. Because of the low concentration applied and the spectral properties of egg-oil a protective role against sunburn cannot depend on light absorption.

HaCaT cells proved to be relatively insensitive against irradiation and a single cell type in culture may be too far

away from the events in real skin. Therefore we have chosen a full thickness skin model (FTSM) (12, 13). This *in vitro* model mimics the *in situ* situation by several important parameters: The epidermis is stratified with a cornified layer (Fig. 2) on top and a reproductive layer (stratum germinativum) at its basis. The epidermis sits on a loose connective tissue with a few fibroblasts only which produce an irregular network of collagen fibrils embedded within an extracellular matrix. These two layers stabilize each other's differentiation. Histologically cross sections through these preparations closely correspond to living skin, although without vascularization. These full skin models are cultured as circular discs with a diameter of about 25 mm. To induce a situation mimicking sunburn these pieces have been exposed to a UVB lamp until they received 150 or 250 mJ/cm² of UVB. The preparation starts with a punch specimen of human skin from healthy volunteers. Epidermis and dermal parts were separated and the dermal fibroblasts seeded into a collagenous matrix which becomes replaced by ECM material produced by the fibroblasts themselves. After several days the epidermis cells which first have been propagated in culture flasks are seeded on top of the ECM layer with the fibroblasts which facilitates the formation of a basal lamina. After 8 d in culture the cultures were elevated to the air liquid interface to allow for stratification and differentiation of the epidermis equivalent. The emission of the UV 3003K illumination cabin (Waldmann, Villingen-Schwenningen, Germany) is in the range between 285 nm to 350 nm with a maximum between 310 and 315 nm. During irradiation the culture medium was replaced by phosphate buffered saline to avoid formation of phototoxic substances. Immediately after irradiation fresh medium was added together with the test substances ("Crème du ciel" which is an ointment containing 4.5% egg-oil, ointment without egg-oil and Ecural®) pipetted on top of the FTSM, each condition on 3 different samples. Ecural was used as a positive control. It is a corticosteroid with well established anti-inflammatory properties. After further 24h the medium was collected, centrifuged at 1200× g at 4° for 10 min and stored at –80 °C until further processing to determine the inflammatory interleukins IL-6 and IL-8 content. These were determined with the Elecsys 2010 kit from Roche Pharmaceuticals (Grenzach-Wyhlen, Germany).

For histological examination the FTSM were kept alive for further 5 days. Histological examination was performed on samples fixed in 4.5% formalin on day 1 or day 6 of the experiments. Staining was done using hematoxylin/eosin.

IL-6 as well as IL-8 content of the culture medium of irradiated samples were up to 8× that of non-irradiated. No significant difference was found between controls and egg-oil treated FTSMs, only Ecural reduced IL-6 secretion significantly (by 26%). 150 mJ/cm² irradiation were as efficient as 250 mJ/cm² irradiation in raising interleukin production and release.

Day 1 after irradiation the histological image in all conditions except those treated with egg-oil showed many

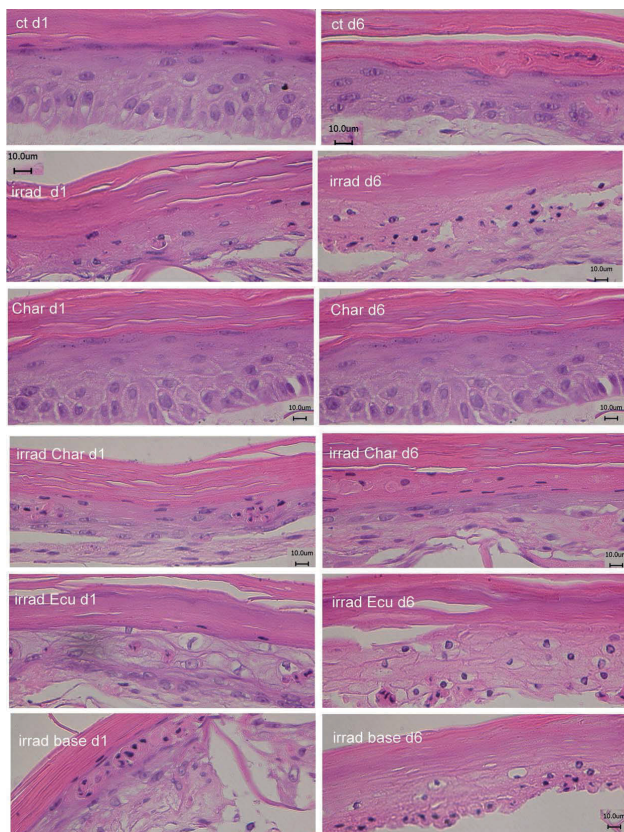


Fig. 2: Histological sections of FTSM (human skin models) stained with hematoxylin-eosin after fixation with 4.5% formalin. Conditions: Irradiated samples (irrad) have been exposed to 150 mJ/cm² UVB and were fixed either 24h or 6d after exposure to light. Controls (ct) were either non-irradiated or irradiated. Char marks specimen treated with egg-oil (15 μ L “crème du ciel” on top of the FTSM), Ecu designates Ecural[®] (15 μ L) treated samples either after irradiation or without, again 24h or 6d after onset of the experiments. For further control purposes FTSM were treated with crème du ciel without egg-oil, thus the base of the ointment. In ctd1, ct d6, Char d1 and Char d6 healthy and well developed stratified epidermis, covered by a thick layer of cornified tissue can be recognized. The images at “ct d 6” and “base 1d” also show some of the underlying dermal layer which normally is present but not shown. 24h and 6d after irradiation epidermis cells in controls and those treated with ointment base have shrunk, dense nuclei indicating a pycnotic state of nuclei typical for cell death. Egg-oil treatment (irrad Char) ameliorates epidermal appearance after 24h as can be seen by the many nuclei which are a bit shrunk but not as dense as those of control or treated with base. 6 days after irradiation egg-oil treated FTSM had almost normal epidermis, previously damaged cells with very flat and dense nuclei are found within the proximal stratum corneum. Ecural[®] induces extensive cell swelling when irradiated and widespread cell death (pycnotic nuclei) 6d after irradiation. Thus egg-oil strongly promotes recovery of epidermis from irradiation. This is neither achieved by ointment without egg-oil nor by Ecural[®].

epidermal cells with small dense nuclei (pycnotic nuclei) or bullous cells indicating “sunburn damages”. Epidermis of egg-oil treated FTSM also appeared injured but less pycnotic nuclei were found (Fig. 2: irrad Char 1d). At day 6 under control conditions (nothing added or only the ointment base of crème du ciel) exhibited extensive cell death, in the intermediary cell layers dominated by pycnotic nuclei (Fig. 2), Ecural treated samples showed reduced stratum corneum, sometimes containing cells with nuclei (which indicate insufficient differentiation) and many bullous epidermal cells. Those FTSMs treated with egg-oil (the full crème du ciel preparation) looked almost normal, most epidermal cells in the basal layers had large normal nuclei, flat compact nuclei were found in the intermediate layers undergoing differentiation. Thus at this condition a strong protective action of egg-oil became evident (Fig. 2).

Summing up, although the inflammatory response as indicated by interleukin release and the immediate cellular reaction was not altered by egg-oil a strong protective action became evident after prolonged exposure.

4. Cellular processes influenced by egg-oil.

Protection of cells against the deleterious effects of irradiation could either result from direct photoprotection or may have physiological reasons connected to the mechanism of action of light on cells. A main source for phototoxicity is the production of ROS due to interaction of light with cytochromes. Insofar light induces aging in a mode comparable to that by mitochondria-based ROS production. Therefore we investigated the influence of egg-oil on ROS production and scavenging in the human epidermis-derived HaCaT cells. If these cells are exposed for a short time to elevated concentrations of hydrogen peroxide, elevated ROS concentrations can be measured for up to two days. This corresponds to the behaviour of other cell types which change mitochondrial dynamics and undergo increased apoptosis on short time H₂O₂ exposure (14).

HaCaT cultures were treated for 10 min with 0.02% H₂O₂ (a concentration which may occur temporarily on strong physical exercise), then the medium was replaced by fresh culture medium either without or with egg-oil dissolved by sonication to different concentrations. The determination of ROS was achieved by staining cells with dihydro-rhodamine 123 (DHR; Molecular Probes; final concentration 5 μ M) for 30 min. This test is based on the principle that non-fluorescent DHR (dihydrorhodamine 123) can be oxidized by hydrogen peroxide to rhodamine 123, forming a green fluorescent compound, which can be quantified by fluorometry: Four slides were used per condition and six frames were measured per slide. Pictures of the fluorescent samples were taken with constant microscopic settings and the micrographs were analysed using the program ImageJ, to evaluate the integrated fluorescence corrected for cell density. The result is shown in Fig. 3.

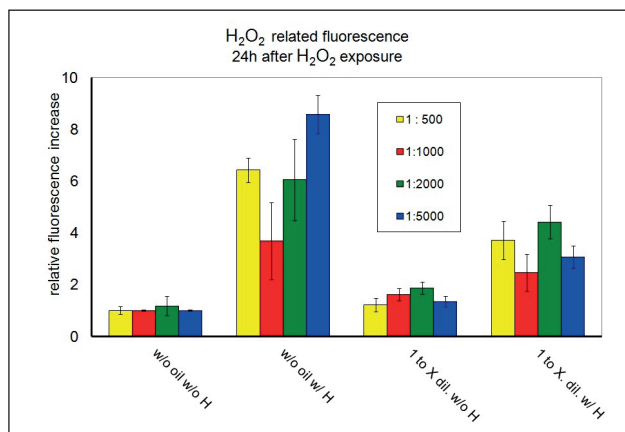


Fig. 3: ROS content of HaCaT cultures 24h after 10 min exposure to 0.02% H_2O_2 . ROS was monitored using rhodamine 123 fluorescence. Cultures were exposed to one of the 4 indicated concentrations of egg-oil (1 to X dil.) and either pretreated with H_2O_2 (w/ H) or without (w/o H). The corresponding controls were either untreated (w/o oil w/o H) or treated with H_2O_2 but without egg-oil (w/o oil w/H). The cultures without egg-oil exhibit the highest ROS amount, but also egg-oil itself slightly enhances ROS production of the cells (1 to X dil. w/o H) although egg-oil reduces ROS production in H_2O_2 treated cultures (1 to X dil. w/ H) at all the applied concentrations. For each egg-oil concentration a separate trait of experiments has been performed therefore all conditions are marked with the respective colour, also for those conditions which have not been exposed to egg-oil.

In all cases 24h after short time treatment with H_2O_2 ROS levels are significantly higher than in controls. At all concentrations used for egg-oil – treatment relative ROS levels are lower than without egg-oil, although egg-oil itself slightly increases ROS levels in cell cultures. Short term experiments investigating ROS production 1h after H_2O_2 exposure revealed a strong but transient increase of ROS production by egg-oil treatment in HaCaT cells but not in HUVEC (15). Thus, the egg-oil action is cell type and time dependent, first elevating ROS production and then reducing it. This corresponds to the well-known phenomenon of hormesis which has also been described for metabolism generated ROS production (16, 17, 18). Increase of respiration and ROS production by free fatty acids is a well known phenomenon (19–21). This may become counteracted by the slight increase in superoxide dismutase 1 (SOD1) (15). An increase of oxygen consumption after 3–6h egg-oil treatment is a further reaction of cells to this preparation. The increased respiration goes in parallel with a reduction of mitochondrial membrane potential and thus results from partial uncoupling of the respiratory chain (15).

Conclusions

The typical difficulties in evaluation of the action of natural mixtures by means of established scientific methods become obvious also in the present study. These include a

broad range of reactions evoked on the cellular level, but these reactions may be relatively weak with limited concentration dependence. That some of the reactions cannot be clearly related to the concentrations applied opens the possibility for criticism. However, this type of criticism may prevent an adequate understanding of the synergisms relying in a complex medication. Weak responses have the big advantage to cause fewer side effects than strong inhibitors of biochemical reactions. The full spectrum of such weak reactions finally may produce very beneficial results. The interpretation of experimental results achieved in cell cultures has to consider the strict reduction of possibilities of the experimental system to respond, and the concentrations used in cell cultures cannot be related directly to the concentrations which are present e.g. in an ointment. The egg-oil containing ointment is called “crème du ciel” and contains 4.5% of egg-oil. Such a high concentration of this extremely hydrophobic preparation cannot be reached in aqueous culture medium and it is not known how much of the substance finally diffuses into skin covered with crème du ciel.

Because the main clinical effect of egg-oil found, was promotion of wound healing, of chronic wounds in particular, interpretation of our results obtained with cell cultures will follow the explanation of this extremely complex process. Skin wound healing involves keratinized epithelia, dermal fibroblasts and extracellular matrix, targeting and action of a variety of lymphocytes and monocytes and vascular endothelia. The physiological connection between all these players is provided by chemokines (i.e. interleukins, $TNF-\alpha$, $TGF-\beta$ and many others). Amelioration of some key events by egg-oil can be derived from our experiments.

Closing wounds by re-epithelialization is based on epithelial migration, followed by cell proliferation and differentiation. In our experiments only transient stimulation of migration was observed by egg-oil, but in healing tissue hypoxia is present which may activate this migration (22) and increased oxygen consumption due to partial uncoupling may further support this effect.

More profound alterations are induced in interleukin release. IL-8 release from keratinocytes (HaCaT cells) was the only interleukin which was strongly stimulated by egg-oil. All the interleukins released from stimulated lymphocytes (PBML) became reduced by egg-oil up to 70%. Egg-oil induced IL-8 release is larger in undifferentiated keratocytes corresponding to basal epidermal strata, than in those starting differentiation (table 3). By this IL-8 reaches peak concentrations directly beyond the epidermis recruiting neutrophils, lymphocytes and macrophages which then become stimulated by $TNF-\alpha$ and IL-1 to produce IL-8 by themselves (23). IL-8 stimulates epithelial cell migration and proliferation (24, 25). Thus interleukins in wound healing are involved not only in inflammatory responses but also in regulation of cell growth and migration. Strong increase in release of these interleukins initiates inflammation and is evoked by bacterial toxins (e.g. lipopolysaccharides) via complex signaling pathways (26). Thus upregulation of ep-

ithelial cell based IL-8 production certainly adds to wound closure and attracts neutrophils and macrophages. But it is the balance of factors and their sequence of action which determines whether wound healing is fast or slowed down, because IL-8 also inhibits epithelial cell proliferation and activates inflammation (27). During the early phase of wound healing leukocyte attraction is essential for debridation and fighting bacterial infections, while prolonged inflammation favors the development of chronic wounds. Therefore egg-oil – based reduction of interleukins produced by activated leukocytes promotes wound healing by acting against too extensive inflammatory responses.

Lymphedema is a frequent situation in chronic wounds preventing wound closure, which could be improved by down-regulation of IL-10 (28), a process also supported by egg-oil (Fig. 1).

ROS, including free radicals have been implicated in several pathogenic events including impaired wound healing due to the release of free radicals from neutrophils trapped within the wound area (29). Therapeutic intervention with hyperbaric oxygen additionally increases ROS levels and impairs wound healing. This negative effect can be abolished by α -lipoic acid mediated ROS scavenging (30). Chronic wound fluid was reported to contain more antioxidants than acute wounds, indicating an adaptive response (31), similar to the hormetic response found for egg-oil action which may potentiate this physiological reaction.

The next topic we have to address is aging: Increased age is a major risk factor for impaired wound healing. It may be responsible for delayed wound healing and altered inflammatory responses including reduced macrophage phagocytic activity (32). Interestingly physical exercise which is well established by its anti-ageing power also improves wound healing due to an anti-inflammatory response in the wound (cited from Guo and DiPietro, 2010). In our “sunburn model” using FTSM the early events were not altered by egg-oil, also interleukins seemed to remain unaffected, however the later phases of damage were considerably reduced yielding almost healthy epidermis after 6d, whilst under control conditions severe cellular damage was seen. This process cannot be explained by increased apoptosis and regeneration, because the FTSM are devoid of macrophages which would be needed for debridation from the dead cells. An alternative mechanism may be activation of autophagy of dysfunctional cellular components, but this possibility we did not investigate. Indeed, the general feature of concentration dependent reduction of PBML derived interleukins by egg-oil could exert an anti-aging effect, because it reduces the pro-inflammatory state without interfering with infection defense (because the inhibitory action does not exceed about 65% with the exception of IL-6 which might be reduced to 10% (Fig. 1)). Promotion of a post-proliferative status in HUVEC together with the appearance of markers for senescence are not in favor of maintaining tissues for a prolonged time in juvenile state, but IL-8 stimulation might overcome this shortage and accelerate cellular turnover in

situ. This is suggested by the protection of FTSM against irradiation damage and the reduction of ROS after ROS induction with H_2O_2 .

The complex constitution of egg-oil will be responsible of several and sometimes opposing effects. This finally corresponds to the complexity of reactions within the human body, i.e. during healing of chronic wounds or while ageing. Compound drugs as well as human physiology have to be considered in a holistic way.

Acknowledgements and Comments

Continuous support of these studies by Phameres e.V. (Pharmaceutical Medical Research Society e.V.) (Wiesloch, Germany) is gratefully acknowledged. Charismon® was a generous gift from Dr. W. Nawrocki (Frankfurt am Main). None of the authors has any conflict of interest regarding the Charismon® product. The interests in this product are purely scientific reasons to investigate its action.

The experiments using cell cultures are not an issue for ethical commissions, however for the skin models the ethics commission of the Medical Faculty of the Goethe University accepted “The usage of tissue routinely derived from operation material for the cultivation of skin and tumor cells” (report 12/97) and “Validation and testing autologous 3-dimensional skin equivalents for covering severe burn wounds and for comparative studies” (report 112/06).

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Received: 24/10/2013

Accepted in revised form: 24/04/2014

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