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Comparative Evaluation of Two Different Novel Formulations of Quercetin Against Non Melanoma Skin Cancer in Human Subjects

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Abstract

Nonmelanoma skin cancer (NMSC) is the steadily increasing cancer in the India. Various studies have put forward strong evidences of NMSC incidence due to UV radiation exposure. Keratinocyte macrophage cells express macrophage which represents both type of receptor viz. folate binding receptor having affinity for folic acid and mannose-binding receptor which are having affinity towards mannose. This study was designed to identify the correlation parameter in evaluation of *in vivo* doses and epidermal changes following UVB irradiation using noninvasive techniques. We in our study have developed two different formulations embedded with endogenous ligands conjugated nanoparticles of same drug (quercetin) and have analyzed its effect on human volunteers against the release of MIF and MED analysis for which prior permission from Human Ethical Committee has been obtained accordingly. The result of the study showed that skin biopsies sections of human subjects showed that there was a dose dependent effect on human skin in response to the UV radiations. The pattern was nearly same for NL-4C and NL-5C for MED analysis. While for MIF release study, for first 12 hours the MIF level ranged from 12.34 ± 9.90 ng/ml to 79.4 ± 23.65 ng/ml which was the result of acute exposure of UV radiations. For next 24-48 hours, the MIF levels was found to be increased from 22.43 ± 12.56 ng/ml to 102.34 ± 20.4 ng/ml which shows that the prepared formulations were significant in inhibiting the release of MIF.

Keywords: Macrophage; MED; MIF; Ultraviolet radiations; Nonmelanoma skin cancer (NMSC)

Introduction

Nonmelanoma skin cancer (NMSC) is the steadily increasing cancer in the India. Various studies have put forward strong evidences of NMSC incidence due to UV radiation exposure. The UV radiations are well absorbed by the DNA and cell proteins and act as initiator as well as promoter in the formation of mutagenic photoproducts inside DNA. These photoproducts are formed between the adjacent thymine (T) and cytosine (C) base pairs and between the pyrimidine base pairs. These dimmers formed are known as cyclobutane dimmers and the pyrimidone (6-4) dimmers respectively. The 6-4 photoproducts are less mutagenic than CPD which is also termed as "hot spot mutation". In addition, they also interfere with the immune system of the body, cause immmosuppression and activate those genes which are directly responsible for causing mutation in DNA [1-3].

Moreover, keratinocyte macrophage cells express macrophage which represents both type of receptor viz. folate binding receptor having affinity for folic acid mannose-binding receptor which is having affinity towards mannose. These receptor is unitly termed as mannose receptor (MR, CD206 or MRC1) which is a transmembrane glycoprotein belonging to the C-type lectin family and is expressed largely by various tissue macrophages, dendritic cells (DCs) and selected lymphatic or liver endothelial cells [4-6]. Due to predominant expression on macrophages these receptors are also well explored for treating skin infectious diseases.

Macrophage migration inhibitory factor (MIF) is a cytokine which remains in association with numerous cancer cells at all stages [7]. MIF is a monitor to macrophages which regulates various activities of macrophages like phagocytosis, adherence, spreading, and metabolism [8]. The release of Macrophage migration inhibitory factor is triggered by gluccocoticosteroids from monocytes/macrophage. They also play crucial role in the activation, stimulation and expression of the various cytokines like TNF-α etc. [9].

In earlier conducted *in vivo* studies have potentiated the role of macrophage migration inhibitory factor (MIF) as an activator of macrophages. MIF is readily shown in the epidermal keratinocytes and fibroblasts of the skin. The MIF have a direct link with inflammation as well as with the progress of tumour. When skin is exposed UV irradiation (280-320 nm) the production of MIF increases and on persistent exposure leads to the inhibition of the p53-dependent apoptotic processes which ultimately leads to skin cancer [10,11]. This mechanism proves that the macrophage migration inhibitory factor has innate role with immunity and its complete understanding could yield fruitful therapeutics in case of skin cancer.

In earlier studies we have gone through a series of experiments to confirm the potentiality of the prepared ligand conjugates, their pharmaceutical production, there *in vitro* profile, their development into an acceptable and economical preparation and the confirmation of their potentiality through animal studies [12]. Up to this stage we have analyzed those contributing factors or markers which are found predominantly during the progress of skin cancer on exposure to Ultraviolet radiations e.g. MMP-1, MMP-2, TNF-α, MAO, ROS, Intracellular GSH, 4-HNE and COX-2 [13]. Each of the markers was

prominent in different stages of proliferation of cancer and show characteristic activity when found in abundant amount inside the cell.

Minimal erythema dose (MED), which is the minimal dose of UVR required to produce a clearly marginated erythema at the irradiated skin site is an important evaluation parameter employed to assess the cutaneous response of skin towards UV radiations. It becomes highly crucial for those patients which are having photodermatitis and in the epidemiological investigation of skin carcinoma.

Materials

Selection of human subjects

The human study experiments were performed in accordance with the approved institutional protocol (IEC Ref.No: 118/IEC/PRSU/2014). All volunteers participated in the study gave written informed consent, which was approved by the Institutional Human Ethical Committee of Pt.Ravishankar Shukla University, Raipur, India. Six groups of human subjects (n=3), both male and female, ranging from 25 to 55 years old, were recruited for this study. Only those individuals were selected which were in good health and having no sign of acute or chronic diseases and having no photoallergic tendency. Lactating Mothers and Pregnant women were excluded.

MED analysis

Firstly their MED was taken. The procedure to carry out this study was as per Katiyar et al. with slight modifications. MED was determined by taking six sites in sequence and exposing them to UV radiation with gradual increase in incident time from 30 seconds. After 24 hours whichever site expresses redness at a definite UV irradiation dose was considered as MED. Thereafter, base line readings were taken and thighs skin sites were exposed for irradiation with or without pretreatment with NL-4 and NL-5 formulations [14]. Volunteers were asked to visit the next day for evaluation of erythema and skin biopsies. Skin Biopsies were taken from each site from every volunteer and snap frozen immediately and stored at -80°C for further use.
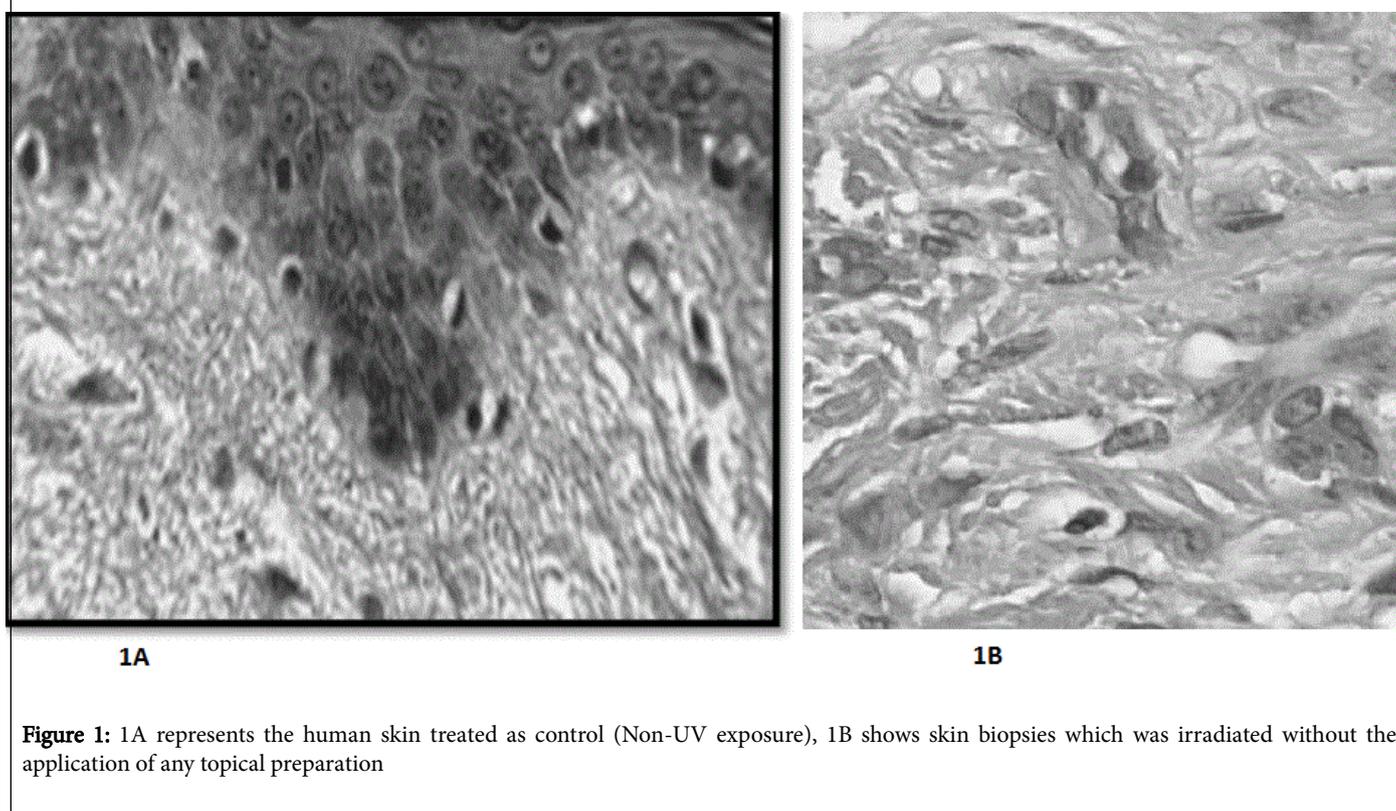
MIF estimation

For Macrophage migration inhibitory factor determination, method described by Tadamichi et al. was adopted with slight modifications. For MIF estimation, all the six groups were exposed to UV radiations once [15]. Before and after receiving exposure to UV radiations (240-280 mJ/cm^2), 2 ml blood was withdrawn from antecubital vein from every volunteer at a duration of 12, 24 and 48 hours and analyzed further for increase in MIF levels.
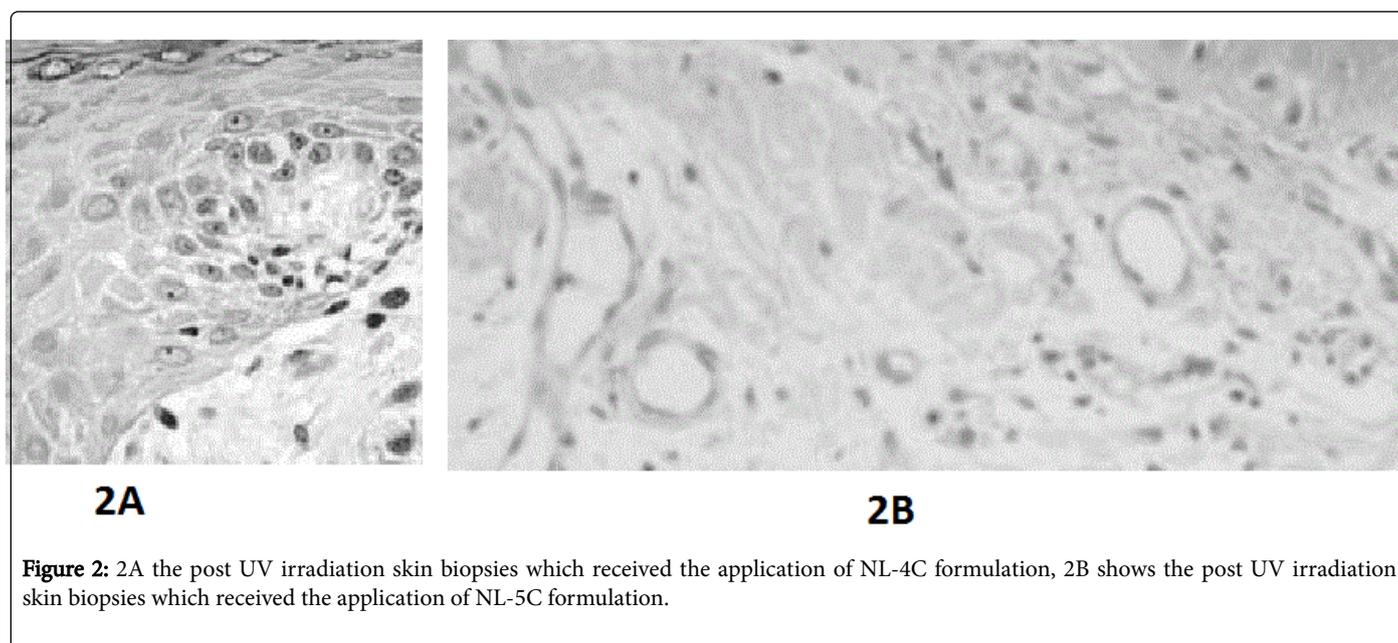
Results

Human biopsies study

The objective behind carrying out this study was to evaluate the inhibitory potentials of NL-4C and NL-5C formulations against UV radiations in human models. The skin biopsies sections showed that there was a dose dependent effect on human skin in response to the UV radiations. The pattern was nearly same for NL-4C and NL-5C for MED. Human biopsies were taken in order to confirm the activity profile of the novel formulation.

Figure 1: 1A represents the human skin treated as control (Non-UV exposure), 1B shows skin biopsies which was irradiated without the application of any topical preparation

The results of skin biopsies study showed that there was a dose dependent reduction in erythema formation in all epidermis samples. The human skin sections showed a decrease level of inflammation as well as an absence of lesion formation on repeated dosing of UV radiations to thighs skin in case of NL-5C formulation. No sign of skin sensitivity or allergic reactions were observed on the application of NL-5C (Figure 1). The results also draw our attention towards the fact that, on applying NL-5C on human skin, incidents like epidermal thickening or formation of wrinkles were not observed. The samples treated with NL-4C also showed absence of certain clinical features observed in UV treated epidermal samples like lesion formation, inflammation etc. The most characteristic feature observed on topical application of NL-4C was reconstitution of collagen and elastin network which was totally disturbed when compared to treated epidermal samples and was not so rominent in NL-5C application (Figure 2). The standard drug also showed no lesion formation but the percentage of reduction in inflammation was lower in comparison to NL-4C and NL-5C. Thus, the study confirms the role of NL-4C and NL-5C as a desired topical formulations for delivering any synthetic or phytoactive containing cargoes for dermal application. In a study conducted by Rai et al. in 2004, an assessment of MED was done against UVR in normal Indian skin. They observed in their study that the mean MED for UVB was 61.5 ± 17.25 J/cm^2. They observed that MED was lower in fair skinned individuals and higher in darker skinned individuals. Their observations helped us to draw the conclusion that MED value is of great significance in the study of impact of ultraviolet radiations in normal Indian skin. The formulation formulated by us showed three fold decrease in MED value in fair coloured skin individuals and two fold decrease in dark coloured skin type individuals. The formulation NL-5C was found to be very highly significant ($p=0.0001$) while the value of p for NL-4C was also significant ($p=0.005$). This study was designed to identify the correlation parameter in evaluation of *in vivo* doses and epidermal changes following UVB irradiation using noninvasive techniques. The overall study results laid to conclusion that the NL-4C and NL-5C has potent anticancer activity against NMSC and could also prove beneficial in reducing skin complications synergistically.

Figure 2: 2A the post UV irradiation skin biopsies which received the application of NL-4C formulation, 2B shows the post UV irradiation skin biopsies which received the application of NL-5C formulation.

NL-4C and NL-5C inhibits the MIF levels

The level of MIF was found to nominal (12.5 ± 1.25 ng/ml) before UV irradiation in human volunteers. But there was a sudden increase in the level of MIF after exposure to UV radiations within 0-12 hours. For first 12 hours the MIF level ranged from 12.34 ± 9.90 ng/ml to 79.4 ± 23.65 ng/ml which was the result of acute exposure of UV radiations. For next 24-48 hours, the MIF levels was found to be increased from 22.43 ± 12.56 ng/ml to 102.34 ± 20.4 ng/ml, which was the delayed response exerted by the body and is visible on the skin in the form of erythema, sunburn or redness (Figure 3). There was a slight variation in the level of MIF in different volunteers. Results are represented here in the form of mean ± SD levels.
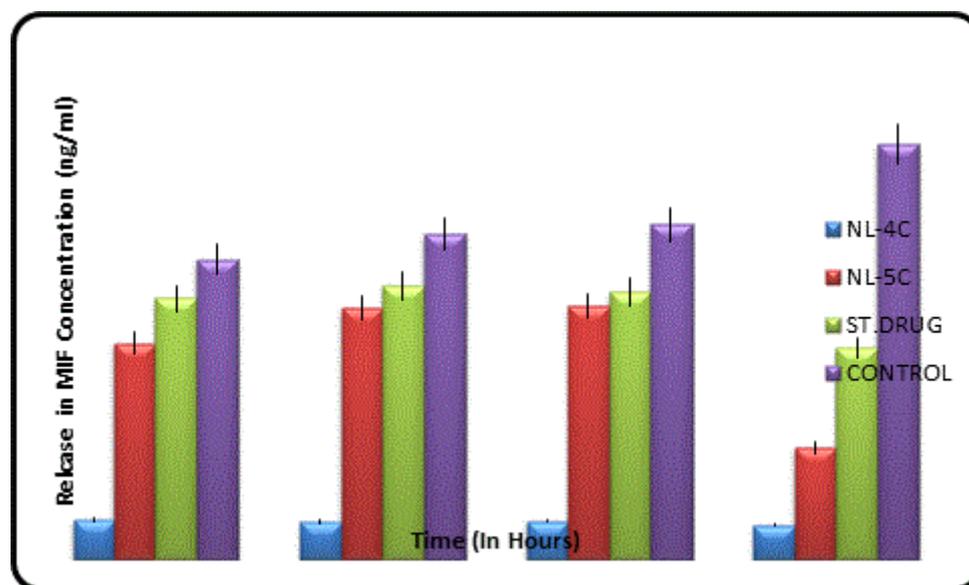
Figure 3: Showing the effects of NL-4C and NL-5C on inhibition the MIF levels. MIF specific ELISA was used to measure serum MIF levels pre and post UV irradiation. Results are represented here in the form of mean ± SD levels (n=3).

From above studies, we can conclude that NL-4C and NL-5C have the anti-MIF activity. Various studies have reported the activity of macrophage migration factor to be crucial in the activation of TNF-α and other cytokinines which stimulates inflammation. The lower level of MIF on application of prepared formulation clears that the objective to deliver a targeted moiety at site of action of dermis has been achieved.

Conclusion

Skin cancer is a disease which has acquired numerous forms in these years. Several drug delivery systems have been made to deliver the therapeutic drug at desired site but due to constant weir and tier of keratinized layer of the skin, lipophilicity of membrane, presence of enzyme system and many other contributing factors, drug delivery to skin is not such easy, as it pretends to be. Targeted drug delivery system employing the mode of active targeting by conjugating the ligands that are specifically recognized by surface receptors of target cells can overcome this problem. To contribute this aspect of active targeting, nanoparticles embrace unique characteristics in terms of appearance and application. Presently, a huge range of synthetic and herbal drugs, biological, enzymes, hydrophilic and hydrophobic small drugs, vaccines, macromolecules can be loaded or encapsulated in the nanoparticles for effective delivery.

In the study presented here two different endogeneous ligand-drug delivery system were prepared, formulated them into a suitable formulation and quantified their effects after preclinical investigation and getting approval from Human ethical committee of the Institute. Quercetin has established anticancer activity, but the approach of the present study was to facilitate the entry of quercetin as a drug inside the cell, since quercetin is a lypophilic drug. The rationale to design a targeted drug delivery system was achieved by designing a ligand conjugated nanocarrier encapsulating quercetin and its targetability and efficacy was confirmed by *in vitro* cell line studies. The

nanocargoes was then converted into nanolotions (nanocargoes loaded lotions) and were evaluated against skin carcinoma in animal models, in which the formulations NL-4C and NL-5C showed promising results. The last step was to get the activity performance of the formulations in human subjects. The crucial involvements of macrophages have been proved in several skin infectious diseases but in skin cancer it is still unclear. Through the study depicted above an attempt has been made to potentiate the role of mannose receptors as well as folate receptors in skin cancer through macrophage targeting. Although some more investigations are required to confirm the underlying mechanism of MIF in skin cancer, we hope that our study would potentiate the development of site specific delivery system of phytoactives in future.

Acknowledgements

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