Original Article

Impeding Minocycline Induced Hyperpigmentation by Pomegranate Extracting the Epidermis of Guinea Pig

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ABSTRACT:

Objective: This study was planned over the hypothesis that pomegranate extract rich in ellagic acid used with minocycline could decrease its adverse effect and prolong its therapeutic use and efficacy.

Study design and Setting: This experimental study was done in the department of anatomy, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Center, Karachi,

Methodology: We acquired 40 guinea pigs (male, adult, 450 – 550 gm), randomly divided them into 4 groups.Group B received 0.0003mg/g bodyweight of minocycline only, group C was given 0.0003mg/g bodyweight of minocycline with 0.4mg/g bodyweight of pomegranate, group D was given 0.4mg/g bodyweight pomegranate only; with keeping group A with no intervention at laboratory diet for 8 weeks. After the experimental period, the animals were sacrificed, H & E and DOPA-OXIDASE staining was done on harvested skin tissues for morphometric observations under light microscopy.

Results: The results showed that minocycline induced reduction in mean thickness of epidermis and increased melanin pigment deposition. Mean number of melanocytes decreased with pomegranate use though the difference was insignificant (P-value > 0.05) but consistent and measurable.

Conclusions: It was proven that by including pomegranate in our daily diet, the process of hyperpigmentation of skin induced by the broad spectrum tetracycline particularly minocycline, can be slow down by decreasing the activity of tyrosinase enzyme, thus it provides a novel pathway to fight against any other drug induced hyperpigmentation occurring due to increase activity of tyrosinase enzyme.

Keywords: Pomegranate, Minocycline, Epidermis, Skin, Hyperpigmentation, Tyrosinase inhibitor, Ellagic acid.

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INTRODUCTION:

Skin is composed of epidermal units that are responsible for the production and distribution of melanin, on which the colour of skin depends. These epidermal units are composed of melanocytes surrounded by keratinocytes. Melanin synthesis occurs in melanocytes under paracrine control. Hyperpigmentation of skin can result from increased melanin synthesis[1]. Drug-induced hyperpigmentation represents about 20% to 40% of all cases of acquired pigmentation. Commonly used drugs such as pain killers and antibiotics particularly sulphonamides and tetracylines[2], can also stimulate melanocytes causing increased melanin production, thus causing hyperpigmentation of skin[1].

Minocycline, a broad-spectrum antimicrobial tetracycline, was mainly accepted for the treatment of tetracycline resistant inflammatory acne. It is still prescribed for acne vulgaris as oral or in topical gel formulations in moderate to severe acne cases[3]. It has been used as an effective, well tolerated therapy in rheumatoid arthritis[4]. The drug is approved by FDA (US) for the treatment of gram negative bacteria in immune-compromised and seriously ill ICU patients[5].

Cutaneous hyperpigmentation is a well-recognized adverse effect of chronic minocycline therapy. Various studies have reported hyperpigmentation induced by chronic administration of minocycline in patients with acne, rosacea, and rheumatoidarthritis. It is a cosmetically concerning side effect that is also noted to occur in subcutaneous fat, nails, teeth, gingivae, oral mucosa, lips, conjunctiva, sclera, as well as various internal organs[6].

Melanin pigment is synthesized by a cascade of enzymatic

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and chemical reactions.Its production is mainly controlled by the activation of tyrosinase enzyme. Thus, tyrosine as inhibitors of natural sources can be used as an essential constituent of de-pigmenting agents for the treatment of hyperpigmentation disorders[7].

Natural sources including plants have recently gained interest for their anti-tyrosinase activity. Researchers do prefer inhibitors from natural sources due to their less toxicity and better bioavailability, especially for food, cosmetics and medicinal purposes[8].

Pomegranate (Punicagranatum) is a tropical fruit and is a rich source of polyphenols such as ellagitannins and ellagic acid. Many studies have documented the beneficial effects of pomegranateconsumption in the treatment of various disorders[9]. Recently, it was demonstrated that the skin-whitening effects of ellagic rich pomegranate are due to the inhibition of melanocyte proliferation and melanin synthesis by tyrosinase in melanocytes[10]. Although, other commercially available tyrosinase inhibitors such as glutathione are now used as skin whitening product intravenously for almost a decade[11], pomegranate being an edible fruit, has the potential for skin whitening through inhibition of tyrosinase leading to the decreased melanin content[10].

In this experimental study, it is investigated that pomegranate impedes melanin pigmentation thus reduces epidermal hyperpigmentation, a commonly occurring adverse effect observed when minocycline is used therapeutically for prolonged period.

METHODOLOGY:

This experimental study was done in the department of anatomy, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Center, Karachi, after Ethical Review Committee approval No. F.1-2/2019/BMSI-E.COMT/075/JPMC obtained from the institute. The duration of experiment was 8 weeks.The study was conducted from April 2012 to May 2012. Forty male adult guinea pigs weighing 450 – 550 g were randomly divided into 4 groups (A, B, C and D) comprising of 10 guinea pigs in each. The animals were kept under standard conditions in the Animal House of BMSI, and were given standard laboratory diet consists of fresh cucumbers and water ad libitum. The animals were acclimatized for one week prior to the experiment. All were on 12 hrs fasting prior to the administration of compound.Group A served as control. Group B received Minocycline (Minocin, Stiefel Laboratories Pakistan Pvt. Ltd; Stiefel Laboratories Inc. Coral Gables, FL 33134.USA) 0.0003 mg/g body weight per oral, through gastric lavage, once daily for 8 weeks in the morning after 12 hours fasting. The dose was calculated based on the optimum recommended human dose 200 mg/day in 375 days being used in evaluation of its efficacy in Primary Sclerosing Cholang it is patients for 1 year without any toxic effects.[12] Group C received Minocycline 0.0003 mg/g body weight per oral and Pomegranate (Just Vitamins, England, Company No. 3991727; UK) 0.4mg/g body weight per oral after grinding and mixing in the daily early morning diet, after 12 hours fasting. The dose was calculated and based on the experimental study done by Yoshimura et al.[13] and prepared on recommended human dose of Pomegranate tablet of 250 mg/100g body Weight. Group D was negative control group and received only pomegranate (Just Vitamins, England, Company No. 3991727; UK) 0.4mg/g body weight orally daily mixed in the diet.

At the end of the experimental period all animals were an aesthetized in a glass container under ether anesthesia and the skin was dissected out after shaving.Two fragments of two inches of skin,one from upper limb and one from lower limbs, were fixed in two types of fixatives. First fragment was fixed in 10% formalin for H&E staining and second one was fixed in 10% formalin in pH7.4 buffer (v/v) plus 0.44 M sucrose for DOPA OXIDASE Method for two days. The tissue of skin fragments were processed in automated tissue processor and sectioned then stained.

In H&E stained sections,morphology of epidermal cells was observed at 400X magnification and thickness of epidermis was measured at 100X magnification using ocular micrometre; at 5 different fields and mean thickness of epidermis was calculated. In Dopa Oxidase nuclear fast red stained sections, melanin pigment deposition in epidermis was observed at 100X magnification and melanocytes were counted at 400X magnification in Stratum Basale and other strata under light microscope with the help of ocular counting reticule in 5 different fields and mean number of melanocytes was calculated.

The melanin deposition was also observed and was graded as per following design:

Grade I: Normal melanin deposition limited till Stratum Basale with scattered distribution (+)

Grade II: Increased melanin deposition limited till Stratum Spinosum with patchy distribution (++)

Grade III: Increased melanin deposition limited till Stratum Corneum with uniform distribution (+++)

The data was entered in SPSS v.23. Mean thickness of epidermis; mean number of melanocytes per reticule for each group was calculated. The difference in the means was calculated with the use of one way ANOVA with post-hoc tukey.The results were considered statistically significant at the P –value of less than 0.05.

RESULTS:

In H & E stained slides, the epidermis appeared normal in all four groups with not much difference at 400X magnifications. (Figure-1a) The epidermis comprises of keratinocytes, containing rounded nuclei, arranged in five strata, from which Strata Basale, Spinosum, Granulosum and Corneum are easily identifiable; Langerhans cells with clear

cytoplasm and rounded nuclei are also seen. (Figure-1a)

The mean thickness of epidermis in upper and lower limbs was 67.30±0.47 67.50±0.48 µm respectively in Group A. (Table-2, column 2 & 3) It was decreased 66.10±0.47 and 66.50±0.38 µm respectively in Group B in comparison to Group A. (Table-2, column 2 & 3) It was increased 67.30±0.47 and 67.5±0.47 µm respectively in minocycline plus Group C in comparison to Group B. (Table-2, column 2 & 3) It was increased 66.70±0.48 and 66.30±0.49 µm respectively in Group D in comparison to Group B.(Table-2, column 2 & 3) But the difference remained statistically insignificant (P-value > 0.05) between all groups.

The melanin pigment deposition was also observed in DOPA OXIDASE with red nuclear fast red stained at 100X magnification, which was grade I that is normal (+) in Group A(Figure-1b, Table-1), was Grade II (+++) in Group B (Figure-1c, Table-1), was grade I (++) in Group C (Figure-1d, Table-1) and was normal (+) in Group D. (Table-1, column 4 & 5). In DOPA OXIDASE with red nuclear fast red stained sections, the shape of the melanocytes was ovoid to round with sparsely stained dendrites spread between keratinocytes of Strata Basal and Spinosum in Group A.(Figure-1b) The morphology of melanocytes observed in the Group B, C and D found to be similar to the Group A.(Figures-1c & 1d).

The mean number of melanocytes per reticule was also counted in upper and lower extremities in DOPA OXIDASE stained sections; which was 2.26 ± 0.29 and 2.21±0.24 respectively in Group A (Table-2, column 4 & 5), was decreased 2.25±0.35 and 2.20±0.30 respectively in Group B in comparison to Group A (Table-2, column 4 & 5); was decreased 2.20±0.28 and 2.22±0.23 respectively in Group C in comparison to both Group A and B (Table-2, column 4 & 5);And was almost same 2.24±0.28 and 2.20±0.25 in Group D as compared to Group A (Table-2, column 4 & 5). Though the differences between the groups were not significant (P-value > 0.05).

DISCUSSION:

Minocycline, a broad-spectrum antibiotic from the group of tetracyclines, is frequently prescribed for acne vulgaris and rosacea. Hyperpigmentation is a relatively common side effect of this drug and can lead to multiple unpleasant skin lesions, which are not always reversible.[14].

The prevalence of pigmentation secondary to Minocycline ingestion varies between 2.4% to 41% of treated individuals and is highest in patients with rheumatoid arthritis[15].

Table 1: Melanin deposition and extension in epidermal layers in different groups

Group	Treatment Given	Treatment Period	Melanin Deposition	Extension
A Control	None diet Ad labitum	8 Weeks	+	Grade I*
B Minocyline Treated	0.0003 mg/g body weight / day orally	8 Weeks	+++	Grade III***
C Minocycline + Pomegranate protected	0.0003 mg/g body weight / day orally + 0.4 mg/g body weight / day orally	8 Weeks	++	Grade II**
D Pomegranate only	0.4 mg/g body weight / day orally	8 Weeks	+	Grade II*

*Melanin deposition till stratum basale
** Melanin deposition till stratum spinosum
***Melanin deposition till stratum corneum
+ scattered distribution
++ patchy distribution
+++ uniformed distribution

Table 2: Mean thickness of epidermis and mean number of melanocytes per reticule in upper and lower limbs of guinea pigs of different groups

Groups (8 weeks)	Mean Thickness of Epidermis (µm)		Mean number of Melanocytes per reticule	
	Upper Limb	Lower Limb	Upper Limb	Lower Limb
A Control With Diet Ad Labitum	67.30±0.47	67.50±0.48	2.26±0.29	2.21±0.24
B Minocyline Treated	66.10±0.38*	66.20±0.39*	2.25±0.35*	2.20±0.30*
C Minocycline + Pomegranate Protected	67.30±0.40*	67.5±0.47*	2.20±0.28*	2.22±0.23*
D Pomegranate Only	66.70±0.48*	66.30±0.49*	2.24±0.28*	2.20±0.25*

KEY * insignificant = 0.05

Figure 1a: Photomicrograph of H & E stained skin section from upper limb of control Group A showing epidermis comprising of keratinocytes with darkly stained cytoplasm and round nuclei, arranged in distinguishable stratum basale (SB), strarumspinosum (SS), stratum granulosum (SG), stratum corneum (SC) along with langherhan's cells (Lc) containing clear cytoplasm and rounded nucleus.400X.

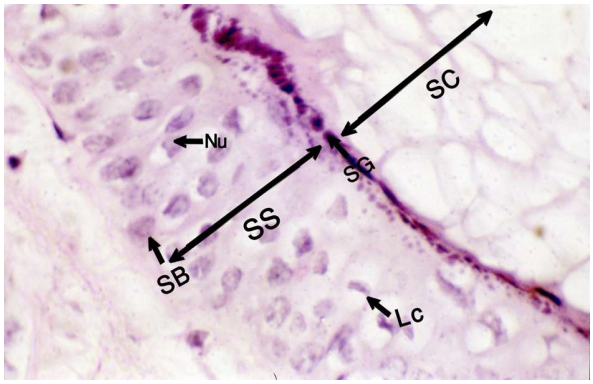


Figure 1b: Photomicrograph of DOPA OXIDASE with Nuclear Fast Red stained skin section showing melanocyte (M) located in stratum basale and melanin pigmentation (mp) limited till stratum basale in the epidermis of upper limb fromcontrolguinea Group A. 100X

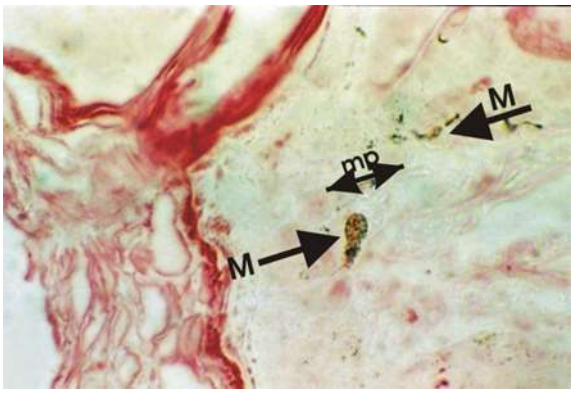


Figure 1c: Photomicrograph of DOPA OXIDASE with Nuclear Fast Red stained skin section showing melanocyte (M) located in stratum basale and melanin pigmentation (mp) limited till stratum corneum in the epidermis ofupper limb from Minocycline treated Group B.100X.

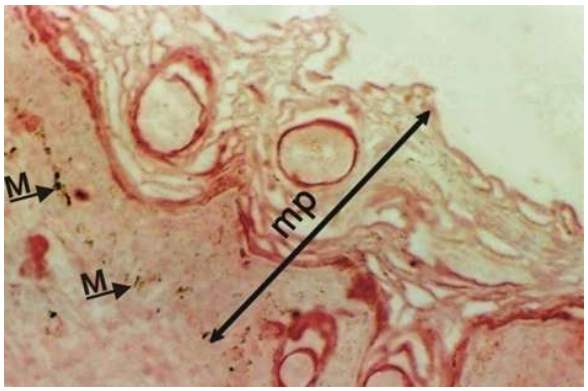
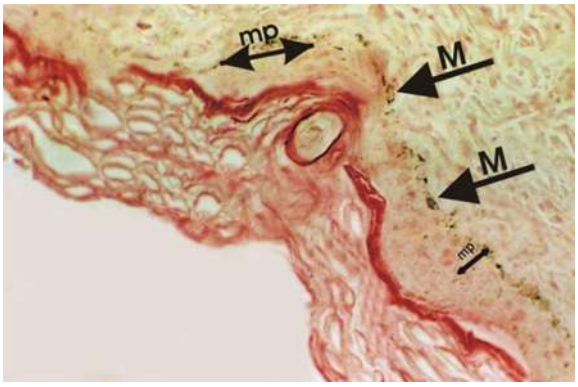


Figure 1d: Photomicrograph of DOPA OXIDASE with Nuclear Fast Red stained skin section showing melanocyte (M) located in stratum basale and melanin pigmentation (mp) limited till stratum spinosumin the epidermis of upper limb from Minocycline treated plus prevented with Pomegranate Group C. 100X



Pomegranate fruit have been used for centuries in ancient cultures for its medicinal purposes and its benefits has been attributed to the content of hydrolysable tannins (ellagitannins) including ellagic acid and ellagic acid derivatives[16]. Ellagic acid is present in the extract of pomegranate has been proven to exhibit the potent antioxidant and tyrosine inhibitor properties[17].

In this study, the given dose of 0.0003mg of minocycline per gram body weight of a Guinea Pig was based on cumulative dose given in the clinical trial conducted by Silveriaet al.[12] This dose is less than the cumulative dose of 0.0139 mg per gram body weight being prescribed for animals with mean bioavailability 62% any without toxic effects[18]. The dose of ellagic acid rich pomegranate of 0.4 mg per gram body weight was based on the experimental study conducted on mice by Youshimura et al.[13]A randomized controlled trial of pomegranate juice and extract was also conducted in 2019 based on the experimental animal study of Yoshimura et al on the healthy women to observe the effects against Ultraviolet B induced erythema produced in their skin without any adverse effects[17]

In the present study, the minocycline treated group B showed decrease in epidermal thickness compared to control group A. This may be explained by the fact that minocycline inhibits the release of cytochrome C from mitochondria by reducing the tumor suppressing gene p53[19], which in turn decreases the pro-apoptotic factors such as BAX(BCL 2-Associated X protein; B Cell Lymphoma associated),thus inhibiting the activation of caspase-9. It also decreases TNF (Tumor Necrosis Factor). These are the reasons of suppression of apoptosis as described by Kumar et al.[20]. That is why there is no alteration in the epidermal thickness. This is also being the findings of Guneyet al. that demonstrate the anti-apoptotic effects of minocycline on fat graft and observed statisticallysignificant apoptosis inhibition[21]. This has also being proven in the study conducted by Abbaszuddenet al.

who found that minocycline protects neurons from caspase activation through decrease release of cytochrome C from mitochondria and also reduces the levels of TNF[22].

In minocycline treated group B, the DOPA OXIDASE nuclear fast red exhibited melanin pigment deposits till Stratum Corneum when compared to control groupA.This is in accordance with the findings of Hanada et.al. who reported high incidence of minocycline induced hyperpigmentation in patients with orthopaedic infections receiving long term minocycline[23]. In another study, the histopathological examination of skin showed increased melanin pigment deposition extending in all layers of epidermis[24]. The mean number of melanocytes was insignificantly increase in treated Group B as compare to control group A,this could be due to the minocycline's ability to scavenge free radicals.This have a protective effect on melanocytes against H_2O_2 induced apoptosis[25].

In pomegranate treated group C, the change in epidermal thickness is insignificant compare to control group A, due to the down regulation of TNF levels by pomegranate rich ellagic acid extract[26], thus there is no significant difference between the epidermal thicknesses of pomegranate treated and control groups.

The melanin deposition in the epidermis was markedly decrease and limited till Stratum Basale (++) in Group C as compared to Group B due to the inhibitory effects on tyrosinase enzyme by ellagic acid of pomegranate preventing the process of melanogenesis. This is being proven experimentally on melanoma cells by the metabolites of dietary ellagic acid derivatives[27].

The number of melanocytes was significantly decreased in Group C,(Table -2,column-4 & 5), compare to control group A. This was due to inhibition of melanocyte proliferation by ellagicacidrichpomegranate[28] proving that ellagic acid possesses strong inhibitory effects on proliferation of melanocytes.

The negative control group D was included in this experiment to exclude out all the probable effects of pomegranate that could occur due to its administration, on the epidermal thickness, melanin pigmentation and number of melanocytes. It was observed that no significant effects were exhibited on the mean thickness of epidermis, melanin pigmentation deposition and extension as well as number of melanocytes when compared to control group A.[26,27,28]

CONCLUSION:

This experimental study had taken into account the single optimum recommended human dose of minocycline that had already been proven from the previously conducted studies, in resulting to producedhyper pigmentary effects in human epidermis, without causing any toxicity. For these reasons, this experimental study was aimed to determine the impeding effects of ellagic acid-rich pomegranate extract, which is in human consumption for quite sometimes, against the cutaneous hyper pigmentary effects due to tyrosinase enzyme inhibition.The results obtained so far, had proven to be quite optimistic in this regard, and open a novel corridor for further research in this aspect.

CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

Author Contribution:
Sarwath Fatimee: Conceptualization, Methodology, Formal Analysis, Investigations, Writing-Original Draft, Referencing
Nadia Younus: Methodology, Validation, Writing- Review & Editing Final Draft
Sadia Sundus: Methodology, Investigations, Resources
Yasmeen Mahar: Supervision, Resources
Syed Munawar Alam: Conceptualization, Resources
Syeda Bushra Ahmed: Investigations, Writing-Original Draft

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