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IN-VITRO ANALYSIS ON THE EFFECTS OF UV-B RADIATION ON THE DORSAL SKIN MELANOPHORES OF INDIAN BULLFROG HOPLOBATRACHUS TIGERINUS

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ABSTRACT

In vitro studies on the dorsal integumental melanophores of amphibian *Hoplobatrachus tigerinus* revealed that the artificial UV-B radiation (313nm) at a dose range from 20.0 - 24.6 μW/cm ^{2} for a continuous period of 60- 120 minutes caused an irreversible damage and disruption to the melanophores resulting into permanent clumping and aggregation of cells. However, at lower duration of exposure, the melanophores showed gradual pigment dispersion, most likely as an innate mechanism of defense to attenuate the UVB causing damage.

KEYWORDS

Ultraviolet- B radiation; melanophores; amphibians; *Hoplobatrachus tigerinus;* UV defense.

INTRODUCTION

Numerous anthropogenic reports suggest that population of amphibians are experiencing dramatic decline. This marked abatement in amphibian population has been noted since the 1980s across the globe and is perceived as one of the most discerning threats to global biodiversity. Recent experiments provide evidence that increasing ambient levels of UV-B radiation has been one of the leading causes for debilitating many amphibian species [52]. Ultraviolet B radiation is the energy from the sun which ranges from 280 to 320 nanometers (nm) in wavelength. The thinning of the stratospheric ozone layer has allowed an increase in the level of UVB that reaches both temperate and tropical regions of the earth's surface [1, 2, 3]. It is quite acceptable that UV-B radiation can kill amphibians and can cause sub lethal damage to them [4]. However, most studies that have examined the effects of UV-B radiation on amphibians have focused on developing embryos. There is little information on how UV-B radiation affects amphibians at later stages of development. At post metamorphic stages sub lethal consequences after UV-B exposure have been documented to cause hampered growth and development, physiological aberrancies, physical deformities and behavioral changes [4]. These effects occur in later life stages even in species, whose embryos appear to be unaffected after being exposed to UV-B radiation [5, 6, 7]. Despite emphasizing the importance of studies focused on investigating the deleterious effects of UV-B after metamorphosis by several workers [6], there has been no substantial research undertaken so far on the subject. Fite *et al*., [8] showed that prolonged exposure to ambient levels of UV-B radiation in adult frogs resulted in severe retinal damage. This effect was observed in wild captured frogs (*Rana cascadae*) and was experimentally induced in *R. pipiens*, also juvenile frogs of several species have been reported to display physiological abnormalities after being exposed to ‘‘modest’’ levels of UV-B radiation[9].

Post-metamorphic mortality could be one of the notable factors contributing to amphibian population declines [10, 11]. The behavioral alteration perceived as a consequence to the increase in UV-B radiation may emerge into greater complexities, for instance; alter an amphibian’s ability to locate food or escape predation [8, 12]. After metamorphosis, amphibians may seek sunlight for thermoregulation. Yet, prolonged exposure may actually damage amphibian eyes [8,] resulting into immune dysfunction and may cause skin lesions [4].

As far as effects of UV on the skin are concerned, most of our knowledge is based on epidemiological studies rather than experimental work. Moreover, there is a cogent connection between the pigment, melanin and UVB radiation, since melanin is considered extremely effective at absorbing UVB and preventing its penetration to deeper layers of the skin. Animals can cope with potentially dangerous UV-B radiation either by preventing damage from occurring or by repairing damage once it had occurred [13]. Although the mechanisms involved in repairing UV-B-induced damage to amphibian eggs and embryos have received considerable attention [14, 15]. Behavioral avoidance of areas with high levels

of UV-B is one way for larval and adult amphibians to prevent damage [16, 17]. In addition, pigments in the skin, such as melanin, provide some protection from UV-induced damage in mammals [18]. This finding has gained ample attention in mammals and humans, and is still under considerable research [19], but unfortunately, it has not been given much importance in the case of amphibians.

Keeping the preceding facts in mind our present study was undertaken to investigate the responses of the pigment cells; melanophores of a commonly found tropical amphibian *Hoplobatrachus tigerinus*, on exposure to UVB radiation at varying time duration. The present investigation provides some understanding of the harmful effects of UVB radiation from a constant source for varying duration of exposure. The immediate mitigatory response of the melanophores in concurrence with the increasing duration of the UVB exposure is most likely a factor for permanent damage and rupture of pigment cells melanophores.

MATERIALS AND METHODS

The study species

The commonly found Indus valley bullfrog, *Hoplobatrachus tigerinus* also called *Rana tigerina* has a widespread inhabitance in the Indian subcontinent and Ceylon-from the Indus of Pakistan to the base of the Himalayas in Kashmir, and from China to the Malay Peninsula and Archipelago [20]. It is mainly aquatic-inhabiting mostly in freshwater wetlands, also found in dry areas which are endowed with abundant sunlight for the year round. This, particular species was chosen since the adults' exhibit diurnal nature and are one of the most commonly found amphibians in the subcontinent.

The study was performed during the months of July till September in the city of Bhopal, North Latitude 23º16' and East Longitude 77º36' in the state of Madhya Pradesh, India.

Adult *H. tigerinus* frogs approximately weighing 150-200 gms were acquired legally from an authorized dealer following the legislation of the country where the study was conducted. The animals were approved by the appropriate animal care review committee at the institution where the experiments were carried out and no damage was caused to the environment or wild animal populations.

The animals were kept in laboratory froggery for 3-4 days with 12:12 hrs of light-dark phase and temperature maintained between 20-25˚C. A mud substratum was made at the base of the tank which was kept wet. The tank was covered with a wire mesh that had a provision for withdrawing and introducing frogs. Food, temperature, humidity and sanitation were taken care of properly. Overfeeding was avoided and any animal with signs of disease or lethargy were left out. Suitable care was taken to minimize any causes of stress to the animals.

The Experimental Design for in vitro studies

For preparation of skin pieces the healthy frogs were withdrawn from the froggery and carefully decapitated. Thereafter, the skin from the dorsal region was carefully removed and immediately immersed in Amphibian Ringer Saline (ARS) composed of NaCl, 111 mM; $NaHCO_3$, 2 mM; KCl, 2mM; $CaCl_2$, 1 mM at pH 7.4 according to the method by Ali et al. [21]. For examination and further treatment, the skin pieces were cut into 2-4 mm size with the help of fine scissors. The skin pieces were kept in small petri dishes with Amphibian Ringer Saline (ARS) and aerated regularly. The temperature of the experimental setup remained between 20^0C - 25^0C.

Artificial UV-B radiation source

Phillips 40 W UV tube - 313 nm (USA) was employed as the artificial UV radiation source, with the maximum emission at 313 nm and emitting no radiation below 280 nm. UV-B levels

ranged from 20.0 - 24.6 μW/cm ^{2}. The UV-B levels subjected to treated cells were within the range of ambient UV-B under field conditions where frogs were found [22, 23], Salim unpublished data).

Calculation of Mean melanophore size Index (MMSI)

The untreated controls without UVB exposure as well as the treated skin pieces were observed under light microscope and the responses of frog skin melanophores were assayed by the mean melanophores size index (MMSI) assay [24, 21], based on the method by Hogben and Slome [25]. In this method, each skin piece having approx. 50-100 melanophores of equal size are randomly selected and their actual diameter (i.e. length x breadth with the dendritic processes) were measured with the help of a Leitz ocular micrometer calibrated previously with Stage micrometer. The value was then multiplied by the unit of the micrometer which was 15μ. Thereafter the arithmetical mean was calculated and the value was divided by hundred to obtain the values in a digit with two decimal points. This was the mean melanophore size index (MMSI). As per the method, the increase or decrease of the melanophore index from the control value represented the dispersion and aggregation of melanophores respectively. The mean was of 9 experiments on untreated as well as treated melanophores from different frogs i.e. n=9.

Control

Control skin pieces without any UVB exposure were kept separately in amphibian ringer saline and aerated frequently and MMSI was measured. Comparison of the MMSI of Control skin at a given time duration with the exposed skin were made in order to draw the differences in the potential physiological activity.

Statistical Analysis

Statistical data analyses were presented as mean ± SEM (standard error of mean). Comparisons were made between the control groups by use of student's t test. All data were analyzed using Graph Pad Prism software (UK). $P<0.05$ indicates statistically significant difference.

The Protocol

After 10 minutes of equilibrium in the ARS each skin piece was pre-examined under the microscope and the damaged pieces with any signs of disrupted melanophores were discarded, whereas only the clear, smooth and undamaged skin pieces were selected. There after, the skin pieces were carefully transferred to petri dishes labeled serially with time of UVB radiation (control, 7 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min and 120 min) to avoid confusion. The samples were then subjected to monochromatic UV-B radiation with wavelength 290-320 nm from a distance of 10 cms for durations ranging from 7 to 120 minutes.

Total of 9 petri-dishes were prepared. One was kept as control subjected to no UVB treatment and the rest as experimental test samples was required for 8 different irradiation periods. The petridishes for UVB irradiation were kept directly under the UVB source and the initial time i.e. t=0 was noted. Clock was set for monitoring the time of petri-dish withdrawals. After 7 minutes i.e. t=7 one petridish was withdrawn. The skin piece was gently picked up and mounted on a glass slide with some ARS and observed under the light microscope fitted with SLR camera. The actual diameter of 10 randomly selected melanophores was measured with the ocular micrometer as described in the method above. Thereafter, another petridish was withdrawn after 10 minutes of irradiation t=10 and observed under the microscope. The petri-dishes were withdrawn at the end of irradiation times at 15 mins (t=15), 20 mins (t=20), 30 mins

(t=30), 45 mins (t=45), 60 mins (t=60) and 120 mins (t=120) and the melanophore responses were observed in the similar way as mentioned above.

It is known that the melanophores of lower vertebrates are considered extremely sensitive in their responses and exhibits a profoundly coordinated and prompt ability to respond to external factors [26]. Melanophores are known to respond to light directly in diverse animals [27, 28, 29]. Therefore, responses due to photoreception at the cellular level can be easily detected by visible movements of melanosomes in the cytoplasm. Putting forth, it is definite that the responses exhibited by the melanophores are the explicit result of the irradiation. However, it is contemplated to ascertain whether UVB radiation has caused any irreversible damage to the melanophores and that time of exposure which posed maximum damage to the cells. To test the physiological viability of melanophores after each irradiation; the skin pieces were re-immersed in fresh ARS. After repeated careful washings, the pieces were observed again under the microscope and their MMSI calculated. The purpose of re-immersing the skin pieces in ARS is to provide physiologically supportive conditions to the cells and to check if they are physiologically active. The cells which are viable revive back to their original state. This step provided data to reveal the most critical time of UVB exposure which caused maximum damage to the cells. During the course of study photographs were taken to capture the striking changes displayed by cells. Also petridishes were aspirated frequently and the ARS pH monitored.

The experiments were repeated in the sets of 9 with different animals and the MMSI was calculated. The calculated MMSI values of all the 9 sets of experiments for the controls at a given time duration and the treated melanophores were finally compared and interpretations of the results were done.

RESULTS

The overall effect of UVB exposure taken together for a period of 120 minutes yielded interesting results. The striking changes on the melanophores at different time intervals of UVB irradiation displayed that the melanophores at first show pigment dispersion most likely as a mechanism for defense but as the duration of irradiation proceeds the pigment granules aggregated. The aggregation attained a state of complete contraction. The irradiation time of 30 minutes and more exhibited maximum changes in melanophore responses.

The comparative responses (in terms of MMSI) of the untreated (control) and treated melanophores with respect to increasing duration of UVB irradiation are summarized under:

Figure A

Time response curve depicting the effect of UVB irradiation on the dorsal skin melanophores of Hoplobatrachus tigerinus

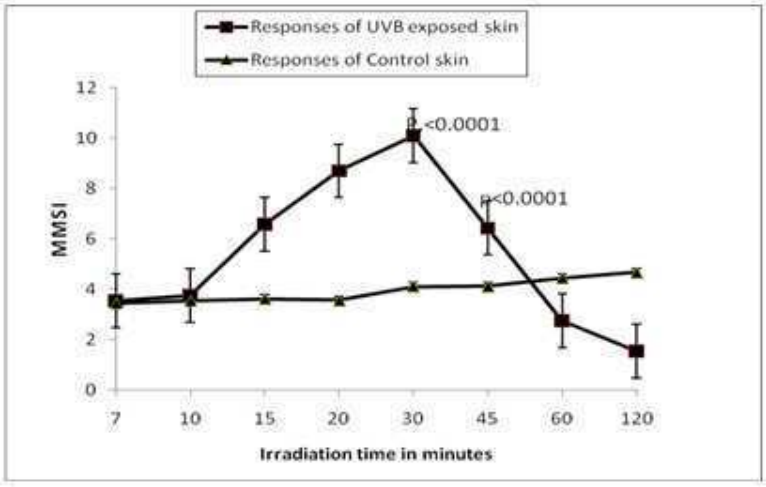

Figure A. Time response curve depicting the effect of UVB irradiation on the dorsal skin melanophores of *Hoplobatrachus tigerinus*. The responses of skin exposed to UVB radiation at varying duration are shown by (■) closed squares. The responses of control skin melanophores without any UVB exposure are shown by (▲) closed triangles. Abscissa: Irradiation time in minutes. Ordinate: Responses of melanophores (MMSI). Vertical bars represent standard errors. $p<0.0001$ signifies level of significance

a. After 7 minutes of UVB irradiation, the melanophores appeared unaffected. The value of MMSI (3.54 ± 0.063) as compared to the control (3.45± 0.087) (Fig 1) MMSI was marked without significant difference. (Fig 2)

Melanophores at Control Position

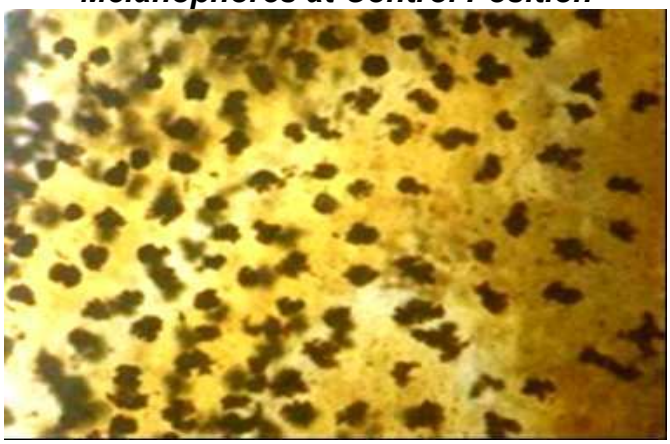

Figure – 1

Control: melanophores without any UV treatment

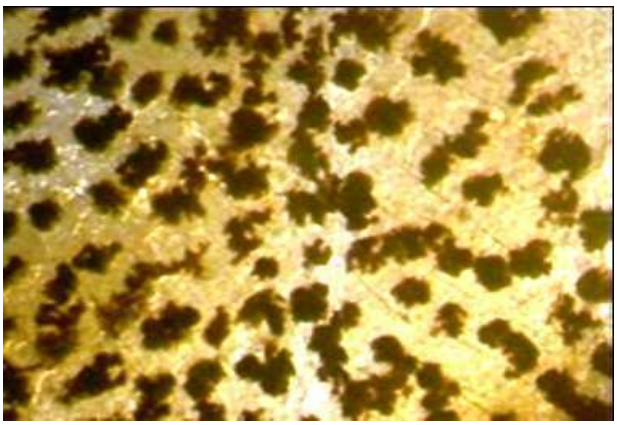

Figure - 2
Melanophores with UV-B Irradiation time 7 minutes (t=7)

b. After 10 minutes of UVB irradiation, the melanophores looked slightly affected. The pigment granules showed a certain degree of dispersion; however the response was very negligible. This was confirmed with a feeble increase in MMSI (4.69 ±0.038) from the control value at that given time (3.55± 0.062). (Fig 3)

Irradiation Time 10 minutes

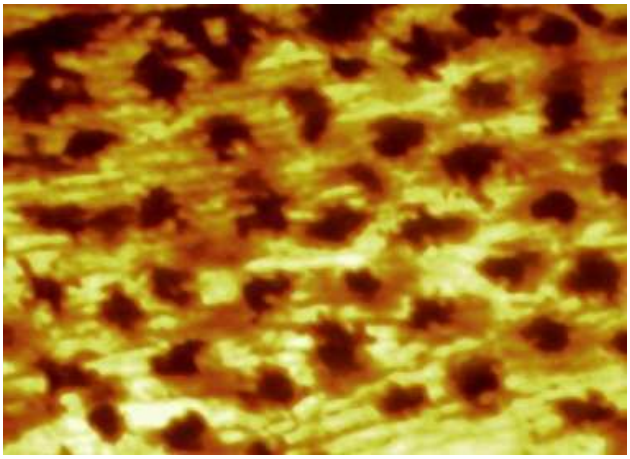

Figure 3
Melanophores with UV-B Irradiation time 10 minutes (t=10)

c. After 15 minutes, the melanophores continued to show dispersion with the MMSI (6.57± 0.037) on a gradual increase from the control (3.63±0.064). (Fig 4)

Irradiation Time 15 minutes

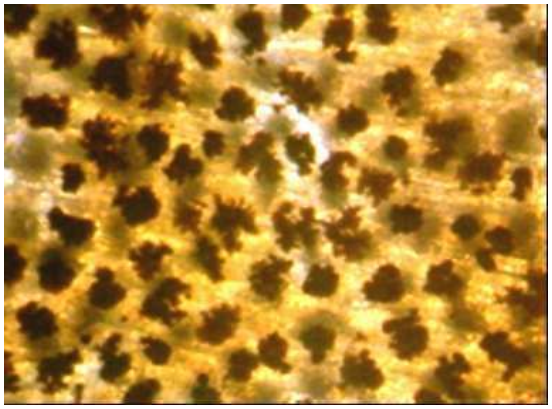

Figure - 4
Melanophore with UV-B irradiation time 15 minutes (t=15)

d. After 20 minutes, the dispersion continued. At this time, the melanophores appeared to be expanded and the dendritic processes dilated outwards. This stage is referred as "Stellate". The MMSI reached (8.7±0.144) with respect to control (3.57±0.078) (Fig 5).

Irradiation Time 20 minutes

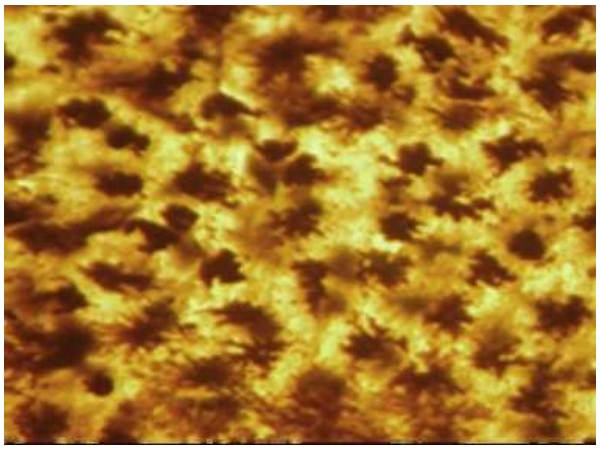

Figure - 5
Melanophores with UV-B Irradiation time 20 minutes (t=20)

e. After 30 minutes, the dispersion kept on growing and the MMSI reached the value of (10.1±0.098) and control value (4.11±0.013). At this duration, the melanophores showed relatively prominent transition from the control. The cells were re-immersed in fresh ARS (10-12 minutes) and again examined. The dispersed melanophores showed regression in their size index after re-immersion and the MMSI was recorded as (4.50± 0.035) (Fig 6).

Irradiation Time 30 minutes

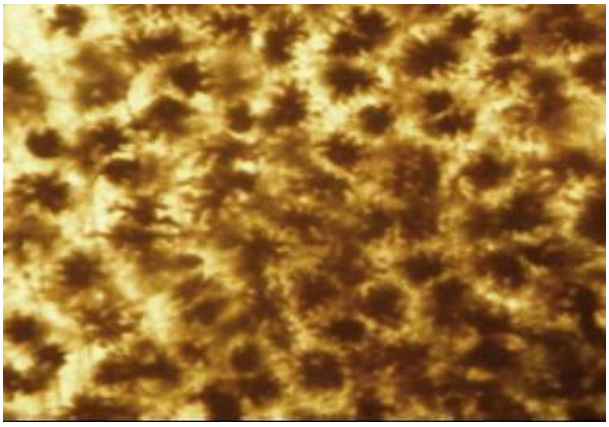

Figure - 6
Melanophores with UV-B Irradiation time 30 minutes (t=30)

f. After 45 minutes, it was surprising to see that the melanophores started to revert back into their peri-nuclear spaces. This regression in the pigment granules within the melanophore was marked as a very interesting development. The MMSI was calculated at (6.43±0.162). The probable outcome of UVB irradiation at this duration could be a critical point. Therefore, we re-immersed the cells in fresh ARS (10-12 minutes) and after several washings we found that the cells could revive back to the control state. The re-immersed cells had the MMSI of (4.46±0.120), suggesting that the cells were physiologically viable. (Fig 7).

Irradiation Time 45 minutes

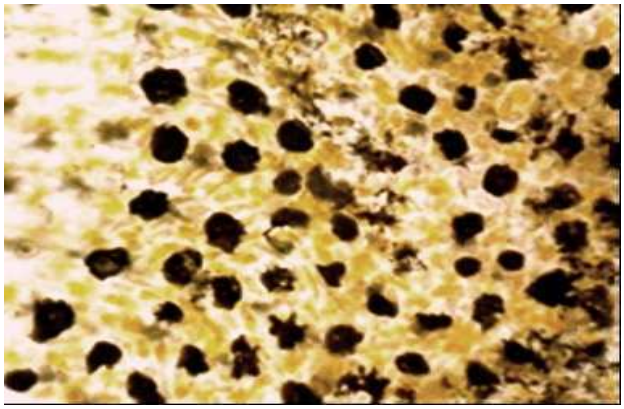

Figure – 7
Melanophore treated with UV-B irradiation time 45 minutes (t=45)

g. After 60 minutes, the melanophores were observed to be further aggregated. The aggregation implied to be of high order as the melanophores appeared like tiny dots. This state is referred as punctuate state. The MMSI was calculated at (2.75±0.098). Thereafter, the cells were re-immersed in ARS. This time the MMSI of the re-immersed

cells scaled remarkably lower than the control (2.80± 0.0378), and the cells did not revive back to the control position, indicating that the melanophores have been physiologically affected. (Fig 8).

Irradiation time 60 minutes

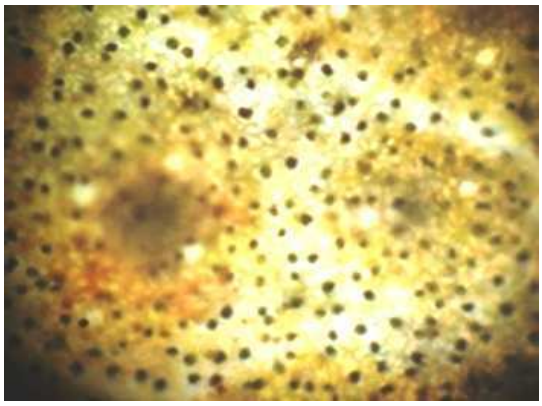

Figure – 8
Melanophore treated with UV-B irradiation time 60 minutes (t=60)

h. After 120 minutes, the cells appeared much distorted and their appearance looked significantly afflicted. The MMSI calculated was (1.55±0.121) from the control (4.67±0.325). (Fig 9)

Irradiation Time 120 minutes

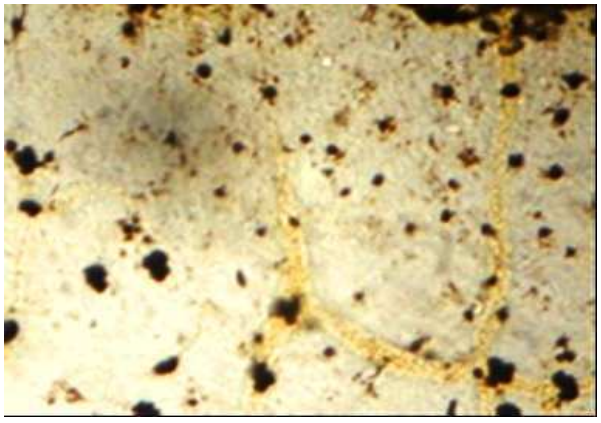

Figure – 9
Melanophore treated with UV-B irradiation time 120 minutes (t=120)

Now, at this stage the massive aggregation brought by the UVB irradiation was a point of concern and the physiological viability of the cells was in question. Therefore, the cells were re-immersed and found that they had completely lost the ability to regain their original state with the MMSI (1.59±0.157). This decidedly was the result of the duration of

irradiation causing permanent damage to the melanophores.

Figure A depicts the comparative responses of Control and UVB exposed skin at different time durations. Figure B depicts the comparison of responses of control; UVB exposed and Reimmersed skin at time 30, 45, 60 and 120 minutes.

Comparative Analysis of responses of control; UVB exposed and Reimmersed skin at time 30, 45, 60 and 120 minutes

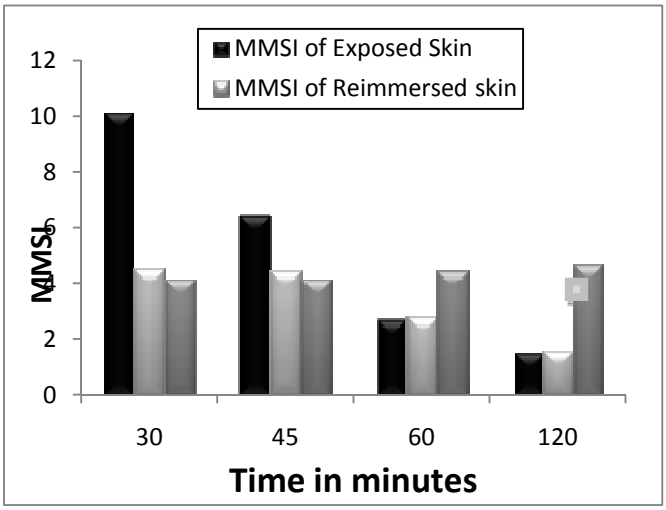

Figure B. Comparative analysis of the responses of dorsal skin melanophores of *Hoplobatcrachus tigerinus*. UVB exposed skin shown by (■) black columns. Re-immersed skin is shown by (■) lighter grey, and the control unexposed skin is shown by grey color (■).

Abscissa: The time in minutes. Ordinate: Responses of melanophores (MMSI). It showed the maximum changes in melanophore responses. Note the decline in the MMSI of the UVB exposed melanophores from the exposure time of 30 minutes to 120 minutes. The melanophores at 120 minutes showed maximum aggregation. However, the trends in

the re-immersion buffer shows that after 30 and 45 minutes the melanophores remain physiologically active as they revert back to their control position. But at 60 and 120 minutes of exposure after re-immersion the

melanophores remain in their aggregated state, indicating their inactivity.

DISCUSSION

One of the cardinal causes of worries in today's ecology is to figure out the factors that limit the distribution and abundance of organisms [30]. The most alarming factor, being the range reductions and population declines of many organisms, gives rise to ecological imbalance and an overall loss in biodiversity [31, 32]. As part of this "biodiversity crisis," many amphibian populations have been declining and undergoing range reductions [33-39]. Due to the great ecological importance of amphibians in the environment, UV effects on the post

metamorphic stages have been the subject of concern in view of the expanding ozone hole in the stratosphere. Even though ample amount of reports are available on the influence of UV radiation on eggs and embryonic stages of amphibian, studies focused on the later stages of the amphibian have not been substantially undertaken so far with a few reports [52]. It is a known fact that melanin pigment within the cells has an innate capacity to attenuate the effects of UV [40], but how long can that effect be curbed was interrogated.

The amphibian *H tigerinus* is one of the most commonly found anuran in the Indian subcontinent and holds a very significant position in the food chain. Also this particular species has been included in Schedule IV of the Indian Wildlife (Protection) Act, 1972 (as amended in 1991), owing to the subsisting decline in population over the years. The results of our experiments are especially relevant regarding the natural habitat conditions of Indian bullfrogs. The adult as well as the juvenile frogs are exposed to prolonged levels of solar radiation after their emergence from hibernation in spring to early fall.

The effect of UVB studied on the dorsal skin melanophores of the amphibian reveals that it does have a defense mechanism to attenuate the effect of UVB, which is displayed by dispersion of pigment granules within the cells with respect to the increase in UVB duration(up till 30 minutes). Interestingly, it was reported by Gloster and Neal [41] that dark skinned mammals, which contain more eumelanin than fair skin was better, protected against UV-induced damage. In general, mammals with darker skin are less prone to UV-induced skin damage than those with lighter skin [42]. This relationship has not been well studied in amphibians. This finding holds significance in our case study and it can be assumed that the pigment dispersion as exhibited by longer duration of UVB exposure could be a protective phenomenon to maintain the integral state of cells from damaging UV rays. Jablonski [43] suggests that melanin production may protect developing amphibian embryos from neural tube defects by acting as a natural sunscreen. It is suggested that melanin is relatively an inexpensive way to prevent critical metabolites, such as folate, from being degraded by UV light during development. Other evidence suggests that UV-B irradiance may induce skin darkening in some amphibians (embryonic and larval *Hyla versicolor* and *Xenopus laevis*, [44] larval *Hyla arborea*, [45]. All these findings strongly support our results. However, the longer duration i.e. beyond 45 minutes, UVB irradiation posed quite detrimental effects on the melanophores and consequentially ended up into their complete rupture and apoptosis. Interestingly it has been reported by Zhai *et al*.[46] that in pure cultures melanocytic cells undergo characteristic apoptosis after physiologic UV exposures. The causes are still unknown. The melanophore aggregating effect of UVB radiation is seen at longer duration. It indicates that photo inactivation at the cellular level brings about a change in the melanin pigment driving machinery. This melanin aggregation effect may be due to stimulation of the adrenergic nerves which may liberate adrenaline or nor adrenaline as a neurotransmitter at the nerve melanophore junction, which causes the melanophore aggregation. It is also understood that adrenaline is released at the time of stress response [47]. This speculation needs further clarification. It is assumed that UV rays structurally alter the molecular components in the outer and inner leaflets of the melanophore nerve membranes, the site of which may be rather the nerves than the receptors. Earlier findings of Spaeth [48] and Fujii *et al*. [49] reported aggregation of melanophores of the teleost fish, *Fundulus* and *Chasmicthys gulosus* by UV radiation respectively. Also recently we found UV radiation to cause melanophore dispersion

in fish, *Channa punctatus* [50]. The contrasting change in the melanophore response is probably due to species difference and offers an interesting example of phylogenetic variation and development of lower species onto higher ones.

Our findings suggest that UVB rays are fairly attenuated by pigment melanin as a protective phenomenon. However, chronic exposure, with the increase in duration of exposure i.e. after 45 minutes resulted into irreversible damage and rupture. The cause of melanophore apoptosis needs to be further investigated. Nevertheless, our results add to the increasing experimental evidence that amphibians are harmed by UV-B radiation.

Moreover, it has been reported that UVB radiation causes skin sores and cataracts in the tadpoles of *R. aurora* and *H. regilla* [51].Animals with cataracts are incapable of forming a clear image in the retina due to scattering of light within the lens, hence their foraging and predator avoidance capabilities are greatly reduced. In addition, the skin sores can be infected by parasites, thereby further increasing tadpole mortality. In our present finding, the damage caused to melanophores is due to enhanced UVB exposure that would surely render the amphibians susceptible to the UV rays and may lead to greater adversities like skin sores, dermal infections and even carcinogenesis resulting into premature mortality.

CONCLUSION

The world is witnessing an alarming decline in amphibian population. There are several underlying factors acting alone or in combination. One contributing factor appears to be increasing is UV-B radiation. UV-B radiation has been reported to cause serious effects at the embryonic, larval as well as the adult stages of amphibians. Although the effects of UVB radiation have been quite detrimental, there have been mechanisms of defense which allow them to cope with UV-B radiation. These include behavior changes, avoidance of the areas of high UV, pigments, jelly coats that surround eggs, and mechanisms of repairing UV-induced DNA damage. Nevertheless, these coping mechanisms against UV-B radiation in many species may not be adequate owing to the persistent, inescapable, global, anthropogenic changes in UV-B levels. It is therefore suggested that UV-B radiation will have significant inimical and deleterious impact on populations of amphibian species that are especially vulnerable to UV-B radiation. The consequential abatement of UVB sensitive species would result into definite imbalance within the ecological community.

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