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Original Article

Melatonin effects on the melanophores in adults and tadpoles of *Rana cyanophlyctis* (Schneider)

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Abstract

Introduction: Effects of melatonin (MT) were comparatively examined on melanophores of isolated skin in adults and tadpole's tailfin of a frog *Rana cyanophlyctis*. MT is generally considered as a potent melanophores aggregating hormone besides regulating the sleep wake cycle in vertebrates.

Methods: Melanophore size index (MSI) was chosen as a recording parameter of the responses. Concentration-response curve was obtained by application of MT to the frog skin. Against this MT, antagonists were employed to observe their blocking effects on aggregatory responses of frog melanophores.

Results: MT has induced aggregation in a wide dose-range on spotted and non-spotted regions in adults as well as in the tailfin of tadpoles. MT induced aggregation was somewhat independent to the applied concentrations of MT and beyond the dose 4.31×10^{-8} M of MT, aggregation of melanophores was decreased. Phenomenons of auto-desensitization and auto-antagonism have been observed. For tadpoles, the sensitivity to MT was higher than that of the adult skin melanophores as evident with the lowest threshold dose of MT to induce a discernible response. MT induced aggregatory responses were effectively inhibited by the specific MT antagonists luzindole and K-185 and also by the Ca^{++} channel blocker verapamil. Seasonal variation in inhibition of MT receptors by K-185 is being reported in this species.

Conclusion: Tadpole melanophores of *Rana cyanophlyctis* were more sensitive towards MT aggregation than their adult counterparts. Seasonal variations and auto-desensitization are all expressed through the specific MT_1, MT_2 receptors and Ca^{++} channels.

Keywords:
Autoantagonism;
Autodesensitization;
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Melanosomes;
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Introduction

Melanophores are the melanin containing chromatophores that appear black or dark brown in color. In adult amphibians, melanophores represent a relatively small percentage of total chromatophore population, but a much higher relative number is found in early developmental stages in young tadpoles (Bagnara, 1966).

Physiological color changes are introduced by light in dermal melanophores either through a direct action on light-responsive melanophores or indirectly by inducing hormonal changes affecting melanophores appearance. Melanocyte stimulating hormone (MSH) mediates light induced dispersion of pigment

granules, while melatonin (MT) induces a rapid aggregation of melanin containing pigment granules in perinuclear zone resulting in blanching of the cell. Exposure to light reduces MT concentration within tissue fluids causing dispersion of pigment granules and darkening of the cell (Vanecek and Klein, 1992; Vanecek, 1998).

In amphibians, MT has been reported as a potent lightening agonist at 1.6×10^{-9} M and completely reversed in a dose-dependent manner the MSH-induced darkening of *Rana pipens* skin. In toads, MT in a wide range of concentrations from 2.5×10^{-10} to 10^{-6} M behaved as a dose-dependent lightening agonist in *B. ictericus* skins previously darkened with alpha-MSH (Filadelfi and Castrucci, 1994). Within these melanophores, pigment granules are translocated along microtubules either towards or away from the cell center. Aggregation causes most of these pigment melanosomes to concentrate towards cell center resulting in animal to appear lightly colored. While a uniform dispersion of the granules within those mealnophores causes the animals to appear dark in color (Haimo, 1998).

Investigating with subtypes of MT receptors, Sugden et al., (1999) revealed that the drug K185 is devoid of agonist activity, but acts as a competitive antagonist for MT_2 receptors in amphibian melanophores. Likewise for the last 60 years since MT discovery, several chemicals have also been identified to act as MT agonists like GR128107 which induces a rapid pigment aggregation in a clonal line of *Xenopus laevis* melanophores through the MT_1 or MT_2 receptors. Also prolonged *in vitro* treatments decline the potency of MT to produce pigment aggregation due to degradation of MT by deacetylation and subsequent deamination and also there is loss of membrane MT receptors (Teh and Sugden; 1999, 2002).

Color change phenomenon involves either hormonal or neural control of various types of chromatophores but the mechanisms at different levels are not yet fully understood (Sköld et al., 2013). In the tropical fish *R. daniconius* aggregatory receptors may belong to the conventional α-MT type. Dispersion of dorso-lateral and band region melanophores induced by MT in presence of various antagonists and on denervated band region could be the result of activation of β-MT receptors of dispersive nature (Ovais et. al., 2015). Aim of the present investigation was to make a comparative study on effects of MT and its signaling receptors/channels on the spotted and non-spotted region skin melanophores of the adult frog *Rana cyanophlyctis* along with its younger developing counterpart i.e., the tadpole. Along with several investigations, we need to ascertain whether the receptors in tadpoles have attained functional modifications in early age or not with further emphasis on their seasonal variations and auto-desensitization.

Materials and methods

Experiments were performed on isolated skin melanophores of adults and tailfins of tadpoles of the aquatic frog *Rana cyanophlyctis.* This frog is grayish to brownish in color, spotted or marbled with black or dark markings which are present in the form of few scattered fine dots. Experiments were performed on both marbled and non-marbled region melanophores of the skin to observe the effects of various drugs. Frogs were procured from Central Institute of Fisheries Education - Powaarkheda, near Bhopal, M.P. (India). Tadpoles were collected from the same pond from where the adults were collected. They were transported to the laboratory in oxygen packed containers and acclimatized for 48 h in the laboratory. They were transferred to the mesh covered glass aquaria (24”×12”×12” where 12” is normally the depth of aquarium). Water of aquaria was changed twice a week. Sand and plants were affixed in aquaria to match a maximum with the pond habitat. Thermocol floats and hollow pipes were also provided in order to attain a platform away from water or hiding within the shade provided by hollow pipes. Source of light was through lab windows maintaining a natural 12 hour light-dark cycle. Being carnivorous frogs and tadpoles were fed with live grasshoppers of minute to medium size. Healthy frogs of both sex (20-40 mm in length and 10-15 g of body weight) and tadpoles (25-30 mm in total length and 3-5 g of body weight) were randomly selected for experiments. Apparatus and fresh reagents were prepared in advance so that the investigations could begin at 11:00 am. Tadpoles belonged to the stages of 41-44 according to the normal table of development (Mohanty-Hejmadi and Dutta, 1977; Jangir, 1996).

Frogs were pinned and skin from back region and hind limbs was excised and immediately placed in the

frog Ringer solution. Skin was further cut into smaller pieces of 2-3 mm^2. For this study we performed experiments with the frog Ringer solution and 0.8% NaCl solution, but Ringer gave better results than 0.8% NaCl solution therefore, further experiments were performed in the frog Ringer solution.

MT solution was prepared by dissolving it in 3-5 drops of absolute ethanol along with deionized water. A concentration-response curve was obtained by the application of this agonist to the frog skin. Against this MT, antagonists were employed to observe their blocking effects on the aggregatory responses of this frog melanophores. For the sake of brevity and to conserve space only selected figures are given. For each concentration of MT separate Petri-dish was used. Contact time of MT with the skin was 10 minutes. When antagonists were employed, skin pieces were first incubated in the antagonist for 10 min and then MT was added for a further incubation of 10 min. Control as well as treated skin pieces were placed on a glass slide with a little incubation medium and covered with a glass cover-slip. Individual melanophores were measured with an ocular micrometer (Erma, Japan) in low power microscope and mean melanophore size index (MSI) was calculated according to the method of Bhattacharya et al., (1976). Increase or decrease of MSI from the control value represents dispersion or aggregation of the melanophores respectively. Mean values of MSI from 5 different frogs/tadpoles were calculated. Experiments on adult frogs were conducted in the months of April and May at room temperature around 35 °C; however water temperature of the animal aquaria was kept fixed at 20 °C. In the case of tadpoles, experiments were performed during the rainy season of July-August.

Drugs used

Following drugs and chemicals were used: Melatonin (MT, Aristo-Pharma Pvt. Ltd. Mumbai, India), Luzindole (Luz, Sigma, USA), K-185 (N-Butanoyl 2-(5,6,7-trihydro-11-methoxybenzo [C] cyclohept [2,1-a] indol-13-yl) ethanamine) (Sigma, USA) and Verapamil (VPL, Samarth Pharma Pvt. Ltd. Mumbai, India).

Statistical analysis

Statistical analysis was performed with the Student's t-test.

Results

Effects of MT were observed on the spotted and non-spotted region skin melanophores of the frog *Rana cyanophlyctis*. The control size of the melanophores in spotted region was found to be larger than that of non-spotted region. Further, in the non-spotted area, agonist and antagonists treated melanophores show their magnitude or MSI values to be significantly lower than the spotted region melanophores.

Experiments were also performed on tailfin melanophores of tadpoles for a detailed study on the effects of MT. Melanophores were treated in different

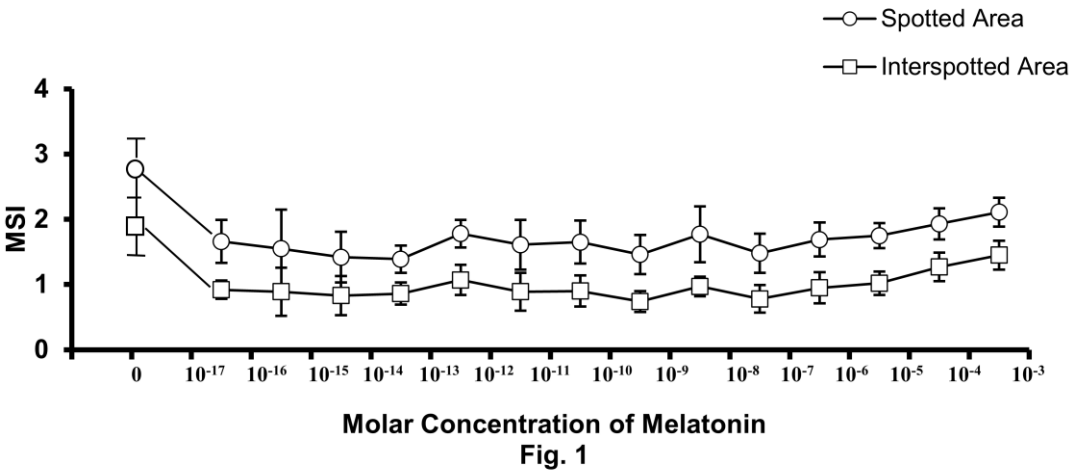

Fig. 1

Fig.1. Graph showing concentration-response curves of melatonin (MT) on isolated skin melanophores of dorsal, spotted as well as inter-spotted region of the frog *Rana cyanophlyctis*. Abscissa: Molar concentrations of MT. Ordinate: Responses of melanophores as melanophore size index (MSI). Each point is the mean SE (vertical bars) from experiments on different frogs, n = 5.

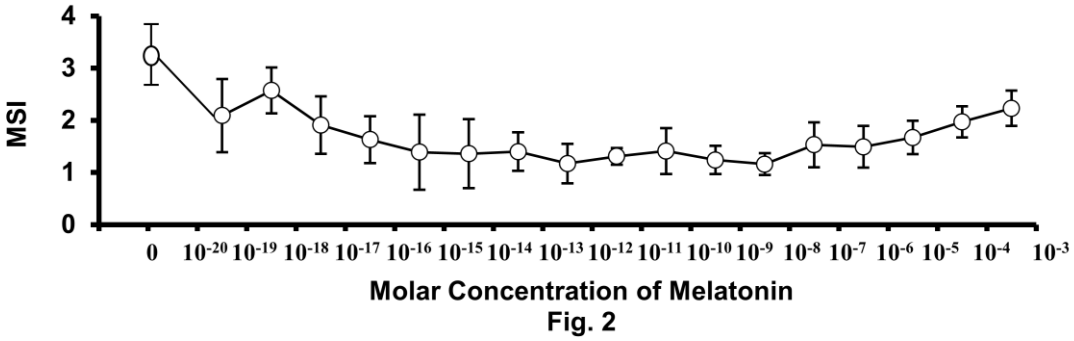

Fig.2. Graph showing concentration-response curves of MT on isolated skin melanophores of dorsal region of tadpole larvae of *R. cyanophlyctis*. Abscissa: Molar concentrations of MT. Ordinate: Responses of melanophores as melanophore size index (MSI).

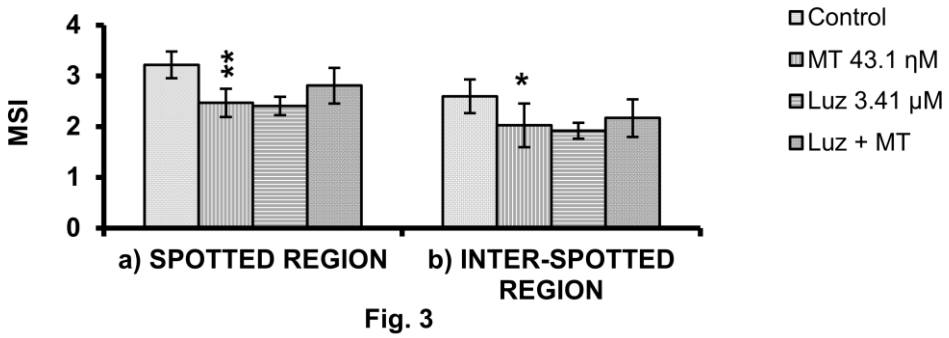

Fig.3. Histograms showing the effect of MT (43.1 ηM) and of Luz (3.41 μM) + MT (43.1 ηM) on both spotted (a) and inter-spotted (b) regions. *P* values were calculated between concentration-response histograms of MT and pre Luz treated melanophores response towards the agonist MT. ***** *P*< 0.05, ***P*<0.01

concentrations and concentration-response curves of melanophores of spotted and non-spotted regions were plotted separately. To find out the site of action of MT in these frog melanophores, several specific and non-specific MT antagonists were employed. MT in a dose-range of 4.31×10^{-17} M to 4.31×10^{-4} M has induced aggregation in the frog melanophores. The aggregation was somewhat independent of the concentrations employed, increase in concentration resulted in gradual inhibition in the sensitivity of melanophores to MT and the highest concentration of MT (4.31×10^{-4} M) gave the lowest degree of aggregation (Fig. 1).

For tailfin melanophores of tadpoles, the threshold dose of MT to induce a discernible response was much lower than that of the adult frogs' skin. MT in its dose-range of 4.31×10^{-20} M to 4.31×10^{-4} M has consistently induced aggregation in the tailfin melanophores of tadpoles. MT up to a concentration of 4.31×10^{-13} M has induced aggregation in a somewhat dose-dependent manner. Further increase in the concentrations of MT gradually inhibited the degree of aggregatory responses of the tailfin melanophores (Fig. 2) which is an example of auto-desensitization. Results confirm that MT induced aggregation in adults as well as in tadpoles is considerably variable in threshold doses to induce discernible response and sensitivity of spotted and non-spotted area melanophores to MT is certainly variable.

Luzindole (Luz) 3.41×10^{-6} M (i.e., 3.41 μM), the general MT_1/MT_2 receptor antagonist, was selected to block the effect of MT 4.31×10^{-8} M (i.e., 43.1 ηM). Luzindole inhibited aggregation in melanophores of spotted and inter-spotted regions. In spotted region, aggregation induced by MT was partially inhibited by luzindole up to 10-15% (Fig. 3a). Whereas in the case of inter-spotted region, blocking effect of luzindole is lower (up to 8-10%) than the spotted region melanophores (Fig. 3b).

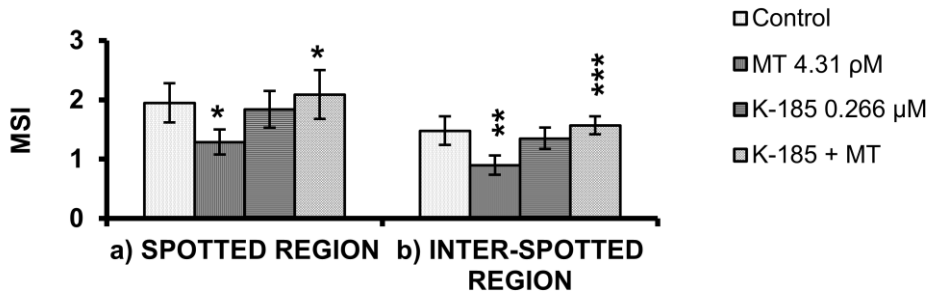

Fig. 4

Fig.4. Histograms showing the effect of MT (4.31 ρM) and of K-185 (0.266 µM) + MT (4.31 ρM) on both spotted (a) and inter-spotted (b) regions. *P* values were calculated between concentration-response histograms of MT and pre K-185 treated melanophores response towards the agonist MT. * *P*< 0.05, ** *P* < 0.01, *** *P* < 0.001

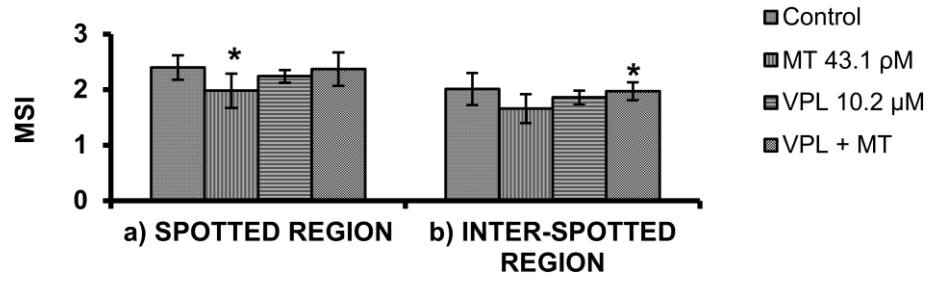

Fig. 5

Fig.5. Histograms showing the effects of MT (43.1 ρM) and of VPL (10.2 µM) + MT (43.1 ρM) on both spotted (a) and inter-spotted (b) regions. *P* values were calculated between concentration-response histograms of MT and pre VPL treated melanophores response towards the agonist MT. * *P*<0.05

K-185 the specific MT_2 receptor antagonist was also employed in this study. Two sets of experiments were performed for this drug in which there was an interval of 20 days. In the first set of experiments, K-185 completely inhibited the aggregation induced by MT 4.31×10^{-12} M (i.e., 4.31 µM) (Fig. 4a and b). K-185 *per se* also induced aggregation of the frog melanophores but the degree of aggregation was less than that of luzindole as described above. In the second set of experiments, which was performed after 20 days of previous experiment, K-185 2.66×10^{-7} M (i.e., 0.266 µM) inhibited the aggregation induced by MT in two concentrations 4.31×10^{-14} M and 4.31×10^{-12} M. In this experiment the aggregatory responses were not completely blocked i.e. the antagonist partially inhibited the aggregation of the melanophores of both the regions up to a level of 30-35%.

Verapamil 1.02×10^{-5} M (i.e., 10.2 µM) the calcium channel blocker partially inhibited the aggregation of the spotted and inter-spotted region melanophores of this frog. In this experiment only in inter-spotted region the inhibitory effect of verapamil was significant (Fig. 5a and b).

Discussion

Melatonin on *R. cyanophlyctis* isolated skin melanophores has induced significant aggregatory responses. MT induced aggregation was somewhat independent to the concentration of MT applied. In teleosteans, amphibians and reptiles MT induced effects may vary with species, dose concentrations, their developmental stages and location of pigment cells (Filadelfi and Castrucci, 1994; Teh and Sugden, 2001). In earlier studies on various amphibian melanophores, it was found that the effect of MT was concentration related, therefore, many bioassay techniques using amphibian melanophores have been developed (Lerner et al., 1959; Lerner and

Wright, 1960; Szmuskovicz et al., 1960; Camargo et al., 1999). Ontogenical changes due to the hormone MT have been found in amphibian *Xenopus laevis*. Color pallor results from stimulation of the pineal by absence of light accompanying a release of MT or a similar melanophore contracting substance (Bagnara, 1963; Bagnara and Hadley, 1973a,b). In frogs, MT causes melanin granule aggregation only within a small number of localized dermal melanophores and is without any effect on iridophores (Hadley and Bagnara, 1969). It was proposed that the 5-methoxy group on indole ring of MT could be essential for the hormonal action over specific receptors for MT in the melanophores (Fujii and Miyashita, 1978). Thus we find that rapid physiological color change has been a principal research subject for cell physiologists (Sköld et al., 2016).

In the present study the threshold dose of MT, which elicited a discernable response in adult frogs was 4.31×10^{-17} M while in earlier works of Lerner and Wright (1960) the minimum quantity of MT causing aggregation on frog skin was 1×10^{-12} M. Studies have found that 10^{-11} M and 10^{-9} M of MT caused a concentration related aggregation in *Xenopus laevis* isolated melanophores (Teh and Sugden, 2001). Earlier studies mentioned above and the studies done on MSH have induced dispersion where MT gave concentration related aggregation of the amphibian melanophores (Filadelfi and Castrucci, 1994). Here, the effects of MT were investigated separately on spotted and non-spotted area skin melanophores of the frog which has not been reported by earlier workers so far in the literatures.

MT induced aggregation was found to be somewhat independent to the applied concentrations of MT and beyond the concentration of 4.31×10^{-8} M the aggregation of melanophores was decreased, this phenomenon too has been described earlier by a few workers as auto-desensitization and auto-antagonism (Filadelfi and Castrucci, 1994; Sherbrooke et al., 1988; Rollag and Lynch, 1993)

It was discovered that sensitivity of tailfin melanophores of tadpoles was much higher than that of adult frog melanophores towards MT. Present results are in agreement with earlier reports, which show that sensitivity of the frog melanophores gradually decreases as the tadpoles transform into adult frogs (Bagnara, 1964). Further, current study shows that phenomenon of auto-desensitization has been observed in developmental stages too. Hence, this phenomenon may be of universal occurrence in the amphibian species.

Luzindole, a MT receptor antagonist (Dubocovich, 1988), has partially antagonized aggregation of the frog melanophores induced by MT. Blocking effect was a little higher in spotted area than the non-spotted area melanophores. Results indicate that effects of MT are partially mediated through the activation of specific MT receptors; hence, the presence of MT_1 and/or MT_2 receptors in both the region melanophores is confirmed in this study. In a recent study conducted on *L. rohita*, luzindole showed a profound blocking effect (up to 143.93%) of MT on all its concentrations employed (Mubashshir et al., 2011). A study on a fish *G. morhua* (Aspengren et al., 2003) showed that luzindole inhibited the MT induced aggregation of melanophores up to 90% in that fish. Our study further revealed that there also exists a difference in the level of antagonism between spotted and non-spotted region melanophores; therefore, population of luzindole responsive melanophores can be said to be variable.

Another MT_2 specific receptor antagonist K-185 (Faust et al., 2000) was also employed to find out the antagonistic effects if any. These experiments were performed in two different sets and second set was performed after 20 days of the first set of experiments. It was observed that in first set of experiments, K-185 inhibited the aggregatory effects of MT on some individual frog melanophores up to 100%, while in second set of experiments this blockade was up to 30-35% only. The results of these experiments indicate that K-185 is a better antagonist, getting a strong support from the results of Sugden et al., (1999), on these frog species melanophores than luzindole and the proportion of MT_2 receptors can be more than MT_1 receptors as the findings by Teh and Sugden (1999, 2002). Results of the second set of experiments indicate that there exists a great variability in this type of experiments which may be either due to individual variations within frogs or by the change of environmental conditions, because in the present case temperature has drifted a bit from April to May (i.e. 35 - 40 $^{\circ}$C). Finally, this change in sensitivity of melanophores to the antagonist may be attributed to the seasonal variations in sensitivity towards MT receptors which has also been reported in the fish

Zacco temmincki (Takabatake et al., 1986).

Calcium channel blocker verapamil employed in its concentrations partially inhibited the MT induced aggregation of the frog melanophores, which suggests that there is definitely a role played by Ca^{++} channels in pigment translocations. These effects have also been observed in melanophores of *G. morhua* where there is involvement of Ca^{++} ions in melanosome aggregation, thus it can be indicated that MT-mediated pathway is highly sensitive to Ca^{++} depletion (Aspengren et al., 2003).

Conclusion

Tadpoles of *Rana cyanophlyctis* possess more sensitive melanophores towards MT aggregation than that of their adult frogs. Spotted regions possess larger melanophores than non-spotted regions. Aggregation of both spotted and non-spotted region melanophores due to MT is being mediated through two subtypes of receptors, MT_1 and MT_2, where concentration of MT_2 receptors can be more than MT_1 receptors in this species. Seasonal variations and auto-desensitization in sensitivity of these receptor subtypes do occur and a free shipment of Ca^{++} ions is necessary for pigment aggregation under the influence of MT.

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Conflict of interest

The authors report no declarations of interest.

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