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Localization of Orexin and its Receptor in Corpus Luteum and *in vitro* Effect of Orexin on Progesterone Production from Luteal Cells in Buffalo

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ABSTRACT

Background: Orexin is a hypothalamic neuropeptide involved in regulating various biological functions, including reproduction. The study aimed to investigate the immunolocalization of orexin and its receptors in the corpus luteum in buffalo (*Bubalus bubalis*) ovary and the effect of orexin on progesterone production.

Methods: The study was carried out on buffalo ovaries, each with a different developmental stage of corpus luteum (CL) (n=10 for each stage). The CLs were separated out from ovaries for immunolocalization of orexin and its receptors in CL. In the *in vitro* study, luteal cells were cultured and treated with recombinant orexin at 0.1, 1 and 10 ng/mL doses for 48h and P_4 concentration in the spent media was determined by ELISA.

Result: Results of the present study revealed that orexin and its receptors are localized in the cytoplasm of large and small luteal cells of CL. The immunoreactivity varied with a stage-specific difference in CL. The present study also revealed that orexin inhibits P_4 production in cultured luteal cells. The findings indicate orexin affects luteal dynamics and reproductive functions in buffalo (*Bubalus bubalis*).

Key words: Buffalo, Corpus luteum, Orexin, Ovary.

INTRODUCTION

Mammalian reproduction consists of the development, differentiation and conversion of ovarian follicular cells, followed by establishing and regressing the corpora lutea (CL) cyclically. The safeguarding of pregnancy is carried out mainly by progesterone (P4), the primary steroid hormone synthesized by CL. Pituitary gonadotropins well regulate the functions of CL. However, a number of local factors have also been found to regulate luteal dynamics (Gupta *et al.,* 2014; Gupta *et al*., 2019; Thakre *et al*., 2021).

Orexin A (OXA) and Orexin B (OXB) are the neuropeptides discovered in the rat hypothalamus by Anwar *et al*. (2008). Both orexins are derived from cleavage of a large molecule, pre-pro-orexin (PPO), having 130-amino acids. The biological actions of orexins are mediated *via* G-protein-coupled receptors: orexin receptor type 1(OX1R) and orexin receptor type 2 (OX2R) (Sakurai *et al*., 1998).

Orexin was identified as a regulator of feeding behavior and energy homeostasis (Sakurai *et al*., 1998). Later on, evidence has also shown the involvement of orexin in the modulation of different endocrine axes, including the hypothalamic-pituitary-ovarian axis (Kaminski *et al*., 2010; Nitkiewcz *et al*., 2010).

Apart from the other organs, orexin, OX1R and OX2R have been detected in the hypothalamus and ovary in porcine (Ciccimarra *et al*., 2018; Kaminski *et al*., 2018), ovary of canine and feline (Levanti *et al*., 2015) and rat ovary (Dobrzyn *et al*., 2018). Expression of orexin has also been documented in the placenta and uterus (Smolinska *et al*., 2015). Pieces of evidence also exist regarding orexin's role on steroid secretion from granulosa cells, theca interna cells and luteal cells (Nitkiewicz *et al*., 2014). The above works suggested the role of orexin in the regulation of reproduction at the central and peripheral level; however, the reports regarding bovine and bubaline species are lacking, hence, the present study was conducted to explore the localization of orexin and its receptors in the corpus luteum (CL) and evaluate the effect of orexin on progesterone production from luteal cells of buffalo.

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MATERIALS AND METHODS

Collection of corpus luteum (CL) and their classification

Buffalo ovaries were collected from a local abattoir and transported on ice within 10-20 min to the laboratory. For separation of CL, ovaries (*n*=40), each with the CL, were used (10 CL per group) for studies. The CLs from each ovary were classified in the following stages of functionality: CL1, early luteal phase (days 1-4); CL2, mid-luteal phase (days 5-10); CL3, late luteal phase (days 11-16) and CL4, regressing CL (days >17) of the estrous cycle (Gupta *et al*., 2019).

Immunohistochemistry

The intact CL of each stage was carefully dissected from the ovaries. Fixation and processing of the slide were performed as per the method described by Gupta *et al*. (2019). Five representative CLs of each group were used for immunohistochemistry. Deparaffinization, dehydration and antigen retrieval and quenching of endogenous peroxidase activity was performed as per Thakre *et al*. (2021). Non-specific background was eliminated by blocking with 3% BSA in PBS for 2 h at 37°C. Sections were probed with primary antibodies (Orexin A, Cat. No. SC-80263 and Orexin R 1/2, SC 166111) at 1:100 dilutions. Sections were washed twice for 5 min in PBS and were incubated for 1-1.5 h at 37°C by Mouse HRP conjugated secondary antibody (SC-516102) and were washed with PBS and stained using an AEC staining kit (AEC 101). In the negative control, isotype IgG replaced the primary antibody. Finally, sections were washed with distilled water and observed under a microscope (Axio Lab A1 from Zeiss). The valuation of immunoreactivity was done in a semi-quantitative method (Thakre *et al*., 2021).

Luteal cell culture

Luteal cells were cultured for determining the effect of orexin on progesterone by the method described by Gupta *et al*. (2019). The minced luteal tissue was washed three times for 5 min at 350×g with a dispersing medium. The cells were dispersed by incubating the luteal tissue in DMEM/F12 medium (Himedia, India) containing 2 mg/mL collagenase I type 1A, 25 µg/ml DNase I and 0.5% BSA Fraction V for 2×45 min in an incubator at 37°C and shaking at 10 min interval. The dispersed cells were filtered through a 70 µm cell strainer and the filtrate was washed twice for 5 min at 250×g with DMEM/F12 media. The supernatant was discarded and erythrocyte lysis was performed with RBC lysis buffer. Cells were resuspended in DMEM/F12 medium containing 10% FBS (Thermo Fisher Scientific) and Antibiotic and Antimycotic solution. After counting cell viability, the cells were seeded in a 24well plate in a humidified CO_2 (5%) incubator at 37.5°C having approximately 1.5×10^5 viable cells per well. The luteal cells were allowed to attach and grow (75-80% confluence) for 48 h. Later on, the cells were maintained in DMEM/F12 media with 1% FBS for 24 h and treated with fresh media (with 1% FBS) containing different concentrations (0.1, 1.0 and 10 ng/mL) of orexin-A and maintained for 48 h. Control cells were grown in similar conditions as other cells except the addition of orexin. Each experimental condition consisted of four replicates. After 48 h, the spent media was collected and stored for P_4 assay and RNA was isolated from cells by TRIZOL reagent and reverse transcribed to quantify StAR, CYP11A1 and HSD3B1 gene.

Progesterone (P_4) ELISA

Concentrations of P_4 in spent culture media of luteal culture were projected using the P_4 ELISA kit (Cat No. 402310) supplied by Neogen Life Sciences, USA. The results are expressed as the concentration of P_4 (ng/mL). The intra- and inter-assay coefficients of variation were less than 10%. Results are given as mean ± SEM. Data were obtained from three independent cultures and each treatment had four replicates.

RNA isolation and RT PCR

Total RNA isolation was carried out from luteal cells using TRIZOL reagent (Invitrogen, USA). DNase I treatment was given to RNA to remove DNA contamination. RT-PCR was used to detect gene expression in luteal cells of buffalo. RNA integrity and purity was checked. The isolated RNA samples were having OD 260: OD 280 values were greater than 1.8. One µg of total RNA was reverse transcribed in 20 µL of final volume of a reaction containing 4 µL 5X reaction buffer, 3 µL $MgCl_2$, 1 µL PCR nucleotide mix, 1 µL RNase inhibitor, 0.5 µL reverse transcriptase, 1.5 µL oligo- (dt 15) primer, 9 µL RNA template (1 µg) + nuclease-free water followed by incubation for 15 min at 50°C and 2 min 30 sec at 42°C and with storage at 4°C.

Primers

The RT-qPCR procedure was used to detect CYP11A1, StAR and HSD3B1 in luteal cells of buffalo. Published primers were used for StAR, HSD3B1 (Kumar *et al*. 2012), β-actin (ACTB), ribosomal protein 15 (RPL15) and cytochrome P450 (CYP11A1) (Gupta *et al*. 2019). Details of the primers used, primer efficiencies and annealing temperature are provided in Table 1.

Quantitative RT-PCR (RT-qPCR) analysis

The quantification of targeted cDNAs was done by real-time PCR using GoTaq® qPCR master mix (Promega, USA) and specific primers in a total volume 15 µL reaction containing 7.5 µLGoTaq® qPCR master mix, 0.5 µL forward primer (0.5 mM), 0.5 µL reverse primer (0.5 mM), 1.0 µL cDNA template at a 50 ng/µL concentration, 5.5 µLnuclease-free water. PCR was performed with specific primer pairs of StAR, CYP11A1, HSD3B1, ACTB and RPL15. The samples were initially denatured at 95°C for 2 min, then 40 PCR cycles were processed denaturation at 95°C for 20 sec, annealing at 60°C for HSD3B1 and RPL15, 62°C for ACTB for 25 sec, extension at 72°C for 30 sec, with a final extension at 72°C for 15 sec and final hold at 4°C. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. The

tissue with the lowest expression (high Ct) was taken as a calibrator. ACTB and RPL15 were utilized as an internal control (reference genes).

Statistical analysis

All experimental data have been shown as mean ± SEM. The statistical significance of the difference in mRNA expression of HSD3B1, StAR and CYP11A1 in cultured luteal cells and P_4 concentration in spent culture media was assessed by one-way ANOVA followed by Duncan's multiple comparison test.

RESULTS AND DISCUSSION

Immunolocalization of orexin-A and Orexin receptor 1/2 (OX1/2 R)

The immunohistochemical localization of orexin A in CL is presented in Fig 1 and immunolocalization of orexin receptor

Table 1: Details of primers, amplicon length and annealing temperature.

Gene	Sequences 5′ -3′	Amplicon length (bp)	Annealing temperature (°C)
β-actin	For: TCTCACGGAGCGTGGCTACAG	100	62
	Rev: CTGCTCGAAGTCCAGGGCCACGTA		
RPL15	For: TGGGCTACAAGGCCAAACAA	140	60
	Rev: GCTTCGAGCAAA CTTGAGCTGG		
STAR	For: CTGCGTGGATTAACCAGGTTCG	84	60
	Rev: CCAGCTCTTGGTCGCTGTAGAG		
CYP11A1	For: AGTTCGAGGGATCCTACCCAGA	146	60
	Rev: AGCCATCACCTCCGTGTTCAG		
HSD3B1	For: GATCATCTGCCTGTTGGTGGA	191	60
	Rev: GTGGATGACCACTGAGGTGC		

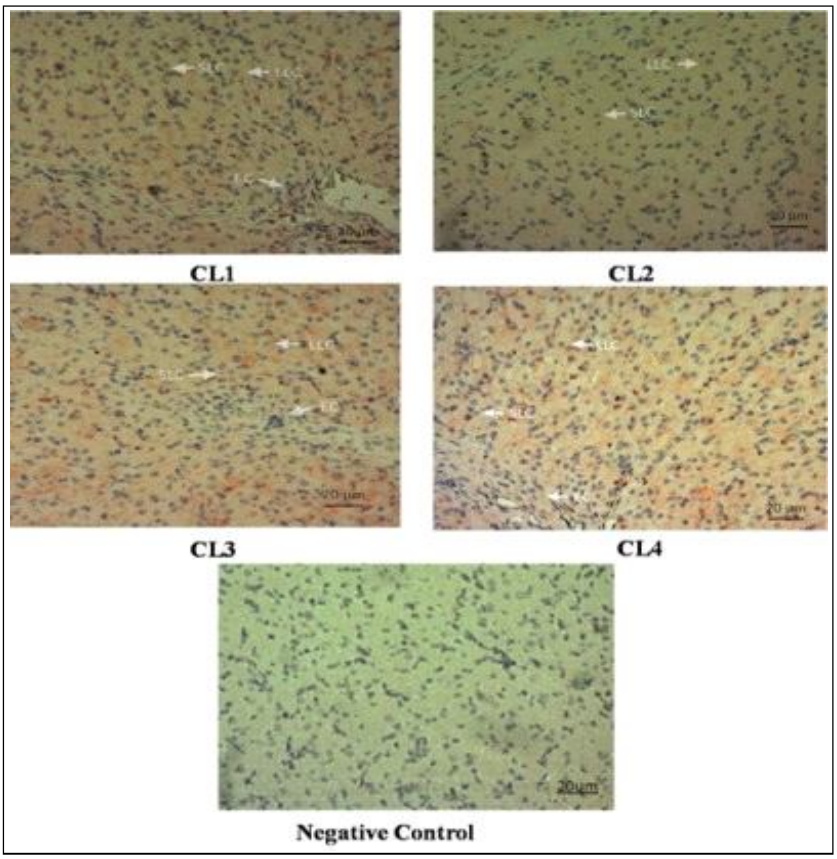

Fig 1: Localization of orexin-A in CL of water buffalo. AEC-immunoperoxidase staining was performed on paraffin-embedded buffalo CL using antibodies against Orexin A in dilution of 1:100. Immuno-specific staining is red-brown. Hematoxylin was used for counterstaining the sections. Orexin A was localized predominantly in the cytoplasm of large luteal cells of early and regressing CL (score 2) while weak/low immunoreactivity (score 1) was seen in mid and late luteal stages. Scale bar = 20 µm.
Abbreviations: LLC, large luteal cell; SLC, Small luteal cell; EC, endothelial cells.

1/2 (OX1/2R) is presented in Fig 2. The orexin and the receptors were localized in the cytoplasm of small and large luteal cells. The reactivity varied with a stage-specific difference. The intensity of immunostaining varied from phase to phase and the number of positive cells was influenced by the different stages of CL. The intensity of immunostaining of orexin and OX1/2R was moderate (score 2) in the early and regressing (CL1, CL4) and was lower (score 1) in the mid and late luteal stages (CL2 and CL3). The negative control showed only a weak background staining (score 0). The result of the present study is consistent with the observation of Ciccimarra *et al.* (2018) wherein immunoreactivity of orexin A was noted in the cytoplasm of luteal cells and for the receptors, it was recorded in nuclear envelope. In another study, orexin A was immunolocalized in the cytoplasm and on the nucleus of luteal cells of CL (Ragionieri *et al.,* 2018). The expression of orexin-A and its receptors in luteal cell is well documented by Nitkiewicz *et al.* (2014) and it was observed that CL showed the highest intensity during the mid-luteal phase (days 10-12) of the estrus cycle. Immunoreactivity in ovarian cells was described as OXA is having local auto or paracrine effects in porcine ovaries as reported by Ciccimarra *et al.* (2018).

Effect of orexin on progesterone (P_4) secretion and StAR, CYP11A1 and HSD3B1expression

The concentration of P_4 in the spent media of the control culture of luteal cells was 2.34 ng/mL. The addition of orexin A to the media 0.1 ng/mL did not affect, however, at 1 and 10 ng/mL orexin-A decreased (P<0.05) P_4 concentration from cultured luteal cells (Fig 3).

The expression of key factors for P4 production *i.e.*, StAR, CYP11A1and HSD3B1, is presented in Fig 4. The relative expression of StAR, CYP11A1 and HSD3B1 in treated samples was compared with that of control samples. The expression of StAR, CYP11A1 and 3β-HSDin cultured cells varied significantly according to the given treatment. mRNA expression of StAR gene was comparable between control at 0.1 ng/mL dose of orexin, however, it significantly decreased (P<0.05) with increased doses. The expression of CYP11A1 was similar to StAR. The mRNA expression of 3β-HSD was significantly (P<0.05) declined in orexin-treated cells in a dose of 0.1 and 1.0 ng/mL and 10 ng/mL, as compared to control. The result of the *in vitro* study is in agreement with the findings observed by Li *et al.* (2019) in ovine where in it was recorded that the expression of StAR,

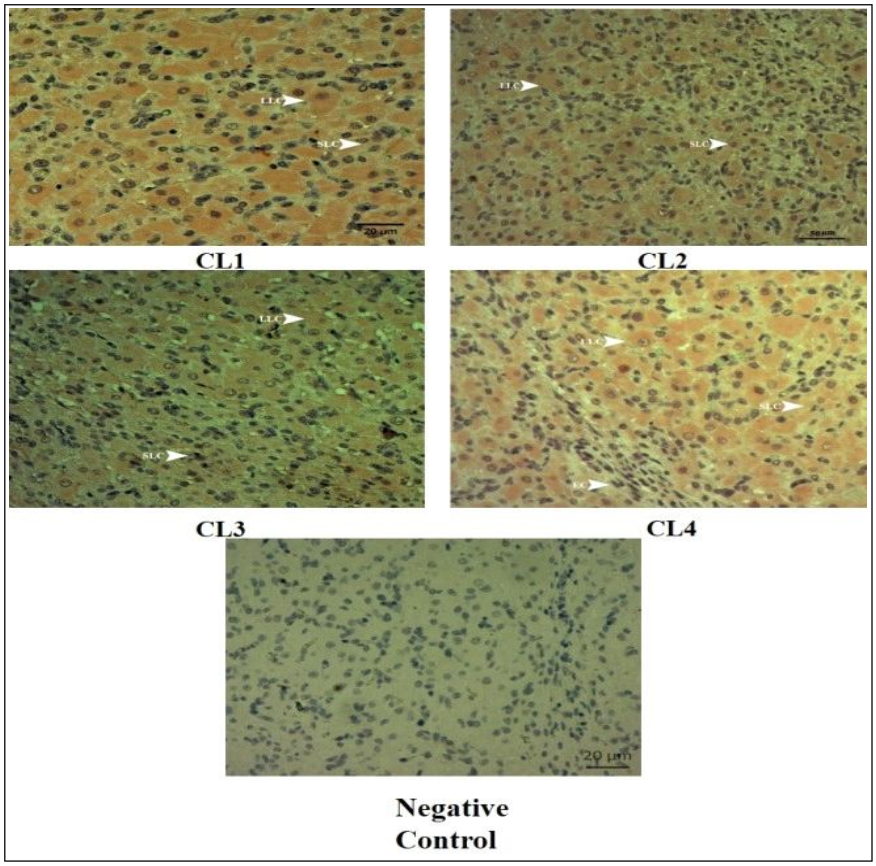

Fig 2: Immunohistochemical of orexin receptor 1/2 (OX1/2 R) in CL of water buffalo. AEC-immunoperoxidase staining was performed on paraffin-embedded buffalo CL using antibodies against OX1/2R in dilution of 1:100. Immuno-specific staining is red-brown. Hematoxylin was used for counterstaining the sections. Orexin receptor was localized predominantly in the cytoplasm of small and large luteal cells of early and regressing CL (score 2) while weak/low immunoreactivity (score 1) was seen on mid and late luteal stages. Scale bar = 20 µm. Abbreviations: LLC: large luteal cell; SLC: Small luteal cell; EC: Endothelial cells.

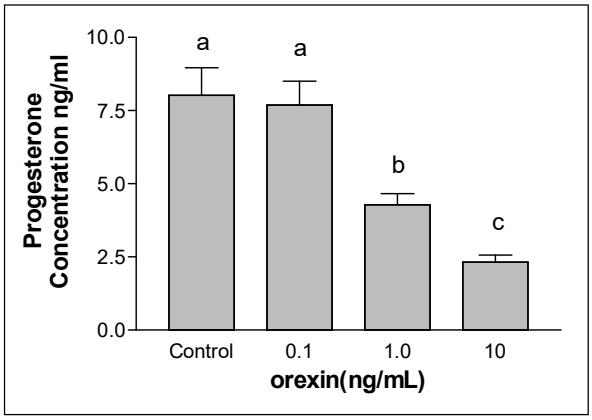

Fig 3: The concentration of progesterone (P_4) in spent media of bubaline luteal cell culture in response to orexin A at the dose of 0.1, 1 and 10 ng/mL for 48 h. Results are means±SEM of four replicates. Different superscripts denote statistically different values (P<0.05).

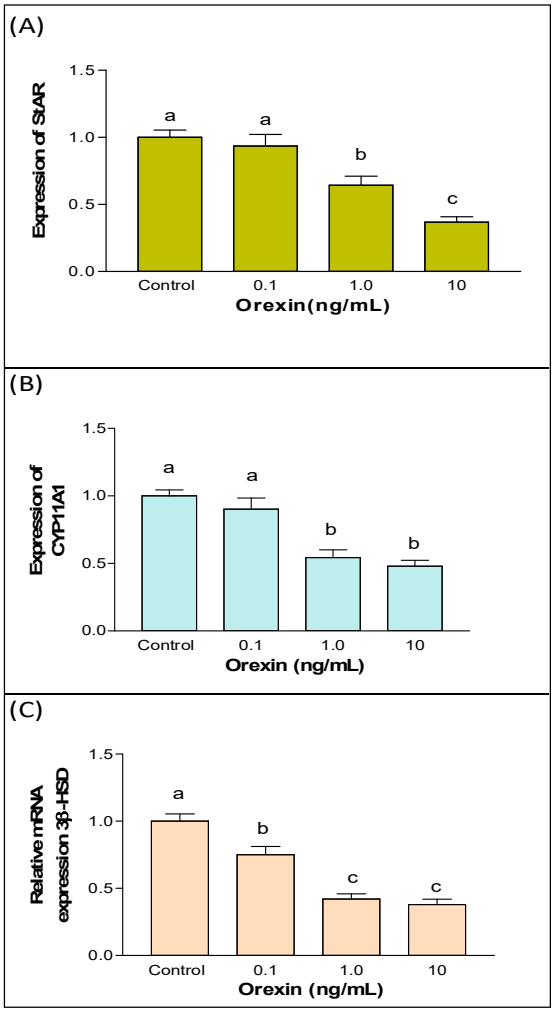

Fig 4: mRNA expression of StAR, CYP11A1and HSD3B1 from cultured luteal cells treated with orexin-A for 48 h at 0, 0.1, 1 and 10 ng/mL in DMEM/F12 medium with 1% FBS (n=4 per dose). Results are shown as mean±SEM. Different superscripts indicate differences between values (p<0.05).

3β-HSD and CYP11A1 decreased with increasing concentration of orexin. However, the present findings do not agree with the observation of Kisielewska *et al*. (2019) in porcine species, in which dose of orexin increases the concentration of 3β-HSD. The decline in P_4 secretion/ production from luteal cells in response to orexin treatment is probably due to a decrease in expression of genes associated with P_4 synthesis (*i.e*. StAR, CYP11A1 and 3βHSD).

It is well known fact that the corpus luteum, ephemeral endocrine structure that grows after ovulation, exerts a crucial role in the hormonal events occurring during the estrous cycle. *In vitro* studies on the effects of orexin in luteal cells of ovary has been carried out earlier in swine Nitkiewicz *et al*. (2014), Ragioniei *et al*. (2018) and in ewes Kirsz *et al*. (2019). In these studies, it was observed that orexin affects steroidogenesis in ovarian cells and the effects varies with concentration. Earlier studies have confirmed that OXA can interfere with steroidogenesis in several tissues (Kiezun *et al.,* 2017). The OXA and its receptors in luteal cells and their role in P_4 production was recorded by Mesen *et al.* (2015). In ewes, centrally administrated OXA decreased the secretion of progesterone in the luteal phase (Kirsz *et al*., 2019). Effect of orexin on P4 secretion from luteal cells of rats (Cataldi *et al*., 2012) and swine (Basini *et al*., 2018) has been recorded. However, Kaminski *et al*. (2010) and Smolinska *et al*. (2014) noted that the porcine ovary had the highest OXA immunoreactivity and higher OX1R expression during luteal phase, suggesting the stimulatory role of progesterone. In porcine species, OXA inhibits P_4 secretion from corpus luteum (Nitkiewicz *et al*., 2014) and similar effects have been observed in rat ovary (Su *et al*., 2008). The expression of P450scc gene was inferior in cells treated with 10 nM OXA, confirming an effect of this molecule on the pathway of P_4 synthesis. Hence, in the present study, the lower level of orexin and its receptors in fully developed and functional corpora lutea could probably help in inhibition of progesterone synthesis during regressing phase. These findings indicate that orexin and its receptors have a directing role in steroid synthesis; however, the effects may vary with species.

CONCLUSION

The results of the present study indicate that the orexin system exists in the buffalo ovaries. The result indicated that orexin and its receptors are present in buffalo CL throughout the estrous cycle. Furthermore, the current study also revealed the modulatory effects of orexin on progesterone production from luteal cells. From the study it can be concluded that orexin has a possible regulatory function in luteal dynamics and reproductive function in water buffalo (*Bubalus bubalis*).

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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