http://jbcp.shahed.ac.ir 10.22070/jbcp.2023.17497.1169

Open Access Research Article

Journal of Basic and Clinical Pathophysiology
Print ISSN: 2322-1895, **Online ISSN:** 2345-4334
Vol. 11, No. 1, June 2023

A Semiannual Publication of
Shahed University Press

The effect of orexin B receptor antagonist and steroid hormones on milk lactose synthesis in the lactating rats

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Abstract

Background and Objective: Prolactin is a necessary factor for lactation and synthesis of milk constituent. Progesterone (P4) and 17β-estradiol (E2) are inhibitory factors for lactation. Orexin is involved in regulating the metabolism and lipid synthesis. Present study investigated the orexin B receptor antagonist and steroid hormone effects on milk lactose synthesis.

Materials and Methods: Thirty Wistar lactating rats were used. Lactating animals in the group 1-4 received saline, 1, 2 or 4 µg of orexin antagonist. Lactating animals of the groups 5 and 6 received 4 µg of orexin antagonist plus 1 µg of 17-β estradiol (E2) or 4 mg of progesterone (p4). Blood and tissue samples were collected at 60 and 180 minutes of injections. Blood samples were measured for prolactin concentrations and tissue samples were examined for alpha lactalbumin (Lalba) and beta-1,4-galactosyltransferase 1 (B4galt1) gene expression in the mammary gland by RT-PCR technique.

Results: Injection of orexin antagonist significantly increased the percentage of milk lactose, plasma prolactin and Lalba gene expression in comparison to control group. Injections of E2 or P4 inhibited the increased effects of orexin antagonist on mean milk lactose percentage, prolactin and Lalba gene expression in comparison to orexin antagonist group. Injections of all drugs did not alter the mean B4galt1 gene expression.

Conclusion: Stimulatory effects of orexin antagonist on milk production may be partly due to the increased prolactin concentration and Lalba gene expression. The mechanism by which the steroid hormones supress the orexin antagonist-induced lactose synthesis may be mediated partly via inhibiting the prolactin production.

Keywords: Orexin B receptor antagonist, prolactin, Beta-1,4-galactosyltransferase 1, Alpha lactalbumin

1. Introduction

It is well established that milk production depends on lactose synthesis of the mammary gland epithelial cells in the most mammals (1-2). Also, lactose synthesis is controlled by the concentrations of glucose, galactose and along with the activation of two key enzymes including alpha lactalbumin (Lalba) and beta-1,4-galactosyltransferase 1 (B4galt1) of the mammary gland epithelial cells (2-3). Many hormones like prolactin, orexinergic and steroid hormones affect the regulation of milk production (2, 4).

Orexinergics are the hormones that their concentration rise in the plasma during negative energy balance. Orexins is recognized as a neuropeptide involved in food intake, energy metabolism lipid synthesis (5-6). Orexins activate various signaling pathways by binding to their G protein-coupled receptors (GPCRs), which are named orexin type 1 receptor and orexin type 2 receptor (OX1R and OX2R, or the hypocretin receptors). Orexin-A binds to OX1R with high affinity, whereas both orexin-A and orexin- B bind to OX2R with equal affinity. Both types of receptors are widely distributed in different organs, such as the hypothalamus, pituitary, and ovaries (5-6). Our previous study showed that the injection of orexin-A decrease the lipid synthesis via deactivation of related enzyme of milk in the lactating rat (7-8).

CCL4 in the liver is metabolized into trichloromethyl radicals by cytochrome P450 with final formation of

Submitted: 21 July 2022**, Revised:** 21 November 2022, **Accepted:** 16 December 2022
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reactive and damaging trichloromethyl peroxy radicals. Such agents alter lipid metabolism and reduce membrane permeability which finally leads to degeneration and necrosis of tissue cells (5).

The increased level of progesterone (P_4) and 17β-estradiol (E_2) suppresses the milk production (9), while ovariectomy is associated with improving lactation (9). Consuming oral contraceptives containing estrogen and progestin inhibit the milk production (9). Exogenous injection of P_4 and E_2 decline milk production and supporting the idea that steroid hormones are important inhibitory factors for lactation (9-10). Prolactin is the most important factor for mammary development, lactation and synthesis of milk constituent in mammals11-12). So that, blocking prolactin synthesis suppress lactation. Also, it has been established that steroid hormones suppress the lactogenic influence of prolactin in mammary gland (9-10). As the effect of orexin signaling pathway on milk production and constituent is not completely clear, the present study aimed to determine whether orexin B receptor antagonist and its interaction with steroid hormones could alter the gene expression of lactose synthesis in the lactation rats.

2. Materials and Methods

2.1. Experimental design

In this experimental study, thirty lactating Wistar rats (weighing 220 to 250 g) were randomly divided into 6 groups. Lactating animals were kept at 22 ± 2°C in a 12 h light/ 12 h dark cycle. Animals had free access to water and food. All procedures for the maintenance and the use of experimental animals were approved by the research ethics committees of Shahid Beheshti University (Code: IR.SBU.REC.1401.125). Lactating animals in 6 groups received either saline, 1, 2, 4 µg orexin B receptor antagonist, 4 µg orexin B receptor antagonist plus 1 µg E_2 or 4 µg orexin B receptor antagonist plus 4 mg P_4 respectively.

2.2. Blood and milk collection

Blood samples were collected at one and three hours after injections. Plasma was collected using sodium citrate solution (40 µl sodium citrate solution/ml blood) before centrifugation to prevent clotting. Plasma was stored at -20 ° C until assayed for determination of prolactin concentration. As previously described, milk samples were collected at one and three hours after injections from the mammary gland of the animals by massaging, Milk samples were kept at – 4 ^{0} C until further assay for the determination of the percentage of milk lactose (7-8).

2.3. Hormone assays

Plasma rat prolactin was measured by a homologous double-antibody radioimmunoassay (RIA). For prolactin assay, prolactin was provided by Tabeshyarnoor Co. (Industrial City of Bu-Ali, Hamadan, Iran). Prolactin was used for iodination. A seven-point standard curve ranging from 0.04 to 10 ng prolactin was used. An average assay binding of 40% was achieved using an initial 1:20000 dilution of prolactin antiserum for prolactin assays. The inter- and intra-assay variations were 6% and 9%. respectively. Milk lactose concentrations were measured by Meyers analyser.

2.4. Tissue collection for Real-time polymerase chain reaction (RT-PCR)

As previously described, the mammary gland tissue samples were collected at one and three hours after injections (7-8). Tissue samples were kept at -80º C until assayed for molecular analysis for alpha lactalbumin and beta-1,4-galactosyltransferase 1 enzymes. Total RNA of samples was extracted based on the acid guanidinium thiocyanate-phenol-chloroform method according to the instructions of the PureZol kit (Bio Rad Co., U.S.A.). Then, 1µg of RNA was used for cDNA synthesis according to the instructions of a cDNA synthesis kit (Thermo Scientific Co., U.S.A). Corbett rotor gene 6000 (Qiagen Co, Germany) and SYBR Green I kit (Takara Bio Inc., Japan) were used to determine the alteration of gene expression levels. The first denaturation 95 Cº for 2 min, followed by 40 cycles of denaturation at 95 Cº for 5 sec, annealing at 60 Cº for 20 sec and extension at 60 Cº for 25 sec was used for PCR cycling. The sequences used for sense and antisense primers were as follows: B4galt1 sense: 5′-TATTTGCATCCAGTCTTTCAGC-3′ and B4galt1 antisense: 5′-CAGCTTAGCTCGATTAAACATGG-3′ (13), Lalba sense:5′-GATGACATAGTATGTGCCAAGA-3′ and Lalba antisense:5′-GAGAAGCTGGAACAGTGGCGCT-3′, GAPDH sense: 5'-AAGTTCAACGGCACAGTAAG-3' (14) and GAPDH antisense: 5'-CATACTCAGCACCAGCATAC-3' (15). Calculation of relative gene expression levels of the target mRNAs were calculated by the equation $^{2-\Delta\Delta CT}$.

2.5. Statistical analysis

The results are presented as mean ± SEM. The data were analyzed by using SPSS software, the one- way ANOVA followed by post hoc Tukey test. In all cases, significance was defined by $p < 0.05$.

3. Results

Figure 1 shows mean milk lactose percentage of the animals receiving either saline, 1, 2, 4 µg orexin B receptor antagonist, 4 µg orexin antagonist plus E_2 or P_4 after one or three hours of treatments. Injection of 4 µg orexin B receptor antagonist significantly increased the mean percentage of milk lactose of the animals in comparison to control group after one hour of injection ($P<0.05$). Injections of E_2 or P_4 inhibit the

increased effects of 4 µg orexin antagonist on mean milk lactose percentage after one hour in comparison to 4 µg orexin antagonist group. Injection of 1 or 2 µg orexin did not significantly change the milk lactose percentage in comparison to control group after one hour. Also, the mean milk lactose percentage of the animals were not altered three hours after injections of all drugs in comparison to control group.

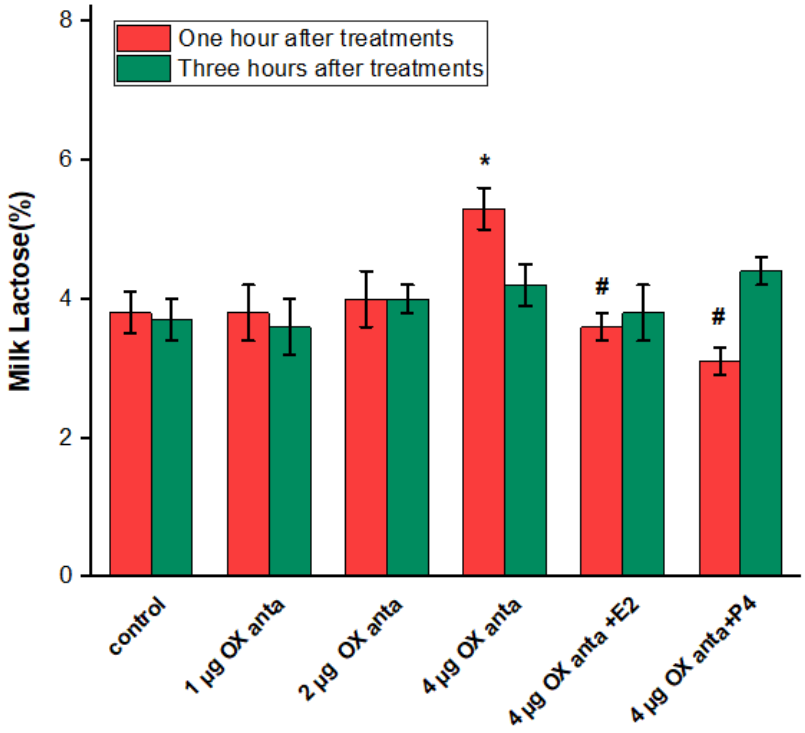

Fig. 1. Effect of orexin receptor antagonist (OX anta), orexin antagonist plus 17-β estradiol (E_2) or orexin antagonist plus progesterone (P_4) on the mean milk lactose percentage of the lactating animals (n=5 in each group) at one or three hours after injections. The results are presented as mean ± standard error of mean (SEM) and significance was defined by $P<0.05$. * compared to control group; # compared to 4 µg orexin antagonist group.

Figure 2. shows mean *Lalba* gene expression of the animals receiving either saline, 1, 2, 4 µg orexin B receptor antagonist, 4 µg orexin antagonist plus E_2 or P_4 after one or three hours of treatments. Injection of 4 µg orexin receptor antagonist significantly increased the mean *Lalba* gene expression of the animals in comparison to control group after one hour of injection ($P<0.05$), whereas injections of E_2 or P_4 inhibit the increased effects of 4 µg orexin antagonist on mean Lalba gene expression after one hour in comparison to 4 µg orexin antagonist group. Injection of 1 or 2 µg orexin did not alter the Lalba gene expression in comparison to control group after one hour. Also, the mean *Lalba* gene expression of the animals was not altered three hours after injections of all drugs in comparison to control group.

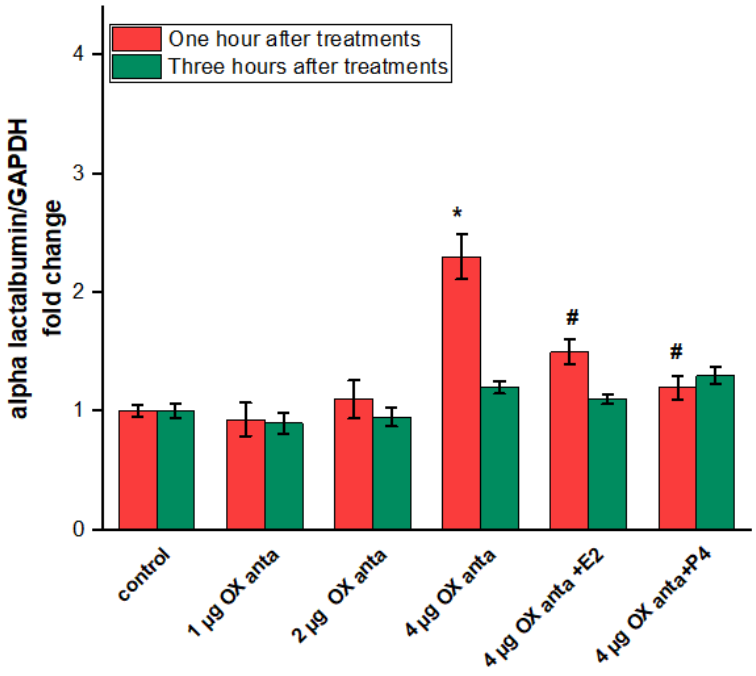

Fig. 2: Effect of orexin receptor antagonist (OX anta), orexin antagonist plus 17-β estradiol (E_2) or orexin antagonist plus progesterone (P_4) on the *mean alpha lactalbumin (Lalba)* gene expression of the lactating animals (n=5 in each group) at one or three hours after injections. The results are presented as mean ± standard error of the mean (SEM) and significance was defined by P<0.05. * compared to control group; # compared to 4 µg orexin antagonist group.

Figure 3. shows mean *B4galt1* gene expression of the animals receiving either saline, 1, 2, 4 µg orexin receptor antagonist, 4 µg orexin antagonist plus E_2 or P_4 after one or three hours of treatments. Injections of all drugs did not alter the mean *B4galt1* gene expression one and three hours after injections.

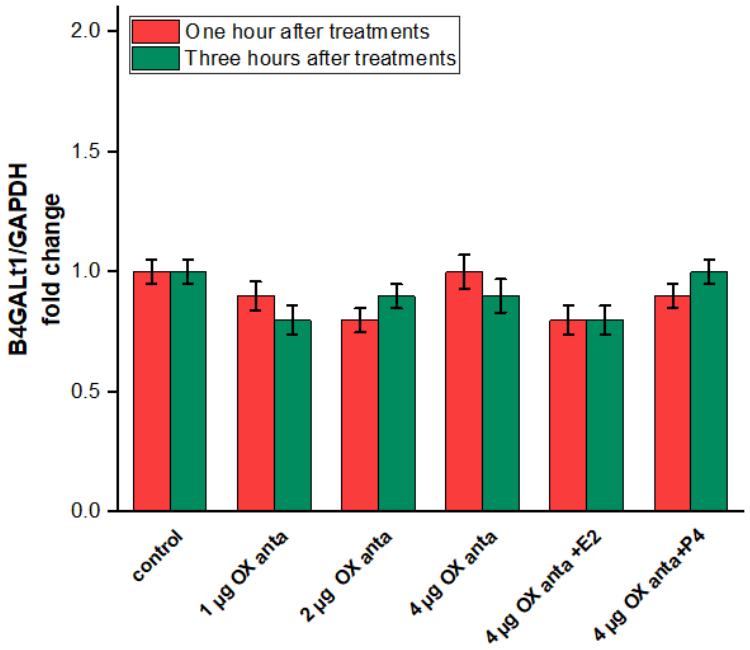

Fig. 3. Effect of orexin receptor antagonist (OX anta), orexin antagonist plus 17-β estradiol (E_2) or orexin antagonist plus progesterone (P_4) on the mean *beta-1,4-galactosyltransferase 1 (B4galt1)* gene expression of the lactating animals (n=5 in each group) at one or three hours after injections. The results are presented as mean ± standard error of mean (SEM) and significance was defined by P<0.05.

Figure 4. shows mean plasma concentrations of prolactin of the animals receiving either saline, 1, 2, 4 µg orexin antagonist, 4 µg orexin antagonist plus E_2 or P4 after one or three hours of treatments. Injection of 4 µg orexin antagonist increased significantly the mean plasma prolactin concentrations of the animals in comparison to control group after one hour of injection ($P<0.05$), whereas injections of E_2 or P_4 inhibit the increased effects of 4 µg orexin antagonist on mean plasma prolactin concentrations after one hour in comparison to 4 µg orexin antagonist group. Injection of 1 or 2 µg orexin did not alter the mean plasma prolactin concentrations in comparison to control group after one hour. Also, the mean plasma prolactin concentrations of the animals were not altered at three hours after injections of all drugs in comparison to control group.

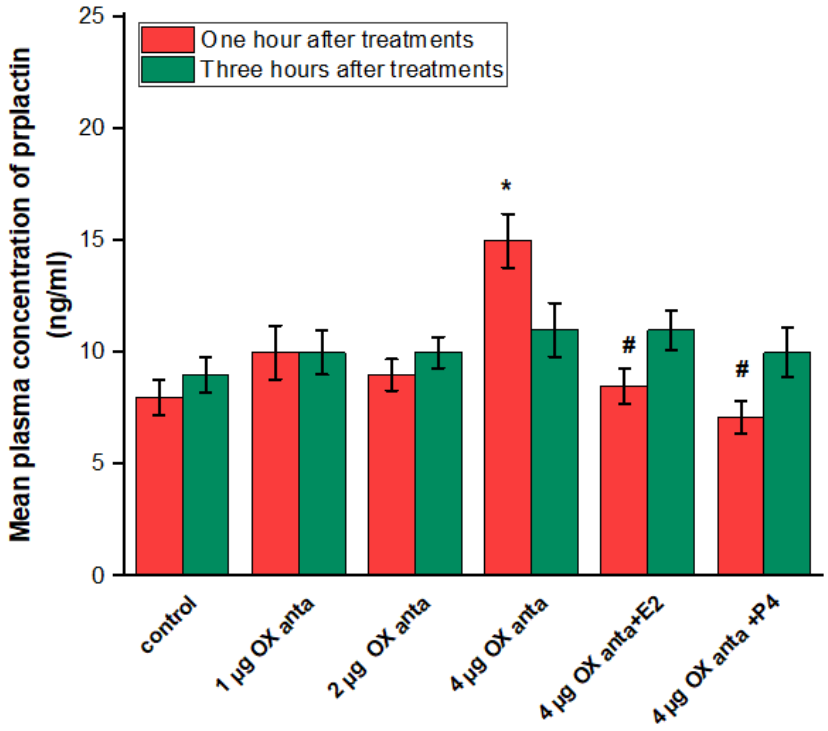

Fig. 4. Effect of orexin receptor antagonist (OX anta), orexin antagonist plus 17-β estradiol (E_2) or orexin antagonist plus progesterone (P^4) on the mean plasma prolactin concentrations of the lactating animals (n=5 in each group) at one or three hours after injections. The results are presented as mean ± standard error of the mean (SEM) and significance was defined by $P<0.05$. * compared to control group; # compared to 4 µg orexin antagonist group.

4. Discussion

The results of this study showed that injection of orexin B antagonist increases the mean plasma prolactin concentration at one hour following injection in the lactating animals. The effect of orexin antagonist on prolactin was studied for the first time in the present study. However, the results are in agreement with the previous ones which focused on orexin influences on prolactin. An in vitro study has shown that orexin supresses the prolactin synthesis through suppression of endogenous bone morphogenetic protein- 4(BMP-4) signalling pathway (16). Intra cerebral ventricular injection (ICV) of orexin exerts an inhibitory effect on prolactin secretion and ICV administration of orexin supresses the dopamine receptor antagonist – induced secretion of prolactin (4). An electrophysiological study established that the OX2R receptor activation results in the activating dopaminergic neurons and following suppression of prolactin synthesis (17), so that in their study, by blocking orexin receptor using OX2R antagonist TCSOX229, the stimulatory effects of orexin on dopaminergic neuron was abolished but antagonism of OX1R by antagonist SB334867 do not exert significant effect (17). Also, a declined hypothalamic mRNA levels of orexin has been shown during lactation due to the elevated prolactin concentration in rats (18-19).

Also, the present results demonstrated that injection of orexin B antagonist augmented the mean percentage of milk lactose at one hour following injection. One

possible mechanism for stimulatory effect of orexin antagonist on milk lactose synthesis may be due to its effects on prolactin secretion. It has been established that prolactin increases the lactose synthesis by increasing the components of lactose synthetase enzyme including alpha lactalbumin (Lalba) and beta-1,4-galactosyltransferase 1 (B4galt1) of the mammary gland (20). To examine this hypothesis, the effects of orexin B receptor antagonist was investigated on the gene expression level of Lalba and B4galt1 in the mammary gland of lactation rats. Injection of orexin receptor antagonist increased significantly the Lalba gene expression whereas it did not alter the B4galt1. As percentage of milk lactose, the possible mechanism for orexin receptor antagonist to induce Lalba gene expression may be related to its stimulatory effects on prolactin secretion. Different previous studies have shown the positive relation between prolactin and the level of Lalba and B4galt1 gene expression (2). They established that prolactin binds to its receptor to activate the Janus kinase 2/Signal transducer and activator of transcription 5 (STAT5) and phosphatidylinositol 3-kinase/Akt signalling pathways. Then Phosphorylated STAT5 binds to other transcription factors such as the glucocorticoids receptor to activate the synthesis of enzymes involved in the milk production (2, 21). So, orexin receptor antagonist may direct lactose synthesis via stimulating prolactin secretion and following upregulation of Lalba gene expression in the mammillary gland.

The results of second part of the present study showed that injection of 17-β estradiol (E_2) or progesterone (P_4) blocked the stimulatory effects of orexin receptor antagonist on percentage of milk lactose, plasma prolactin concentration and Lalba gene expression at one hour following injection. These results are in accordance with the previous ones which demonstrated the inhibitory effect of steroid hormones on lactose synthesis in ovariectomized, pregnant or lactating rats. (20). During pregnancy the circulating E_2 and P4 inhibit milk secretion by supressing the prolactin release from the pituitary gland and by making the mammary gland cells unresponsive to prolactin. Administration of E2 block the lactogenic influences of prolactin on Lalba production (22). So that, the inhibitory effect of E_2 on milk production may be partly related to direct antagonism between E2 and prolactin (22). There are evidences that E_2 or P_4 supress lactose synthesis during lactation. In fact, blood concentration of P_4 should decline to initiate the lactose synthesis during lactation (2). It has been revealed that Lalba and B4galt1 gene expression increased significantly in ovariectomized rats whereas the injection of P_4 to them supresses the Lalba and B4galt1 gene expression (2, 23).

It has been found that the orexin signaling pathways interact with the hypothalamus- pituitary- gonadal (HPA) axis activity. in induction stress condition. Orexins induce stress-related behaviors and sympathetic activity (2, 24). Corticotrophin releasing hormone (CRH) neurons express the OX2R (24-25) and injection of orexins activates the CRH neurons. Whereas OX1R or OX2R antagonists block the effects of orexin on HPA axis (24). Also, induction of stress activates the orexins neurons and results in augmentation of orexin gene expression (24, 26).
Administration of glucocorticoids to lactating cows results in significant reduction of milk lactose (27). Injection of hydrocortisone to rat dam during lactation declined protein and lactose concentration in milk (28). Also, the lactose synthesis and milk production decrease during stress and increased cortisol secretion (29). A suggestive mechanism underlying the negative effect of glucocorticoids on milk production may involve the suppression of Lalba synthesis (2). Based on these previous studies, suppressing HPA axis may be involved in stimulatory effects of orexin B receptor antagonist on Lalba gene expression in the mammary gland.

Conclusion

In lactating animals, injection of the orexin B receptor antagonist increases the mean plasma prolactin concentration at one hour following injection and 17-β estradiol (E_2) or progesterone (P_4) blocked the stimulatory effects of orexin B receptor antagonist on percentage of milk lactose, plasma prolactin concentration and Lalba gene expression. The B4galt1 gene expression did not alter following injection of orexin B receptor antagonist, E_2 or P_4. The mechanism by which the steroid hormones suppress the orexin B receptor antagonist-induced lactose synthesis may be mediated partly via inhibiting the prolactin production or suppressing hypothalamus- pituitary- gonadal axis.

Acknowledgment

The authors appreciate the Shahid Beheshti University for financial support and supplying the apparatus.

Conflict of interest

There is no conflict of interest in this article.

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