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ARTICLE

Effect of intraductal drug delivery of orexin receptor antagonists into lactating rat mammary gland on milk cholesterol metabolism by regulating *Fas* and *Hmgcr* genes

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ABSTRACT In recent years, many studies have demonstrated that the system of orexin plays a pivotal role in regulating lipogenesis enzymes. However, its effect on the mammary glands is not entirely known. This study answers the question of whether intra-ductal injection of orexin antagonists (OX1RA and OX2RA) into the mammary glands can result in the expression of fatty acid synthase (*Fas*) and HMG-CoA reductase (*Hmgcr*) genes and the secretion of cholesterol in lactating female rats or not. To this end, 42 Lactating rats were randomly divided into experimental groups including a control group and groups receiving OX1RA and OX2RA intraductal (with doses of 5, 10, and 20 µg/kg, i.duc). Milk samples were collected for cholesterol testing. Using specific primers for each gene, the target genes were measured via real-time PCR. Data differences were considered significant with P <0.05. PCR exhibited that the injection of orexin antagonists significantly reduced *Fas* and *Hmgcr* gene expression. Moreover, the injection of antagonists significantly reduced milk cholesterol. Intra-mammary injection of orexin antagonists reduces milk cholesterol levels by affecting the expression of *Fas* and *Hmgcr* genes.
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Introduction

Milk is a quintessential component on which all the nutritional needs of the baby are dependent (Wilde et al. 1999). Proteins, fatty acids, and other nutrients are abundant in milk (Grigor et al. 1986). The quantity and quality of milk fat greatly influence the quality and energy of dairy products (Heesom et al. 1992). Mammalian epithelial cells of lactating animals are highly active in the synthesis of the lipid center (Rohlfs et al. 1993).

Fatty acids are extracted from the two sources of the breakdown of blood lipids and *de novo* synthesis. In the *de novo* synthesis, the enzyme fatty acid synthase (FAS), which is an enzymatic complex present in the cytoplasm of the cells (Menendez and Lupu 2007), plays an essential role in elongating the fatty acid chain and is used to produce 16-carbon Palmitoyl- CoA, as well as other short-chain fatty acid products (Nakamura et al. 2014). The FAS enzyme is made up of two identical polypeptide chains that contain 7 essential domains (Yang et al. 2015).

There exists an interaction between glucose and fatty acid (FA) biosynthetic pathways; many enzymes are involved in this metabolism, too (Wang et al. 2014). Animal nutrition studies have unveiled that a low-fat, high-carbohydrate diet increases fat synthesis in both obese and thin individuals and that adipose tissue is a major site of fat synthesis (Ritterhoff et al. 2020). The induction of FAS is also involved in obesity, and reducing its expression prevents obesity (Ježek et al. 2018). The FAS enzyme is required in the first pathway of cholesterol precursor production (Jensen-Urstad and Semenkovich 2012).

Milk acts as a transport medium for vital molecules such as vitamins, minerals, bioactive fats, and cholesterol (Keim et al. 1982). Milk is beneficial if cholesterol is present in an adequate amount, as it plays an important role in membrane biosynthesis, steroid hormone production, and bile production (Kessler et al. 2014; Myant 1973). The enzyme 3-hydroxy-3-methylglutary-CoA (HMG-CoA) reductase is important in the synthesis of cholesterol in the mammary gland (Vaziri and Liang 1995). Also, this enzyme converts HMG-CoA to mevalonate in the cholesterol biosynthesis pathway (Liu and Yeh 2002), which is needed for DNA synthesis and cell proliferation (El-Sohemy and Archer 2000).

Orexin, or hypocretin, is a neuropeptide produced in the lateral hypothalamus and is derived from prepro-orexin (PPO) 130 amino acid peptide in rats with a 33-amino acid peptide, orexin A, along with a 28-amino

acid peptide, orexin B (Wang et al. 2018). These two peptides have two types of receptors called orexin-1 (OX1R) and orexin-2 (OX2R) bound to G-protein (Scammell and Winrow 2011). The high distribution of orexinergic neurons and orexin receptors in various tissues and regions shows its role in regulating neuroendocrine functions such as nutrition, metabolism, sleep and wakefulness, and controlling reproductive processes (Steiner et al. 2013). Orexins have different affinities for these two receptors; while OX1R binds to OXA with more specificity, OX2R shows the same inclination to both OXs. To this end, the antagonists designed and developed to date for OXRs also exhibit such properties (Goforth and Myers 2017). These antagonists include antagonists that act selectively like OX1R (such as SB-334867 compound) or OX2R (such as JNJ-10397049 compound) and dual non-selectively (such as ACT-078573 compound) (Sakurai 2014).

Studies have shown that orexin reduces prolactin through dopamine (Fujisawa et al. 2019). The decline in the expression of orexin during pregnancy and lactation increases prolactin and after 48 to 72 hours of starvation during lactation, the NPY mRNA levels decrease and MCH mRNA and PPO increase, resulting in increased appetite and decreased prolactin and lactation (Hsueh et al. 2002).

Given the importance of milk availability during lactation and proper nutrition of infants, as well as the prominent role of two enzymes, namely FAS and HMG-CoA reductase in the milk lipid synthesis in the mammary gland, this study is aimed at investigating the effect of OX1R and OX2R antagonists on the levels of cholesterol as well as the expression of FAS and HMG-CoA reductase (*Hgmcr*) genes in the mammary gland.

Materials and Methods

Animals

In this study, 42 lactating Wistar rats that were having their second pregnancy (Sutherland et al. 1986) and weighed 200 to 250 g were randomly selected and used. These rodents were maintained at a temperature of 22 ± 2 °C under twelve-hour dark/light period conditions with a relative humidity of 50%. At this stage, the animals had free, unlimited access to water and food and were transferred to this place one week prior to the experiments to acclimate to the laboratory environment. The animals were treated according to the international standards of working with animals. Moreover, the ethics committee of Shahid Beheshti University supervised the whole process and approved it with the code IR.SBU.REC.1399.043.

The materials used in this study included SB-334867 antagonist (Orexin1 receptor antagonist, purchased from Tocris) and JNJ-10397049 (Orexin2 receptor antagonist, purchased from Tocris) which were diluted in a volume ratio of 1:1 (v/v).

Table 1. Primers used in this study (From left to right 5´-3´).

Gene	Forward	Reverse
β-actin	GTGGGGCGCCCCAGGCACCA	GTCCTTAATGTCACGCACGATTTC
FAS	CCTCTTCCCTGGCACTGGCTACCT	ACTCGGCGGGGATCGGGACTT
Hmgcr	ACCGTGGGTGGTGGGAC	GCCCCTTGAACACCTAGCATC

β actin = beta actin; Fas = Fatty acid synthase; Hmgcr = HMG-CoA reductase

General procedure, milking, and surgical methods

Rats were randomly divided into control group and groups receiving OX1R and OX2R antagonists (doses of 5, 10, and 20 μg/kg of B.W.). The doses were selected based on our experience and further optimized using pilot studies (Hosseini and Khazali 2018; Faedo et al. 2012). In order to inject into the mammary gland, all 12 pairs of mammary glands received an injection (Kenyon et al. 2019). Anesthesia of the animal was performed using isoflurane by the inhalation of 2-4% of the concentration of this substance through the nasal passage. Meanwhile, their eyes were oily and closed; subcutaneously, meloxicam (5-10 mg/kg) was used as analgesia in the animals. The hair around the nipple was removed with a cream and the nipple was cleansed with alcohol. Using a pair of micro-dissecting tweezers, we lifted the skin around the nipple to open the nipple. Then, a 20 μL insulin syringe with a gauge of 31, OX1R, and OX2R antagonists was gently injected into the nipple duct. After the injection, the animals were isolated from isoflurane to regain consciousness. 6 h after the injection, rat milking was performed with a rat milking device (WAT-2006, Tokyo, Japan) and fresh milk samples were collected and stored at -20 °C until cholesterol measurement. Then the animal went under deep anesthesia and subsequently the mammary gland tissue was removed and kept at -80 °C for later analysis (Krause et al. 2013). The rats were euthanized after the experiment using a chamber prefilled with carbon dioxide (CO_2) gas with a concentration of 70% (Conlee et al. 2005).

Cholesterol measurement

Using Rat Cholesterol Kit (CHOD/PAP method - Bambolim Complex, INDIA) with intra-assay CV%: <10% and inter-assay CV%: <12%. Briefly, we pour the contents of bottle of L2 (Enzyme Reagent 2) into bottle of L1 (Enzyme Reagent 1). This working Reagent is stable for at least 8 weeks when stored at 2-8 ℃. Mix well and incubate at 37 ℃ for 5 min or at room temperature (25 ℃) for 15 min. Measure the absorbance of the standard and test sample against the blank, within 60 min. We measured choles-

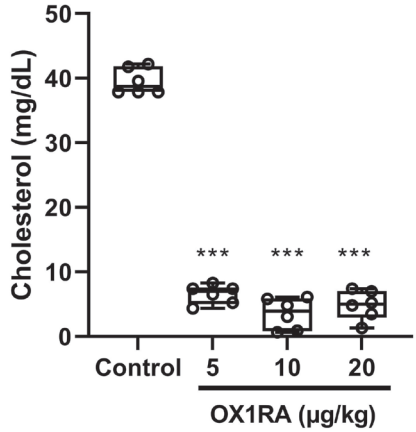

Figure 1. Milk cholesterol levels in different groups (OX1RA5, OX1RA10, and OX1RA20). *** P <0.001 compared with the control group. Data were presented as median (min, max) (n = 6).

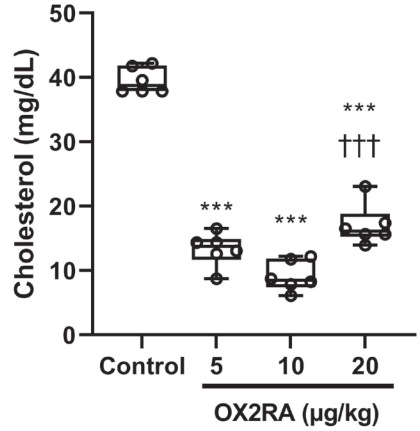

Figure 2. Milk cholesterol levels in different groups (OX2RA5, OX2RA10, and OX2RA20). *** P <0.001 compared to the control group, ††† P <0.001 compared to the OX2RA10. Data were presented as median (min, max) (n = 6).

terol level according to the company's instructions with ELISA leader BioTek 800TS (USA) at 505 nm wavelength and express as mg/dL.

RNA extraction and gene expression

Gene JET RNA Purification Kit (Thermo Scientific, USA) was used to extract RNA from breast tissue. A DNase treatment (Thermo Scientific, USA) was used to remove possible DNA contamination from the whole RNA prior to the cDNA synthesis using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). All steps were performed according to factory instructions. To measure each gene, we used specific primers for each gene in a real-time PCR assay (Table 1). A Corbett-RG 6000X device (Corbett Research, Australia) was used to measure the relative gene expression for each sample using the SYBR Premix Ex TaqTM II kit from Takara (Japan). In each sample in the strips, the samples are duplicated and their volume is 20 µL. Cycle threshold (CT) then recognizes the reaction and calculates the CT genes based on it. Finally, the target genes are calculated relative to the reference gene using the formula $2^{-\Delta\Delta CT}$ (Hosseini and Khazali 2018).

Data analysis

Findings were analyzed using GraphPad Prism 8 software, the results were analyzed as median (min, max) from the mean of the evaluation. The data normality condition was assessed by the Shapiro-Wilk test; then, the data were measured by the one-way test of ANOVA and the Tukey post hoc. GraphPad Prism 8 software was used to plot the data and $P < 0.05$ was considered significant.

Results

The effect of injecting orexin receptor antagonists on milk cholesterol levels

In this study, injections of OX1R antagonists at doses of 5, 10, and 20 g/kg provided a significant reduction in milk cholesterol levels in comparison with the control group (P <0.001) (Fig. 1).

Likewise, the injection of OX2R antagonist with doses of 5, 10, and 20 µg/kg compared to that of the control group showed a significant difference in reducing milk cholesterol levels (P <0.001); Injection of 10 g/kg of OX2R compared with a dose of 20 g/kg also resulted in significant decreases (P <0.001) (Fig. 2).

The effect of injecting orexin receptor antagonists on FAS gene expression

As can be observed from the results of this study, mice that were injected with an OX1R antagonist at a dose of 10 g/kg compared to the control group showed significantly lower expression of the *FAS* gene (P <0.001) (Fig. 3).

Compared to that of the control group, the injection of OX2R antagonist with a dose of 10 µg/kg showed a significant difference in decreasing the expression of the *FAS* gene (P <0.001); in a similar vein, the injections of OX2R with a dose of 5 µg/kg compared to a dose of 10 µg/kg and a dose of 10 µg/kg compared to a dose of 20 µg/kg significantly reduced the gene expression of *FAS* (P<0.001) (Fig. 4).

The effect of injecting orexin receptor antagonists on Hmgcr gene expression

Results of this study indicate that administration of OX1R antagonist at a dose of 10 g/kg showed a significant re-

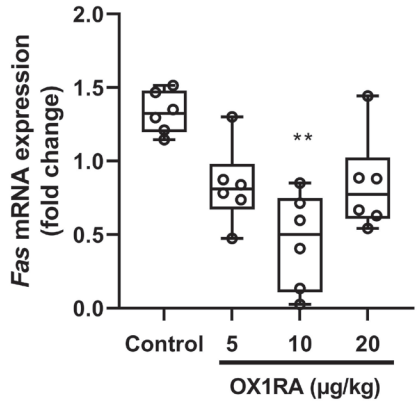

Figure 3. Expression of *Fas* gene in the groups of control, OX1RA5, OX1RA10, and OX1RA20. ** P <0.01 compared to the control group. Data were presented as median (min, max) (n = 6).

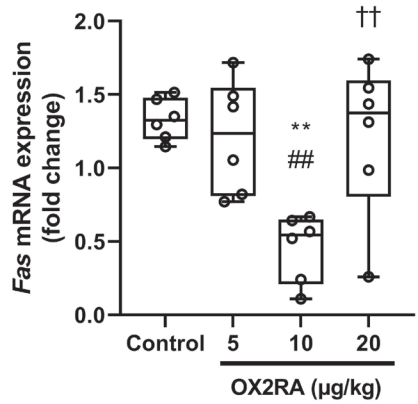

Figure 4. Expression of *Fas* gene in the groups of control, OX2RA5, OX2RA10, and OX2RA20. ** P <0.01 compared to the control group, ## P <0.01 compared to the OX2RA1, †† P <0.01 compared to the OX2RA10. Data were presented as median (min, max) (n = 6).

duction in *Hmgcr* gene expression when compared to the control group (P <0.001); the injection of OX1R at a dose of 10 µg/kg compared to a dose of 5 µg/kg and 20 µg/kg also revealed a higher expression of the *Hmgcr* gene (P <0.001) (Fig. 5).

Injection of OX2R antagonist at a dose of 10 µg/kg revealed a significant difference in terms of decreased expression of the *Hmgcr* gene compared to the control group (*P*<0.001). Moreover, OX2R injection at a dose of 5 µg/kg compared to a dose of 10 µg and a dose of 10 µg/kg compared to a dose of 20 µg/kg resulted in a significant decrease in expression of the *Hmgcr* gene (P <0.001) (Fig. 6).

Discussion

The present study was the first scrutiny in the literature on the intra-ductal injection of receptor antagonists (OX1RA)1 and (OX2RA)2 into the mammary gland and the effects of their interaction on gene expression of some important enzymes in lipid synthesis such as FAS and HMG-CoA reductase. The impacts of this injection on insulin measurements were also discussed. The results of the current study reveal that these injections reduce the expression of *FAS* and *Hmgcr* genes and reduce cholesterol.

An intraductal injection of OX1RA and OX2RA was studied in the present study in order to investigate its effects on cholesterol synthesis enzyme gene expression and the results showed that with the injection of either OX1RA or OX2R, the gene expression of *FAS* and *Hmgcr* are reduced. There has not been much study on the effect of OXS on cholesterol synthesis enzymes. Liu and Yeh (2002) showed that organic sulfur compounds reduce cholesterol synthesis (Liu and Yeh 2002). Digby et al. (2006) demonstrated the role of orexin in fat metabolism by isolating adipocytes and treated with OXA and OXB (Digby et al. 2006). Skrzypski et al. (2011) showed that isolating rats' OXA fat cells increased TAG and lipogenesis and decreased lipolysis (Skrzypski et al. 2011).

In this study, OX1RA and OX2RA antagonists were used to examine the effect they had on FAS gene expression. The elicited findings demonstrated that such injections diminish the expression of the *FAS* gene. Previous studies documented that the expression of the *FAS* gene is elevated by nutrition and increased insulin sensitivity is associated with an elevation in the expression of the *FAS* gene in the adipose tissue (Ranganathan et al. 2006; Xie et al. 2019). Madison (2016) showed that the expression of genes such as FAS and HMG-CoA reductase plays an important role in cholesterol biosynthesis (Madison 2016).

As part of this study, the impact of injecting antago-

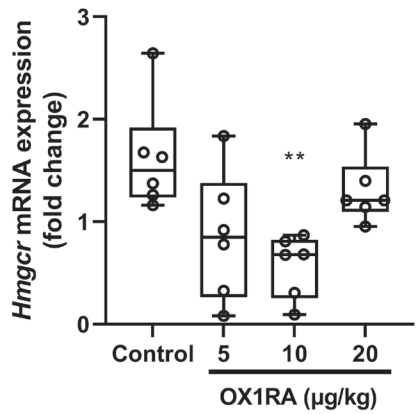

Figure 5. *Hmgcr* gene expression in the groups of control, OX1RA5, OX1RA10, and OX1RA20. ** P <0.01 compared to the control group. Data were presented as median (min, max) (n = 6).

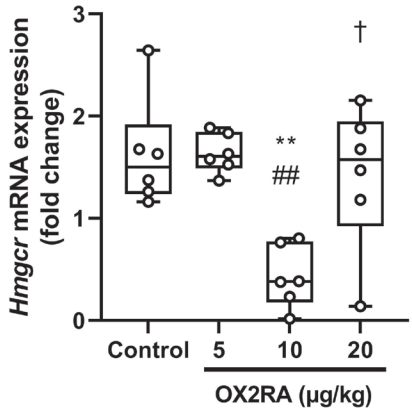

Figure 6. *Hmgcr* gene expression in the groups of control, OX2RA5, OX2RA10, and OX2RA20. ** P <0.01 compared to the control group, ## P <0.01 compared to the OX2RA1, † P <0.05 compared to the OX2RA10. Data were presented as median (min, max) (n = 6).

nists on HMG-CoA reductase gene expression was also evaluated, demonstrating that the injection reduces the expression of this enzyme. The role of orexin in this enzyme has not been reported yet. EL-Sohemy and Archer (2000) showed that this enzyme in the biosynthesis pathway of cholesterol converts HMG-CoA to mevalonate and is very important in lactation (El-Sohemy and Archer 2000). Viturro et al. (2009) using the RT-PCR technique showed that cholesterol is one of the key enzymes in the synthesis of HMG-CoA reductase (Viturro et al. 2009).

This is the first study to be conducted on the mentioned gap and has a number of limitations. Further research avenues are warranted for testing more genes and paving the way for a more vivid picture, especially in the immunohistochemical areas.

Conclusion

Overall, our results exhibited that the intra-ductal injection of antagonists into the mammary gland decreased milk cholesterol levels. Moreover, the result of the injection of OX1RA and OX2RA antagonists was found to be reduced *FAS* and *Hmgcr* genes.

Acknowledgment

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