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(56) Documents Cited:
Molecules (Basel, Switzerland), vol. 19, no. 7, 2014, Zhang Xia et al., "GSK3beta regulates milk synthesis in and proliferation of dairy cow mammary epithelial cells via the mTOR/S6K1 signaling pathway.", p. 9435-9452.

(58) Field of Search:
Other: **BIOSIS, MEDLINE**

(54) Title of the Invention: **Application method for regulating lithium chloride in bovine mammary epithelial cell**
Abstract Title: **Application method for regulating lithium chloride in bovine mammary epithelial cells.**

(57) The use of lithium chloride (LiCl) in a bovine mammary epithelial cell (BMEC), including the use of LiCl in the synthesis of milk protein and milk fat, and the use of LiCl and trans-vaccenic acid (TVA) in the synthesis of conjugated linoleic acid (CLA) in the BMEC. In the present disclosure, the lithium chloride can effectively activate HIF-1α and Wnt/β-catenin signalling pathways and downstream signalling pathways thereof, and activate JAK2/STAT5, mTOR, and SREBP1 signalling pathways to promote the synthesis of the milk protein in the BMEC and regulate the synthesis of the milk fat and the CLA, so as to improve the lactation of mammary glands of a dairy cow and increase economic benefits.

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DRAWINGS

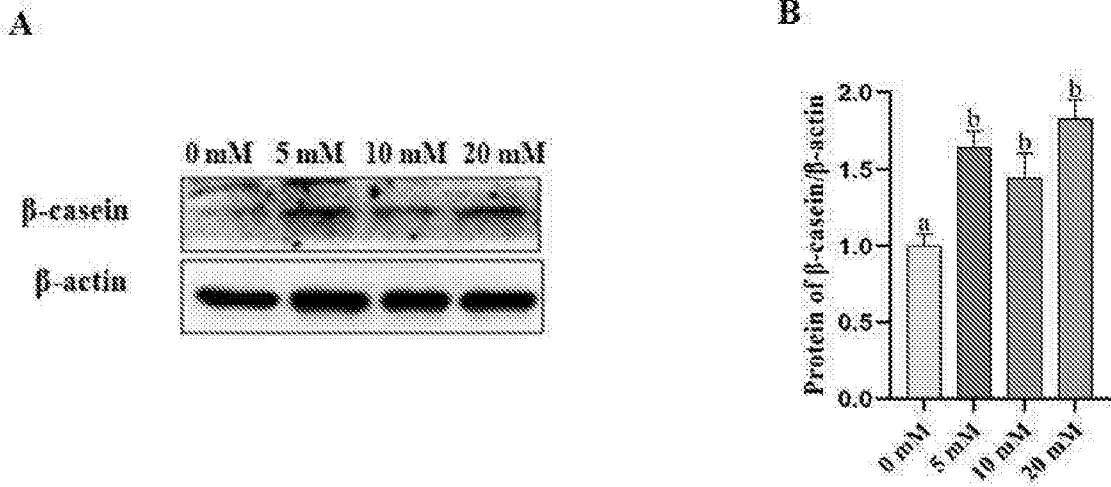


FIG. 1

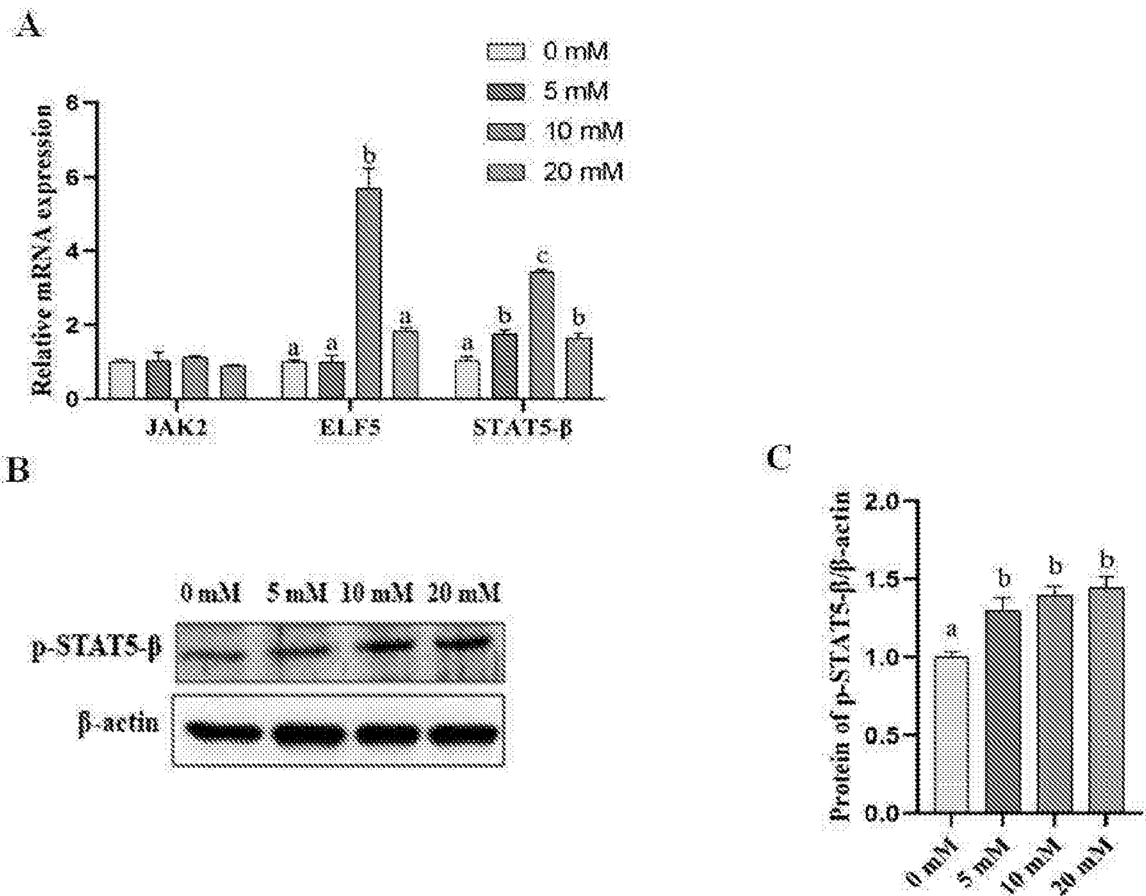
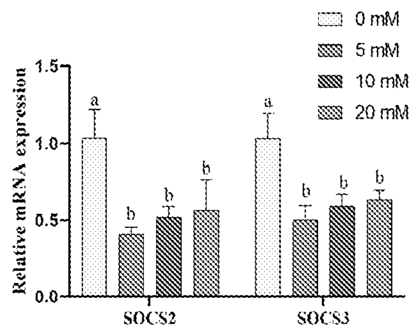


FIG. 2

A



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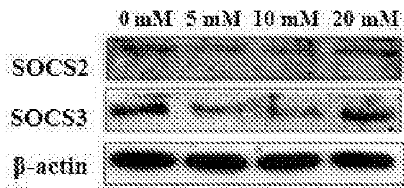

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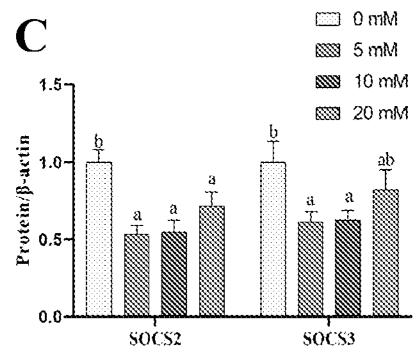
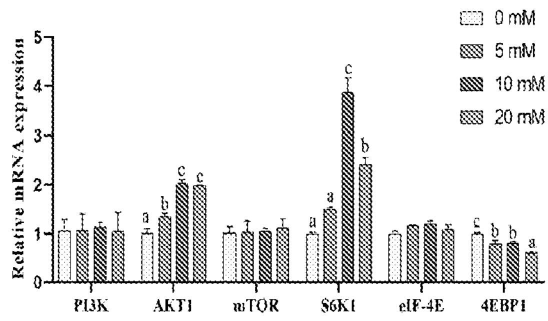


FIG. 3

A



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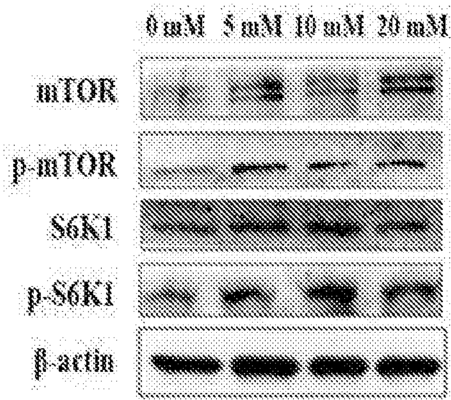

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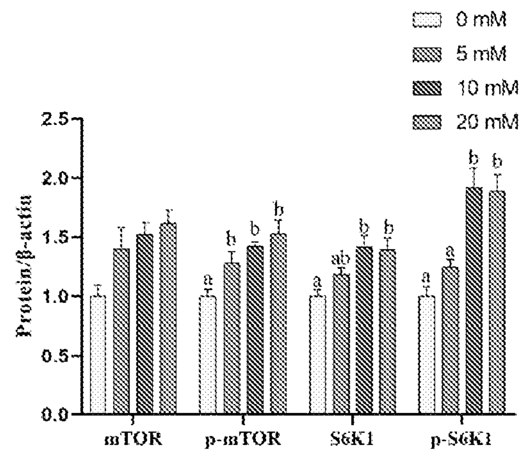

FIG. 4

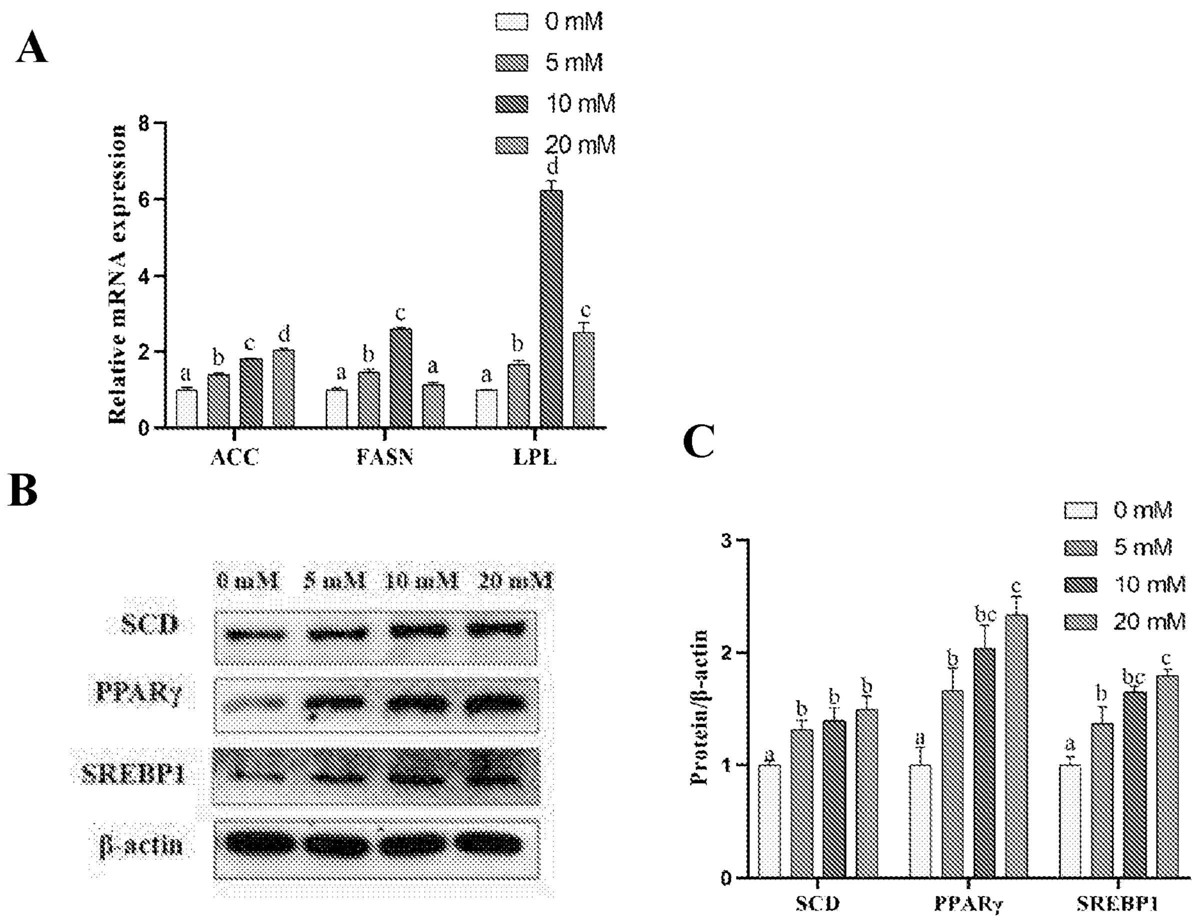

FIG. 5

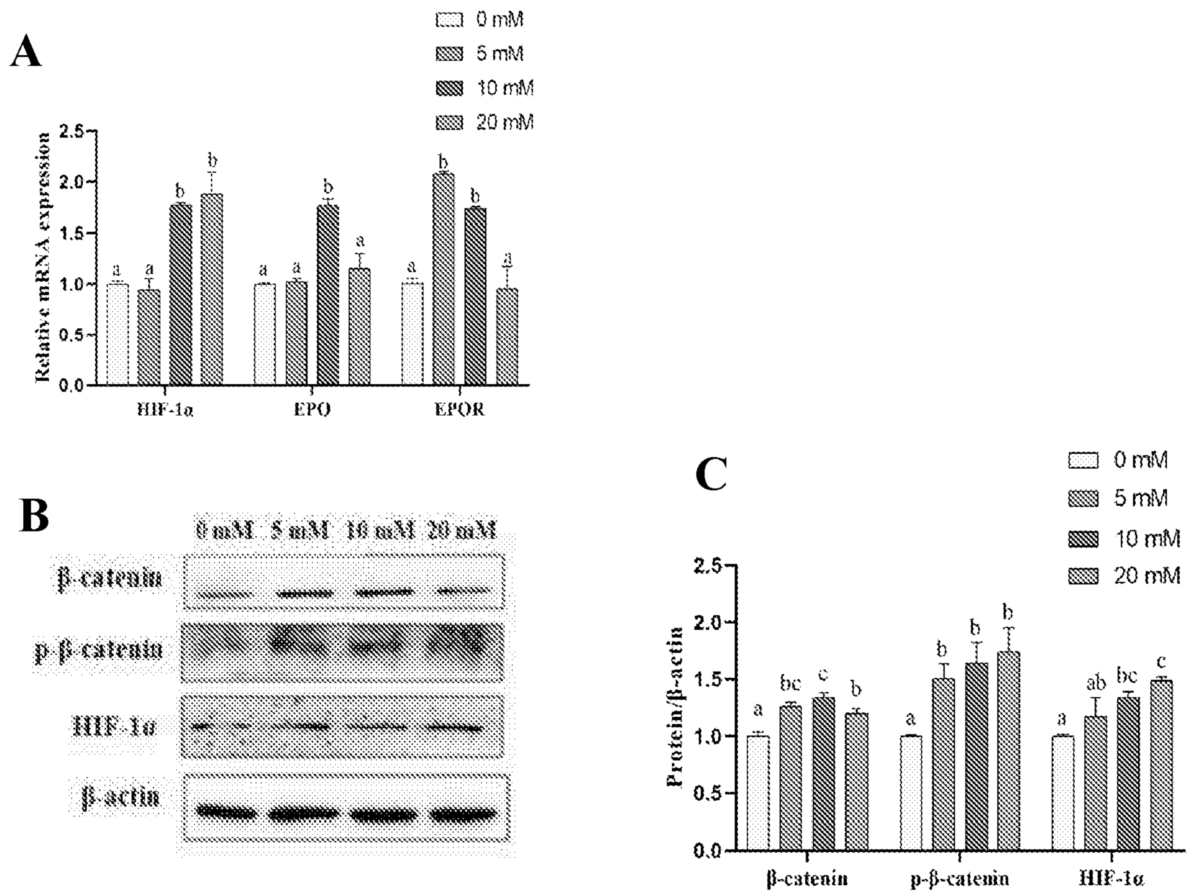

FIG. 6

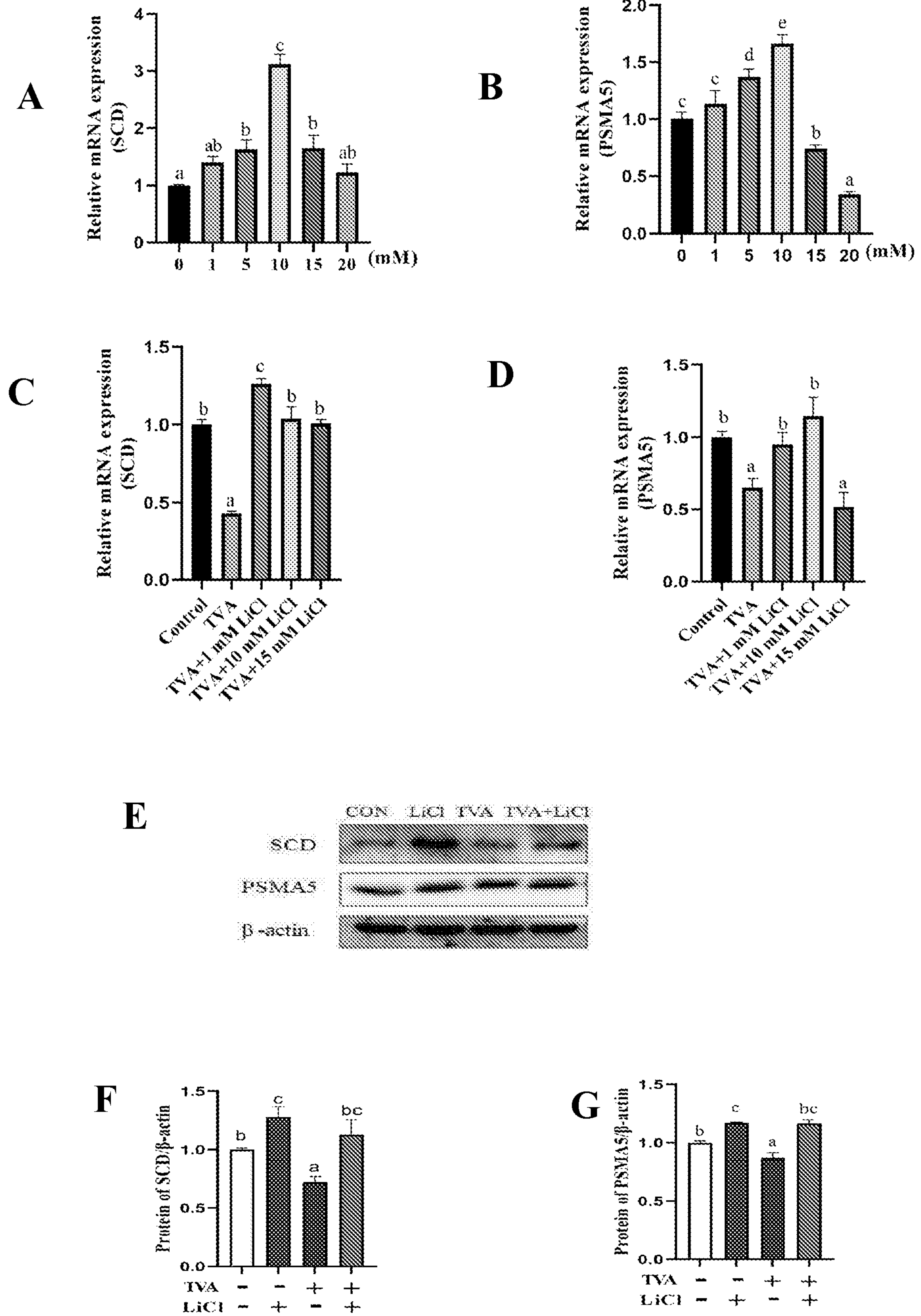

FIG. 7

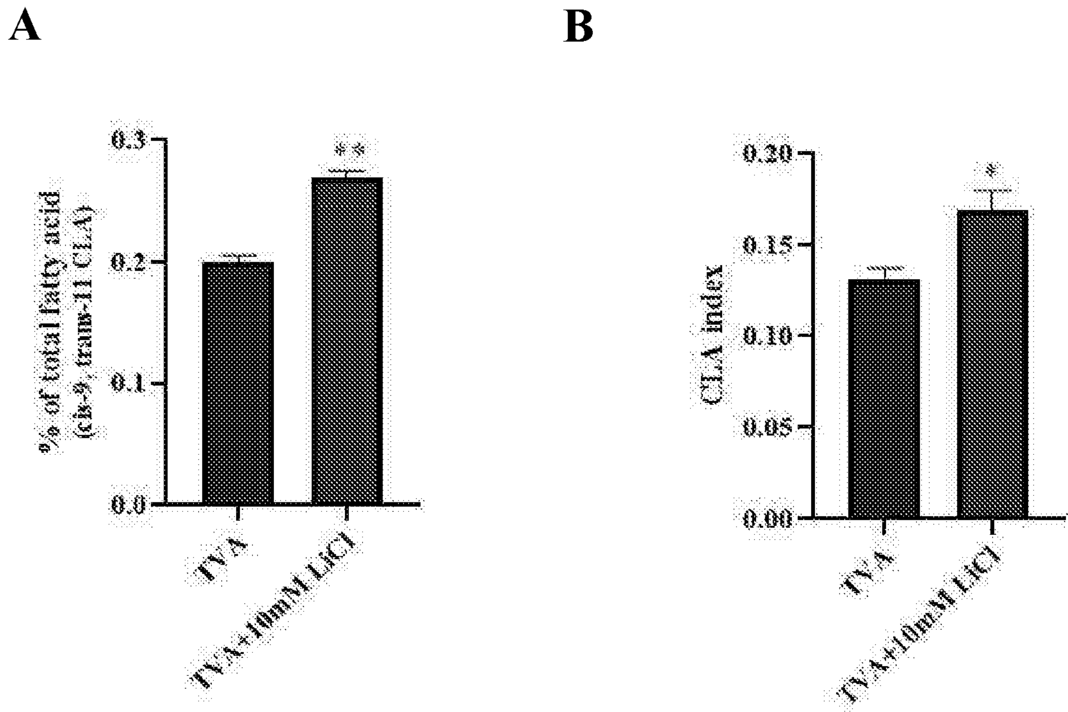

FIG. 8

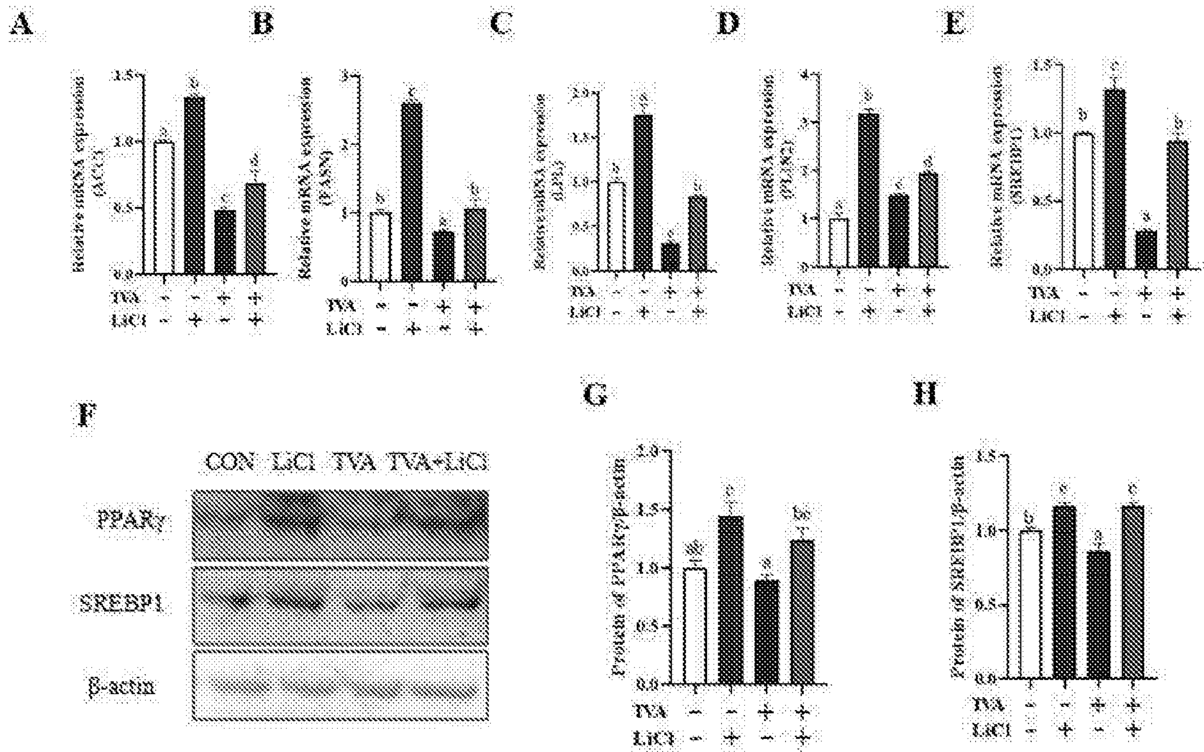

FIG. 9

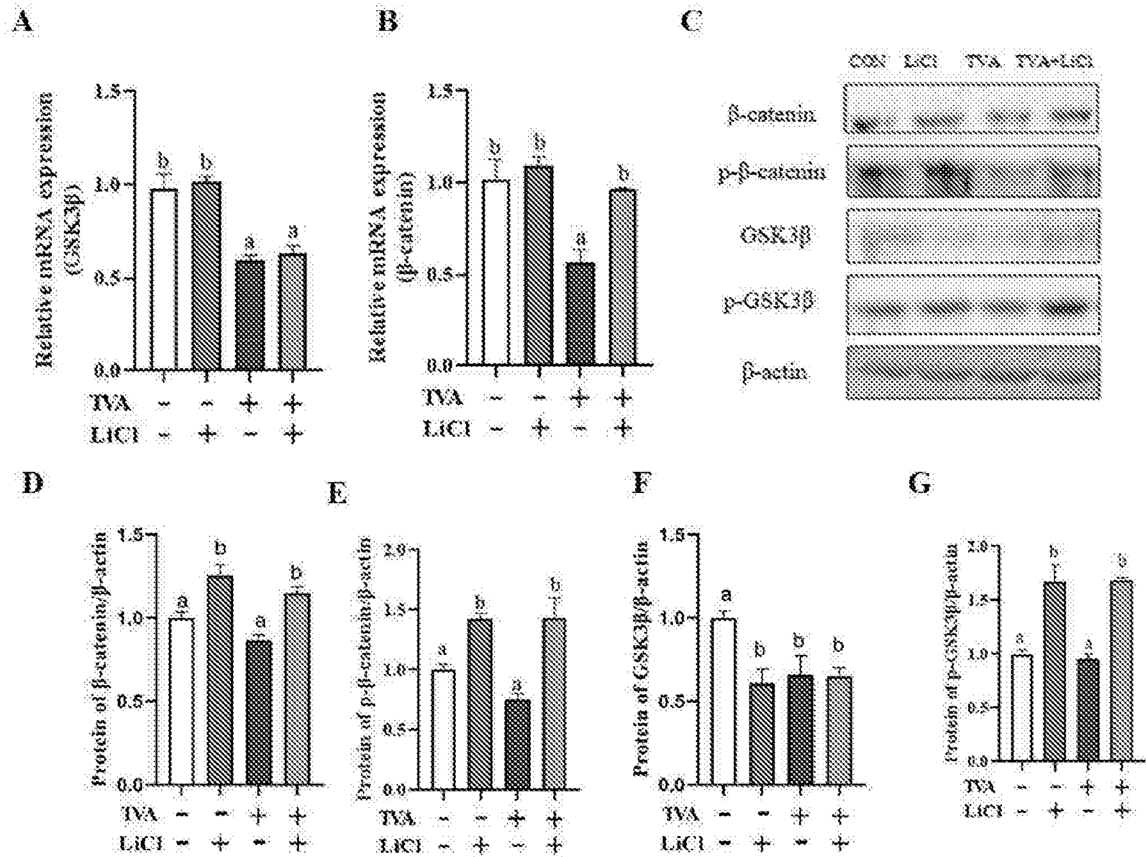

FIG. 10

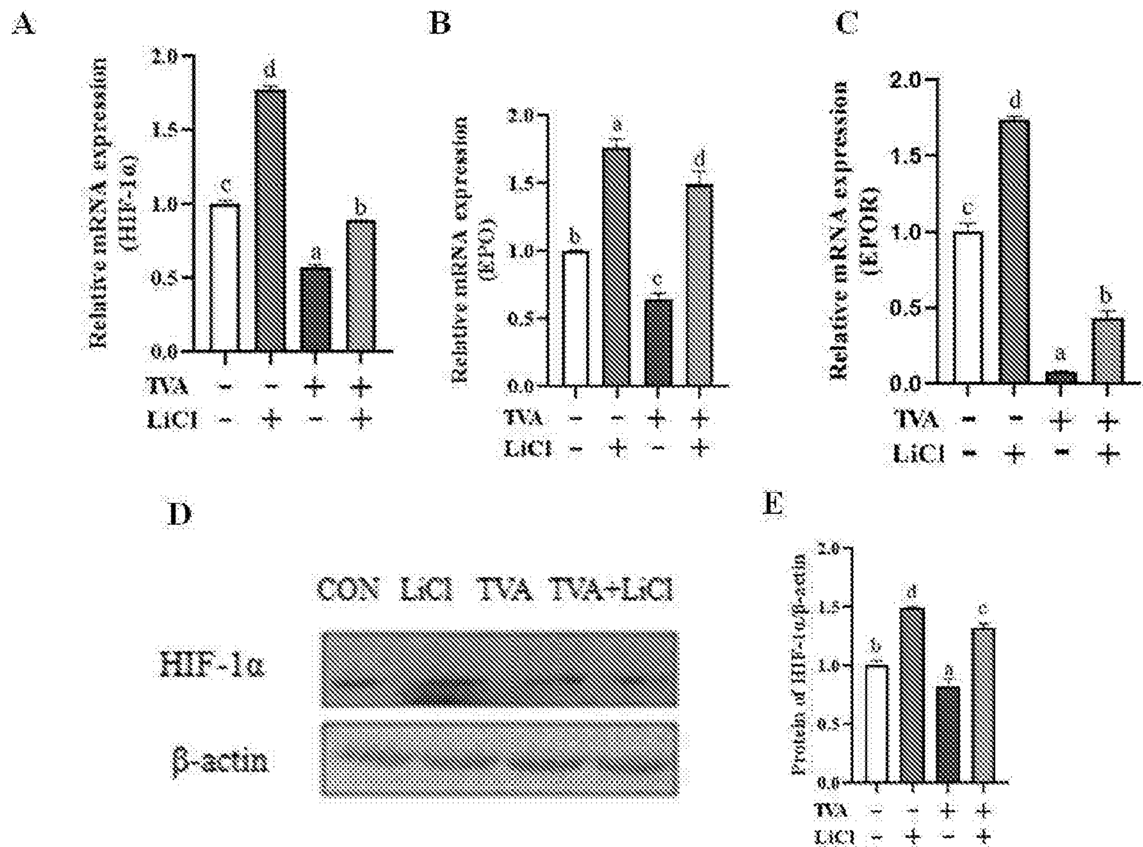

FIG. 11

APPLICATION METHOD FOR REGULATING LITHIUM CHLORIDE IN BOVINE MAMMARY EPITHELIAL CELL

TECHNICAL FIELD

[0001] The present disclosure relates to the use of lithium chloride, in particular to an application method for regulating lithium chloride in a bovine mammary epithelial cell (BMEC).

BACKGROUND

[0002] With the continuous improvement of people's living standards and the increasing emphasis on health concepts, the national requirements for milk quality are getting higher and higher, and the demand is also increasing. High-quality milk and dairy derivatives rich in nutrients have attracted people's attention. Over the past decades, the research on improving milk composition and milk performance of dairy cows through feed nutritional regulation has attracted more and more attention in China, especially improving the protein and fatty acid composition of milk has become a highlight of research.

[0003] Conjugated linoleic acid (CLA) is an isomer of linoleic acid, a functional unsaturated fatty acid that is found in ruminants and is beneficial to humans. CLA has a plurality of biological functions such as anti-cancer, anti-arteriosclerosis, antidiabetic, immunity-boosting, bone tissue metabolism improvement, and fat deposition suppressing functions. In nature, CLA mainly exists in the dairy and meat products of ruminants in the form of *cis*-9, *trans*-11 CLA. It is found that the synthesis of CLA in ruminants is mainly carried out in the rumen and mammary tissue. In lactating ruminants, by ingesting feed containing linoleic acid or linolenic acid, the linoleic acid produces *cis*-9, *trans*-11 CLA through hydrogen bond transfer in the presence of rumen microorganisms, and the resulting *cis*-9, *trans*-11 CLA is further hydrogenated to *trans*-vaccenic acid (TVA, *trans*-11 C18:1) in the presence of rumen microorganisms, and finally hydrogenated to stearic acid (C18:0). However, linolenic acid does not produce *cis*-9, *trans*-11 CLA in the rumen, but produces TVA through hydrogenation by rumen microorganisms. These unhydrogenated CLA and TVA are absorbed and transported into the mammary tissue by blood, and TVA is endogenously re-synthesized into *cis*-9, *trans*-11 CLA under the desaturation of stearoyl-CoA desaturase (SCD, considered to be a Δ9-fatty acid desaturase). On the whole, at most 30% of CLA is synthesized in the rumen, and at least 70% or even more than 90% of CLA is endogenously synthesized in the mammary tissue through the desaturation of TVA. It is known that at least 70% of CLA is endogenously synthesized in the mammary tissue through the desaturation of TVA, but the conversion efficiency of TVA into CLA is still very low, which

cannot meet the exertion of the effective function of the CLA. Since the body fat and milk fat of ruminants are dominated by *cis*-9, *trans*-11 CLA and dairy products are the best products for ingesting CLA, how to increase the content of *cis*-9, *trans*-11 CLA synthesized in ruminants has become an important research hotspot in recent decades. If the expression of genes related to the synthesis of CLA can be regulated, the synthesis efficiency of CLA in the mammary gland will be improved, more milk or goat milk rich in CLA can be produced, and the defect of failure to exert the physiological functions of CLA due to low CLA content in the existing dairy products can be improved, providing the nation with functional dairy products rich in CLA. Research on whether exogenous supplementation of nutrients affects the synthesis of endogenous CLA in ruminants is still lacking.

[0004] Lithium is a silver-white alkali metal element and the content thereof ranks 27th on the earth. Lithium is commonly found in the earth's crust and various mineral water, and a quantity of lithium is further found in plants. In nature, lithium often exists in the form of lithium salts. As a clinical mood stabilizer, lithium has been used in the treatment of mental disorders for more than 60 years. In addition, lithium further has anti-cancer, anti-viral, anti-inflammatory, antioxidant, immunomodulatory, and osteogenesis-promoting effects. Lithium deficiency can lead to growth retardation and reduced reproductive performance of goats. However, there are fewer studies on lithium in the nutrition of dairy cows, and it is not clear whether additional lithium should be added to dairy cow feed.

[0005] In the feeding process of dairy cows, lithium chloride can be additionally supplemented in the form of feed additives. In the present disclosure, lithium chloride of different concentrations is additionally added to BMECs to investigate an optimal concentration of lithium chloride and the effect thereof on milk synthesis.

SUMMARY

[0006] An objective of the examples of the present disclosure is to provide an application method for regulating lithium chloride in a BMEC, aiming to solve the problem of how to increase the content of milk protein and the synthesis of *cis*-9, *trans*-11 CLA in ruminants.

[0007] The examples of the present disclosure are realized by an application method for regulating lithium chloride in a BMEC, mainly including the following steps:

[0008] step 1: use in effects of the lithium chloride on the synthesis of milk protein and milk fat in BMECs: inoculating the BMECs in a Petri dish supplemented with a growth medium, and culturing the BMECs in a 37°C cell culture incubator; conducting differentiation induction on the BMECs for four days, adding different concentrations of the lithium chloride at the same

time, and extracting and collecting RNAs and proteins after four days; detecting regulatory effects of the lithium chloride on β-casein in the BMECs, PI3K/AKT1/mTOR and JAK2/STAT5 signaling pathways, and enzymes related to milk fat synthesis by quantitative polymerase chain reaction (qPCR) and Western blot; preliminarily investigating a mechanism of regulation of lactation of the BMECs by the lithium chloride by the qPCR and the Western blot; and

[0009] step 2: use in effects of the lithium chloride on the synthesis of CLA in the BMECs: inoculating the BMECs in a Petri dish supplemented with the growth medium, and culturing the BMECs in the 37°C cell culture incubator; conducting differentiation induction on the BMECs for four days, screening out an optimal concentration of the lithium chloride, adding 50 μM TVA, and extracting and collecting the RNAs and the proteins; detecting an effect of the lithium chloride on key marker genes for the synthesis of the CLA in the BMECs by the qPCR and the Western blot, and detecting CLA content in the BMECs to determine the effect of the lithium chloride on the synthesis of the CLA in the BMECs by gas chromatography (GC); preliminarily investigating a mechanism of regulation of the synthesis of the CLA in the BMECs by the lithium chloride by the qPCR and the Western blot.

[0010] In a further technical solution, the growth medium is a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 1% penicillin-streptomycin (100 IU/mL penicillin + 100 μg/mL streptomycin).

[0011] In a further technical solution, a differential medium used for the differentiation induction is a DMEM supplemented with 10% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 5 μg/mL prolactin, and 1% penicillin-streptomycin.

[0012] In a further technical solution, the differential medium is placed in 6-well plates, and a quantity of the differential medium is 2 mL.

[0013] In a further technical solution, the lithium chloride in step 1 may have three concentrations: 5, 10, and 20 mM.

[0014] In a further technical solution, detection of the regulatory effects of different concentrations of the lithium chloride on genes and proteins related to the synthesis of the milk protein and the milk fat in the BMECs in step 1 includes the following steps:

[0015] step i. inoculating the BMECs at the growth phase into 6-well plates, and conducting static culture on the BMECs in each well with the growth medium until the BMECs reach 70%-80% confluence;

[0016] step ii. grouping the 6-well plates as follows,

[0017] treatment groups: aspirating and discarding the culture medium, washing the BMECs with phosphate-buffered saline (PBS), adding differential media supplemented with the lithium chloride at three concentrations of 5, 10, and 20 mM, respectively, culturing the BMECs in the 37°C cell culture incubator for four days, and changing the culture medium every day,

[0018] a control group: aspirating and discarding the culture medium, washing the BMECs with the PBS, adding the differential medium, culturing the BMECs in the 37°C cell culture incubator for four days and changing the culture medium every day; and

[0019] step iii. collecting BMECs cultured for four days from each group, extracting the RNAs and the proteins, and detecting the expression of genes related to the synthesis of milk protein and milk fat by the qPCR and the expression of proteins related to the synthesis of the milk protein and the milk fat by the Western blot.

[0020] In a further technical solution, the detection of lactation signaling pathways of the BMECs, JAK2/STAT5, PI3K/AKT1/mTOR, and SREBP1, by the qPCR and the Western blot in step 1 includes the following steps:

[0021] step a, inoculating the BMECs in 6-well plates and dividing the BMECs into four groups, namely a control group and three lithium chloride treatment groups, where lithium chloride concentrations of the three lithium chloride treatment groups are 5, 10, and 20 mM, respectively, and all groups are treated for four days;

[0022] step b, detecting effects of the expression of *JAK2*, *ELF5*, *STAT5-β*, *SOCS2*, *SOCS3*, *PI3K*, *AKT1*, *mTOR*, *S6K1*, *eIF-4E*, *4EBP1*, *ACC*, *FASN*, *LPL*, *HIF-1α*, *EPO*, and *EPOR* genes in the four different groups by the qPCR; and

[0023] step c, detecting effects of the protein expression of β-casein, p-STAT5-β, mTOR, p-mTOR, S6K1, p-S6K1, SCD, PPARγ, SREBP1, HIF-1α, β-catenin, and p-β-catenin in different groups by the Western blot.

[0024] In a further technical solution, the optimal concentration of the lithium chloride screened in step 2 may be 10 mM.

[0025] In a further technical solution, the use in effects of the lithium chloride on synthesis of CLA in the BMECs includes the following steps:

[0026] step i. inoculating the BMECs at the growth phase into 6-well plates, and conducting static culture on the BMECs in each well with the growth medium until the BMECs reach 70%-80% confluence;

[0027] step ii. grouping the 6-well plates as follows,

[0028] treatment groups: aspirating and discarding the culture medium, washing the BMECs with PBS, adding a differential medium supplemented with 10 mM lithium chloride, culturing

the BMECs in the 37°C cell culture incubator for four days, and changing the culture medium every day; after four days, treating the BMECs with 50 μM TVA for 4 h, where there are the following treatment groups: a 10 mM lithium chloride group, a TVA group, and a TVA + 10 mM lithium chloride group;

[0029] a control group: aspirating and discarding the culture medium, washing the BMECs with the PBS, adding the differential medium, culturing the BMECs in the 37°C cell culture incubator for four days, and changing the culture medium every day; and

[0030] step iii. collecting the BMECs, extracting the RNAs and the proteins, detecting the expression of genes related to the synthesis of the CLA by the qPCR, detecting the expression of proteins related to the synthesis of the CLA by the Western blot, and detecting the CLA content in the BMECs by the GC.

[0031] In a further technical solution, detection of the lithium chloride against genes and proteins related to the synthesis of the CLA in the BMECs by the qPCR and the Western blot includes the following steps:

[0032] step a. inoculating the BMECs into 6-well plates and dividing the BMECs into four groups, namely a control group, a 10 mM lithium chloride group, a TVA group, and a TVA + 10 mM lithium chloride group;

[0033] step b. detecting effects of the expression of *SCD*, *PSMA5*, *ACC*, *FASN*, *LPL*, *PLIN2*, *SREBP1*, *GSK3β*, *β-catenin*, *HIF-1α*, *EPO*, and *EPOR* genes in four different groups by the qPCR; and

[0034] step c. detecting effects of the protein expression of SCD, PSMA5, PPARγ, SREBP1, β-catenin, p-β-catenin, GSK3β, p-GSK3β, and HIF-1α in different groups by the Western blot.

[0035] An application method for regulating lithium chloride in a BMEC provided in the example of the present disclosure detects expression levels of genes related to the synthesis of milk protein and fatty acids by real-time fluorescent quantitative PCR, related proteins by Western blot , and the content of CLA in cells by GC. It is found that 10 mM lithium chloride promotes the synthesis of the milk protein and fatty acids in BMECs, and enhances the lactation of the BMECs. In the present disclosure, the lithium chloride can effectively activate HIF-1α and Wnt/β-catenin signaling pathways and downstream signaling pathways thereof, and activate JAK2/STAT5, mTOR, and SREBP1 signaling pathways to promote the synthesis of the milk protein in the BMEC and regulate the synthesis of the milk fat and the CLA, to improve the lactation of mammary glands of a dairy cow and increase economic benefits. The present disclosure further provides a new direction for the mechanism underlying the regulation of milk synthesis in dairy cow mammary tissue by lithium chloride for the first time.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] FIG. 1 is a schematic diagram showing the effects of different concentrations of lithium chloride on the expression of β-casein in BMECs;

[0037] FIG. 2 is a schematic diagram showing the effects of different concentrations of lithium chloride on a JAK2/STAT5 signaling pathway in BMECs;

[0038] FIG. 3 is a schematic diagram showing the effects of different concentrations of lithium chloride on the expression of SOCS family in BMECs;

[0039] FIG. 4 is a schematic diagram showing the effects of different concentrations of lithium chloride on a PI3K/AKT1/mTOR signaling pathway in BMECs;

[0040] FIG. 5 is a schematic diagram showing the effects of different concentrations of lithium chloride on the expression of genes related to milk fat synthesis in BMECs;

[0041] FIG. 6 is a schematic diagram showing the effects of different concentrations of lithium chloride on Wnt/β-catenin and HIF-1α signaling pathways in BMECs;

[0042] FIG. 7 is a schematic diagram showing lithium chloride in the upregulation of the expression of *SCD* and *PSMA5* during the endogenous synthesis of CLA in BMECs;

[0043] FIG. 8 is a schematic diagram showing the improvement of the endogenous synthesis of CLA in BMECs by lithium chloride;

[0044] FIG. 9 is a schematic diagram showing the upregulation of the expression of genes and proteins related to fatty acid synthesis by lithium chloride during the endogenous synthesis of CLA in BMECs;

[0045] FIG. 10 is a schematic diagram showing the effects of lithium chloride on the upregulation of the expression of genes and proteins of the Wnt/β-catenin pathway during the endogenous synthesis of CLA in BMECs.

[0046] FIG. 11 is a schematic diagram showing the effects of lithium chloride on the upregulation of the expression of genes and proteins of the HIF-1α pathway during the endogenous synthesis of CLA in BMECs.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0047] In order to make the objectives, technical solutions, and advantages of the present disclosure clearer, the present disclosure will be further described in detail below with reference to the accompanying drawings and examples. It should be understood that the specific examples described herein are merely intended to explain the present disclosure, rather than to limit the present disclosure.

[0048] The specific implementation of the present disclosure will be described in detail below with reference to the specific examples.

[0049] Experiment 1: Use of effects of lithium chloride on the synthesis of milk protein and milk fat in BMECs

[0050] Example 1: BMEC culture

[0051] BMECs. BMECs were cultured in growth medium [DMEM, 10% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 1% penicillin-streptomycin (100 IU/mL penicillin + 100 μg/mL streptomycin)]; after 2-3 passages, the BMECs were inoculated in 6-well plates supplemented with 2 mL of differential medium [DMEM, 10% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 5 μg/mL prolactin, and 1% penicillin-streptomycin (100 IU/mL penicillin + 100 μg/mL streptomycin)] for differentiation induction for four days, and cultured with different concentrations of lithium chloride (5, 10, and 20 mM).

[0052] Example 2: Use of lithium chloride in promoting lactation of dairy cows — the effect of lithium chloride on the secretion of β-casein by BMECs

[0053] 1. BMECs at the growth phase prepared in Example 1 were inoculated into 6-well plates, and static culture was conducted on the BMECs in each well with the growth medium until the BMECs reached around 70%-80% confluence;

[0054] 2. after step 1 was completed, the 6-well plates were grouped as follows:

[0055] treatment groups: the culture medium was aspirated and discarded, the BMECs were washed with PBS, the differential medium supplemented with lithium chloride (5, 10, and 20 mM) was added, and the BMECs were cultured in a 37°C cell culture incubator for four days (the culture medium needed to be changed every day);

[0056] control group: the culture medium was aspirated and discarded, the BMECs were washed with PBS, the differential medium was added, and the BMECs were cultured in the 37°C cell culture incubator for four days (the culture medium needed to be changed every day).

[0057] BMECs cultured for four days were collected from each group, proteins were extracted, and the expression of β-casein was detected by Western blot.

[0058] The gene expression of β-casein is shown in FIGS. 1A and 1B. The results showed that compared with the control group, all lithium chloride (5, 10, and 20 mM) treatment groups showed significantly upregulated protein expression of β-casein ($P < 0.05$).

[0059] Example 3: Effect of lithium chloride on pathways related to milk protein synthesis in BMECs

[0060] 1. Detection of a lactation signaling pathway, JAK2/STAT5 signaling pathway, in BMECs by qPCR and Western blot

[0061] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group and lithium chloride (5, 10, and 20 mM) treatment groups, where all groups were treated for four days. The effects of the expression of *JAK2*, *ELF5*, *STAT5-β*, *SOCS2*, and *SOCS3* genes in four different groups were detected by qPCR. The results are shown in FIG. 2A. The mRNA expression levels of *JAK2* in the lithium chloride (5, 10, and 20 mM) treatment groups were significantly different from those in the control group ($P > 0.05$). In addition, the mRNA level of ELF5 in BMECs of the 10 mM lithium chloride-treated group was significantly higher than that in the control group ($P < 0.05$). In the BMECs treated with lithium chloride (5, 10, and 20 mM), as shown in FIG. 2A, the mRNA level of *STAT5-β* was significantly higher than that in the control group ($P < 0.05$). Notably, in FIGS. 2B and 2C, the level of p-STAT5-β protein was significantly upregulated in the BMECs of the lithium chloride treatment groups (5, 10, and 20 mM) compared with the control group. The expression of STAT5-β inhibitors *SOCS2* and *SOCS3* was detected at the same time (FIG. 3A). Herein, the mRNA levels of JAK2/STAT5 pathway inhibitors (*SOCS2* and *SOCS3*) were significantly inhibited in the lithium chloride (5, 10, and 20 mM) treatment groups compared with the control group. Moreover, Western blot showed that the protein expression of SOCS2 was significantly inhibited in the lithium chloride (5, 10, and 20 mM) treatment groups compared with the control group, and the protein expression of SOCS3 was significantly inhibited in the lithium chloride (5, 10, and 20 mM) treatment groups compared with the control group (FIGS. 3B and 3C).

[0062] These findings suggested that lithium chloride could activate the JAK2/STATA5 signaling pathway by inhibiting the expression of *SOCS2* and *SOCS3*.

[0063] 2. Detection of a lactation signaling pathway, PI3K/AKT1/mTOR signaling pathway, in BMECs by qPCR and Western blot

[0064] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group and lithium chloride (5, 10, and 20 mM) treatment groups, and all groups were treated for four days. The effects of the expression of genes related to the PI3K/AKT1/mTOR pathway in four different groups were detected by qPCR. The results are shown in FIG. 4A, there was no significant difference in the mRNA expression level of *PI3K* between the lithium chloride (5, 10, and 20 mM) treatment groups and the control group ($P > 0.05$). The mRNA level of AKT1 in the BMECs was significantly upregulated in the lithium chloride (5, 10, and 20 mM) treatment groups compared with the control group ($P < 0.05$). The mRNA expression level of *S6K1* in the BMECs was significantly upregulated in the 10 and 20 mM lithium chloride treatment groups compared with the control group ($P < 0.05$). There was no significant difference in the mRNA expression level of *eIF-4E* between the lithium chloride (5, 10, and 20

mM) treatment groups and the control group ($P > 0.05$). The expression level of *4EBP1* was significantly lower in the lithium chloride (5, 10, and 20 mM) treatment groups than in the control group ($P < 0.05$). Results of Western blot (FIGS. 4B and 4C) showed that there was no significant difference in the protein expression level of mTOR between the lithium chloride (5, 10, and 20 mM) treatment groups and the control group ($P > 0.05$). In addition, lithium chloride (5, 10, and 20 mM) significantly upregulated the protein expression of p-mTOR compared with the control group ($P < 0.05$), and the protein expression levels of S6K1 and p-S6K1 were significantly upregulated in the 10 and 20 mM lithium chloride treatment groups compared with the control group ($P < 0.05$). Finally, no significant differences in protein expression levels of S6K1 and p-S6K1 were observed between the 5 mM lithium chloride treatment group and the control group ($P > 0.05$).

[0065] These findings suggested that lithium chloride activated the JAK2/STAT5 and mTOR signaling pathways by inhibiting the expression of *SOCS2* and *SOCS3*, influenced the levels of phosphorylated STAT5-β, p-mTOR, S6K1, and p-S6K1, and ultimately regulated the expression of β-casein.

[0066] Example 4: Effect of lithium chloride on cytokines related to milk fat synthesis in BMECs

[0067] 1. Detection of cytokines related to milk fat synthesis in BMECs by qPCR

[0068] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group and lithium chloride (5, 10, and 20 mM) treatment groups, and all groups were treated for four days. The effects of the expression of fatty acid uptake gene (*LPL*), activation and transport gene (*FASN* and *ACC*) in four different groups were detected by qPCR. The results are shown in FIG. 5A. The expression levels of *ACC* and *LPL* genes were significantly upregulated in the lithium chloride (5, 10, and 20 mM) treatment groups ($P < 0.05$), and the expression level of the *FASN* gene was significantly upregulated in the 10 and 20 mM lithium chloride treatment groups compared with the control group ($P < 0.05$).

[0069] 2. Detection of cytokines related to milk fat synthesis in BMECs by Western blot

[0070] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group and lithium chloride (5, 10, and 20 mM) treatment groups, and all groups were treated for four days. The protein levels of SCD, PPARγ, and SREBP1 in four different groups were detected by Western blot. The results are shown in FIGS. 5B and 5C. The protein levels of SCD, SREBP1 and PPARγ were significantly upregulated in the lithium chloride (5, 10, and 20 mM) treatment groups ($P < 0.05$).

[0071] These findings suggested that lithium chloride influenced enzymes related to milk fat synthesis and network regulators, ultimately regulating the lactation capacity of BMECs.

[0072] Example 5: Lithium chloride regulating Wnt/β-catenin and HIF-1α signaling pathways to influence the expression of genes related to milk protein and milk fat

[0073] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group and lithium chloride (5, 10, and 20 mM) treatment groups, and all groups were treated for four days. The effects of the expression of genes and proteins of Wnt/β-catenin and HIF-1α signaling pathways in four different groups were detected by qPCR and Western blot. The results are shown in FIG. 6A. The expression of the *HIF-1α* gene was significantly upregulated by 10 and 20 mM lithium chloride ($P < 0.05$); the mRNA expression of *EPO*, a downstream gene of *HIF-1α*, was significantly upregulated by 10 mM lithium chloride ($P < 0.05$); the mRNA expression of *EPOR*, a downstream gene of *HIF-1α*, was significantly upregulated by 5 and 10 mM lithium chloride ($P < 0.05$). The results of Western blot in FIGS. 6B and 6C showed that the protein expression of β-catenin and p-β-catenin was significantly upregulated in the lithium chloride (5, 10, and 20 mM) treatment groups ($P < 0.05$), and the protein expression of HIF-1α was significantly upregulated by 10 and 20 mM lithium chloride ($P < 0.05$).

[0074] These findings suggested that lithium chloride could influence the expression of genes and proteins related to milk protein and milk fat by activating the Wnt/β-catenin and HIF-1α signaling pathways.

[0075] Experiment 2: Use in the effect of lithium chloride on the synthesis of CLA in BMECs

[0076] Example 1: BMEC culture

[0077] BMECs BMECs were cultured in growth medium [DMEM, 10% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 1% penicillin-streptomycin (100 IU/mL penicillin + 100 μg/mL streptomycin)]; after 2-3 passages, the BMECs were inoculated in 6-well plates supplemented with 2 mL of differential medium [DMEM, 10% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 5 μg/mL prolactin, and 1% penicillin-streptomycin (100 IU/mL penicillin + 100 μg/mL streptomycin)] for differentiation induction for four days; concentration screening was conducted by adding different concentrations of lithium chloride (1, 5, 10, 15, and 20 mM); after the optimal concentration of lithium chloride was screened out, the BMECs were treated with 50 μM TVA for 4 h.

[0078] Example 2: Effect of lithium chloride on the upregulation of the expression of *SCD* and *PSMA5* genes related to the endogenous synthesis process of CLA in BMECs

[0079] 1. BMECs at the growth phase prepared in Example 1 was inoculated into 6-well plates, and static culture was conducted on the BMECs in each well with the growth medium until the BMECs reached around 70%-80% confluence.

[0080] 2. After step 1 was completed, the 6-well plates were grouped as follows:

[0081] Treatment group 1: the culture medium was aspirated and discarded, the BMECs were washed with PBS, the differential medium supplemented with (1, 5, 10, 15, and 20 mM) lithium chloride was added, and the BMECs were cultured in a 37°C cell culture incubator for four days (the culture medium was changed every day); after four days, RNAs and proteins were extracted, the expression of *SCD* and *PSMA5* was detected by qPCR, and the concentration was preliminarily screened.

[0082] Treatment group 2: the culture medium was aspirated and discarded, the BMECs were washed with PBS, the differential medium supplemented with (1, 10, and 15 mM) lithium chloride was added, and the BMECs were cultured in the 37°C cell culture incubator for four days (the culture medium needed to be changed every day); after four days, the BMECs were treated with 50 μM TVA for 4 h, RNAs were extracted, and the expression of *SCD* and *PSMA5* was detected by qPCR and Western blot, and the optimal treatment concentration was screened.

[0083] Control group: the culture medium was aspirated and discarded, the BMECs were washed with PBS, the differential medium was added, and the BMECs were cultured in the 37°C cell culture incubator for four days (the culture medium needed to be changed every day).

[0084] BMECs cultured for four days were collected from each group, RNAs were extracted, and the expression of *SCD* and *PSMA5* was detected by qPCR.

[0085] The expression of *SCD* and *PSMA5* is shown in FIGS. 7A and 7B. The results showed that compared with the control group, lithium chloride (5, 10, and 15 mM) treatment groups showed significantly upregulated mRNA expression of *SCD* ($P < 0.05$). The mRNA expression of *PSMA5* was significantly upregulated in the lithium chloride (5 and 10 mM) treatment groups ($P < 0.05$). Therefore, after different concentrations of lithium chloride (1, 10, and 15 mM) were screened for differentiation for four days, the BMECs were treated with 50 μM TVA for 4 h. As shown in FIGS. 7C and 7D, the mRNA expression of SCD and PSMA5 were significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the mRNA expression of *SCD* was significantly upregulated in the TVA + lithium chloride (1, 10, and 15, mM) groups compared with the TVA group ($P < 0.05$); the mRNA expression of *PSMA5* was significantly upregulated in the TVA + lithium chloride (1 and 10 mM) groups compared with the TVA group ($P < 0.05$), among which 10 mM lithium chloride treatment had a better effect. Therefore, 10 mM lithium chloride was selected as the optimal treatment concentration. The effects of lithium

chloride on the upregulation of the protein expression of *SCD* and *PSMA5* genes related to the endogenous synthesis of CLA in BMECs were detected by Western blot. As shown in FIGS. 7E, 7F, and 7G, the protein expression of *SCD* and *PSMA5* was significantly upregulated in the lithium chloride treatment groups compared with the control group ($P < 0.05$), and the protein expression of *SCD* and *PSMA5* was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the protein expression of *SCD* and *PSMA5* was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$).

[0086] These findings suggested that lithium chloride could upregulate the expression of *SCD* and *PSMA5* genes related to the endogenous synthesis of CLA in BMECs.

[0087] Example 3: Improvement of lithium chloride in the endogenous synthesis of CLA in BMECs

[0088] 1. Detection of lithium chloride in the endogenous synthesis of CLA in BMECs by GC

[0089] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group, a TVA group, a 10 mM lithium chloride group, and a TVA + 10 mM lithium chloride group, and after four-day treatment, the BMECs were treated with 50 μM TVA for 4 h. GC detected lithium chloride in the endogenous synthesis of CLA in BMECs. The results are shown in FIG. 8. The endogenous synthesis of *cis*-9, *trans*-11 CLA ($P < 0.01$) , and CLA index ($P < 0.05$) were significantly increased in the TVA + lithium chloride group compared with the TVA group.

[0090] These findings suggested that lithium chloride could improve the endogenous synthesis of CLA in BMECs.

[0091] Example 4: Effects of lithium chloride on the expression of genes related to fatty acid synthesis during the endogenous synthesis of CLA in BMECs

[0092] 1. Detection of cytokines related to milk fat synthesis in BMECs by qPCR and Western blot

[0093] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group, a TVA group, a 10 mM lithium chloride group, and a TVA + 10 mM lithium chloride group, and after four-day treatment, the BMECs were treated with 50 μM TVA for 4 h. The effects of the expression of fatty acid uptake gene (*LPL*), activation and transport gene (*FASN* and *ACC*) in four different groups were detected by qPCR, as well as the effects of the expression of the adipose differentiation-related protein (*PLIN2*) and fatty acid network regulatory gene (*SREBP1*). The results are shown in FIG. 9. The mRNA expression of *ACC*, *FASN*, *LPL*, *PLIN2*, and *SREBP1* was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); the mRNA expression of *ACC*, *FASN*, *LPL*, and

SREBP1 was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the mRNA expression of *ACC*, *FASN*, *LPL*, *PLIN2*, and *SREBP1* was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$). The protein expression of SREBP1 and PPARγ was detected by Western blot. The results are shown in FIGS. 9F, G, and H. The protein expression of PPARγ was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); the protein expression of PPARγ was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$); the protein expression of SREBP1 was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); the protein expression of SREBP1 was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the protein expression of SREBP1 was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$).

[0094] These findings suggested that lithium chloride could upregulate the expression of genes and proteins related to fatty acid synthesis in the endogenous synthesis of CLA in BMECs and that lithium chloride could interact with TVA and alleviate the inhibitory effect of TVA on these genes.

[0095] Example 5: Study of effects of lithium chloride on the upregulation of the expression of genes related to Wnt/β-catenin and HIF-1α during the endogenous synthesis of CLA in BMECs

[0096] The BMECs were inoculated in 6-well plates and divided into four groups, namely a control group, a TVA group, a 10 mM lithium chloride group, and a TVA + 10 mM lithium chloride group, and after four-day treatment, the BMECs were treated with 50 μM TVA for 4 h. The effects of the expression of genes and proteins of Wnt/β-catenin and HIF-1α signaling pathways in four different groups were detected by qPCR and Western blot. The results are shown in FIGS. 10A and B. The mRNA expression of the Wnt/β-catenin pathway inhibitor *GSK3β* was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the mRNA expression of *β-catenin*, a representative protein downstream of the β-catenin pathway, was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the mRNA expression of *β-catenin* was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$). Also, genes related to HIF-1α signaling pathway were detected. As shown in FIGS. 11A, B, and C, the mRNA expression of *HIF-1α*, *EPO,* and *EPOR* was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the mRNA expression of *HIF-1α*, *EPO*, and *EPOR* was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); the mRNA expression of *HIF-1α*, *EPO*, and *EPOR* was significantly upregulated in the TVA + lithium chloride group

compared with the TVA group ($P < 0.05$). The protein expression of genes related to Wnt/β-catenin and HIF-1α signaling pathways was detected by Western blot. The results are shown in FIGS. 10C, D, E, F, and G. The protein expression of β-catenin was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); the protein expression of β-catenin was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$); there was no significant difference in the protein expression of p-β-catenin between the TVA group and the control group ($P > 0.05$); the protein expression of p-β-catenin was significantly upregulated in the TVA +lithium chloride group compared with the TVA group ($P < 0.05$); the protein expression of GSK3β, an inhibitor of Wnt/β-catenin signaling pathway, was significantly inhibited in the lithium chloride group, the TVA group, and the TVA + lithium chloride group compared with the control group (all $P < 0.05$); the protein expression of p-GSK3β was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); there was no significant difference in the protein expression of p-GSK3β between the TVA group and the control group ($P > 0.05$); the protein expression of p-GSK3β was significantly upregulated in the TVA + lithium chloride group compared with the TVA group and the control group ($P < 0.05$). For the HIF-1α signaling pathway, as shown in FIGS. 11D and E, the protein expression of HIF-1α was significantly inhibited in the TVA group compared with the control group ($P < 0.05$); the protein expression of HIF-1α was significantly upregulated in the lithium chloride group compared with the control group ($P < 0.05$); the protein expression of HIF-1α was significantly upregulated in the TVA + lithium chloride group compared with the TVA group ($P < 0.05$).

[0097] These findings suggested that lithium chloride could improve the endogenous synthesis of CLA in BMECs by activating the Wnt/β-catenin and HIF-1α signaling pathways.

[0098] The above descriptions are only preferred embodiments of the present disclosure and are not intended to limit the present disclosure. Any modifications, equivalent substitutions and improvements made within the spirit and principles of the present disclosure shall be included in the protection scope of the present disclosure.

WHAT IS CLAIMED IS:

1. An application method for regulating lithium chloride in a bovine mammary epithelial cell (BMEC), comprising the following steps:

step 1: use in effects of the lithium chloride on the synthesis of milk protein and milk fat in BMEC: inoculating the BMEC in a Petri dish supplemented with a growth medium, and culturing the BMEC in a 37°C cell culture incubator; conducting differentiation induction on the BMEC for four days, adding different concentrations of the lithium chloride at the same time, and extracting and collecting RNAs and proteins after four days; detecting regulatory effects of the lithium chloride on β-casein in the BMEC, PI3K/AKT1/mTOR and JAK2/STAT5 signaling pathways, and enzymes related to milk fat synthesis by quantitative polymerase chain reaction (qPCR) and Western blot; preliminarily investigating a mechanism of regulation of lactation of the BMEC by the lithium chloride by the qPCR and the Western blot; and

step 2: use of effects of the lithium chloride on the synthesis of conjugated linoleic acid (CLA) in the BMEC: inoculating the BMEC in a Petri dish supplemented with the growth medium, and culturing the BMEC in the 37°C cell culture incubator; conducting differentiation induction on the BMEC for four days, screening out an optimal concentration of the lithium chloride, treating the BMEC with 50 μM *trans*-vaccenic acid (TVA) for 4 h, and extracting and collecting the RNAs and the proteins; detecting an effect of the lithium chloride on key marker genes for the synthesis of the CLA in the BMEC by the qPCR and the Western blot, and detecting CLA content in the BMEC to determine the effect of the lithium chloride on the synthesis of the CLA in the BMEC by gas chromatography (GC); preliminarily investigating a mechanism of regulation of the synthesis of the CLA in the BMEC by the lithium chloride by the qPCR and the Western blot.

2. The application method for regulating lithium chloride in a BMEC according to claim 1, wherein the growth medium is a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 1% penicillin-streptomycin.

3. The application method for regulating lithium chloride in a BMEC according to claim 1, wherein a differential medium used for the differentiation induction is a DMEM supplemented with 10% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 5 μg/mL prolactin, and 1% penicillin-streptomycin.

4. The application method for regulating lithium chloride in a BMEC according to claim 3, wherein the differential medium is placed in 6-well plates, and a quantity of the differential medium is 2 mL.

5. The application method for regulating lithium chloride in a BMEC according to claim 1, wherein the lithium chloride in step 1 has three concentrations: 5, 10, and 20 mM.

6. The application method for regulating lithium chloride in a BMEC according to claim 2, wherein detection of the regulatory effects of the lithium chloride on the β-casein and genes and proteins related to the milk fat synthesis in the BMEC in step 1 comprises the following steps:

step i. inoculating the BMEC at the growth phase into 6-well plates, and conducting static culture on the BMEC in each well with the growth medium until the BMEC reaches 70%-80% confluence;

step ii. grouping the 6-well plates as follows,

treatment groups: aspirating and discarding the culture medium, washing the BMEC with phosphate-buffered saline (PBS), adding differential media supplemented with the lithium chloride at three concentrations of 5, 10, and 20 mM, respectively, culturing the BMEC in the 37°C cell culture incubator for four days and changing the culture medium every day,

a control group: aspirating and discarding the culture medium, washing the BMEC with the PBS, adding the differential medium, culturing the BMEC in the 37°C cell culture incubator for four days, and changing the culture medium every day; and

step iii. collecting BMEC cultured for four days from each group, extracting the RNAs and the proteins, and detecting the expression of genes related to the synthesis of milk protein and milk fat by the qPCR and the expression of β-casein and proteins related to the synthesis of the milk protein and the milk fat by the Western blot.

7. The application method for regulating lithium chloride in a BMEC according to claim 2, wherein detection of lactation signaling pathways of the BMEC, JAK2/STAT5, PI3K/AKT1/mTOR, and SREBP1, by the qPCR and the Western blot in step 1 comprises the following steps:

step a, inoculating the BMEC in 6-well plates and dividing the BMEC into four groups, namely a control group and three lithium chloride treatment groups, where lithium chloride concentrations of the three lithium chloride treatment groups are 5, 10, and 20 mM, respectively,

and all groups are treated for four days;

step b, detecting effects of the expression of *JAK2*, *ELF5*, *STAT5-β*, *SOCS2*, *SOCS3*, *PI3K*, *AKT1*, *mTOR*, *S6K1*, *eIF-4E*, *4EBP1*, *ACC*, *FASN*, *LPL*, *HIF-1α*, *EPO*, and *EPOR* genes in the four different groups by the qPCR; and

step c, detecting effects of the protein expression of β-casein, p-STAT5-β, mTOR, p-mTOR, S6K1, p-S6K1, SCD, PPARγ, SREBP1, HIF-1α, β-catenin, and p-β-catenin in different groups by the Western blot.

8. The application method for regulating lithium chloride in a BMEC according to claim 1, wherein the optimal concentration of the lithium chloride screened in step 2 is 10 mM.

9. The application method for regulating lithium chloride in a BMEC according to claim 2, wherein the use in effects of the lithium chloride on the synthesis of CLA in the BMEC in step 2 comprises the following steps:

step i. inoculating the BMEC at the growth phase into 6-well plates, and conducting static culture on the BMEC in each well with the growth medium until the BMEC reaches 70%-80% confluence;

step ii. grouping the 6-well plates as follows,

treatment groups: aspirating and discarding the culture medium, washing the BMEC with PBS, adding a differential medium supplemented with 10 mM lithium chloride, culturing the BMEC in the 37°C cell culture incubator for four days, and changing the culture medium every day; after four days, treating the BMEC with 50 μM TVA for 4 h, where there are the following treatment groups: a 10 mM lithium chloride group, a TVA group, and a TVA + 10 mM lithium chloride group;

a control group: aspirating and discarding the culture medium, washing the BMEC with the PBS, adding the differential medium, culturing the BMEC in the 37°C cell culture incubator for four days, and changing the culture medium every day; and

step iii. collecting the BMEC, extracting the RNAs and the proteins, detecting the expression of genes related to the synthesis of the CLA by the qPCR, detecting the expression of proteins related to the synthesis of the CLA by the Western blot, and detecting the CLA content in the BMEC by the GC.

10. The application method for regulating lithium chloride in a BMEC according to claim 2, wherein detection of genes and proteins related to the synthesis of the CLA in the BMEC by the

qPCR and the Western blot in step 2 comprise the following steps:

step a. inoculating the BMEC into 6-well plates and dividing the BMEC into four groups, namely a control group, a 10 mM lithium chloride group, a TVA group, and a TVA + 10 mM lithium chloride group;

step b. detecting effects of the expression of *SCD*, *PSMA5*, *ACC*, *FASN*, *LPL*, *PLIN2*, *SREBP1*, *GSK3β*, *β-catenin*, *HIF-1α*, *EPO*, and *EPOR* genes in four different groups by the qPCR;

step c. detecting effects of the protein expression of SCD, PSMA5, PPARγ, SREBP1, β-catenin, p-β-catenin, and HIF-1α in different groups by the Western blot; and

step d. detecting fatty acid content in the BMEC by the GC, and comparing an effect on the CLA content.

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Application No:	GB2215781.2	**Examiner:**	Dr Sam Stokes
Claims searched:	1-10	**Date of search:**	15 May 2023

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-10	Molecules (Basel, Switzerland), vol. 19, no. 7, 2014, Zhang Xia et al., "GSK3beta regulates milk synthesis in and proliferation of dairy cow mammary epithelial cells via the mTOR/S6K1 signaling pathway.", p. 9435-9452. see abstract and results section 2.2

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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The following online and other databases have been used in the preparation of this search report

BIOSIS, MEDLINE

International Classification:

Subclass	Subgroup	Valid From
A61K	0033/14	01/01/2006
C12N	0005/07	01/01/2010
C12N	0005/071	01/01/2010