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RESEARCH ARTICLE

Expression of Stanniocalcin-1 in the Gastrointestinal Tract and Kidney of Neonatal Calves

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ABSTRACT

Stanniocalcin-1 (STC-1) is a novel calcium- and phosphate-regulating hormone that was originally found in bony fish and subsequently in mammals. This study mainly aimed to outline the distribution and relative expression levels of STC-1 in the gastrointestinal tract and kidney of neonatal Holstein calves. The localization of the STC-1 protein/mRNA in these sites was investigated by immunocytochemistry and *in situ* hybridization staining. Impressively, the results showed that the STC-1 mRNA was mainly distributed in the distal segments of the kidney, whereas the protein was mostly found in the proximal and distal segments, including the glomerulus. In the gastrointestinal tract, the STC-1 mRNA was expressed in the *lamina propria* and mucosa epithelial cells, whereas the protein was found in the cytoplasm of goblet cells, chief cells of the fundic glands, gastric pits, and intestinal glands. Quantitative analyses indicated that the highest levels of both STC-1 mRNA and protein were expressed in the kidney. The highest STC-1 mRNA and protein levels in the stomach were expressed in the abomasum. In addition, the duodenum and colon exhibited the highest STC-1 mRNA relative expression ratios among the intestine, whereas the highest protein expression levels were found in the duodenum and jejunum. In addition, The STC-1 protein detected in this experiment may be a STC50 rather than a big STC. Thus, STC-1 may be involved in digestion and/or absorption in the alimentary canals and the kidney in those sites with active digestion and absorption.

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INTRODUCTION

Stanniocalcin (STC) is a homodimer glycoprotein hormone was first identified in bony fish, where it is synthesised/secreted by corpuscles of Stannius (CS), an endocrine gland unique to bony fish (Huang *et al*., 2014). STC-1 is the mammalian homologue was first identified from a human cell line (Roddy *et al*., 2017) and subsequently in other mammalian species (Roch *et al.*, 2011). Unlike in fish where STC counteracts hypercalcemia in a classical endocrine fashion by inhibiting the gill and intestinal Ca^{2+} uptake and the simultaneous stimulating the renal inorganic phosphate (Pi) reabsorption (Huang *et al.*, 2015), mammalian STC-1 is normally undetectable in the blood (Deol *et al.*, 2001) with the exception of during pregnancy (Tremblay *et al.*, 2009) and tumorigenesis (Fang *et al.*, 2014; Ifeacho *et al.*, 2016). In addition, STC-1 ubiquitously exists in the highly vascularized tissues (De Niu *et al.*, 2000) and involves in a variety of biological and/or pathological processes, such as Ca^{2+}/Pi regulatory (Madsen *et al.*, 1998), cancer (Brantley *et al.*, 2018; Li *et al.*, 2018; Sobotka *et al.*, 2018), neuroprotection (Durukan Tolvanen *et al.*, 2013; Roddy *et al*., 2017), apoptosis (Ching *et al*., 2012) and anti-inflammation (Liu *et al*., 2016; Mohammadipoor *et al*., 2016). The involvement of STC-1 in the modulation of various processes in multiple tissues and its undetectable level in the blood may underlie its activity as a local mediator of cell function in a paracrine/autocrine fashion (Yeung *et al.*, 2012).

The gastrointestinal tract and kidney are the main sites for metabolism and Ca^{2+}/Pi (re)absorption in which many hormones, such as 1, $25(OH)_2D_3$, various brain-gut peptides, as well as STC-1 (Zhao *et al.*, 2018), play

specific roles. The functions and localization of STC-1 in cows have been extensively studied for decades (Delbecchi *et al.*, 2005; Tremblay *et al.*, 2009; Liu *et al.*, 2012; Muñoz *et al.*, 2017), however, the distribution of STC-1 in the gastrointestinal tract of cattle is still unclear. This lack of information prompted the study of this hormone in these locations of dairy animals because they are well known for their importance in the absorption of nutrients, especially in neonatal calves and lactating cows.

MATERIALS AND METHODS

The experimental protocol was approved by the official Committee on the Ethics of Animal Experiments of Huazhong Agricultural University.

Tissue preparation: Five male Chinese Holstein calves (1-day-old) were anesthetized and euthanized. The stomach, intestine, and kidney were removed and dissected into small pieces. Parts of them were rapidly immersed in liquid nitrogen for RNA/protein isolation, and the others were fixed in pre-cooled 4% paraformaldehyde/0.1 M PBS (pH 7.0-7.6) for 24 h for immunocytochemistry (ICC) and for 1 h for *in situ* hybridization (ISH). The fixed tissues were dehydrated, embedded, cut, and mounted on microscope slides for ICC or on poly-lysine-coated slides for ISH.

Generation of bovine STC-1 antiserum: The cDNA from the cattle kidney was used to amplify the complete CDS of bovine STC-1 (excluding a 48-bp signal peptide sequence) using the PCR primers: 5'-ATGGATCCATG GCAACCCATGAGGCGGAGCA-3' and 5'-ATCAAGCT TCTAGGCACTCTCCTGGGAGG-3'. The amplicon was purified and then subcloned into the pET-32a+ plasmid (Invitrogen, Carlsbad, USA) at *Bam*H I and *Hin*d III sites. The recombinant plasmid was sequenced and transformed into the Rosetta (DE3) bacteria (Invitrogen). After a 3-h induction by 1 mM IPTG, the fusion protein was purified from the bacteria using Ni^{2+}-column chromatography (Tiangen, Beijing, China). Japanese white rabbits were immunized through the subcutaneous injection of 1-2 mg of STC-1 protein, and the antiserum was collected after the animals were anesthetized. Western blot analysis was performed to detect the specificity of the STC-1 antiserum.

Immunocytochemistry: For ICC, the slides were deparaffinized, rehydrated and quenched the endogenous peroxidase activity by a 10-min treatment with 3% H_2O_2, and a 20-min heating in 0.01 M citrate buffer solution (pH 6.0) was utilized to unmask the antigens. After blocking with 5% normal rabbit serum, the slides were incubated overnight at 4°C with diluted STC-1 antiserum (1:500 in PBS) or with an equivalent volume of normal rabbit serum as a control. The sites of immunoreaction were visualized using the biotinylated goat anti-rabbit IgG (Abcam, Cambridge, USA) and DAB system (CoWin, Beijing, China). The slides were counterstained with hematoxylin, the images were captured by an inverted microscope system (Olympus, Japan).

***In situ* hybridization:** The digoxigenin-labelled anti-sense probe for human STC-1 mRNA were synthesized by Boster (Boster, Wuhan, China), and the sequence is 5'-TGTGACACAGATGGGATGTATGACATCTGTAAAT C-3'. In situ hybridization was investigated by using an ISH detection kit (Boster) according to the manufacturer's protocol.

Real-time quantitative RT-PCR: The total RNA and cDNA were prepared using the TRizol reagent (Invitrogen) and a reverse transcription kit (TaKaRa) according to the manufacturer's instructions, respectively. Gene expression was performed using ABi StepOne real-time PCR apparatus (Applied Biosystems, Foster, USA). GAPDH gene served as the internal standard. The PCR primers are as follows: STC-1 (NM_003155.2), 5'-AGTGATTCGCTGCCTCAACA -3' (sense) and 5'- TCT CCAGGCATGCAAAAGCT -3' (anti-sense); GAPDH (NM_017008), 5'-CCTTCATTGACCTTCACTACATGG TCTA-3' (sense) and 5'-TGGAAGATGGTGATGGCCT TTCCATTG-3' (anti-sense). Each sample was assayed in triplicate in 25-μL volumes with 1× SYBR Green I and 0.2 μM forward and reverse primers for each gene. After denaturation at 94°C for 5 min, the reaction followed by 40 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 25 s and a final 5-min extension at 72°C.

Western blot analysis: Each tissue was homogenised with ice-cold RIPA lysis buffer (Beyotime, Wuxi, China) supplemented with a protease inhibitor mixture (Sigma, St. Louis, USA). An 8-min centrifugation was performed at 12,000×g to collect the supernatant whose protein concentration was calculated by a BCA protein assay kit (CoWin). A total of 30 μg of protein from each sample was electrophoresed on 12% SDS/PAGE and transferred to a 0.22-μM PVDF membrane (Biosharpp, Hefei, China). After a 2-h blocking with milk-TBST (5% non-fat milk, 0.5% Tween-20 and 0.15 M NaCl in 0.01 M Tris-HCl), the membranes were incubated with diluted STC-1 antiserum (1:500 in TBST) or rabbit monoclonal against GAPDH antibody (Abcam) for 3 h. The membranes were then incubated with goat anti-rabbit IgG-HRP (1:5000, CoWin). The antigen-antibody reaction was visualized by enhanced chemiluminescence (CoWin).

Statistical analysis: All of the data are represented as the mean±SEM. The significance of the differences between samples was analyzed by one-way ANOVA. Each sample was analyzed in triplicate or quadruplicate in the same experiment, and individual experiments were repeated at least three times. A $P<0.05$ was considered statistically significant.

RESULTS

Localization of STC-1 protein and mRNA: The ICC and ISH results for the kidney are shown in Fig. 1. In kidney, the positive immunoreactivity for STC-1 (STC-1ir) was mainly found in the renal glomerulus (G, A1), the cytoplasm of the proximal tubules (PT, A1), and the distal tubules (DT, A1). And the collecting duct (CD, A1) was lightly stained by STC-1 antiserum. The ISH signal was abundantly present in the renal distal tubule (DT, B1), thick ascending limb (TAL, B1) and collecting duct (CD, B1), whereas the proximal tubule (PT, B1) and glomerulus (G, B1) presented a weak or no signal.

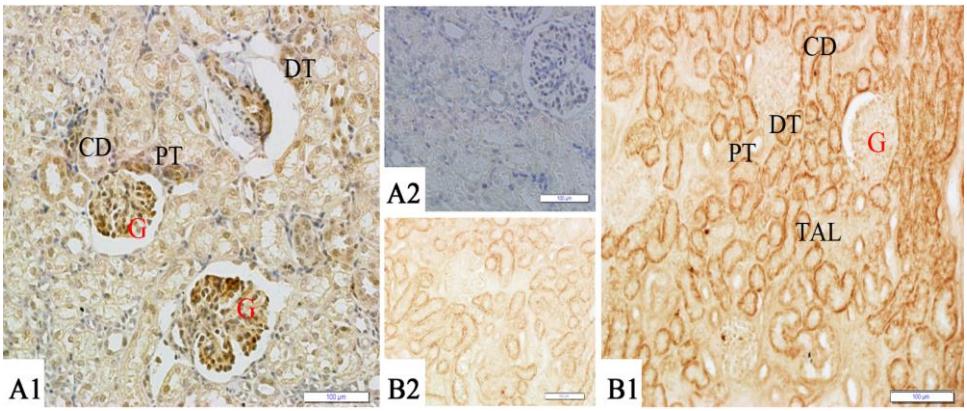

Fig. 1: Immunocytochemical (ICC) and *in situ* hybridization (ISH) analysis of bovine kidney. A1) The tissue section was stained by rbSTC-1 antiserum. A2) An adjacent tissue section incubated with normal rabbit serum in lieu of the STC-1 antiserum. B1) The tissue section was labelled by anti-sense cRNA probe. B2) An adjacent tissue section hybridized with PBS instead of anti-sense probe. G: glomerulus, PT: proximal tubule, DT: distal tubule, TAL: thick ascending limb, CD: collecting duct. Scale bar: 100 µm.

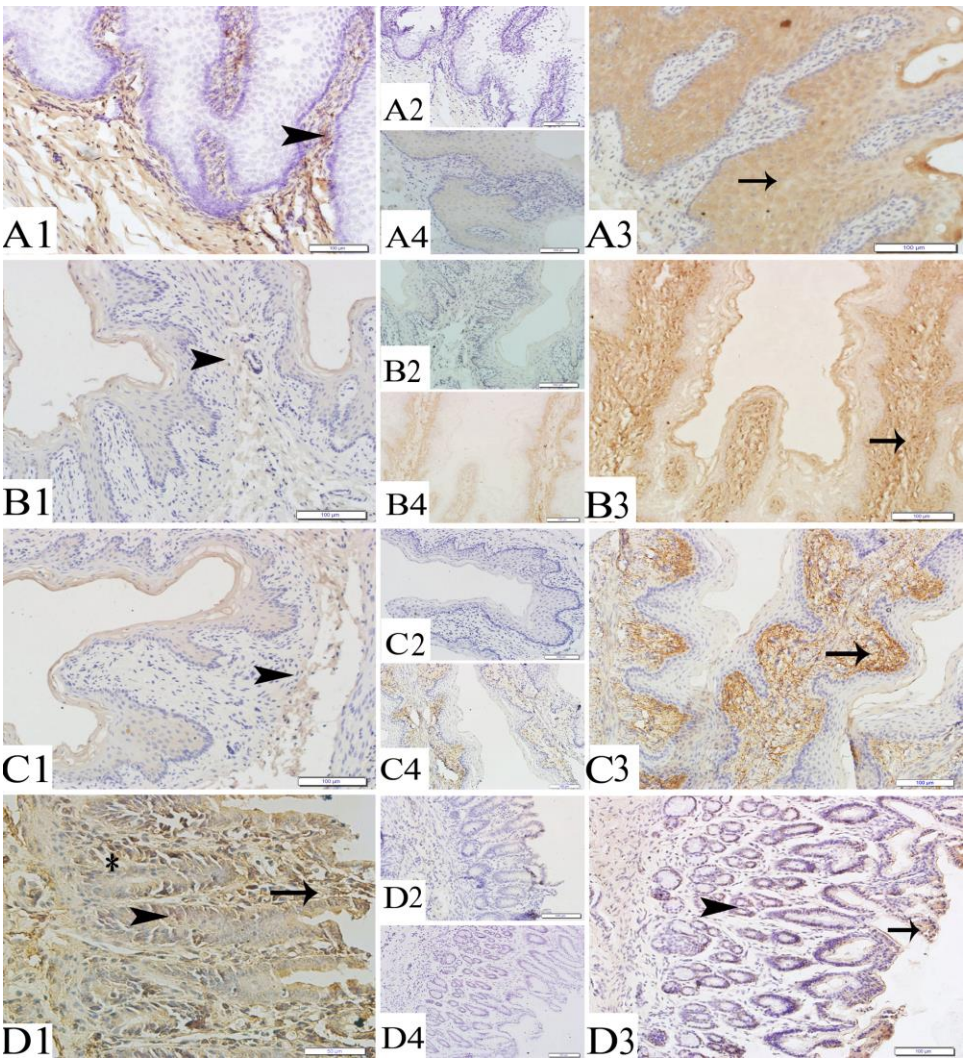

Fig. 2: Tissue distribution of the STC-1 protein and mRNA in bovine stomach. A1) to D1) The rumen, reticulum, omasum, and abomasum tissue sections were respectively stained by ICC using the STC-1 antiserum. A2) to D2) The corresponding adjacent tissue sections of stomach stained by ICC using the normal rabbit serum in lieu of the rbSTC-1 antiserum. A3) to D3) The rumen, reticulum, omasum, and abomasum tissue sections of stomach are stained by ISH using anti-sense probe. A4) to D4) The corresponding adjacent tissue sections of stomach hybridized with PBS instead of the anti-sense probe. Scale bar: 50 µm in D1, 200 µm in C2 100 µm in others.

The results from the stomach tissues are presented in Fig. 2. In the forestomach, STC-1ir was mainly localized in the *lamina propria*, and that in the rumen was stronger than that in the reticulum and omasum (Arrowheads, A1 to C1). In addition, weak immunoreactivity was also observed in the mucous epithelium of the reticulum (B1 and C1) and the *lamina propria* of the abomasums (Arrowhead, D1). However, the STC-1ir in the fundic glands (Asterisk, D1) and gastric pits (Arrow, D1) of the abomasums was more evident than that observed in other segments of the stomach. Hybridization with the anti-sense probe yielded significant positive hybridization signals in the *lamina propria* of the forestomach (Arrows, A3 to C3) and in the fundic glands (Arrowhead, D3) and gastric pits (Arrow, D3) of the abomasum.

Fig. 3 demonstrates the results obtained for the intestinal tissues. Numerous STC-1ir were found in the intestinal tract, and most of these were confined to the cytoplasm of goblet cells in the duodenum, jejunum, and ileum (Black arrows, A2 to C2) and to the cells of the intestinal glands of the duodenum, jejunum, ileum, and cecum (Asterisks, A2 to D2), however, an evident STC-1ir was not observed in the colon (E2). The ISH analysis showed obvious positive hybridization signals only in the duodenum, particularly in the cytoplasm of goblet cells (Hollow arrow, A1). The *lamina propria* of the jejunum,

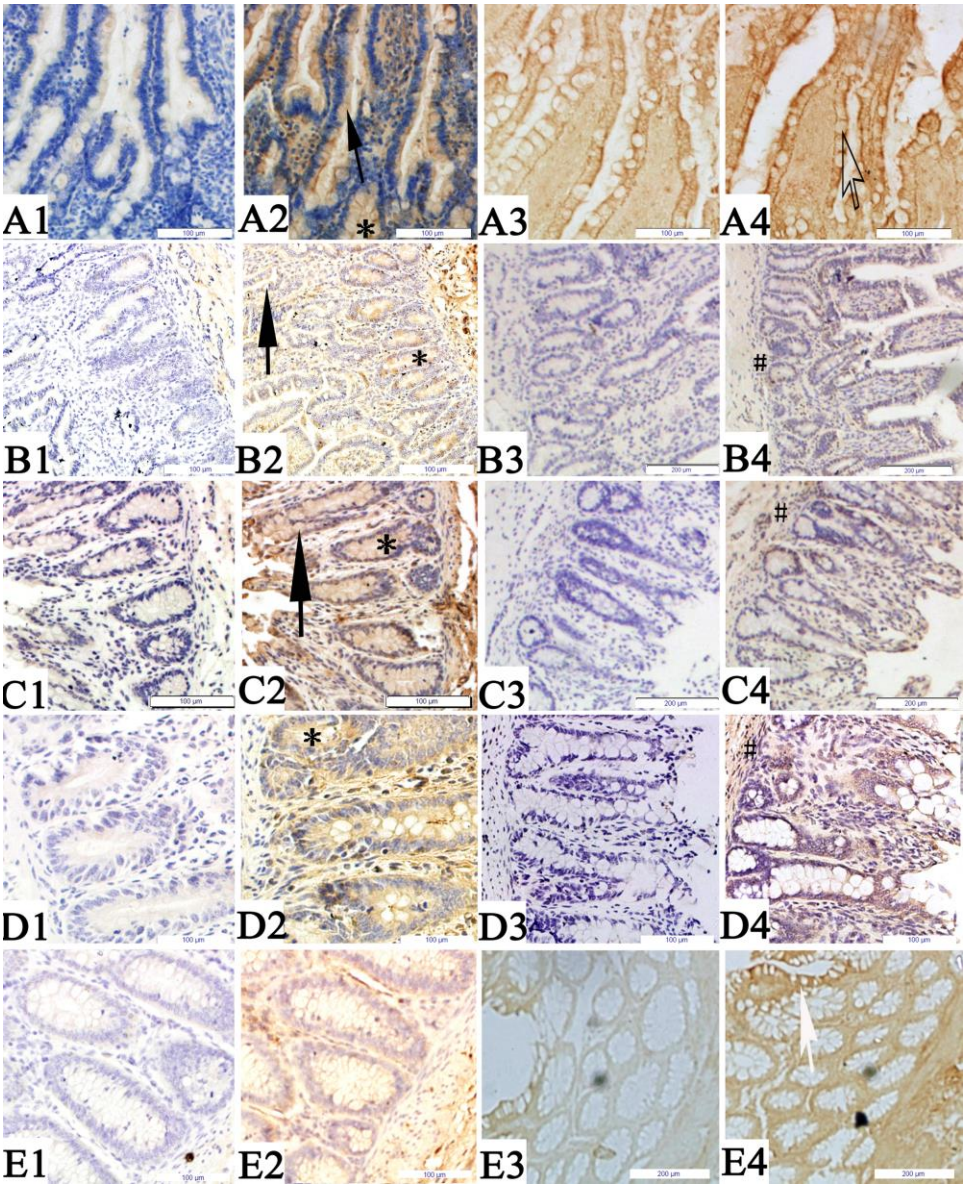

Fig. 3: Tissue distribution of the STC-1 protein and mRNA in the intestine. Panels A to E are the photographs of the duodenum, jejunum, ileum, cecum, and colon, respectively. Numbers 1 to 4 are the tissue sections stained by ICC with normal rabbit serum and STC-1 antiserum, and by ISH with PBS and the anti-sense probe, respectively. Scale bars: 200 μm in B3, B4, C3, C4, E3, E4; 100 μm in others.

ileum, and cecum exhibited weak hybridization signals (Pound signs, B4 to D4). In addition, a small amount of positive hybridization signals were observed in the intestinal epithelial cells bordering the goblet cells in the colon (White arrow, E4).

Quantitative analysis of STC-1 expression in gastrointestinal tract: Real-time RT-PCR using SYBR Green I and Western blot assays were performed to evaluate the quantitative difference in the STC-1 mRNA and protein expression levels in the gastrointestinal tract of neonatal calves. The expression levels of STC-1 mRNA and protein in the kidney were used as the standard values, and all of the data obtained from the gastrointestinal tract were compared with the corresponding levels in the kidney. The data shown in Fig. 4 demonstrated that the abomasum exhibited the highest mRNA expression level (~49% of that in the kidney and ~19.5%, ~19.8%, and ~17.2% of that in the rumen, reticulum, and omasum, respectively) in the stomach. In the small intestines, the highest level of STC-1 mRNA was found in the duodenum (~85% of the level in the kidney), and the levels of STC-1 mRNA in the jejunum and ileum were ~38% and ~27% of that in the kidney. In the large intestine, the level of STC-1 mRNA expression in the colon (~78%) was markedly higher than that in the cecum (~21%). In the intestinal tract, the highest levels of protein were found in the duodenum and jejunum. In addition, the level in the abomasums was higher than that in the forestomach. However, all of the expression levels obtained in the gastrointestinal tract were lower than those found in the kidney. The Western blot analysis detected a band of approximately 30 kDa, which suggests that the STC-1 detected in this study may be STC50.

DISCUSSION

In this study, the STC-1 protein and not the mRNA was clearly observed in the renal glomerulus. This finding is inconsistent with previous reports, which showed only STC-1 mRNA in the glomerulus of rats (Kobayashi *et al.*, 2009) and mice kidney (Deol *et al.*, 2001). This result may be associated with the large amount of STC-1 secreted by maternal ovaries into the blood of neonates during pregnancy and lactation (Tremblay *et al.*, 2009). Thus, many STC-1 proteins flowed through the bloodstream

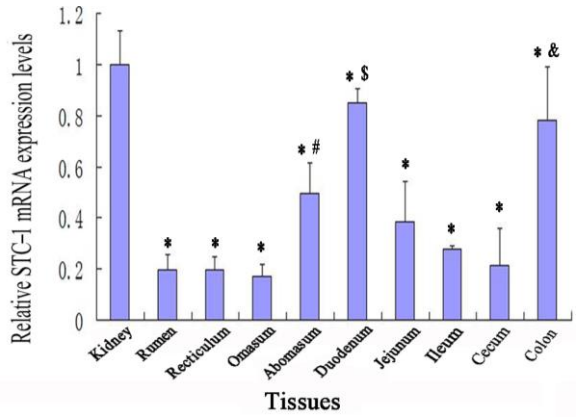

Fig. 4: The stanniocalcin 1 (STC-1) gene expression levels in the kidney and gastrointestinal tracts were detected by real-time PCR and normalized by the levels of GAPDH. *P<0.05 represented statistically less mRNA levels in the gastrointestinal tract compared to the kidney, $^{\#}$P<0.05 represented statistically greater mRNA levels in the abomasum compared to the forestomach, $^{\$}$P<0.05 represented statistically greater mRNA levels in the duodenum compared to the distal small intestine, $^{\&}$P<0.05 represented statistically greater mRNA levels in the colon compared to the cecum.

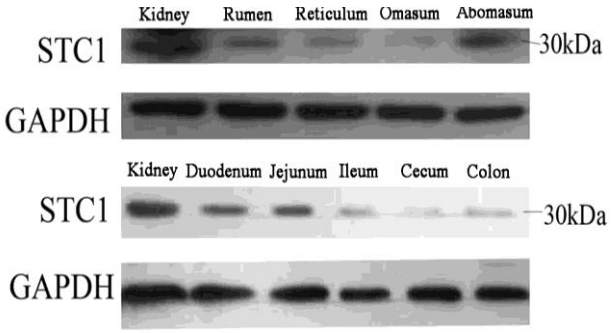

Fig. 5: Western blot analysis of the stanniocalcin 1 (STC-1) protein expression levels in the kidney and gastrointestinal tract.

to reach the glomerulus, which is richly endowed with blood vessels. In addition to the glomerulus, the STC-l protein was also observed in the cell cytoplasm of the distal tubules, proximal tubules, and collecting ducts. However, ISH signal for STC-1 mRNA was evident in the distal tubule, collecting duct, and thick ascending limb of the kidney, only a weak signal was observed in the proximal segments. The observations in the renal tubules were generally consistent with those obtained in previous studies, which showed that STC-l mRNA is expressed in the distal segments of rodent kidney and that the protein is expressed in the proximal and distal segments excluding glomerulus (Lyu *et al.*, 2018). These results suggested that the STC-l expression patterns in cow kidney may differ from that in rodents, and this difference may be related to the fact that the requirement of Ca^{2+}/Pi (re)absorption in new born cows is higher than that in rodent. However, the exact mechanism for this difference requires further study.

An efficient digestion and absorption via the stomach and intestine is vastly important in neonatal calves, which demand a large amount of nutrients during their growth and development. Thus, it is important to perform an in-depth study of the distribution and roles of STC-1 in digestive organs. It has been well documented that treatment with STC-1 can inhibit Ca^{2+} transport across intestinal epithelia (Madsen *et al.*, 1998; Chou *et al.*, 2015), however, not until 2002 did the first mammalian intestinal epithelial cell-derived STC-1 mRNA be found (Yoshiko *et al.*, 2002). This study performed a detailed investigation of the precise localization of STC-1 in the gastrointestinal tract of neonatal calves. The ruminant species like cattle have four stomachs, namely rumen, reticulum, omasum and abomasums. The former three are named forestomach and only responsible for physical digestion and a small amount of chemical digestion. In addition, the abomasum is equivalent to the true stomach of non-ruminant mammals, through which ruminant animals can digest food and secrete gastric juices that contain pepsin and hydrochloric acid. In this study, both STC-1 protein and mRNA are rarely found in the forestomach. This finding suggests that forestomach are not the major sites of actions of STC-1, but it may be involved in the absorption of the lipid (Zaidi *et al.*, 2006) because forestomach play a role in lipid absorption (Ransom *et al.*, 2017). In addition, the highest levels of STC-1 mRNA and protein were found in the abomasum, especially in the epithelial cells of the fundic glands and gastric pits, which suggests that STC-1 mainly acts in the abomasum. All of the data imply that STC-1 plays roles in digestion and/or absorption, however, it remains unclear whether STC-1 is secreted into the lumen of the stomach accompanying pepsin or other mucous as described in a previous report (Kobayashi *et al.*, 2009).

The small intestinal surface is the main site for digestion and absorption and covered with villi. The villi epithelium consists of absorptive epithelial cells, which are responsible for nutrition and ion absorption, and goblet cells that secrete mucus whose main component is glycoproteins. This study showed the cytoplasm of goblet cells in the duodenum, jejunum, and ileum could be stained yellow by ICC, which may imply that the STC-1 protein was included in the mucus that was secreted by goblet cells (Kobayashi *et al.*, 2009). In addition, the STC-1 protein was also localized in the chief cells of the intestinal glands in the small intestine and cecum, which can secrete intestinal juice whose main function is the decomposition and digestion of food. Interestingly, the quantitative analysis showed that the highest STC-1 mRNA level was found in duodenum and colon among the intestinal tract, but the highest protein level was observed in the duodenum and jejunum. The ICC and ISH results confirmed that the colonic intestinal gland cells adjacent to the goblet cells can be stained by the anti-sense probe rather than the STC-1 antiserum. This finding implies that STC-1 may not be secreted under ordinary circumstances in the colon (Kobayashi *et al.*, 2009), although it could be produced. The differences in the spatial distribution between the STC-1 protein and mRNA may be the result of the translation of the STC-1 protein mainly occurs in sites of active digestion and absorption; hence, STC-1 may be involved in these processes.

As a pleiotropic hormone, previous studies on the functions of STC-1 were mainly conducted in rodents and human, and few studies focused on cows. The current study confirmed that STC-l is widely expressed in the gastrointestinal tract of calves by a detailed examination of the localization and quantification of STC-l mRNA and protein in specific segments of the gastrointestinal tract. In addition, the molecular size of the STC-1 monomer as

investigated by this study is 30 kDa under reducing conditions, thus, this homodimer hormone may be STC50 (a higher molecular weight variant of STC-1 was produced by ovary during pregnancy and lactation), but further tests are needed because both STC50 and big STC exhibit common antigenicity, as was previously reported (Varghese *et al.*, 1998). However, the difficulties associated with obtaining samples from adult cows prevented us from studying the localization and quantification of STC-1 in adult male/female calves, all of these problems will be addressed in our future studies.

Conclusions: In summary, this paper outlined the overall spatial distribution and expression pattern of STC-1 in detail. The results suggested STC-1 mRNA and protein were widely expressed in the bovine gastrointestinal tract and kidney, especially in the locations with more active digestion and absorption, namely in abomasum, duodenum and jejunum. Furthermore, STC-1 was abundantly expressed in the epithelial cells producing digestive juice and/or mucus by an investigation at the cellular level, suggesting that it may be involved in digestion and/or absorption.

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Authors contribution: All of the authors participated in the design of this study and completed the experiment. RG kindly provided the samples which were collected by LW and YB. LW was mainly in the charge of the test of STC-1 mRNA and protein expression levels in gastrointestinal tract and kidney. LMW and DG carried out the data analysis, collected important background information and drafted the manuscript. ICC and ISH were completed by YB and YY. Manuscript editing and review were performed by DG and RG. All authors have read and approved the content of the manuscript.

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