

## PATTERNS OF CALCIUM CHANNEL (TRPV6) EXPRESSION IN RABBIT GUT EPITHELIUM

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**Abstract:** The present study was undertaken to explore the immunohistochemical localisation of TRPV6 calcium channels in rabbit gut epithelium that are actively involved in calcium absorption. To undertake the research, twelve apparently healthy adult female rabbits with a body weight between 1.0 to 1.5 kg were procured, acclimatised and divided into two groups: control and test. Both groups were kept on same feed along with exogenous calcium supplementation in test group animals only. The serum calcium level revealed that normally a high value of serum calcium is maintained in the rabbit as compared to other mammals, thus indicating that the homeostatic mechanism might be poorly developed. Immunohistochemistry and reverse transcription polymerase chain reaction analysis revealed that the caecum was the site of maximum calcium absorption in rabbit, followed by the duodenum and jejunum. The expression pattern of TRPV6 protein/mRNA was weaker in test group animals than in the control group, indicating that the channel was functional in low calcium concentration in the gut.

**Key Words:** rabbit, gut epithelium, TRPV6, immunohistochemistry, RT-PCR.

## INTRODUCTION

Two highly homologous members, TRPV5 and TRPV6, constitute the apical  $\text{Ca}^{2+}$  entry in kidney and small intestine, respectively (Dekker *et al.*, 2003). Calcium ( $\text{Ca}^{2+}$ ), a macro-mineral, is involved in many physiological processes such as bone mineralisation, muscle contractions, neuronal excitability, blood coagulation, enzyme activity, gene transcription, cell adhesion and apoptosis (Kovalevskaya *et al.*, 2011). Intestinal  $\text{Ca}^{2+}$  absorption is the main process to obtain  $\text{Ca}^{2+}$  from nutrients. Hoenderop *et al.* (2005) described two mechanisms of intestinal  $\text{Ca}^{2+}$  absorption, *viz.* paracellular and transcellular pathways. Rabbit differs from other mammals in its  $\text{Ca}^{2+}$  physiology. Cheeke (1987) postulated that the regulatory controls of calcium do not seem to operate in rabbits. When dietary calcium levels in rabbit are higher than necessary, the extra calcium is absorbed, the blood calcium level rises and exceeds the kidney threshold, and the excess blood calcium is excreted in the urine. Works have been done on the localisation of epithelial calcium channels and its role in calcium absorption in different species of animals, such as swine (Brandenburger, 2004; Breves *et al.*, 2007), cows (Liesegang *et al.*, 2008), sheep (Wilkins *et al.*, 2009) and horses (Sprekelar *et al.*, 2011), but specific literature on rabbit is still lacking. Hence, the present study was confined to localising the expression patterns of TRPV6 channels in different segments of the rabbit intestine.

## MATERIALS AND METHODS

All the animal experiments complied with the guidelines promulgated by the Institutional Animal Ethics Committee (IAEC) of West Bengal University of Animal and Fishery Sciences, Kolkata, India (No. Pharma/IAEC/138,

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dated: 30.06.2014). To undertake the present research, twelve (n=12) apparently healthy adult New Zealand white female rabbits, with a body weight between 1.0 to 1.5 kg, were procured from an authorised breeder. The animals were numbered with Indian ink and their weights were taken at regular weekly intervals. They were given one week for acclimatisation before the experiment began. The animals were housed in the animal house of the faculty of Veterinary and Animal sciences. Proper ventilation and ad libitum supply of clean water was provided as per standard protocol. Feed in the form of Bengal gram (*Cicer arietinum*) and Water spinach (*Ipomea aquatica*) was given as per the body weight of individual animals. Regular monitoring of their health status was carried out. After acclimatisation, the animals were equally divided into control and test groups. The control group was kept on a normal feeding schedule as indicated above, and the test group was supplemented with an oral administration (syringe fed) of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  at rate of 1 mg/animal daily (SIDS, 2002) for six weeks.

### **Blood sampling**

Blood samples were collected from both the control and test group animals by cardiac puncture at weekly intervals after properly anaesthetising the animal using ketamine-xylazine protocol at rate of 35 mg/kg and 3 mg/kg body weight, respectively, via intramuscular route. The sampling consisted of taking blood samples at hourly intervals from 1<sup>st</sup> h to 12<sup>th</sup> h of feeding in the control group and from 1<sup>st</sup> h to 12<sup>th</sup> h of calcium supplementation in the test group for six weeks. The blood samples were collected and the serum was separated and then centrifuged at 3000 rpm for 10 min. The supernatant obtained was pipetted out and stored in micro centrifuge tubes at  $-20^\circ\text{C}$  for further analysis. Calcium from serum samples was estimated by calcium test kit (Span diagnostic, OCPC method) according to the manufacturer's protocol. The absorbance was measured at 578 nm by spectrophotometer.

### **Immunohistochemistry**

After proper euthanasia and dissection of the animals, tissue samples were obtained from different segments of rabbit intestine from both the control and test groups, then processed by acetone-benzene schedule (Luna, 1968); 5  $\mu\text{m}$  thick paraffin sections were taken on poly-L-lysine coated glass slides (P0425, Sigma) for immunohistochemical identification of calcium channels TRPV6. Citrate based antigen unmasking was done for antigen retrieval for 15 min at  $95^\circ\text{C}$  (cat. no. 3300, Vector). The sections were then incubated for 60 min in 1% goat blocking serum in 0.1 M phosphate buffer saline (PBS) (pH-7.2) at room temperature to block non-specific antibody binding activity. After subsequent washing with PBS, the sections were incubated at  $37^\circ\text{C}$  for 2 h in humid chambers with sheep polyclonal anti-TRPV6 antibody (Cat. no. ab179988; Abcam, England; raised against amino acid sequence 320-370, dilution 1:100). Immunoreactivity was detected by 1 h incubation with secondary antibody (Horseradish peroxidase conjugated donkey anti-sheep IgG H&L, dilution 1:200, Cat. no. ab6900; Abcam, England). Slides were then rinsed three times in PBS for 5 min each. Sections were further treated with 3,3'-diaminobenzidine (DAB) solution for 3 min (DAB peroxidase substrate kit; Cat. no. SK- 4100). The sections were counterstained with haematoxylin, dehydrated in ethanol, cleared in xylene and then mounted in dibutylphthalate polystyrene xylene. Negative controls were carried out by incubating the slide with antibody diluents/blocking serum instead of primary antibody at the same concentration. Photography was done using Leica Qwin Image Analyser software in Leica DM 2000 Microscope.

### **Real-Time Quantitative PCR Analysis**

Immediately after the animals were sacrificed, the tissue samples from different segments of intestine of rabbit (duodenum, jejunum, ileum and caecum) from both the control and test groups were stabilised in RNAlater at  $4^\circ\text{C}$  for initial 24 h and subsequently at  $-20^\circ\text{C}$  till estimation.

The tissue samples were disrupted in liquid nitrogen and ground thoroughly with a mortar and pestle. Total RNA purification was done using Allprep DNA/RNA minikit, Qiagen. RNA quantification was done using Nanodrop 2000 spectrophotometer (Thermo scientific) in  $\text{ng}/\mu\text{L}$ . The obtained RNA was subjected to DNase treatment to prevent genomic DNA contamination. The required amount of RNA was reverse transcribed to get cDNA (Allprep DNA/RNA minikit, Qiagen). The cDNA obtained was used to determine the TRPV6 mRNA expression levels. Expression of TRPV6 mRNA, normalised for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was quantified using SYBR green PCR system (7900 HT Fast Real Time PCR system, Applied Biosystems). Sequence specific PCR primers

(GAPDH: left/ forward primer 5'CCACCATCCATTCTACACG3'; right/ reverse primer 5'GCCGGGATGATGTTCTGATG3' and TRPV6 left/forward primer 5'CCAGTGGACATACGGGCC3'; right/reverse primer 5'GCTTCTTGGTGGTGACGATA3' were purchased from Sigma. NTC was carried out containing all the components except the cDNA. The components were incubated at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min and 72°C for 30 s, followed by dissociation cycle comprising of 15 s at 95°C, 15 s at 60°C and 15 s at 95°C. The ct values thus recorded were used for further analysis of the expression patterns of TRPV6 mRNA in different segments of intestine in both the control and test group animals.  $\Delta$ ct control was calculated by taking the difference between the ct values of experimental gene TRPV6 and housekeeping gene GAPDH in the control group.  $\Delta$ ct test was calculated by taking the difference between the ct values of experimental gene TRPV6 and housekeeping gene GAPDH in test group.  $\Delta\Delta$ ct was calculated by taking the difference between the  $\Delta$ ct test and  $\Delta$ ct control. Finally,  $2^{-\Delta\Delta ct}$  was calculated to find out the gene expression fold change.

### Statistical analysis

Data are expressed as means±standard error of mean. For serum calcium levels obtained in control and test group animals, F-test was done to analyse the variation, if any. The complete statistical analysis was performed using IBM SPSS statistics 20 software.

## RESULTS

### Serum calcium levels

The serum calcium levels of control group and test group animals showed that the blood serum calcium level was higher in the control group as compared to the test group. However, at the last 3 h, i.e. from 10<sup>th</sup> to 12<sup>th</sup> h, the serum calcium concentrations of both groups were almost the same. From Figure 1, at 2-2.5 h interval, it was revealed that the serum calcium level in both the groups were similar. The statistical data revealed that the serum calcium concentration level was found to be non-significant between the groups and amongst the hours of each group, thus there was no statistical difference between the control and test group animals. The serum calcium levels in both the control and test group were found to be normally high as compared to other animals (Figure 1).

### Immunohistochemistry

#### Control group

A very strong immune reaction for TRPV6 antigen was detected throughout the mucosal epithelium of the caecum. The columnar epithelial cells had dark brown immunostaining specifically at the apical membrane region (Figure 2

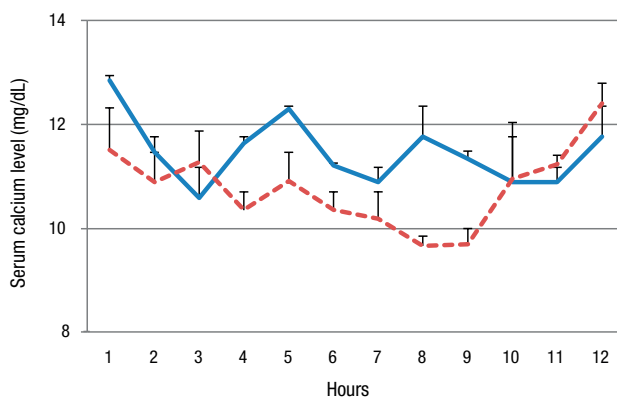
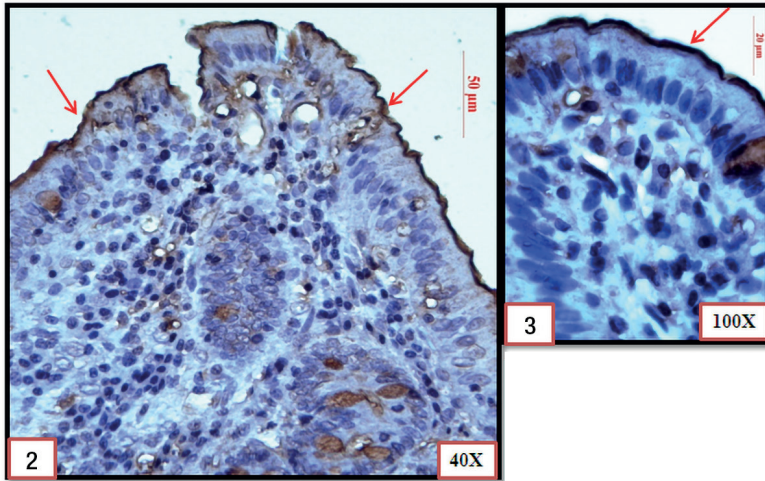
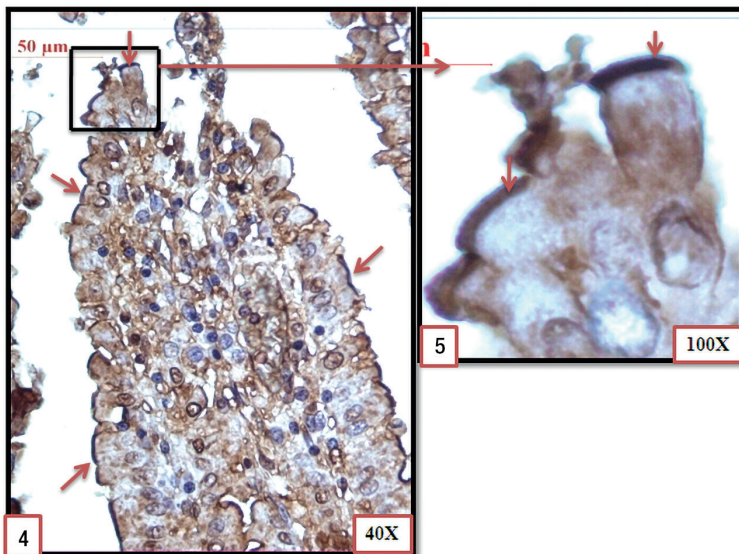


Figure 1: Mean±standard error of mean of serum calcium levels (mg/dL) of rabbit at different feeding times (— Control) and calcium supplementation (--- Test).



Figures 2 and 3: Photomicrograph showing intense immunoreactivity of TRPV6 protein on the apical membrane of enterocytes throughout the length of mucosal fold of caecum from control group animal (red arrow).

and 3). The reaction intensity was even more than that of the duodenum. Duodenal enterocytes also showed a strong labelling of TRPV6 antigen immunoreactivities (columnar absorptive epithelial cells), particularly in the apical membranes as a dark brown precipitate staining throughout the length of the villi, but the intensity of reaction was less than that observed in the caecum (Figures 4 and 5). The TRPV6 immunoreactivity staining was specific only for the enterocytes, whereas other cell types like goblet cells, interspersed between the enterocytes and identifiable by their light supra nuclear cytoplasm and blue counterstained nuclei, were always devoid of any specific brown staining



Figures 4 and 5: Photomicrograph showing strong immunoreactivity of TRPV6 protein on the apical membrane of enterocytes throughout the length of villi of duodenum from control group animal (arrow).

throughout the length of the villi. The cryptal epithelial cells within the lamina propria, at the base of the villi as well as in the deeper parts, were negative for TRPV6 immunostaining. The lining epithelium of the Brunner's gland in the tunica submucosa was also devoid of any brown immune staining for TRPV6 (Figure 6).

In the jejunum, the TRPV6 immunoreactivity showed location-specific reactions. The brown staining was weak to moderate in the apical membrane of enterocytes, specifically at the base of the villi, whereas at the tip of the villi the enterocytes were found to be devoid of any staining for TRPV6 (Figures 7 and 8). The goblet cells and the cryptal epithelial cells were also negative for TRPV6 immunoreactivity. This result suggested that in the jejunum the calcium channel proteins were more active towards the base of the villi as compared to the tip.

The immunostaining for TRPV6 antigen was not observed in the serial section throughout the ileum. The enterocytes throughout the length of the villi, goblet cells and cryptal epithelial cells were all found to be negative for TRPV6 antigen (Figures 9 and 10).

#### Test group

The immune reactivity for TRPV6 antigen in the duodenum of test group animals was of low intensity as compared to the control group animals. The reaction was identified as brown staining, which was present only in the apical membranes of the enterocytes at the tip portion of the villi, while the base of the villi was devoid of any TRPV6 immunostaining, which was in contrast to that of the control group, where the whole length of the villi showed the

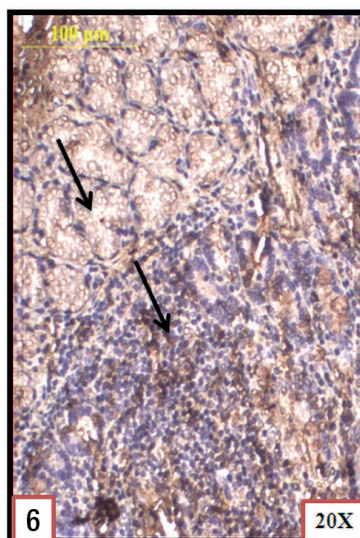
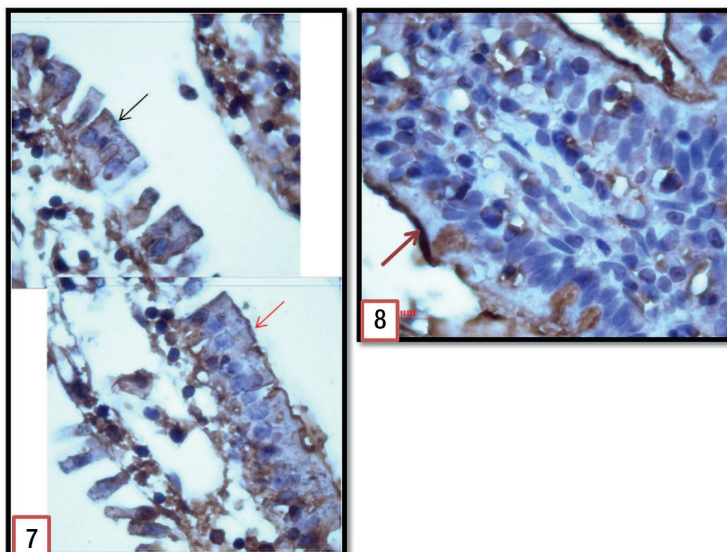
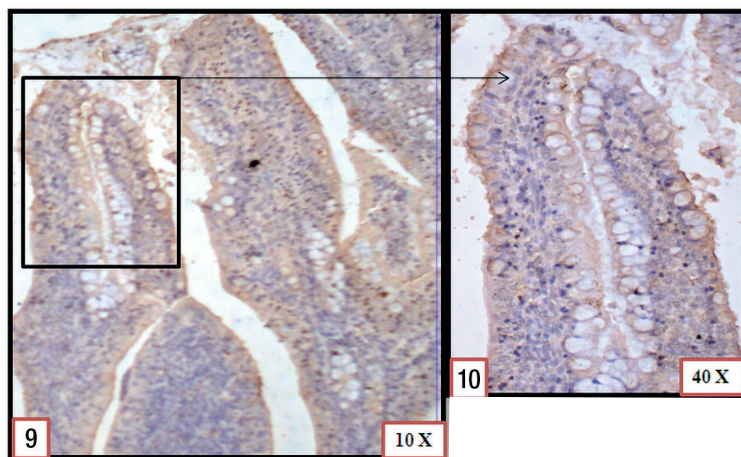


Figure 6: Photomicrograph showing no immunoreactivity of TRPV6 protein in Brunner's and cryptal glands (arrow).



Figures 7 and 8: Photomicrograph showing no immunoreactivity of TRPV6 protein on the apical membrane of enterocytes at the tip of villi of jejunum (Figure 5 above, black arrow), mild reaction at the middle half of villi (Figure 5 below, red arrow) and intense reaction at the base of villi (Figure 6, brown arrow) from control group animal.





Figures 9 and 10: Photomicrograph showing no immunoreactivity of TRPV6 protein on the apical membrane of enterocytes throughout the length of villi of ileum (black arrow) from control group animal.

positive reactions (Figure 11). The rest of the cellular structures such as goblet cells, cryptal epithelial cells and Brunner's gland were devoid of any reaction, as in the control group animals.

The epithelial absorptive cells in the jejunum of the test group animals showed only mild to no immuno-reactions for TRPV6 antigen, which was more or less comparable to that of the control group of animals but of lesser intensity. The reaction, wherever present, was specific for the apical membranes of enterocytes only, whereas the goblet cells and cryptal cells were devoid of any TRPV6 antigen positivity (Figure 12).

The section from the ileum of test group animals was also devoid of TRPV6 antigen immune staining, like that of the control group animals. The enterocytes throughout the length of the villi, goblet cells and cryptal epithelial cells were all found to be negative for TRPV6 antigen (Figures 13 and 14).



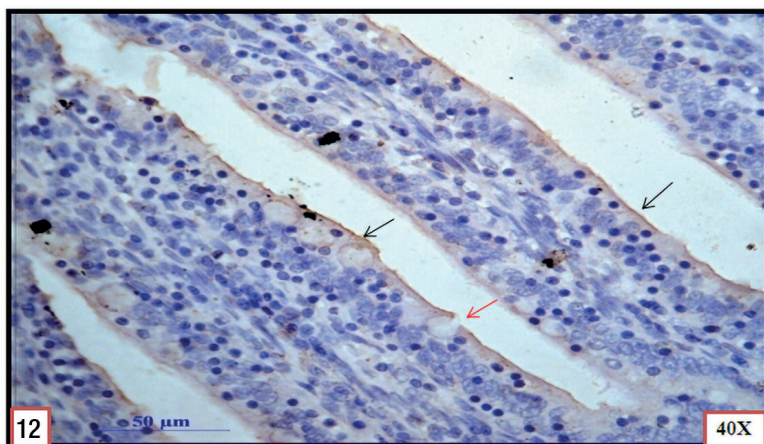
Figure 11: Photomicrograph showing weak immunoreactivity of TRPV6 protein on the apical membrane of enterocytes throughout the length of villi of duodenum from test group animal (arrow).

The mucosal epithelium of the caecum in the test group animals showed positive reaction for TRPV6 antigen, but was of mild intensity as compared to the control group animals. The immunolabelling was restricted to the apical membranes of the enterocytes present at the tip of the mucosal folding, but was absent at its base. The goblet cells and the cryptal epithelial cells were devoid of any reaction for TRPV6 antigen, as in the control group animals (Figures 15 and 16).

#### **RT-PCR**

Expression of TRPV6 mRNA normalised for GAPDH expression was quantified using SYBR green PCR system in the tissue samples collected from both the control and test group animals that comprised duodenum, jejunum and caecum samples. Tissue samples from ileum were not considered, as it showed negative reactions in immunohistochemistry.

Expression of TRPV6 mRNA was greater in the tissue samples from control group animals as compared to the test group animals. In the duodenal, jejunal and caecal tissue samples of

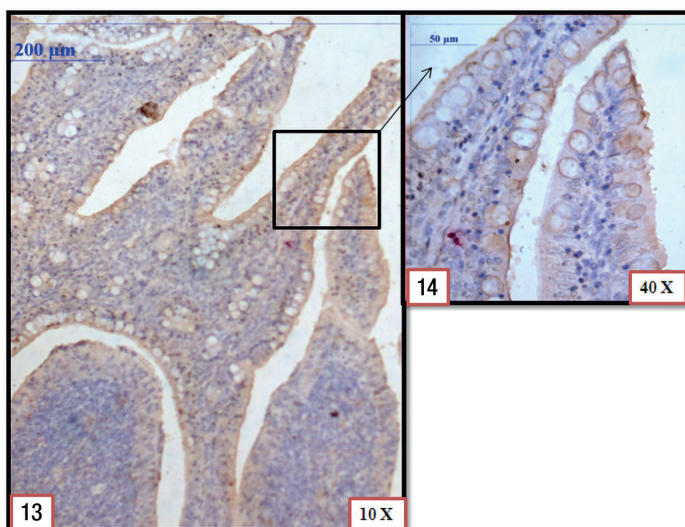


**Figure 12:** Photomicrograph showing weak immunoreactivity of TRPV6 protein on the apical membrane of enterocytes at the base of villi of jejunum from test group animal (black arrow) and no reaction in the goblet cell area (red arrow).

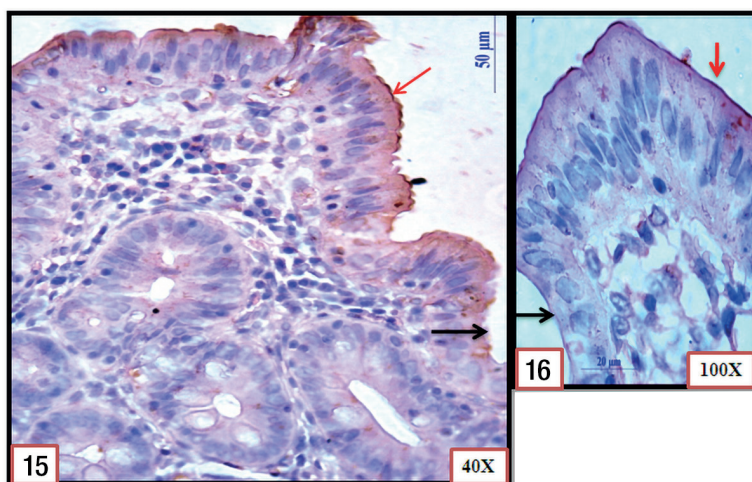
test group animals, the TRPV6 mRNA expression was 1.496, 1.269 times and 7.636 times down-regulated, respectively (Table 1, Figure 17). The result indicated that the maximum down-regulation was noted in the caecal tissue samples from test group animals. This implied that the caecum was the site of maximum calcium absorption in rabbits, followed by duodenum and jejunum, where the maximum effect was due to increased calcium in the diet of test group animals.

## DISCUSSION

Regarding the dietary calcium levels, Chapin and Smith (1967) had concluded that in a well formulated diet, a dietary calcium level of approximately 0.22% (air dry basis) supported maximum rate and efficiency of gain in young,



**Figures 13 and 14:** Photomicrograph showing no immunoreactivity of TRPV6 protein on the apical membrane of enterocytes throughout the length of villi of ileum (black arrow) from test group animal.



**Figures 15 and 16:** Photomicrograph showing weak immunoreactivity of TRPV6 protein on the apical membrane of enterocytes at the tip of mucosal fold of caecum from test group animal (red arrow) but no reaction at the base (black arrow).

growing rabbits. A further increase in the dietary calcium level would not show any significant changes. This might be a reason for the non-significant variation in the serum calcium level in control and test group animals noted in the present experiment.

Rabbits have adapted a unique strategy in which most dietary calcium is absorbed in the intestine, and the excess is excreted in the urine. In this species, calcium levels may vary within a wide range, and in direct proportion to the dietary calcium intake irrespective of the metabolic need. Rabbits exhibit a unique pattern of renal response to parathyroid hormone (PTH). The rabbits' ionised calcium concentration is protected from hyper- and hypo calcaemia by rapidly changing PTH and calcitonin secretion. Changes in PTH secretion are seen only at relatively high calcium concentrations, levels that are the physiologic norm for the rabbit. Despite having a high serum calcium concentration, rabbits have readily measurable levels of PTH, which are dramatically reduced by infusion of calcium. This implies that the parathyroid gland and PTH actively contribute to calcium homeostasis in this species. The physiologic effects of calcitonin in the rabbit remain unclear. Some authors have reported that intramuscular injections of calcitonin decreased serum calcium concentration, while others concluded that there was no change. No consistent effect has been demonstrated on urinary excretion of calcium in the rabbit.

A high serum calcium level in both the control and test group of animals as compared to other animals was in accordance with the reports of Hoenderop *et al.* (2005), with average serum calcium values of 8.5 to 10 mg/dL in other mammals. Different authors also reported a high serum calcium level in rabbit like 13.5 mg/dL (Agkün and Rudman, 1969) and 13.8±0.2 mg/dL (Barlet, 1980) of serum. These results suggested that in rabbits the serum calcium level was maintained at higher level than in other mammals, which was in accordance with the present observations. The author further cited the reason in support of higher serum calcium level that the total serum calcium in the rabbit

**Table 1:**  $\Delta$ ct values of TRPV6 mRNA expression normalised with glyceraldehyde 3-phosphate dehydrogenase in different tissue samples of control and test group animals.

Tissue samples	$\Delta$ ct Control	$\Delta$ ct Test	$\Delta\Delta$ ct (Test-Control)	$2^{-\Delta\Delta$ ct}
Duodenum	7.374±0.159	6.793±0.300	-0.581±0.459	-(1.496)
Jejunum	15.46±0.493	15.116±0.361	-0.344±0.131	-(1.269)
Caecum	12.716±0.278	9.783±0.369	-2.933±0.09	-(7.636)

"-" sign indicates down-regulation of TRPV6mRNA in test group samples.



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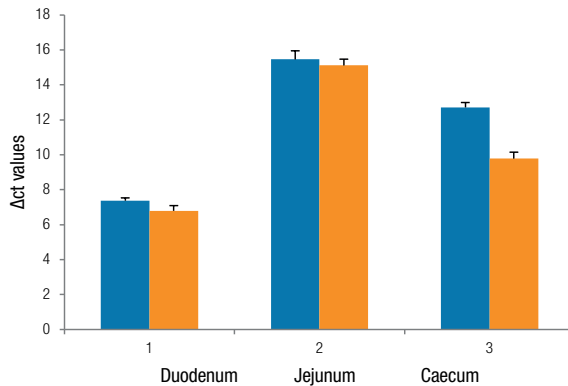


Figure 17: Mean±standard error of mean of  $\Delta ct$  values of TRPV6 normalised with glyceraldehyde 3-phosphate dehydrogenase in duodenum, jejunum and caecum of control and test group animals. ■ Control, ■ Test.

was 30-50% higher than in other mammals and varied over a wide range from 3.25-3.75 mmol/L (13-15 mg/dL). The intestinal absorption of calcium in most mammals involved primarily vitamin D<sub>3</sub>-regulated active transport. In the rabbit, however, calcium was absorbed in direct proportion to the amount ingested in the diet rather than in accordance with metabolic need, and calcium absorption was relatively independent of vitamin D.

From the present study it was found that the serum calcium level even after 12 h of feeding was maintained in higher concentration at almost same value during the feeding time in both groups, which indicated that in the case of rabbit blood serum it was always maintained at a higher level irrespective of dietary calcium. In support of our hypothesis we can cite the report of Eckermann-Ross (2008), who stated that most mammalian species grow only 1-2 sets of teeth in their lifetime. In contrast, a rabbits' teeth constantly erupt at a rate of approximately 2-2.4 mm/wk. The rabbit's increased life-long demand for calcium compared to other mammals was met by its efficient intestinal calcium absorption. In addition, during normal dental wear, calcium was released from the teeth, swallowed, and reabsorbed from the intestine.

In the present study, the strong immunoreactions for TRPV6 in the columnar epithelial cells indicated that the absorptive epithelial cells were the only cell types that play a key role in transcellular transport of calcium from lumen to the interior of the cell, as they were the specific carriers of TRPV6 calcium channel proteins. Other cell types were negative for TRPV6 immuno-reaction, thus indicating that this marker protein was the specific channel protein for enterocytes. The location-specific reaction in the jejunum of controlled group animals might be due to the maximum contact time of the digested food particle at the base of the villi for absorption. Negative TRPV6 immuno-reaction in the ileum indicated that it had no role to play in calcium absorption via transcellular pathway, as it did not have TRPV6 calcium channels that are specific for active transcellular pathways of calcium absorption. Presence or absence of immunoreactivity in any cell/tissue depends upon the presence or absence of a particular channel protein. In support of the present finding, no specific literature was available, although Hoenderop *et al.* (2000) reported ECaC (Epithelial calcium channel) expression in rabbit intestine. Although they did not report it to be TRPV6 specific, the result was in accordance with the present study. They reported the presence of ECaC in rabbit duodenum. Ileum was also negative for ECaC. Thus, the caecum and duodenum was observed to be the principle site of active calcium absorption via the transcellular/TRPV6 pathways in rabbits in the present study. The contradictory result in the duodenum of test group animal as compared to control group might be due to the excess accumulation of feed particle vice-versa excess calcium at the base of the villi, which gave a negative feedback in the expression of TRPV6 channel as compared to the lower calcium concentration in the control group. The present findings indicated that these energy dependent transcellular calcium channels were more active when the dietary calcium was less, as in the control group the reaction was more intense as compared to the test group animals, which were supplemented with exogenous calcium in the diet. These findings were in accordance with the reports of Bronner and Pansu (1999), Van Abel *et al.* (2003)

and Eckermann-Ross (2008). They stated that the paracellular pathway was predominated when the dietary Ca was abundant. If dietary Ca was restricted or Ca demand was increased, the transcellular transport was the essential mechanism for Ca absorption from the diet.

Immunohistochemical localisation of TRPV6 protein revealed a strong reaction in the tissue samples of control group animals as compared to the test group animals, especially in the caecum, followed by the duodenum and jejunum. No literature was available in support of TRPV6 expression pattern in the case of rabbit. Reports regarding the expression patterns of TRPV6 calcium channels are also meagre in other animals. A non-TRPV6 specific channel i.e. ECaC expression was reported by Hoenderop *et al.* (2000) in rabbit, although it was not TRPV6 specific, but the result was in accordance with the present study. Wilkens *et al.* (2009) also reported the immuno-labelling of TRPV6 antigen in the duodenal and jejunal enterocytes of sheep, especially in the apical membrane regions, and concluded that the jejunum was more important than the duodenum for Ca absorption in sheep, but this result was not in accordance with the present expression pattern of TRPV6.

Different workers (Boos *et al.*, 2007 in goat; Liesegang *et al.*, 2008 in cow; Riner *et al.*, 2008 in sheep; Sprekelar *et al.*, 2011 in horse) have reported that the site of active calcium absorption varied in different species of animals by immunostaining of VDR (Vitamin D receptors) and Calbindin (Cb D-9K) in the transcellular pathway of Ca absorption. But none of them reported the importance of TRPV6 channel localisation, which is of prime importance in calcium absorption from the gut to the cytoplasm. The RT-PCR analysis revealed that expression of TRPV6 mRNA was greater in the tissue samples of control group animals as compared to the test group animals. No specific literature was available regarding RT-PCR analysis of TRPV6 mRNA in rabbit. Few authors reported the expression patterns of TRPV6 mRNA in other species of animals, such as in sheep (Wilkens *et al.*, 2009) and in horse (Sprekelar *et al.*, 2011). However, it was observed from these available literatures that the expression varied in different animals. In sheep it was the jejunum that showed maximum expression of TRPV6 mRNA, while in horse the site for maximum expression was the duodenum. Thus the site for Ca absorption varies in different species of animal depending upon their feeding behaviour and gastrointestinal anatomy.

Immunohistochemistry and RT-PCR analysis in the present observation revealed that the caecum was the site of maximum calcium absorption in rabbit, followed by duodenum and jejunum. The expression pattern of TRPV6 protein/mRNA was weak in test group animals with higher calcium concentration than the control group, which was kept on normal diet, indicating that the channel was functional in low calcium concentration in the gut.

All these outcomes suggested that in case of the rabbit, calcium supplementation is neither required nor economical, and judicious use is recommended if required.

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