RESEARCH ARTICLE

# Effects of protein diet on expression of Anoctamin1 of Cajal cell in the nervous plexus of the stomach in male mice

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## Abstract

**Objective:** To determine the association between a protein-rich diet and the expression of anoctamin 1 in Interstitial cells of Cajal within the muscle layer of the stomach wall in male mice.

**Method:** The experimental study was conducted at the Anatomy Department of the College of Medicine, Al-Nahrain University, Iraq, from November 2020 to April 2021, and comprised male adult healthy male mice. They were divided randomly into two equal groups. Group A was fed a high protein diet, while control group B was fed a standard pellet diet. The tissue samples were harvested at day 30 post-surgery. The stomach samplings were placed in 10% neutral formalin for 24 hours to obtain paraffin sections for routine histological and immunohistochemical staining. The protein expression in stomach smooth muscle of each group was detected by immunohistochemical staining. Data was analysed using SPSS 24.

**Result:** Of the 20 mice, 10(50%) were in each of the two groups. Group A exhibited significant weight-gain compared to group B ($p \leq 0.05$). There was significant elevation in muscle wall thickness in group A, compared to group B ($p \leq 0.05$). Tunica muscularis of the stomach in group A significantly thickened compared to group B ($p \leq 0.05$). The expression of anoctamin 1 was significantly more intense in group A compared to group B ($p \leq 0.01$).

**Conclusion:** Unbalanced food had a significant impact on the stomach, affecting the thickness of the muscularis layer and the expression of anoctamin 1 in cajal cells in the muscularis externa.

**Key Words:** Anoctamin, Paraffin, Cajal, Formaldehyde

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## Introduction

Interstitial cells of Cajal (ICC) were originally identified by Cajal, who characterised them as nerve-like cells localised at the terminals of motor neurons within organs innervated by peripheral nerves. He classified these cells as primitive neurons based on distinct staining properties using methylene blue and silver chromate. Subsequent advancements in the field revealed that ICCs express a receptor associated with anoctamin 1 (Ano1), a calcium-activated chloride channel. Ano1 serves as a novel and selective molecular marker applicable to all ICC classes present in the gastrointestinal (GI) tracts of both humans and mice. This discovery enabled the immunochemical identification of ICC independently of the Kit protein. ICCs were initially characterised using morphological criteria, exhibiting close association with nerve varicosities and forming numerous gap junctions among themselves and with smooth muscle cells, creating a network across the gut wall. The classification of ICC was multifactorial, and there exists consensus regarding the morphology, localisation and roles of various ICC types[1]. ICC are denser in corpus/antrum than fundus. This indicates that they likely serve as pacemaker cells regulating gastric motility. ICC are an integral component of the enteric nervous system, playing a pivotal role in modulating gastrointestinal muscle movement and orchestrating contractions facilitating food transit through the digestive tract. Hence, the heightened concentration of these cells in specific stomach regions may underscore their significant involvement in governing intestinal activity and food propulsion.

Scanning electron microscope showed complex ICC arrangement with Auerbach's plexus in guinea-pig small intestine. Reticular fibrils cover plexus, enabling detailed intact nerve and tissue study[2,3]. ICC changes are linked to motility issues. Abnormalities are linked to GI motility problems and diseases[4,5].

The current study was planned to determine the association between a protein-rich diet and the expression of Ano1 in ICCs within the muscle layer of the stomach wall in male mice.

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## Materials and Methods

The experimental study was conducted at the Anatomy Department of the College of Medicine, Al-Nahrain

University, Iraq, from November 2020 to April 2021. Approval was obtained from the institutional animal ethics committee, and the study was conducted in line with the Guidelines on Ethical Treatment of Experimental Animals issued by the Iraq Provincial People's Government according to National Institute of Health guidelines for the care and use of laboratory animals[6].

Healthy adult male albino mice aged about 8 weeks and weighing 20-30g were selected and divided into two groups. Intervention group A was subjected to a high protein diet, while control group B received a standard pellet diet containing 30% protein. The allocation of protein intake percentages was determined in the light of literature[7]. The intervention lasted 30 days during which all animals were treated according to National Institute of Health guidelines for the care and use of laboratory animals[6]. The mice were then dissected. The stomach samplings were engaged in 10% neutral-buffered formalin (NBF) for 24 hours before being histologically processed for paraffin section that included de-waxing, staining and mounting, as well as fixation, dehydration, clearing, impregnation, embedding and sectioning. Tissue preparation for paraffin blocking was done in the light of a study[8]. Immunohistochemical process and monoclonal immunoglobulin M (IgM) antibody were used (Sigma Aldrich, Germany; Abcam, United Kingdom). The procedures were carried out in accordance with the instructions provided by the manufacturers.

Data was analysed using SPSS 24. $P<0.05$ was considered significant.

## Results

Of the 20 mice, 10(50%) were in each of the two groups. Group A exhibited significant weight-gain compared to group B ($p\leq0.05$) (Table 1). In control group B, the stomach's distinct layers were readily distinguishable, comprising four layers: mucosa, submucosa, muscularis and serosa. The muscularis externa comprised two prominent layers of smooth muscle: an inner circular layer and an outer longitudinal layer. The myenteric plexus (Auerbach's plexus) was positioned between these layers of smooth muscle. In protein group A, tunica muscularis of the stomach exhibited an increased thickness, and was composed of multiple layers of smooth muscle fibres. Within the interstitial spaces of the muscle cells, the Auerbach plexus displayed hypertrophy and degeneration, evident by the presence of vacuolated cytoplasm (Figure 1-A-B).

The thickness of the muscularis layer displayed a more increase in Group A compared to group B (Table 2).

The expression of Ano1 was significantly more intense in group A compared to group B ($p\leq0.01$) ((Table 3; Figure 1-C-D; Figure2).

**Table-1:** Weight of the animals before and after using diet variation.

| Group Name | Mean (gm) ± SD | | T-test |
|---|---|---|---|
| | Before | After | |
| Control Group | 22.56 ±1.94 | 22.33 ±1.22 | NS |
| Protein Group | 21.44 ±1.67 | 28.44 ±1.42 | 1.549 ** |
| P-value | 0.110 NS | 0.0001 | |

SD: Standard deviation, NS: Non-significant.

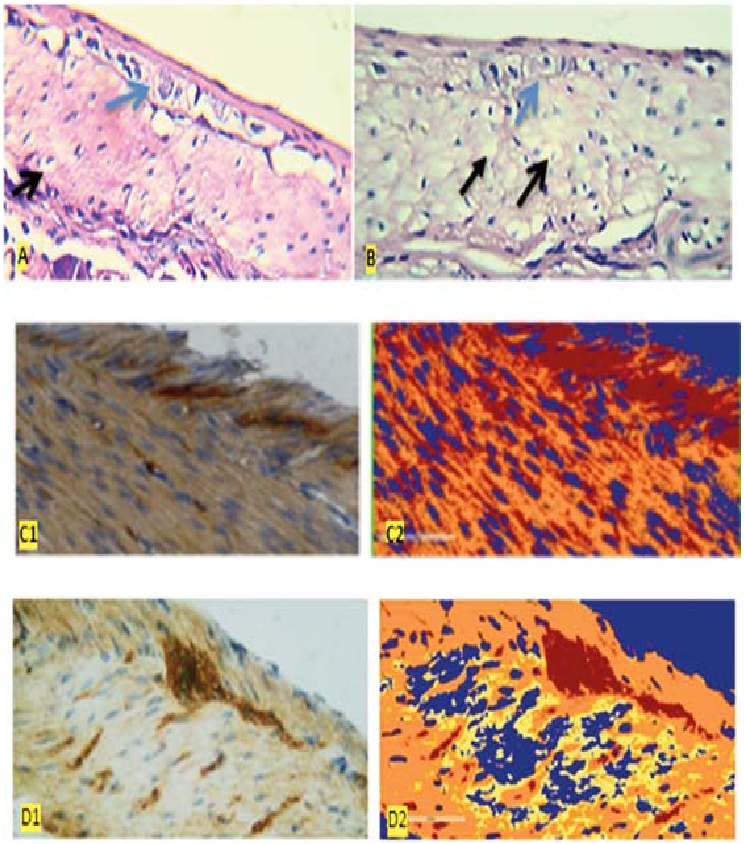


**Figure-1:** (A-B) Cross-section of muscularis layer of stomach showing degenerated myentric plexus (blue arrow), and vacuoles between smooth muscles (black arrow) (Haemotoxylin and Eosin [H&E] X40); (C1) Intensity of the reaction at muscularis externa, (C2) the same tissue analysed by aperio software; (D1) Intensity of the reaction at muscularis externa; ( D2) the same tissue analysed by aperio software.

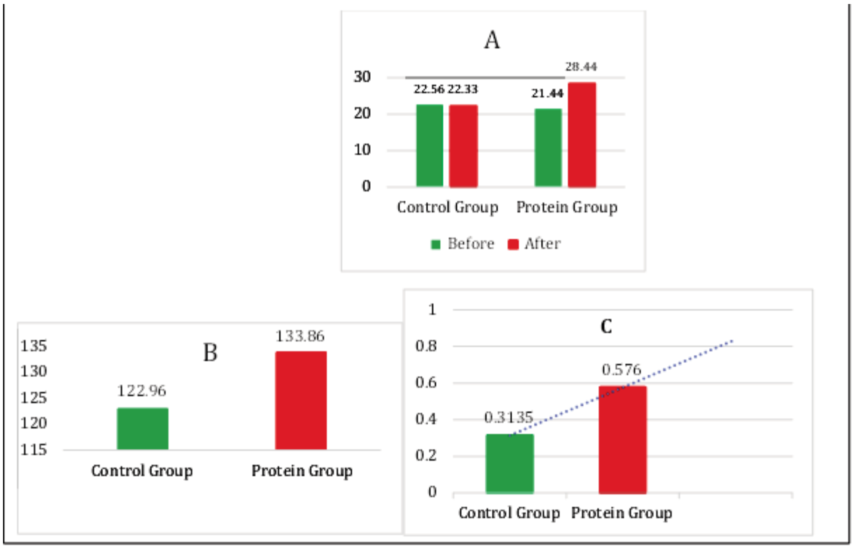


**Figure-2:** (A) Weight of the animals before and after using diet variation; (B) Comparison between difference groups in muscularis thickness; (C) Comparison between diet modality groups in anti-anoctamin 1 (Ano1) expression.

**Table-2:** Comparison related to muscularis thickness.

| Group | Mean (µm) ± SD |
|---|---|
| Control | 122.96 ±23.51 |
| Protein Group | 133.86 ±34.59 |
| | ** P≤0.05 |

SD: Standard deviation.

**Table-3:** Comparison related to anti- anoctamin 1 (Ano1) expression.

| Group | Mean (pixels per square micron) ± SD |
|---|---|
| Control Group | 0.3135 ±0.1363 |
| Protein Group | 0.5760 ±0.0953 |
| | ** P≤0.01 |

SD: Standard deviation.

## Discussion

The present study revealed a notable disparity in expression, with animals on a protein-rich diet displaying heightened aggression and quarrelsome behaviour. Furthermore, the animals subjected to the protein diet exhibited a noteworthy rise in the number of faecal contents collected daily from within their cages, accompanied by an unpleasant odour compared to the control group. This finding is consistent with DeNapoli et al., who observed an increase in dominance despite a decrease in dietary protein content[9]. Moreover, aggression is frequently employed in competitive systems to secure access to limited resources within a social context.

In the current study, the protein diet group gained weight, which was also observed in a prior study[10]. The participants on a high-protein diet had a thick muscularis layer, which contradicts the notion that a high-fibre, low-protein diet enhances the thickness of the muscularis layer[11].

The expression of Ano1 in the muscularis layer of protein-fed animals exhibited a high level of expression, which is consistent with previous research[12]

The protein group animals exhibited a significant increase in Ano1 expression, which is associated with the adjacent ganglionic neurons of the myenteric plexus, but this does not indicate an increase in their numbers, and this is correlated with the same conditions as malnutrition[13]. Unbalanced meals can generate morpho functional alterations in the enteric nerve plexuses, leading to malnutrition-related diseases, such as stomach discomfort, constipation, faecal incontinence, diarrhoea, and malabsorption[14]. This explains and exhibits how a high-protein diet induces stress by altering the muscularis layer, resulting in a decrease in thickness and, as a result, a significant rise in the expression of Ano1 protein in the muscularis layer compared to the control group. This is consistent with reports that starvation is a pathological condition that can manifest as reversible or irreversible organic structural alterations[15]. All normal metabolic processes require protein participation, and protein-loss affects all tissues, but the rate and type of modification are not the same. Protein deficiency affects tissues with high cell refurbishment rates, such as the intestinal mucosa, while protein deficiency affects tissues with low cell renovation rates, such as the myenteric nerve plexus system.[16] In malnourished rats, hypoplasia and hypo trophic mucosa have been detected[17], but other results have also been reported[12,13].

The presence of Ano1 in ICCs of the control group was critical for maintaining a balanced peristaltic smooth muscle activity, which guarantees coordinated stomach motility. Ano1 regulates synchronous peristaltic motor action. Inhibitory Ano1 protein buildup in the stomach and digestive tract may impair normal motility. This can result in increased relaxation and decreased contraction force, potentially resulting in difficulty emptying the stomach and chronic constipation. On the other hand, symptoms like nausea, belching, bloating, heartburn, indigestion, regurgitation, or vomiting might occur when the nerve regulating the stomach muscles is compromised. Gastroparesis, often known as delayed gastric emptying, is a disorder in which the stomach empties itself gradually. Moving partially digested food from the stomach to the small intestine depends heavily on the stomach's muscles.[18].

In comparison to the control group, the expression of Ano1 protein in the muscularis layer of protein diet animals demonstrated elevated expression of Ano1. This increased the motility of ICCs by causing them to innervate the muscularis layer of the gut tube. This was reported earlier as well[19].

The current findings showed that dietary variability, whether high or low in protein, affected Ano1 protein production in the ICCs, which facilitated peristaltic motility in the stomach. This is consistent with a prior study[20]. Other studies have discovered that intraduodenal proteins had load-dependent effect on Antro pyloroduodenal motility as well as energy intake at a subsequent meal in both lean and obese people, implying that small intestinal sensitivity to protein remains intact[21]. GI motor activity has a role in the regulation of blood glucose appetite in both lean and obese adults by decreasing gastric emptying activity and thus appetite[21].

## Conclusion

An imbalanced diet significantly affected the thickness of the muscularis externa layer and the expression of Ano1 by ICCs in the muscularis layer in the stomach. A high-protein diet caused Ano1 levels in the stomach to rise. As a result, gut motility was disrupted, which could result in specific motility abnormalities that are a major contributor to GI illnesses.

**Disclaimer:** The text is based on an academic thesis.

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