Experimental Studies to Define the Role of Calcium (Ca$^{2+}$) and Voltage-Gated Calcium Channel Blockers (vCa-CCB) against Neurosteroid Induced Obesity

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Authors' contributions

This work was carried out in collaboration between both authors. Author VRC preformed all the experimental work and wrote the first draft of the manuscript. Author AA managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Introduction: Obesity is closely associated with various types of illness, primarily caused by more calorie intake than body burn. In adipocytes, Calcium (Ca$^{2+}$) is an important second messenger involved in the regulation of many physiological functions which are essential for survival. In the present research, we have investigated the role of Ca$^{2+}$ ions in obesity by manipulating cytosolic Ca$^{2+}$ ion concentration by selective blocking/advancing the Ca$^{2+}$ ions through the voltage-gated calcium channels. Voltage-gated calcium channel (vCa) plays a key role in regulating intracellular and extracellular Ca$^{2+}$ concentration. Cytoplasmic level of Ca$^{2+}$ was manipulated by supplying calcium carbonate and by using vCa blockers i.e. nifedipine- (N-type vCa-CCB) and ethosuximide (T-type, vCa-CCB).

Methods: Obesity was induced by progesterone in female mice and test drugs were co-administered with progesterone whereas sibutramine was used as standard. The treatment was carried out for 28 days, during and after the treatment period various parameters were studied viz.

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1. INTRODUCTION

At its simplest, obesity results from consuming more calories than bodies’ burn, but it’s a more complex problem than that. Obesity was once the preserve of rich western nations but obesity is now a global epidemic affecting many people in emerging economies even developing nations. Over the last 4 decades, the occurrence of obesity in adults has been rising at a rapid rate with the number of obese adults reaching 671 million in 2016 (390 million in women and 281 million in men) compared to 100 million in the year 1975 (69 million women and 31 million men) [1]. Moreover, the incidence of childhood obesity also rinsing constantly. Childhood obesity is up 54% in 6 to 11 year-olds and 25% of all children in the US are now considered overweight or obese [2]. The longer period a child is overweight, the more likely they are to have permanent weight problems that transcend all ethnic backgrounds [3]. Very few drugs are available for the management of obesity and associated complications, because many new drugs have been recalled due to the unwanted effects due to risk benefit- ratios [4-6]. The increase in weight gain is a consequence of complicated changes in the environment, where food is more readily available and opportunities for physical activity are lacking. Dietary changes are considered to be the best possible way to reduce body weight, one of the most essential micronutrients in our diet is calcium. Calcium is the most abundant divalent cation in our body that governs many functions. Calcium (Ca²⁺) is an important second messenger involved in multiple signalling pathways and mediating diverse physiological functions. The free concentration of the Ca²⁺ ion in the cytosol involves the regulation of mechanisms necessary for the short-term regulation of cell functions like contraction, excitation, secretion which account for many physiological processes [7].

Recently, the role of T/L-type calcium channels was noticed in sleep-vigilant cycle dysfunction-induced obesity and earlier works of the literature suggest that Ca²⁺ channels in the hypothalamus promote the release of orexigenic hormones, affecting the nutritional status and triggering obesity [8]. Moreover, few studies have shown the involvement of calcium channels in controlling the bodyweight but exactly how the calcium and calcium channels are involved in the regulation of energy balance and body weight regulation is still unclear.

Studies have demonstrated that ovarian hormones i.e. estrogen and progesterone plays vital role in energy homeostasis. It’s said that lack of/absence of estrogen during the perimenopause and post-menopausal phase responsible for obesity in women [9]. Progesterone is a neuroactive steroid its level increases during the luteal phase of the menstrual cycle. Past evidence suggests the involvement of progesterone in the pathophysiology of obesity and affective disorders. Some reports suggest the use of progesterone containing preparations as a contraceptive or for hormone replacement therapy to cause sufficient weight gain by causing hyperphagia and increased fat deposition in the body. Reports also suggest that progesterone can produce these effects by declining serotonin level in the hypothalamic neurones. Hypothalamus is the main site governs the food intake, decrease level of serotonin causes hyperphagia and weight gain [10].

Based upon these observations the current study has been undertaken to study the influence of calcium carbonate, calcium-channel blockers (nifedipine- N-type calcium channel blocker, ethosuximide-T-type calcium channel blocker) against progesterone-induced obesity in female Swiss albino mice.

Keywords: Obesity; calcium channels; calcium carbonate; ethosuximide; fecal lipid.

Results: Calcium carbonate treated group has shown promising effects in the decrease in body weight by increasing fecal lipid content and lipolysis which was reflected by an increase in body temperature. Ethosuximide also offered significant protection by decreasing the food intake but has not shown any notable effect on fecal fat content, whereas nifedipine has not offered any protection against the obesity induced by neurosteroid.

Conclusion: Calcium carbonate has significant anti-obesity activity by including thermogenesis, and increasing fecal lipid content.
2. MATERIALS AND METHODS

2.1 Chemicals
Nifedipine, ethosuximide were purchased from Santa Cruz, USA, supplied by Bio Medical Sciences Est. Saudi Arabia. Sibutramine was obtained as gift sample from Ranbaxy laboratories, India and biochemical kits of Span diagnostics. All the chemicals used in the study were of analytical grade.

2.2 Materials
Tomos scientific cooling centrifuge (5427 R), Labomed Inc LB271 trinocular microscope, Shimadzu UV-visible Spectrophotometer (UV1800), rotary microtome- Sfee Germany, and, Stat Fax autoanalyzer (2000), digital balance (ER-180A).

2.3 Experimental Animals
Forty-eight female Swiss albino mice (22-25 gm) were obtained from the central animal house facility (Animal Biosafety Level 1) Northern Border University, Saudi Arabia. Animals were housed in cages in a room maintained under controlled conditions of constant temperature and relative humidity of 21 ± 1°C and 50-55%, 12:12 Hr light/dark cycle ad libitum access to chow diet. The mice were acclimatized for 10 days, had free access to chow and water to check any signs of morbidly. Before commencing and during the experimental procedure national guidelines of bioethics were strictly followed.

2.4 Animal Grouping and Dosing Schedule
Forty-eight female Swiss albino mice were divided into eight groups, each group containing 6 animals. Group 1, served as normal control received standard chow diet, group 2 labelled as negative control treated with progesterone (10 mg/kg, s.c.), while group 3 served as standard treated with sibutramine (10 mg/kg, p.o.) [11,12] along with progesterone, 10 mg/kg, s.c.[13] Group 4 was supplied with calcium carbonate (50 mg/kg,p.o.)[14] and progesterone, 10 mg/kg, s.c. while group 5, 6, 7, 8 were treated with low and high dose of nifedipine (1.5 and 3 mg/kg p.o.)[15] and ethosuximide (5 and 10 mg/kg, p.o.)[16] along with progesterone, 10 mg/kg, s.c. respectively. The entire animals were treated for 4 successive weeks (28 days). Refer Table 1.

2.5 Test and Standard Drug Preparation
Sibutramine is soluble in water so dissolved in distilled water to make the final concentration. Whereas progesterone was dissolved in arachis oil for appropriate doses. Nifedipine was prepared by adding 0.1% DMSO in distilled water to the final concentration. Calcium carbonate has limited water solubility (15 mg/L), so 1% of gum acacia was used to prepare the suspension.

All the drugs including standard and test were prepared freshly just before dosing. All the drugs were administered p.o, through the oral gavages except the progesterone which was administered by s.c. route.

2.6 Induction of Obesity by Neurosteroid
Progesterone vials which were earlier dissolved in arachis oil was used. Every week a fresh preparation of progesterone was made. A dose of 10 mg/kg injected subcutaneously in the dorsal neck region to mice for 4 weeks along with the test drug (test groups) or with Sibutramine (standard group) or with distilled water (negative control). All the test drugs, standards sibutramine were administered for 28 consecutive days along with progesterone where as normal control group has received only vehicle [17].

2.7 Study Parameters and Procedures
2.7.1 Monitoring the food intake
The food consumption studies were carried out five times in this study i.e. at the beginning of every week (day 1, 7, 14, 21) and additionally at the end of the last week (28th day). The test feed was prepared by coating 10% of sucrose on the standard mice chow and dried in shadow 24 hours before the experiment. On the day of the experiment, one hour (0 times) before food and water were withdrawn, and 30 min later, the test/standard drugs were administered. 10 gm of the prepared feed was presented to the group of six mice in a glass petri dish and food intake was observed at various time intervals i.e. at 0.5, 1, and 2 hr. 0.1 gm was considered as a correction factor for any kind of spillage while feeding. The quantity of feed consumed per 20gm body weight was measured. The experiment was carryout in a quiet space to avoid any kind of the noise pollution that many interfere feeding behavior of the mice [18].

2.7.2 Measurement of body weight deviations
The change in the body weight of mice was recorded every week for 4 consecutive weeks in
all groups. Body weight was measured by using an analytical balance of 1 gm sensitivity.

2.8 Measurement of Body Temperature
Measurement of rectal temperature is a common method of knowing the core body temperature in rodents. Rectal temperature was measured on 28th day, before and after drug administration at 1 and 2hr by using a rectal thermometer. The mouse was hand-restrained and placed on a horizontal surface, of cage lid and the tail was lifted up, a yellow spring instrument telethermometer probe which was covered with vaseline was inserted in the anus at a distance of 2 cm and hold in position for 10 seconds. The protocol was done to all the animals in the experiment [18].

2.9 Study on White Adipose Tissue (WAT) Mass
On the 29th day of the experiment, all the mice have sacrificed by carbon dioxide overdose and internal organs were separated. White adipose tissue (WAT) from different regions i.e. mesenteric, perirenal, and periovarian fat pad were extracted. Immediately, wet weight of WAT was measured on an analytical balance and organ to body weight ratio was recorded.

Adiposity index was calculated by using the formula:

\[
\text{Adiposity Index} \ (\%) = \left[ \frac{\sum \text{(Fat pad)}}{\text{Body weight}} \right] \times 100
\]

2.10 Separation of Internal Fat Pad
As stated, WAT was dissected from 4 regions i.e. mesenteric, perirenal, periovarian, and Omental fat.

2.10.1 Mesenteric fat
Mesenteric fat is present in the abdominal region through which blood vessels and nerves supply are passed to the ilium. It begins at the lesser curvature of the stomach and ends at the sigmoidal colon of the large intestines. Its obtained by completely isolating the stomach to the intestine portion and later placed on a dissection tray. White tissue which creates an umbrella-like structure and helps to form a coiled structure of the intestine was dissected out.

2.10.2 Periovarian fat
Both the ovaries from the lower part of the mice were isolated, later by mild pressing ovaries were taken out; the ruminant was considered as periovarian fat.

2.10.3 Perirenal fat
It was separated from both the kidneys by gently squeezing the kidneys through the white tissue around it.

2.10.4 The Omental fat
By separating its large fold which hangs down from the stomach and extends from the stomach to the posterior abdominal without producing any harm to the posterior portion of the tissue [19].

2.11 Determination of Stool Lipid Content
The stool analysis for the determination of fat content was carried out on day 0 and 28th day of the study of after the last drug administration. Faeces of the individual group of the mice have collected over 12 hrs (overnight) and were dried in an oven at 70 ± 0.5°C for 60 min. In the powdered 1,000 mg of faeces, 5 ml of normal saline was added and transferred in a 15 ml plastic tube, later 5 ml of chloroform: methanol (2:1 by v/v) was added and mixed thoroughly. The content was centrifuged at 1000 x g for 10 min at room temperature to separate the two lipid phases; the bottom liquid phase contains separated lipids.

One plastic tube was holed above a glass tube and 22.1/2 G needle was passed through the tube wall into the lower of the two liquid phases; later the needle was removed immediately. Later, another air-filled syringe of the same gauge size was inserted at the top of the lower of the two liquid phases, just below the solid phase and air was injected. The lower liquid phase was drained from the plastic tube and collected in the glass tube. Glass tubes were dried off to evaporate the liquid, and the tubes were weighed on an analytical balance. Empty tube weights were subtracted from the new tube weights to obtain the lipid mass per 1,000 mg of faeces [20].

2.12 Histology of Fat Pad
The separated periovarian fat was rinsed in 0.9% of the normal saline to remove the blood, later dipped in 12% formalin for the next 24 hours. The tissue later washed with distilled water to
remove excessive fixative. Tissue later dehydrated with the graded series of ethanol, cleared with xylene and fixed in paraffin wax. Thickness size of 3 μm sections was cut with the help of rotary microtome- Slee Germany and mounted on a clean glass slide. The slides were stained with hematoxylin and eosin. The glass slides were observed under LabomedInc LB271 trinocular microscope at 200 X and photographed [19].

2.13 Statistical Analysis

The obtained results were compared (test group vs positive control); (positive control vs normal control) by using one way ANOVA followed by Dunnett’s test. All results were expressed in ± SEM. The p value, p<0.05 (95% level) was considered as statistically significant. All the data analysis was carried out by using graph pad prism 6.1.

3. RESULTS

3.1 Monitoring the Food Intake

The food consumption behavior of the mice was observed every week i.e. day 1, day 2, day 14, day 21 (data not shown), and day 28. The presented data represents the study which was carried out on 28th day of the study. The neurosteroid treated group has shown a significant (p<0.01) increase in the food consumption compared to normal saline-treated and standard Sibutramine treated group. There was a significant decline in food consumption by the treatment of Sibutramine at all three intervals of the study. Co-administration of Ethosuximide with progesterone significantly (p<0.01) reduced the food intake compared to plain progesterone treated group in dose-dependent manner (Fig. 1).

3.2 Measurement of Body Weight Deviations

Significant (p<0.01) increase in the body weight was observed by the progesterone treated group compared to the normal control group. Sibutramine, successfully tackled the progesterone-induced increase in the body weight, in fact, the body weight was significantly reduced (p<0.01). This increase in body weight may be because of the thermogenic property of the drug. The calcium carbonate treated group has shown a significant (p<0.01) decline in the

Fig. 1. Effect of calcium carbonate and calcium channels blockers on neurosteroid induced food consumption in female mice

Values are expressed as means ± SEM of five experiments.

*Comparison of test and disease control with normal control
# Comparison test with Disease Control
body weight compared to the progesterone treated group. Ethosuximide at high dose i.e. 10 mg/kg has shown a reduction in body weight compared to plain progesterone treated group, whereas low dose has not shown any significant effects. Calcium carbonate has significant (p<0.01) decline in the bodyweight compared to any other test groups but not as effective as standard sibutramine (Fig. 2).

3.3 Effect on Rectal Temperature

Sibutramine and Ethosuximide have represented a significant (p<0.1) increase in body temperature compared to plain progesterone and normal control group at 1 hr and 2 hrs of post-treatment. Even plain progesterone treated group also has shown significant (p<0.1) increase in body temp, may be because of food-induced thermogenesis (Fig. 3).

3.4 Effect on WAT

Weight of white adipose tissue and adiposity index were studied. There was significant (p<0.01, p<0.1 respectively) decline in both the parameters that were observed in the calcium carbonate treated group compared to plain progesterone. In contrast, there was a significant (p<0.01) increase in body adiposity index and wet weight of WAT in plain progesterone treated group. Treatment with Ethosuximide, the high dose also represented a decline in both the parameters compared to the disease control group. Whereas Nifedipine treated (low and high dose) groups have not shown any change (Fig. 4).

3.5 Effect on Faecallipid Content

Fecal lipid content was measured on the day '0' and 28th day of the study. Lipid content in the faeces was significantly (p<0.01) high in calcium carbonate treated group compared to all treatment groups. Treatment groups of Ethosuximide have also shown a slight increase in the stool lipid content but not as good as calcium carbonate treated groups. Other treatment groups have not any notable changes (Fig. 5).

3.6 Histology of Fat Pad

Fig. 6 represents the effect of various treatment groups on the fat cell size. Plain progesterone treated group shows the increase in fat cell size (Fig. 6b) compared to the normal saline-treated group. This effect was attenuated by the treatment of calcium carbonate (Fig. 6d) and sibutramine treated groups. Other treatment groups have not shown any observable effects on the architecture of the fat cell (Fig. 6).

4. DISCUSSION

Obesity is associated with an increase in adipocyte tissue mass, because of both hypertrophy and hyperplasia. Hypertrophy represents the increase in the size of fat cells whereas hyperplasia is increasing in the number of adipocytes. Scientists around the world have developed many methods to reduce hypertrophy and hyperplasia which can then help to reduce the body weight [21]. Even strategies were also made to initiate the programmed cell death mechanism of the adipocyte by apoptosis mechanism which is considered as one of the best options in the current situation. Once an adipocyte has reached its maximum size, the adipocyte weight can increase further by increasing the number of adipocytes. The weight loss can be achieved by decreasing the hypertrophy and/or hyperplasia. By encouraging the apoptosis of the adipocyte it’s quite possible that a person may lose the body weight over time.

Calcium signals are essential for normal cellular functions including cell growth, differentiation and apoptosis, and homeostasis. Around 98% of total body calcium is stored in the bone in the form of calcium phosphate. The calcium concentration of the extracellular fluid (ECF) is about 2.2–2.6 mmol/L in the form of calcium whereas, around 1.3–1.5 mmol/L in the form of free calcium ions (Ca²⁺) [22]. The concentration of calcium in intracellular fluid is about 50–200 nmol/L, depending on the cell types, which is very less than that in the ECF [23]. Though, intracellular concentration plays many important roles includes muscle contraction, neuronal depolarization, regulation of enzyme function, its release and uptake etc. Calcium channels play a vital role to maintain the intracellular and extracellular concentration in the narrow range. A few earlier studies stated that calcium plays an important role in adipocyte differentiation [24]. Based upon these observations present study has been undertaken to understand the role of calcium and calcium channel blockers in obesity. In the present study, calcium carbonate is taken as a source of calcium ions whereas L-type calcium channel
Fig. 2. Effect of calcium carbonate and calcium channels blockers on neurosteroid induced obesity in female mice % change in body weight

Values are expressed as means ± SEM of five experiments.

*Comparison of test and disease control with normal control
# Comparison test with Disease Control

Fig. 3. Effect of calcium carbonate and calcium channels blockers on neurosteroid induced change in body temperature

Values are expressed as means ± SEM of five experiments.

*Comparison of test and disease control with normal control
# Comparison test with Disease Control
Table 1. Treatment schedule

| Animal group | Treatment schedule |
|--------------|--------------------|
| Group 1      | Normal control – Standard Chow diet |
| Group 2      | Vehicle + Progesterone, 10 mg/kg, s.c. |
| Group 3      | Sibutramine (10 mg/kg, p.o.) + Progesterone, 10 mg/kg, s.c. |
| Group 4      | Calcium Carbonate (50 mg/kg) + Progesterone, 10 mg/kg, s.c. |
| Group 5      | Nifedipine (1.5 mg/kg p.o.) + Progesterone, 10 mg/kg, s.c. |
| Group 6      | Nifedipine (3 mg/kg p.o.) + Progesterone, 10 mg/kg, s.c. |
| Group 7      | Ethosuximide (5 mg/kg, p.o.) + Progesterone, 10 mg/kg, s.c. |
| Group 8      | Ethosuximide (10 mg/kg, p.o.) + Progesterone, 10 mg/kg, s.c. |

Fig. 4. Effect of calcium carbonate and calcium channels blockers on neurosteroid induced change in white adipose tissue (WAT)

Values are expressed as means ± SEM of five experiments.

*Comparison of test and disease control with normal control
#Comparison test with Disease Control
blocker i.e. nifedipine and T-type calcium channel blocker ethosuximide were used to manipulate the cytosolic calcium concentration to study its role in obesity.

Steroidal hormones are often considered as to increase in body weight. The steroidal hormones which are involved in the regulation of brain functions are called neurosteroids [25]. Progesterone is one such hormone linked to the pathophysiology of obesity by inducing the hyperphagia and eating disorder. Some reports suggest the use of progesterone-containing preparations as a contraceptive or for hormone replacement therapy to cause substantial weight gain by causing hyperphagia and increased fat deposition [13,26]. Reports also suggest that progesterone can produce these effects by declining the serotonin level in the hypothalamic neurons which governs the feeding behaviour. Moreover, few reports suggest that progesterone can interact with the voltage-sensitive calcium channels in the neuronal circuit of the hypothalamus [27]. By observing all the above facts we have used progesterone as a source to induce obesity.

Our study results demonstrated that progesterone at the dose of 10 mg/kg shown hyperphagia, which was partially reduced by the treatment with ethosuximide high dose but not by nifedipine or calcium carbonate treatment. This data suggests that the eating behaviour of the neurosteroids may be induced through the

![Graph showing effect of calcium carbonate and calcium channels blockers on neurosteroid induced change in faecal lipid content](image)

**Fig. 5.** Effect of calcium carbonate and calcium channels blockers on neurosteroid induced change in faecal lipid content

Values are expressed as means ± SEM of five experiments.

*Comparison of test and disease control with normal control

# Comparison test with Disease Control

![Image of normal control](image)

**Fig. 6a.** Normal control

![Image of vehicle + progesterone, 10 mg/kg](image)

**Fig. 6b.** Vehicle + Progesterone, 10 mg/kg,

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Fig. 6c. Sibutramine (10 mg/kg, p.o.) + Progesterone, 10 mg/kg, s.c.

Fig. 6d. Calcium Carbonate (50 mg/kg, p.o.) + Progesterone, 10 mg/kg, s.c.

Fig. 6. Effect of calcium carbonate and calcium channels blockers on progesterone modulated histological changes of fat pad

Voltage gates of T-type calcium channels in the brain. This effect produced by the ethosuximide in a dose-dependent manner. Co-administration of sibutramine also reduced the hyperphagia induced by progesterone. Hyperphagia produced by progesterone through the progestin receptors by modulating the serotonin level of the hypothalamic serotonergic neurons [28]. Sibutramine inhibits the 5-HT re-uptake in the hypothalamic site which is involved in the feeding behavior; this suggests the possible interaction exists between progesterone and 5-HT. Because of this reason, sibutramine causes significant weight reduction. Weight reduction is also been observed in the calcium carbonate treated group.

It has been stated that cytosolic calcium has been involved in regulating adipocyte differentiation and plays a vital role in the metabolic derangement [29]. As per the earlier literatures a sustained rise in intracellular Ca^{2+} has been shown to activate apoptotic cell death and that Ca^{2+} -mediated apoptosis can be induced in mature adipocytes [30,31]. The same might have happened in our study, high cytosolic Ca^{2+} level more than the normal levels inhibits adipocyte differentiation, and triggered the apoptotic pathway in adipocytes which subsequently causes decreases in the accumulation of lipid drops [32]. This might be a possible explanation that calcium carbonate causes a decrease in body weight. Interestingly, L-type and T-type voltage-gated calcium channels have been reported to be present on adipocytes. Ethosuximide which is a selective T-type calcium channel blocker has shown some reduction in body weight but not by Nifedipine.

An increase in body temperature was observed in standard sibutramine treated and calcium carbonate treated groups. Treatment with calcium carbonate causes lipolysis and thus thermogenesis [33-36]. Measurement of WAT and adiposity index considered as an important parameters in our study which was significantly decreased by the treatment of calcium carbonate which supports our above results.

Faecal lipid content was also measured, which was found significantly high in calcium carbonate treated group. After oral administration calcium carbonate, excrete the lipid by the formation of insoluble calcium soaps and change the interfacial organization of the hydrolyzed lipids which are involved in the calcium-induced decline in fat digestion and absorption to increase in fecal fat excretion [37,38]. This protective effect was not been observed in any other of the test drugs.

5. CONCLUSION

Based upon the above discussion we conclude that calcium carbonate has significant anti-obesity activity by its duel effects i.e. thermogenesis and by enhancing the lipid and fat excretion. A few more studies are required to establish a daily intake of calcium to avoid its
harmful effects. T-type calcium channel blocker (ethosuximide) has also shown some protection against obesity induced by neurosteroid by reducing food intake and hence decrease in body weight, while at our laboratory settings N-type calcium channel nifedipine has not offered any protection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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