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ELUCIDATION OF THYRO-MODULATORY PATHOPHYSIOLOGICAL ACTIONS FOLLOWING PROLONGED CO-EXPOSURE TO EXCESS CALCIUM & MAGNESIUM: A DURATION - DEPENDENT STUDY

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ABSTRACT
Calcium and magnesium, indispensable minerals for survival, are the two chief components of hard water. Previous in vivo (dose and duration-dependent) as well as in vitro studies with high calcium and magnesium invidually had already established the adverse effects caused by these 2 individual minerals. However, the effects of hard water as a combination of calcium and magnesium on thyroid physiology are still unconvincing and scanty.
Experimental animal (rat) models were developed to study the intricate details of $CaCl_2$ - $MgSO_4$ co-exposure induced effects in a duration - dependent study protocol.
Adult, male, albino Wistar strain rats were subjected to $CaCl_2$ - $MgSO_4$ co-exposure for 15, 30 and 60 days with each group receiving 1.5g $CaCl_2$-1.5g $MgSO_4$/100 g diet/day, against respective controls. Daily food consumption, body weight gain%, absolute and relative thyroid weight, histologic and histometric analyses,
enzyme activity patterns including thyroid peroxidase (TPO), thyroidal Na^+-K^+-ATPase, thyroidal 5' -deiodinase type I (DI), hormone profiles including total & free T4, total & free T3, TSH were studied. Significant ($P< 0.05$) alterations were found in almost all parameters, in the Ca - Mg treated groups in a duration -dependent fashion when compared to their respective control groups. So, it can be suggested that, prolonged, excess $CaCl_2$ - $MgSO_4$ co-exposure produces marked structural and functional alterations of thyroid physiology and thus contributes in the thyroid pathophysiological development.

KEYWORDS: Ca, Mg, hard water, thyroid dysfunction, *In vitro*, TPO, Na^+-K^+-ATPase, DI

INTRODUCTION
Calcium and magnesium, the two indispensable, elements for survival. Along with their nutritional resources, these two are highly abundant in hard water. Though usually most people suffer the deficiency of these 2 minerals, a vast majority of population also get exposed to excess level of calcium and magnesium via hard water and other sources. Owing to the ever increasing encounter with hard water via industrial resources, nutritional supplements, living organisms are rendered over exposed to the undesired adverse effects of excess calcium and magnesium. These are the two main components of hard water. Since they are found to be involved in most of the physiological processes and are among the most studied minerals having biological relevance. Though their biological importance for the survival of human is indispensable, any fluctuation from their normal values cause wide spread significant impact which may turn fatal, if left untreated.
On the other hand, thyroid is considered to be one of the most vital organs, an endocrine gland, that regulates nearly all the important physiological activities. Since ancient ages, thyroid glands had been subjected to extensive studies which unveiled the enormous pathophysiological consequences owing to hypo and hyper functioning of this gland. Certain epidemiological studies throughout the world have suspected a probable relation between hard water consumption and development of thyroid dysfunctions leading to the development of goiter and associated disorders.
Our previous studies had already established the detailed mechanism of individually, high calcium and magnesium induced thyroid disruptive actions (Chandra *et al*, 2012; Chandra *et al,* 2014). Also, in vitro studies with high magnesium exposure on thyroid morphology and functional status has also been established (Goswami *et al*, 2024), echoed the in vivo results. However, till date knowledge regarding the detailed mechanism of high calcium magnesium co - induced thyro-modulatory actions is incomprehensive.
The present study was aimed to explore to get insight into this intricate mechanistic aspect.

Experimental Design
Animal maintenance and treatment
Adult (three months' old/90±5 days), healthy, male, albino rats (Rattus norvegicus) of Wistar strain weighing 140±10 g were used in the present study. The animals were maintained as per national guidelines and protocols, approved by the

Institutional Animal Ethics Committee (PHY/CU/IAEC/16/2008 dated 15.05.2008). The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house (temperature: 22±2°C; relative humidity: 40-60%) with constant 12:12 light: dark schedule. The animals were fed on standardized normal diet (20% protein) which consisted of 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined til oil, and 0.25% shark liver oil, 4% salt and water ad libitum (Chandra *et al*., 2010). All the animals were in docile conditions and no mortality was observed.

In the treatment schedule, rats were equally divided into six groups each consisted of eight rats per group. Three groups were kept as respective controls for 15, 30 and 60 days and fed with normal diet. All the treated groups received 1.5g $CaCl_2$-1.5g $MgSO_4$/100 g diet/day as the specified dose was found to exert maximum effects in the dose-dependent study described in previous chapter. First treated group received the above mentioned dose for 15 days, second treated group for 30 days and third treated group for 60 days. All animals were caged individually. Feed consumption, corrected for feed wasted was measured as described in the previous chapter. Animal sacrifice and all other experimental procedures were same as described earlier.

RESULTS

In course of the $CaCl_2$ - $MgSO_4$ treatment for different durations, the food consumption was recorded for each rat. Food consumption was almost same in all the groups (all the control and treated groups) (Table 1).

Table 1. Food consumption of the experimental animals under the influence of $CaCl_2$-$MgSO_4$ for different durations at the dose of 1.5 g%

Parameter	Groups					
	15 days		30 days		60 days	
	Control	$CaCl_2$ - $MgSO_4$ treated	Control	CaCl2-MgSO4 treated	Control	$CaCl_2$ - $MgSO_4$ treated
Food intake/day(g)	25±1.3	26±2.1	25±1.8	24±1.8	25±1.3	23±2.2

Data are presented as mean ± SD, n = 8. Student's t-test was performed. Values bearing asterisk are significantly different at $p < 0.05$ when compared to control.

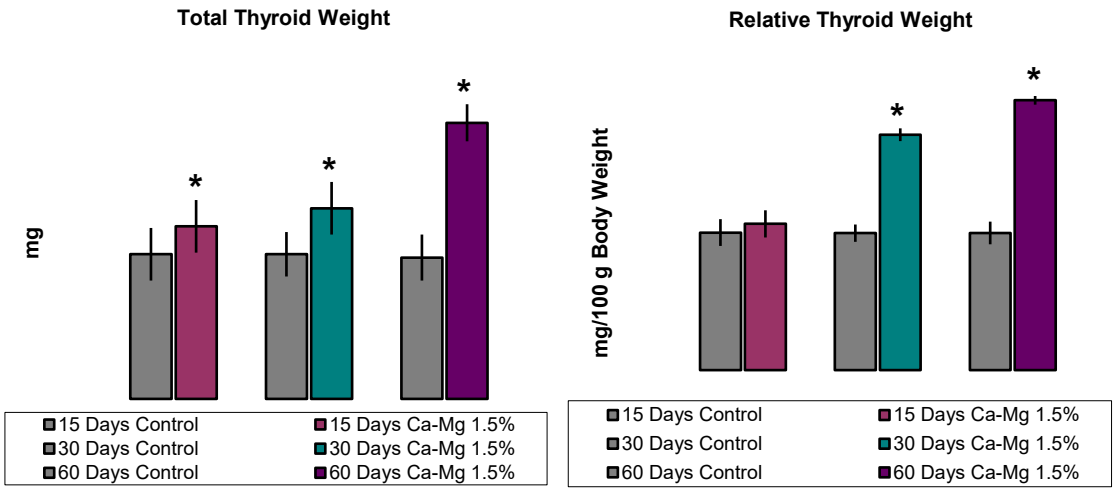
Body weight of all the control animals showed a net gain of 17.2 %, 29.3 % and 32.2 % for 15 days, 30 days and 60 days, respectively, while that of the $CaCl_2$ - $MgSO_4$ treated groups were found to be 11.7 %, 16.9 % and 10.2 % for 15 days, 30 days and 60 days, respectively i.e., net body weight gain (%) were noticeably lowered in duration-dependent fashion (Table 2).

Table 2. Body weight of the experimental animals under the influence of $CaCl_2$ - $MgSO_4$ for different durations at the dose of 1.5 g%

Parameters	Groups					
	15 days		30 days		60 days	
	Control	$CaCl_2$ - $MgSO_4$ treated	Control	$CaCl_2$ - $MgSO_4$ treated	Control	$CaCl_2$ - $MgSO_4$ treated
Body weight (g) (Pre-treatment)	140.0±5.5	143.5±6.9	141.2±4.94	141.0±5.9	140.2±10.4	142.0±6.3
Body weight (g) (Post-treatment)	173.5±5.7	155.5±11.3	191.7±6.88	168.7±8.2	196.5±17.2	152.5±10.5
Body weight gain (%)	17.2	11.5	29.3	14.5	32.2	10.0

Data is presented as the mean ± SD, n=8.

Both total (Fig. 1 A) as well as relative (Fig. 1 B) thyroid gland weights of the experimental groups of rats were found to be significantly ($p < 0.05$) higher in the 30 days and 60 days treated groups but no such changes are found in the 15 days treated group.



(A) (B)

Figure 1. Total (A) and relative (B) thyroid gland weight of the experimental animals under the influence of $CaCl_2$ - $MgSO_4$ for different durations at the dose of 1.5 g%

[Data are presented as mean ± SD, n = 8. Student's t-test was performed. Values bearing asterisk are significantly different at $p < 0.05$ when compared to control.]

The histological features of thyroid glands of both the control as well as $CaCl_2$ - $MgSO_4$ treated group of rats were shown in Plate 1. Thyroid gland section of control rats as usual showed no signs of pathological changes while thyroid gland section of the $CaCl_2$ - $MgSO_4$ (15 days) treated group showed more or less similar features with the control group. However, thyroid gland sections of the $CaCl_2$ - $MgSO_4$ 30 days and 60 days treated groups showed the presence of marked hypertrophy and or hyperplasia of thyroid follicular epithelial cells (Plate 1).

Table 3.Morphometric/Histometric analysis of thyroid gland weight of the experimental animals under the influence of $CaCl_2$ - $MgSO_4$ for different durations at the dose of 1.5 g%

Parameters	Groups					
	15 days		30 days		60 days	
	Control	$CaCl_2$ - $MgSO_4$ treated	Control	$CaCl_2$ - $MgSO_4$ treated	Control	$CaCl_2$ - $MgSO_4$ treated
Area of follicular cells (mm2)	1.45±0.05	1.50±0.03	1.45±0.05	1.66±0.06*	1.45±0.05	1.78±0.04*
Area of colloids (mm2)	1.16±0.04	1.24±0.03*	1.20±0.05	1.34±0.01*	1.19±0.02	1.49±0.06*

Data are presented as mean ± SD, n = 6. One-way analysis of variance (ANOVA) test followed by a multiple comparison t-test was performed. Values bearing asterisk are significantly different by ANOVA at $p < 0.05$ when compared to control.

Morphometric/histometric analysis data of thyroid gland of the control and calcium-magnesium treated groups are depicted in Table 3 depicting increased areas of follicular cells as well as colloid. Semiquantative assessment of thyroid follicles of control and the calcium chloride-magnesium sulfate treated rats (Table 4) showed that, in the treated groups, the relative number (%) of large, medium and small sized follicles were increased.

Table 4. Semiquantitative assessment of thyroid gland weight of the experimental animals under the influence of $CaCl_2$ - $MgSO_4$ for different durations at the dose of 1.5 g%

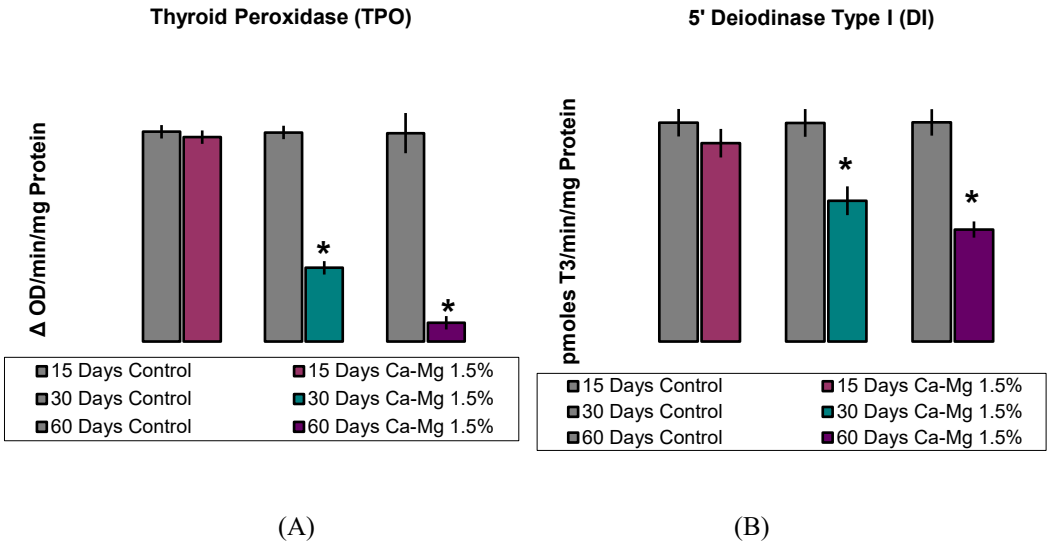
Parameters	Groups					
	15 days		30 days		60 days	
	Control	$CaCl_2$ - $MgSO_4$ treated	Control	$CaCl_2$ - $MgSO_4$ treated	Control	$CaCl_2$ - $MgSO_4$ Treated
Small follicles (%)	46	48	48	31	46	26
Medium follicles (%)	24	24	26	38	23	32
Large follicles (%)	26	24	23	27	26	38

Immature follicles (%)	5	4	3	4	5	2

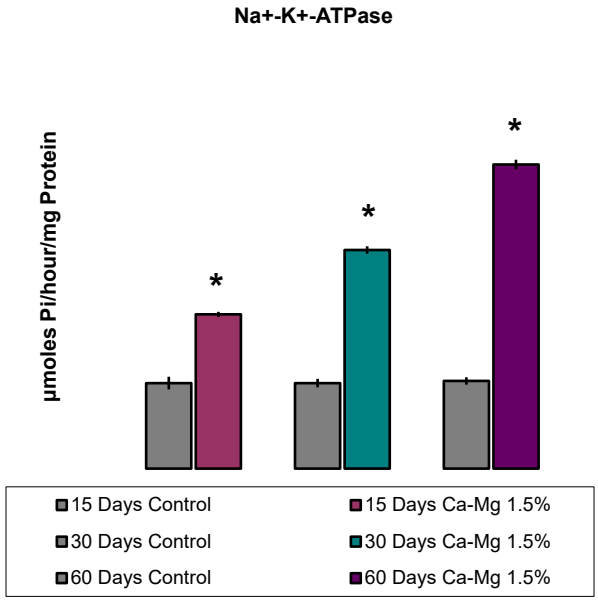
Data is presented as the mean ± SD, n=8. Small follicle = Follicular diameter of 5-10µm; Medium follicle = Follicular diameter of 11-15 µm; Large follicle = Follicular diameter of 16-20µm; Immature follicle = Follicular diameter 0-5 µm.

The control as well as 15 days treated group showed predominance of small and medium sized follicles with relatively few large follicles. In contrast, in 30 and 60 days calcium-magnesium treated groups, the follicles were mainly of large and medium sizes, the numbers being dependent on the duration of high calcium-magnesium exposure, indicating the potential role of calcium-magnesium in the development of histomorphological alterations in thyroid.

The present study depicted remarkable alterations in enzyme activity following calcium-magnesium exposure. In case of both thyroid peroxidase (TPO) as well as thyroidal 5' -Deiodinase Type I (DI)-enzyme activities showed considerable ($p < 0.05$) retardation in all the treated groups. However, thyroidal Na^+-K^+-ATPase enzyme activity of the experimental groups showed a progressive increase followed by gradual, duration-dependent ($p < 0.05$) stimulation in the subsequent 30 days and 60 days treated groups (Table 6).



(A) (B)



(C)

Figure 2. Effects of $CaCl_2$ - $MgSO_4$ for different durations on thyroid peroxidase (TPO) (A), thyroidal Na^+-K^+-ATPase (B) and 5' -Deiodinase Type I (DI) (C) activities in experimental animals at the dose of 1.5 g%

[Data are presented as mean ± SD, n = 8. Student's t-test was performed. Values bearing asterisk are significantly different at $p < 0.05$ when compared to control.]

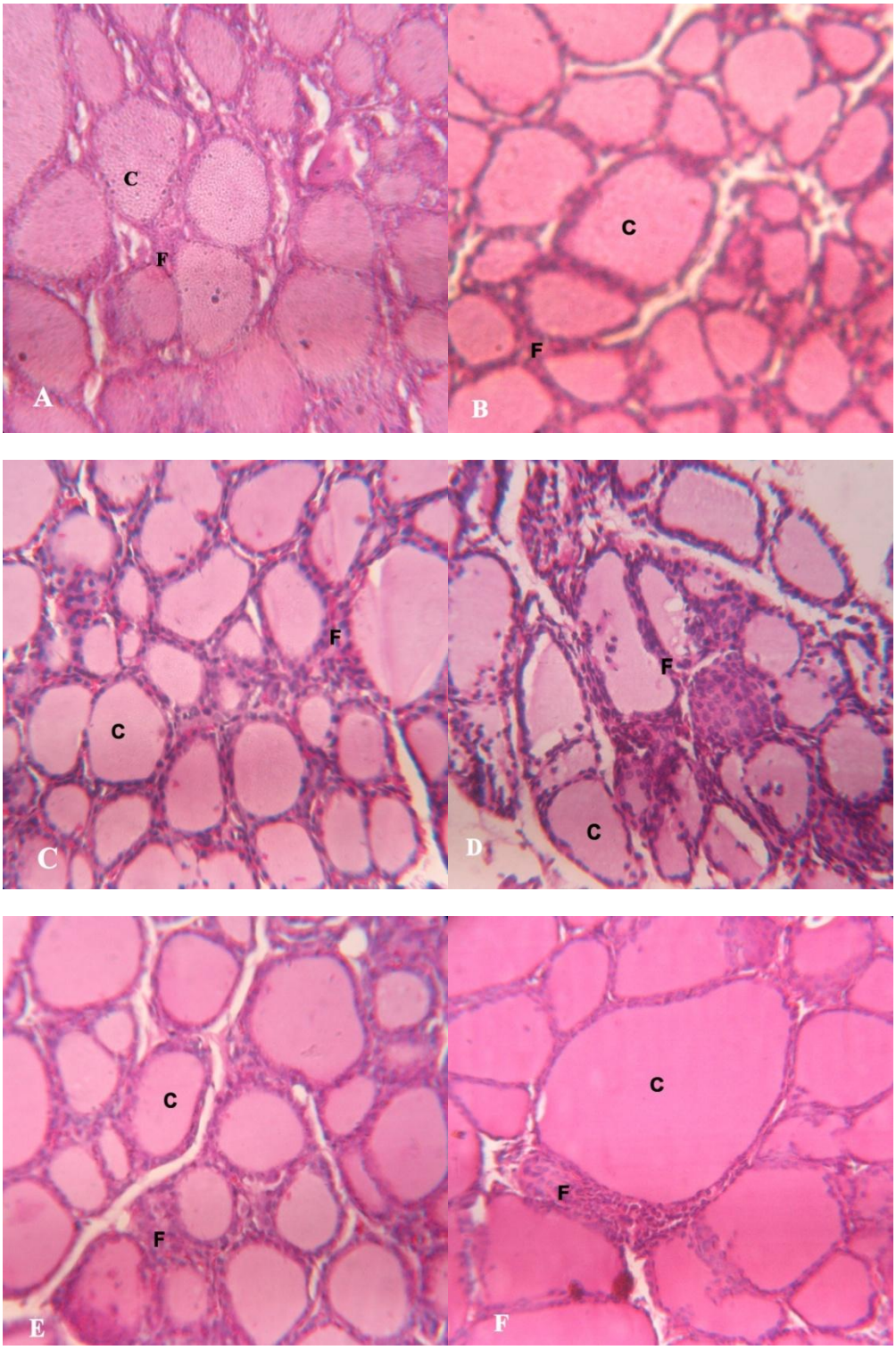


Plate 1.

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hotomicrographs of paraffin-embedded H&E- stained rat thyroid sections (400X) showing duration dependent effects of calcium-magnesium at the dose of 1.5 g %. (A) Thyroid section from control animals. (B) Thyroid section from calcium chloride-magnesium sulfate treated animals (for 15 days). (C) Thyroid section from control animals. (D) Thyroid section from calcium chloride-magnesium sulfate treated animals (for 30 days). (E) Thyroid section from control animals. (F) Thyroid section from calcium chloride-magnesium sulfate administered animals (for 60 days). C = Colloid, F = Follicle.

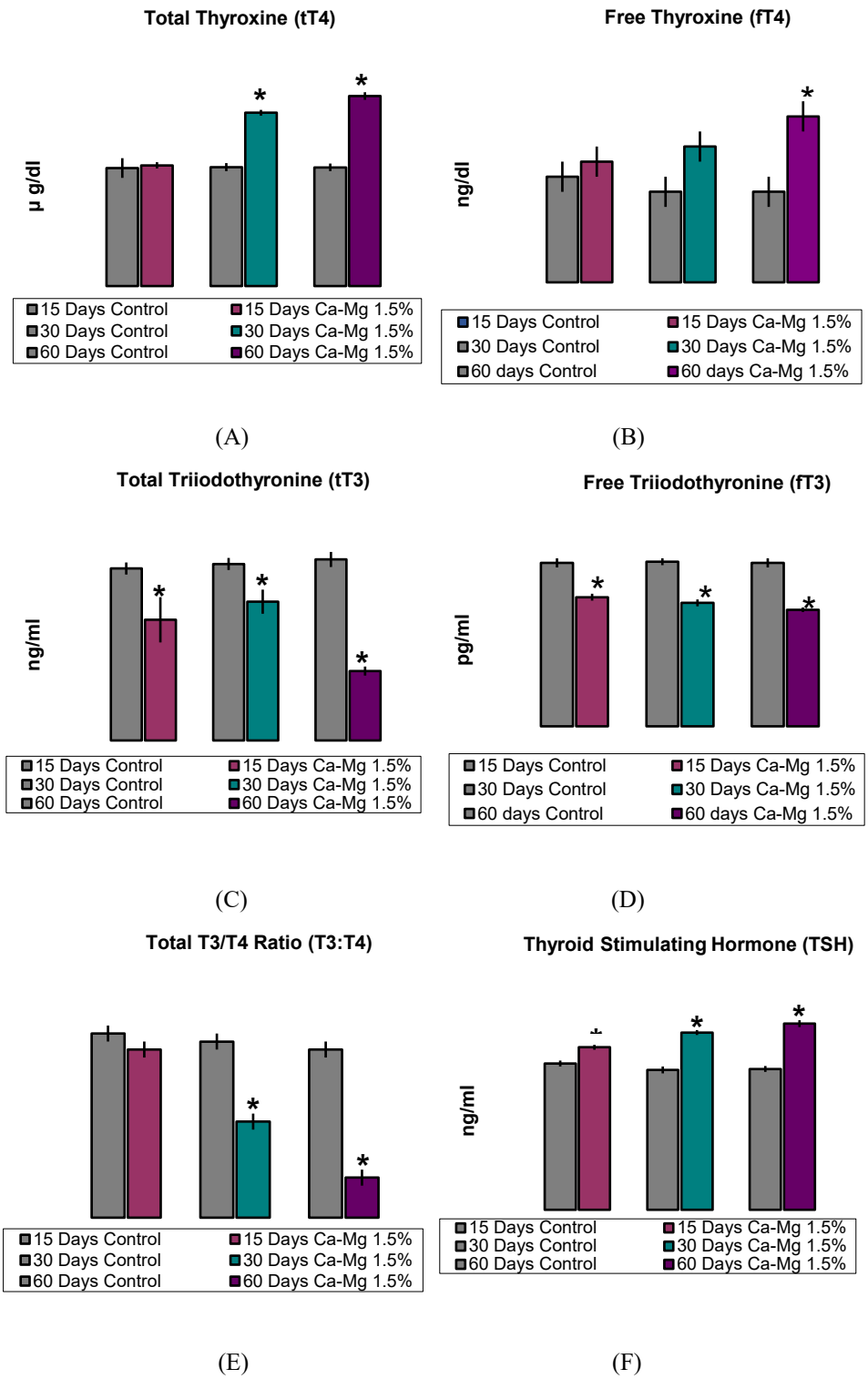


Figure 3. Effects of $CaCl_2$ - $MgSO_4$ for different durations on serum total (A) & free (B) thyroxine (T4), total (C) & free (D) triiodothyronine (T3), total T3:T4 (E) and TSH (F) levels in experimental animals at the dose of 1.5 g%

[Data are presented as mean ± SD, n = 8. One-way analysis of variance (ANOVA) test followed by a multiple comparison t-test was performed. Values bearing asterisk are significantly different by ANOVA at $p < 0.05$ when compared to control.]

Serum hormonal profile was also altered following calcium-magnesium treatment. Serum total thyroxine (T4) level was gradually elevated with the progression of duration of exposure. Total triiodothyronine (T3) level of the experimental rats was found to be lower than their respective control groups. T3/T4 ratio (T3:T4) was also decreased in the calcium-magnesium treated groups as compared to their respective control groups. A considerable increase in thyroid stimulating hormone (TSH) was observed in the calcium-magnesium treated experimental groups in a duration-dependent fashion as compared to the control group (Table 7).

DISCUSSION

The treated groups showed a decrease in net body weight gain (%) in comparison to their respective controls in a duration-dependent manner, after administration of calcium-magnesium in combination, though food intake among all groups remained almost same. The results showed a trend similar with that of the dose as well as duration-dependent studies with both individual calcium and magnesium and also the dose-dependent experiment with their combined form.

The activities of all the major thyroid hormone biosynthesizing hormones, viz., thyroid peroxidase (TPO), thyroidal Na^+-K^+-ATPase and thyroidal 5' -Deiodinase Type I (DI)-were found to be modulated after administration of calcium-magnesium for different durations.

TPO activity showed a considerable decrease with the progression of durations in the calcium-magnesium treated groups, in a similar fashion to that of the dose and duration-dependent experiments with individual calcium. Therefore, the inhibition of TPO activity with calcium-magnesium in combination, as observed in the current study, may be mediated by calcium.

Na^+-K^+-ATPase activities of the treated groups showed a marked duration-dependent increase to ensure greater iodide uptake by the gland, as a compensatory measure, to ameliorate the impairment caused by high calcium and magnesium exposure. This also echoed the results of the previous sections. The Na^+-K^+-ATPase activity has been greatly intensified as compared to the individual treatments, presumably due to the collaborative responses of calcium and magnesium with progression of time.

The duration-dependent inhibition of deiodinase enzyme activity following calcium-magnesium exposure was revealed in the present study. Though the previous sections described the strong antagonistic, i.e., respective inhibitory and stimulatory effects of calcium and magnesium on DI activity, duration-dependent treatment with their combined form showed similarity with that of the individual calcium treatment (Chandra *et al*., 2012) indicating preferential actions of calcium over magnesium in this regard.

In the present study, serum total as well as free thyroxine (tT4 and fT4) and triiodothyronine (tT3 and fT3) levels and total T3:T4- all were exhibited chronicity-dependent modulations as evidenced by an enhancement in total and free T4 with a concomitant retardation in total and free T3 levels as well as a fall in total T3/T4 ratio following exposure to high calcium-magnesium with the progression of exposure. The changes were in a synchronized fashion with enzyme activity status. Though individually magnesium exerted a different kind of response in dose and duration-dependent experiments, the changes following calcium-magnesium combination treatments echoed that of the individual calcium treatments, thus once again establishing the relative predominance of calcium over magnesium on thyroid hormonal profile.

Heightened serum TSH level despite an elevated thyroid hormone level was observed in duration-dependent fashion in all the calcium-magnesium treated groups, compared to their respective controls. The dose and duration-dependent studies with individual calcium and magnesium as well as the dose-dependent treatment with their combined form also revealed augmented release of TSH. Mechanistically, these results could be explained in light of the evidences reporting increased TRH-induced TSH gene expression in presence of calcium or magnesium-induced impairment of TSH functioning-both of which are followed by enhanced TSH release.

Thyroid gland weights (both absolute as well as relative) in calcium-magnesium treated groups were increased significantly than their respective controls, gradually with the progression of exposure. Histopathological as well as morphometric analysis revealed increased number of diffuse thyroid follicles without inflammatory reaction, large as well as small follicles, tall columnar follicular epithelium, with massive hypertrophy and hyperplastic changes with concomitant increase in both follicular and colloidal areas indicating TSH-dependent alterations. Semi quantitative assessment study highlighted the presence of increased relative numbers of medium and large sized follicles in the calcium-magnesium treated groups as compared to their respective control groups, which are mostly made up of small sized follicles, thus pointing at the development of thyroid pathophysiological alterations (Chandra *et al*., 2012). Excess TSH secretion coupled to thyroidal Na^+-K^+-ATPase activity resulted in hypertrophy and hyperplasia that lead to thyroid gland enlargement as observed in the present study resulting thyroid gland enlargement.

It may thus be elucidated that, the combined form of calcium-magnesium gradually developed a state of biochemical hypothyroidism as has been evidenced by sequential alterations in activities of thyroid hormone synthesizing enzymes and thyroid hormone profiles, resulting in cytological/morphological modifications in thyroid causing its enlargement or goiter. Combinations of the two potent individual thyro-modulating agents as well as chronicity of exposure to them are the other prime regulatory factors regarding the execution of their effects.

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