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RESEARCH ARTICLE ON SUBACUTE INTRAMUSCULAR TOXICITY STUDY OF AQUEOUS CHOLECALCIFEROL IN WISTAR RATS

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ABSTRACT
In the market there are multiple choices available for vitamin D supplements. We prepared aqueous cholecalciferol novel drug preparation use in the behalf of oil soluble vitamin-D accretion for more rapidly efficiency. Vitamin-D is a dietary supplement which is using for multipurpose. The main source of cholecalciferol is sunlight and dairy foods. We hypothesized that aqueous cholecalciferol would be beneficial than oil soluble preparations and found out no possible toxicity in wistar rats. Forty-eighth female and male wistar rats were divided into four group (n=6): group1 (placebo-IM), group 2 (62000 IU/kg cholecalciferol-IM), group 3 (93000 IU/kg cholecalciferol-IM) and group 4 (124000 IU/kg cholecalciferol-IM). The result, including body weight, feed intake, hematological, biochemical parameters in all different groups did not reveal any abnormality related to aqueous cholecalciferol.

KEYWORDS: Aqueous cholecalciferol, Calcium, Rickets, Parathyroid hormone, Osteoporosis.

1. INTRODUCTION
It is largely through ancient coincidence that vitamin D_3 has been categorized as a vitamin rather than as a steroid hormone.[1] Vitamin-D is a key factor for phosphorus and calcium absorption for bone health and regulation of metabolism.[2] There are mainly 3 source of vitamin-D are sunlight, dairy products and vitamin D supplements. As per literature there are very limited sources to be precious sources of naturally occurring vitamin D_3 like fish, meat, offal, egg and dairy products.[3] Ergocalciferol and cholecalciferol are two types of vitamin-D its structural distinction lies only on the C-17 side chain, the former having a double bond on C-22 to C-23 and a C-24 methyl group.[3] It has been presumed that when skin is bare to sunlight, amount of the stores of 7-DHC in the epidermis undergoes a photochemical response causing in the eventual formation of vitamin D_3. The higher concentration of 7-DHC per milligram of lipid was located in the stratum Basale.[4] Melanin works as a neutral filter and absorb UV radiation which is responsible for previtamin D_3 synthesis. After previtamin D_3 activated in the skin, it will produce vitamin D3 by isomerization or can absorb solar radiation and photoisomerize to a diversity of products, including lumisterol and tachysterol.[5] As per literature age group between the range of 8-18 year old subjects was produced 2-3 times higher amount of previtamin D3 than 77-82 year old subjects.[6] UVB fall onto skin and 7-dehydrocholesterol penetrates into epidermis layer and further convert into cholesterol. Now cholecalciferol hydroxylated in liver by two enzymes called CYP2R1 and CYP27A1 to form 25-hydroxycholesterol this metabolite is further converted in the renal to a number of other forms, via 1α-globulin and hydroxylation to form 1,25-dihydroxycholecalciferol. So the kidney is main production house for calcitriol, 1, 25-dihydroxycholecalciferol circulates from kidney to small intestine, bone, strained muscles. This calcitriol binds with vitamin D receptors and goes for further process.[7] Prostate, brain, colon and breast as well as immune cells also have a VDR.[8][9] Parathyroid hormone regulates normal calcium level by acting on renal 1-hydroxylase enzyme thus PTH is the major controller of renal 1-hydroxylase.[10] In the occurrence of hypocalcemia, the diminished calcium is sensed in the parathyroid (PTH) glands by the calcium-sensing receptors.[11] The recent discovery suggest that patients with kidney failure increases FGF-23 which is directly effect on 1α hydroxylase enzyme and decreases there production, may be this is a directly effecting parameter in renal failure patients for failing to maintain calcitriol level in the body.[12] Thus the patients with renal failure require calcitriol supplementation.[13] There is such cytochrome that regulates calcium activities. CYP2R1 is the key CYP for human and mice because irregularity in calcium metabolism produced by genetic defect in CYP2R1

gene.[14] Hydrophilic preparation gives rapid action then oil based preparation so this novel formulation may have higher efficiency than oil soluble vitamin D supplements but the main purpose of the study is to find out if any toxicity produces by test item in relevant different doses with respective animal species.

2. MATERIAL AND METHODS

2.1 Animals

Total 48 wistar rats (n=6) from both gender obtained from Cadila Pharmaceutical Pvt Ltd, Animal House. Animals were 4 to 8 weeks old and ± 20 mean body weight was limited for randomization of rodents. All rats were housed in standard polypropylene rat cages in a temperature (18-22°C) and humidity (30-70%) controlled room submitted to a 12 hours light/dark cycle. All procedures were carried out in harmony with the conventional guideline for testing with animals.[15]

2.2 Experimental Method

After acclimatization (7 days), the normal diet provided at *ad libitum* through whole study plan. All animals were divided in eight group (n=6). Rats were administered aqueous cholecalciferol intramuscular at a dose of 0 IU/kg (control), 62000 IU/kg (group II), 93000 IU/kg (group III) and 124000 IU/kg (group IV) once a week with 1 ml disposable syringe fitted with 23-G needle till 28 days. All the observations documented during the study period like mortality, clinical signs, and body weight and food consumption.

2.3 OBSERVATION

All animals were observed once a day for atypical physical or behavioral alteration. The cage side observation and autonomic activity were observed.

2.4 Blood collection

Blood from the all animals collected after overnight fasting after dosing on 28th days. The collection of blood was done through cardiac puncture by keeping the animal under CO_2 anesthesia. The collection of blood was done in a 1.5 ml volume microcentrifuge tube coated with solution of sodium citrate having 3.5% strength as anticoagulant.

2.5 Hematological parameters

At the end of treatment (on 28th day) all groups were fasted overnight. *Ad libitum* water was given during fasting. Blood samples were composed on 29th day from orbital plexus under anesthetic condition and following parameters were assessed as Hemoglobin, RBCs, WBCs, Monocytes, Lymphocytes, Neutrophils, Basophils, Eosinophils, Platelets, Hematocrits, MCV, MCH, MCHC, Reticulocytes. All parameters were investigated using 5 part dissimilar multispecies fully automatic hematology analyzer sysmex Xs 800i.

2.6 Biochemical parameters

At the end of treatment (on 28th day) all groups were fasted overnight. *Ad libitum* water was given during fasting. On 29th day blood was collected from orbital plexus under anesthetic condition and following parameters were assessed Glucose, Cholesterol, AST, ALT, ALP, Triglycerides, BUN, Creatinine, Total Bilirubin, Total Protein, Albumin, Globulin, Sodium, Potassium, Phosphorus, Calcium.All biochemical parameters were evaluated using fully automatic analyzer model Sysmex Xs 800i, Norderstedt, Germany.

2.7 Statistical analysis

Statistical analysis of the data collected from body and organ weight, biochemical, hematological parameter was performed by ANOVA followed by Dennett's Multiple t' test, where $p < 0.05$ has been considered as statistically significant.

3. RESULT

3.1 Clinical signs and Mortality

No mortality and clinical sign noticed in any of the doses tested.

3.2 Body weights and Food Intake

None of the animals in treatment groups showed any statistical significant variation in body weights and food consumption compared to respective control groups indicating that the test item did not had any effect on body weight or food intake.

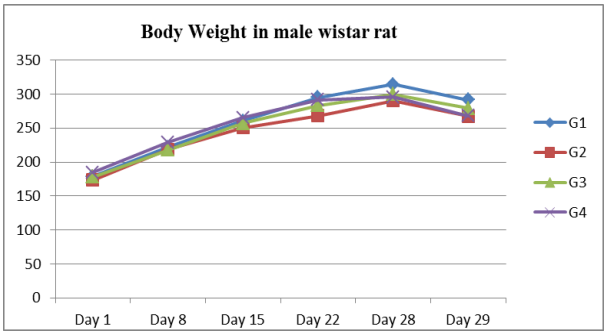

Figure no: 1. Mean values of male wistar rat's body weight in.

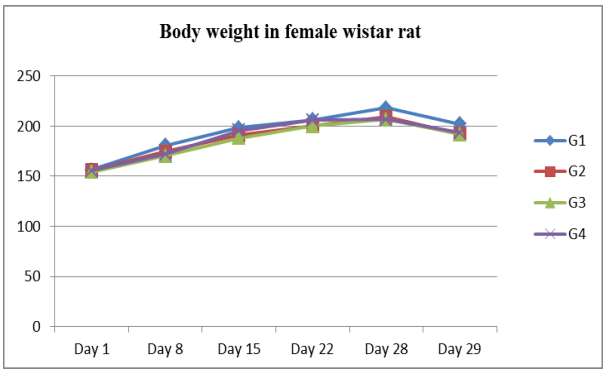

Figure no. 2: Mean values of female wistar rats body weight in.

3.3 Effect of cholecalciferol on organ weight in wistar rat

Different dose treatment groups did not reveal any toxicological effect on organ weight as compared to control groups in total study animals.

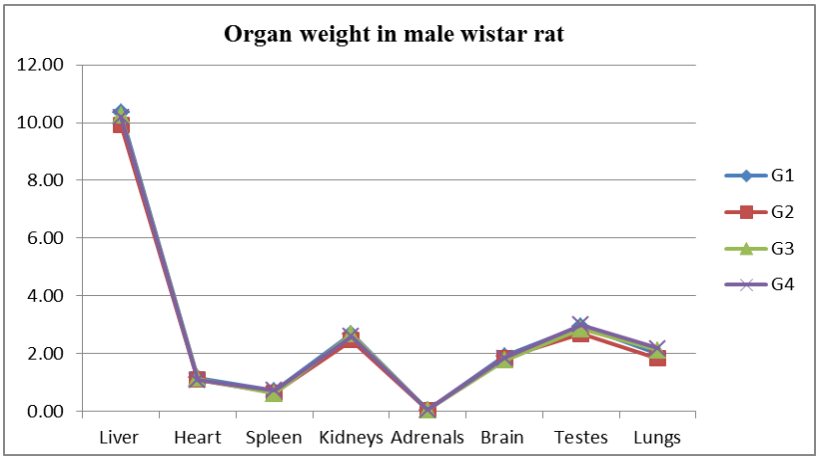

Figure no: 3 Mean value of organ weights in male wistar rats.

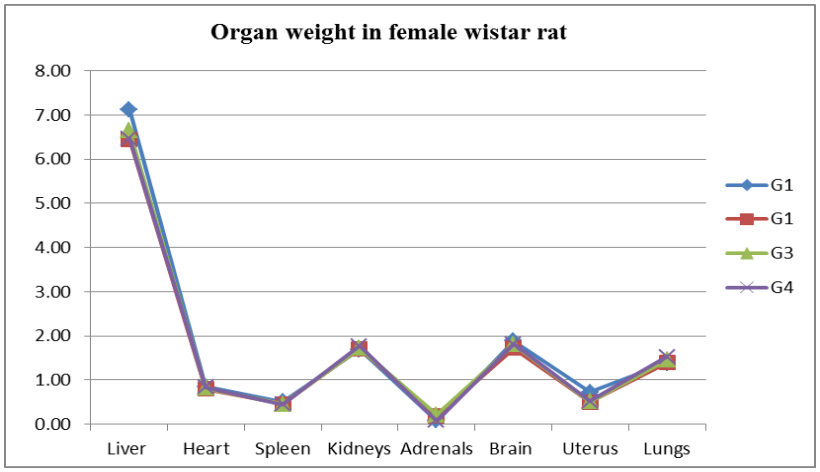

Figure no: 4 Mean value of organ weights in female wistar rat.

3.4 Effect of cholecalciferol on hematological parameter in male rats

Treatment did not effect in hematological parameters estimated in both sexes of treatment groups when compared with control.

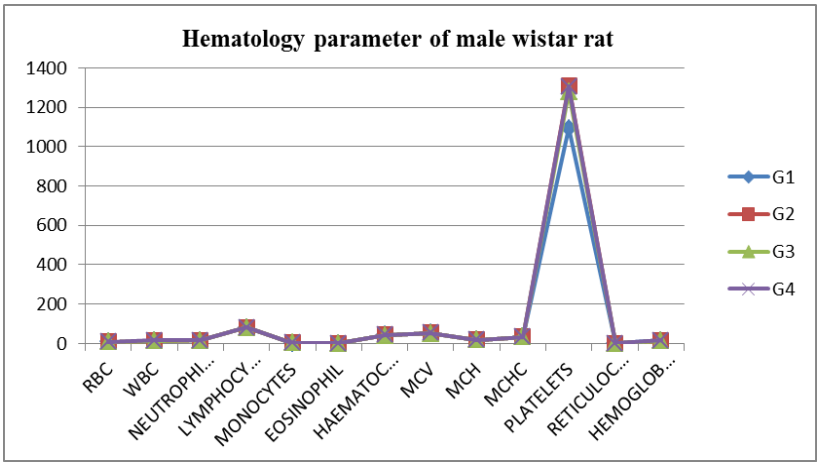

Figure no: 5 Mean value of hematological parameters in male wistar rats.

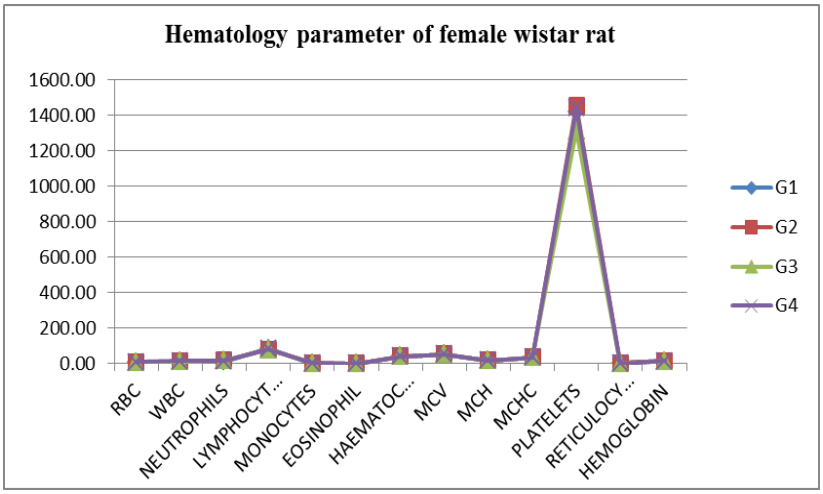

Figure no: 6 Mean value of hematological parameters in female wistar rats.

3.5 Effect of cholecalciferol on biochemical parameter in male rats

Biochemical parameters of the treatment groups were compared with the control groups. There were no treatments related significant differences in biochemical value of study animals.

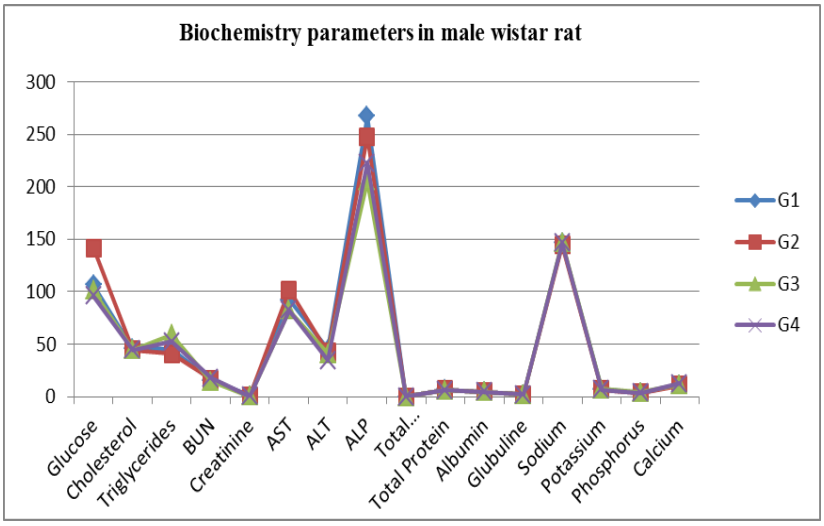

Figure no. 7: Mean value of biochemical parameters in male wistar rats.

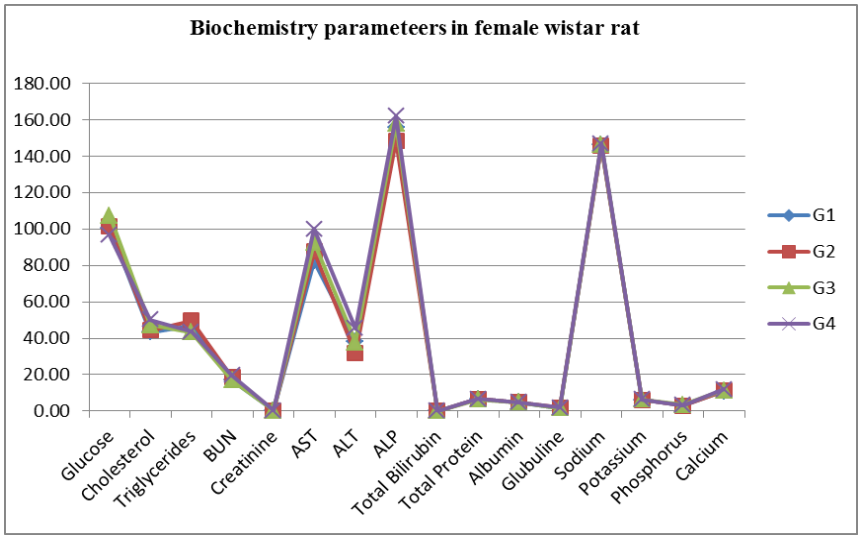

Figure no. 8: Mean value of biochemical parameters in female wistar rats.

4. DISCUSSION

The subacute toxicity study of novel water soluble cholecalciferol was performed for 28 days. Zero mortality was recorded during the whole period of study. The observational data with intramuscular dosing of 640 00IU/kg, 93000IU/kg, and 124000IU/kg cholecalciferol recorded no severe toxicity in any animal.

At fourth week of treatment, all animals were observed for functional and behavioral examinations like body posture, respiration, tonic-clonic involuntary movement, blinking abnormalities, sensory response, approach response, pinna reflex, sound response, tail pinch response were observed. Hand held activities like handling, lacrimation, reactivity, palpebral closure, salivation, abdominal tone, limb tone, pupillary reflex, piloerection, grip strength, proprioception were observed. Open field activities like gait, movements, number of rearing, number of defecation, and number of urination were observed. Functional and behavioral examinations were observed normal in all treated group.

At the second week, food intake noticed decreased in all male animal groups but, at the last week food consumption were normal. In female increased feed intake in second and third group as compared to control group but feed consumption at the last week was normal in both male and female animals so this change was considered as a biological variation.

Body weight was measured at every week to find out the effect of cholecalciferol on the growth rate. At the last week of dosing, body weight was increased in all female groups. In male animals at fourth week third group was decreased as compared to control group. Increased in body weight was normal biological variation. It was not considered as significant changes.

RBCs, WBCs, hemoglobin, MCHC, Hematocrit, MCV, Neutrophils, Lymphocytes, Monocytes, Eosinophils, MCH, Platelets count, Reticulocytes were estimated to find out if any adverse effect produced on hematological parameters by cholecalciferol.

In male eosinophil counts were observed as significant in low dose $p<0.05$ whereas mid and high dose was found $p<0.001$. Monocytes were found $p<0.001$ in low and mid group as high dose value remained normal. MCHC were found $p<0.05$ in mid and high dose. MCHC is a red cell indices and also used to identify hemoglobin content [16]. Reticulocytes was found $p<0.05$ in high group as compared to placebo group but the reticulocytes data was well within the normal laboratory range[17] and female animals were not shown any significant changes in reticulocytes thus this changes considered as a biological variation.. In female lymphocytes low dose was found significant with $p<0.05$ value whereas mid and high doses groups didn't shown any significancy. Whereas monocytes in low and mid group is significant with p value $p< 0.01$ and in high dose ($p<0.05$). Eosinophils were found $p< 0.01$ in mid and high group. According to CPCSEA guideline all above data were well within the normal range thus this changes not considered as an adverse effect of test item.[18] In this study hemoglobin level was normal and MCHC used for identify hemoglobin content so this changes in mean corpuscular hemoglobin concentration considered as a biological variation and did not show any major effect of water soluble cholecalciferol on MCHC. The values of RBCs, WBCs, MCV, and hemoglobin are well within the normal laboratory control data. Hence it is considered as a normal biological variation. All other hematological parameters were normal and did not show any significant variations.

Different biochemical parameters were measured to assess any abnormality occurs due to cholecalciferol toxicity. To find out if any carbohydrate & fat metabolism related abnormality detected due to the test item. Total protein and globulin were used to identify any toxicity occurs due to cholecalciferol in liver.[19] Total bilirubin was measured to find out any toxic effect related to any bile duct in liver. Alkaline phosphate was measured to see if any cellular damage[20][21] occurred due to cholecalciferol. Creatinine & urea are main parameter for examine renal system abnormality[22] if occurs due to cholecalciferol toxicity. Total protein and Bilirubin level was reduced in group-III ($p<0.05$) of male animals but the high doses groups were not show any changes in this hematological parameters and female groups were not show any significance value as compared to placebo group thus this difference considered as sporadic and not related to test item. Phosphorus level in male animals were changed in low doses group ($p<0.05$) as compared to placebo group but this changes was not observed in mid or high doses group thus this abnormality conducted as biological variation. Calcium was shown significant value in high dose group $p<0.01$.

In female animals phosphorus level was vary in group III ($p<0.001$) and group IV ($p<0.01$) as compared to placebo group. The phosphorus level in all females were well within and significancy of this hematological parameter was decreased with dose gradient considered as vary in animal or biological variation not related to test item. In female animals calcium was found significant in high doses group ($p<0.01$) as compared to placebo group. According to cholecalciferol mechanism, level of calcium increased with dose gradient but here calcium level was decreased. This effect may occur due to overacting of calcium and that may started feedback mechanism in animal body and stopped calcium absorption.[23]

AST and ALT indicates liver abnormality.[24] In this study not show any abnormality in these parameters thus that indicates that test item not produced any significant effect in liver. ALP level was normal in all groups as

compared to control group. All other biochemical parameters were comparable to control group.

BUN, AST, ALP, ALT, sodium, potassium, calcium these data were well within normal laboratory control data. It indicates that cholecalciferol at given dose did not produce any adverse effect on above discussed parameter.

Based on the above observations, it is concluded that the intramuscular administration of cholecalciferol up to the high dose as 124000IU/kg did not produced any histopathological changes in wistar rats.

The result of study indicated that when cholecalciferol was intramuscularly administered in different doses to wistar rats for 28 days not produced any high toxic effect.

5. CONCLUSION

The study was executed to know if, any toxic effect was produced due to aqueous cholecalciferol within 28 days of repeated dose. The 3 varying dosed were administered with placebo, 62000 IU/kg, 93000 IU/kg, and 124000 IU/kg aqueous cholecalciferol by intramuscular route.

Food intake and body weight of each female and male group did not reveal any significant changes due to test item. No observed any behavioral or physical abnormality in any treated groups.

After 28 days, blood collection was done on 29th day of study termination for biochemical and hematological investigation related to test item. 3 different doses didn't reveal any abnormalities in biochemical and hematological parameters.

From all collected data and according to discussion the study shows that aqueous cholecalciferol did not produce any marker toxic effect at dose of 124000 IU/kg. So No-Observed-Adverse Effect-Level (NOAEL) of aqueous cholecalciferol is more than 124000IU/kg.

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Conflict of Interest: No conflict of interest.

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