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RESEARCH ARTICLE

ENHANCED EFFECTS OF CHOLECALCIFEROL PLUS PIOGLITAZONE ON LIPOLYSIS BY UP-REGULATION OF PPAR-ALPHA MRNA IN MATURE 3T3-L1 ADIPOCYTES

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Abstract

Introduction: Vitamin D deficiency has been associated with obesity, diabetes mellitus type 2, and hypertension diseases. The active vitamin D binds to nuclear vitamin D receptor (VDR) and regulating lipids and carbohydrates metabolism genes; however, prophylaxis is ruled out because induce hypercalcemia and hyperphosphatemia. VDR and the peroxisome proliferator–activated receptor gamma (PPARγ) cross-talk regulates lipid and glucose metabolism genes.

Methods: In this study was analyze the effect of cholecalciferol (CCF) as vitamin D plus pioglitazone (PIO) on mature 3T3-L1 adipocytes on proliferation, fat accumulation by colorimetric methods and the mRNA genes involved in lipid signaling by RT-PCR.

Results: cellular proliferation in mature 3T3-L1 adipocytes treated with CCF plus PIO combination during 0.5, 6 and 24 hours, were not affected; however, high CCF concentrations decreased cell proliferation. The combined treatment decreased fat accumulation, as well as increases glycerol release with CCF plus PIO-treated adipocytes. Key adipogenic genes were down-regulated (CEBP-α, SREBP-, PPARᵞ) whereas lipogenic genes (PPAR-α) were up-regulated at mRNA level.

Conclusion: These findings imply that CCF plus PIO induce lipolysis through the expression of PPAR-alpha in adipocytes.

Introduction:-

CCF or vitamin D_3 is the active form of vitamin D, or calcitriol (1, alpha-25-dihydroxyvitamin D_3)[1], then binds to its receptor (VDR) activating signaling pathways linked with lipid metabolism [2] and glucose transport[3]. Furthermore, current studies has linked vitamin D insufficiency to chronic illnesses such as obesity and D [4, 5], also has been associated to metabolic dysregulation including insulin resistance and hyperlipidemia[4, 6]. Likewise, has been reported that vitamin D supplementation improves insulin sensitivity, and glucose transport, this because VDR is expressed on pancreatic β cells [7]. Furthermore, calcitriol-bound VDR inhibited adipogenesis in 3T3-L1 adipocytes [8]. However, vitamin D supplementation is not recommended due to the risk of hypercalcemia and

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hyperphosphatemia caused by vitamin D excess[1, 9]. In contrast, hypovitaminosis D has as a risk factor for insulin resistance and metabolic syndrome[4-6].

On the other hand, the peroxisome proliferator–activated receptor (PPAR) regulates genes involved in lipid metabolism and glucose transport. Endogenous or synthetic ligands such as pioglitazone (PIO) can active these receptors[10-12]. Cross-talk between VDR and PPARγ has been reported, both belong to the nuclear receptor group and have been shown to regulate genes involved in the same signaling pathways via molecular cross-regulation of their transcription factors[11, 13, 14]. The retinoic X receptor (RXR) and PPAR Coactivation is hypothesized to have synergistic effects on glucose and lipid metabolism[13]. This might be because vitamin D retains its beneficial effects by avoiding hyperphosphatemia and hypercalcemia. FGF23 is a hormone that regulates phosphate homeostasis; it has been shown that excessive quantities of phosphate high levels are toxic, producing oxidative stress and DNA damage in animal models[15-17]. Hyperphosphatemia also induces autophagy in endothelial cells by inhibiting the Akt/mTOR signaling pathway, which may protect against high phosphate inorganic-induced apoptosis[16, 17].

The goal of this study was to analyze the combined effect of CCF a form of vitamin D and PIO on lipid content, cell viability and gene expression involved in adipogenesis and lipogenesis (CEBP, SREBP and PPARgamma), lipolysis (PPAR-α) in mature 3T3-L1 adipocytes. Previously, it was shown that 3T3-L1 adipocytes express CYP27A1 and CYP27B1 enzymes, which transform cholecalciferol into calcitriol, the active form of vitamin D[18].

Material And Method:-

Culture of mouse fibroblast cell line 3T3-L1

The mouse fibroblast preadipocytes cell line 3T3-L1 was obtained from American Type Culture Collection (ATCC). They were propagated in DMEM medium supplemented with 3.7 g/L sodium bicarbonate, phenol red, supplemented with 10% fetal bovine serum (FBS). The cells were maintained at a temperature of 37°C and a 5% CO_2 atmosphere. The 90% fibroblasts were disaggregated with 0.25% trypsin/EDTA solution and 1 x 10^6 cells were reseeded in 6-well Petri dishes for experiments.

3T3-L1 cell line differentiation

3T3-L1 cells were cultured in DMEM medium in 6-well plates, for 24 hours at a density of 1 x 10^6. When cells reached 80% confluence, differentiation medium (high glucose DMEM, 10 µg/mL insulin and 1 µM dexamethasone) was added. On the second day, maintenance medium (high glucose DMEM and 10 µg/mL insulin) was changed to maintenance medium (high glucose DMEM and 10 µg/mL insulin) for 6 days.

Experimental groups

Cholecalciferol, CCF (≥98% purity), pioglitazone hydrochloride (PIO), dexamethasone (DEX), phosphate buffer saline (PBS), trypsin/ethylenediaminetetraacetic acid (EDTA), and insulin were obtained from Sigma-Aldrich, Mexico. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco®. 3T3-L1 adipocytes were treated with 1, 5 and 10 µM PIO; 0.5, 5, 15 and 50 µM CCF; 1 µM PIO and 0.5, 5, 15 and 50 µM CCF; 5 µM PIO and 0.5, 5, 15 and 50 µM CCF;10 µM PIO and 0.5, 5, 15 and 50 µM CCF. The control group was treated with dimethyl sulfoxide (DMSO). Exposure times were during 0.5, 6 and 24 hours.

Trypan blue assay

Adipocytes were washed with 1X PBS, trypsinized and then stained with trypan blue (Sigma Aldrich Inc.). Stained and colorless cells were observed microscopically and counted. Cell viability was determined as follows: colorless cells/total cells) x 100 = % cell viability.

Oil-red O staining

Fat accumulation was analyzed by oil-red O staining. The mature adipocytes were washed with 1X PBS and fixed in 10% formaldehyde for 30 minutes at room temperature. Formaldehyde was removed, washed twice with 1X PBS and 5% oil red (Sigma-Aldrich) was added for 30 minutes. After two washes with 1X PBS, 98% isopropanol was added and absorbance was determined at 550 nm.

Quantification of cholesterol, triglycerides and free glycerol

A cholesterol kit (Sigma-Aldrich) was used for cholesterol determination. Total cholesterol determination was done by a colorimetric (570 nm) / fluorometric (λex = 535/ λem =587 nm) cholesterol product measurement.

Quantification of triglyceride content was measured using a Triglyceride Quantification colorimetric/fluorometric kit (BioVision). Mature adipocytes were resuspended in 1X PBS containing 5% Triton X-100. The contents were taken and heated at 80°C for 5 min, then placed on ice for 1 min. Each sample was placed in 96-well plates and 50 µL of buffer + 2 µL of lipase standard was added; the plate was incubated in agitation for 20 min at room temperature. Subsequently, 50 µL of the reaction mixture was added and incubated again for 60 min at room temperature. The absorbance was measured in a SpectraMax 190 spectrophotometer at a wavelength of 570 nm. Free glycerol levels in culture media were measured at 540 nm using Free Glycerol Reagent (Sigma-Aldrich).

Determination of mRNA by RT-PCR

Total RNA extraction was performed with TriReagent (Sigma-Aldrich). Cells in Petri dish were spiked with TriReagent and incubated at room temperature. The sample was transferred to 1.5 mL eppendorf tubes and chloroform was added. It was manually mixed and incubated for 10 minutes at room temperature, followed by centrifugation at 12,000 rpm for 5 minutes at 4°C. The aqueous phase was transferred to another eppendorf tube and isopropanol was added, again mixed and incubated for 10 minutes, then centrifuged at 12,000 rpm for 10 minutes at 4°C. The pellet obtained was washed 3 times with 75% cold ethanol, between each wash it was centrifuged at 7,500 rpm for 5 minutes at 4°C. The ethanol was allowed to evaporate and the pellet was re-suspended in sterile water.

Quantification was performed on a multiple volume spectrophotometer, using only 2 µL of the sample at an optical density reading at 260 and 280 nm, to measure nucleic acids and proteins, respectively. RNA integrity was analyzed by horizontal electrophoresis in 2% agarose gel with ethidium bromide as fluorescence marker. The mRNA was synthesized to cDNA, using ReverTra Ace® qPCR RT master mix. Quantitative real-time PCR was conducted with a SensiFAST™ SYBER® No-ROX Kit (SIGMA) in an Eco™ Real-Time PCR System (ROCHE). C/EBPα,forward: 5′-GGATACTCAAAACTCGCT CC-3′, reverse: 5′-CTAAGTCCCTCCCCTCTAAA-3′; PPARγ,forward: 5′-TTTTCAAGGGTGCCAGTTTC-3′, reverse: 5′-AATCCTTGGCCCTCTGAGAT-3′[19]; PPAR-α forward: 5´-TCAGGGTACCACTACGGAGT3´, reverse 5´CTTGGCATTCTTCCAAAGCG-3´; SREBP-1c forward: 5´-GGAGCCATGGATTGCACATT-3´, reverse 5´-CCTGTCTCACCCCCAGCATA-3´[20]; and β-actin,forward: 5′-CCACAGCTGAGAGGGAAATC-3′, reverse 5′-AAGGAAGGC TGGAAAAGAGC-3′. Denaturation was carried out at 94 °C during 3 min, next 40 cycles: 35 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The relative expression was calculated to the housekeeping geneβ-actin normalized as an internal controlwith the $2^{-\Delta\Delta C}{}_{T}$method.

Statistical analysis

Results were analyzed with the statistical program GraphPad PRISM version 6.0, and data were represented as mean ± standard deviation (SD). Student's t-tests, two-way ANOVA and Dunnet's post hoc were performed and p values < 0.05 were considered statistically significant.

Results:-

Effect of CCF plus PIO on the viability and phosphate content of mature 3T3-L1 adipocytes

Total phosphate concentration was measured to assess the negative cellular effects of CCF and PIO on adipocyte viability. The mature 3T3-L1 adipocytes were treated to CCF (5-50 µM) and PIO (1-10 µM) or both, with only with 50 µM CCF causing a significant reduction in cell viability (Figure 1A). CCF and PIO had no effect on cell proliferation as compared to control adipocytes (Figure 1B), even at 50 µM of CCF.

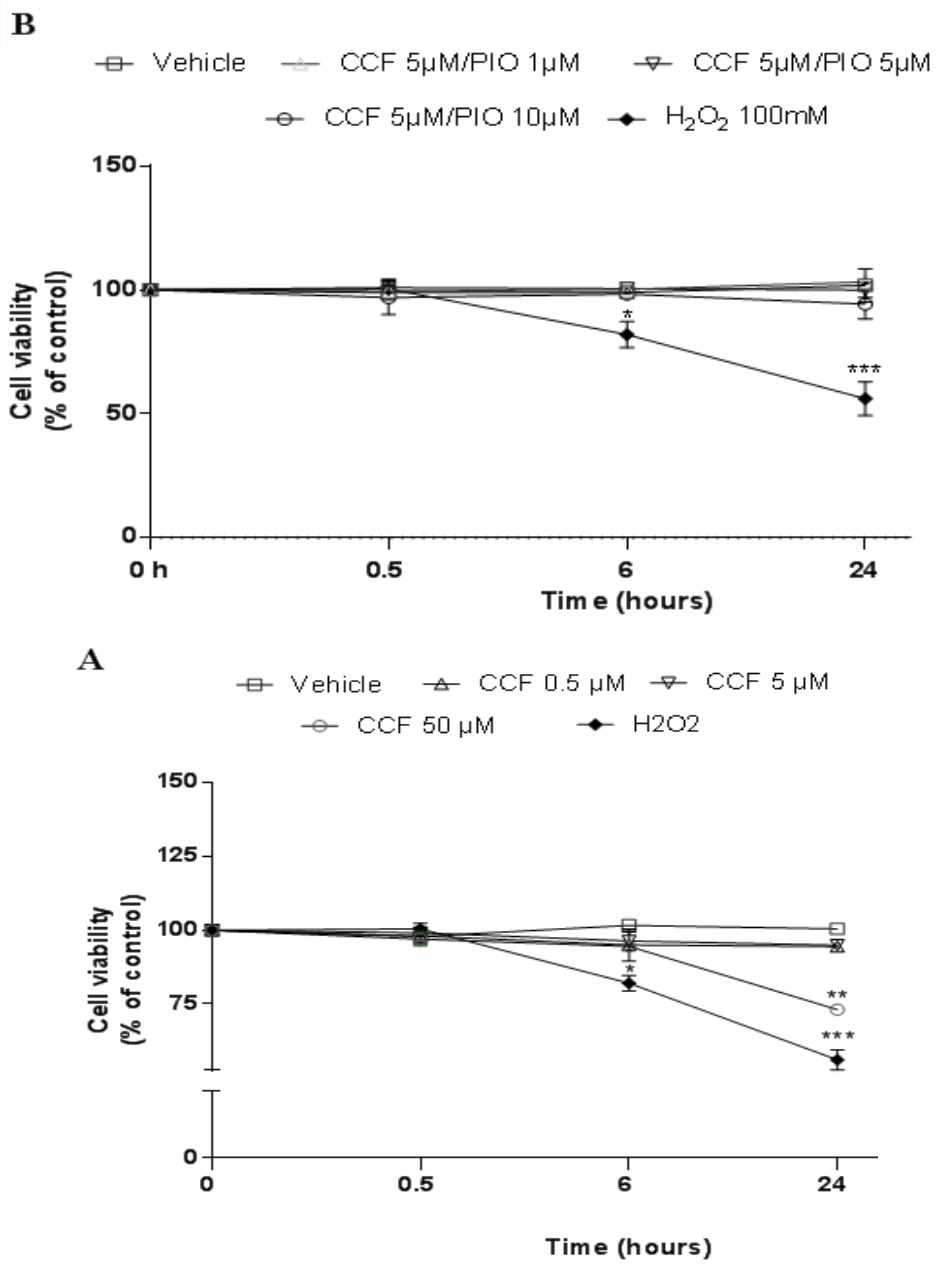


Figure1:-

Figure 1:- CCF and PIO effects on cell viability, total phosphate and FGF23 expression in mature 3T3-L1 adipocytes. Adipocytes were treated with CCF (5, 15 and 50µM) and PIO (5 µM) or both and after of 0.5, 6 and 24-hour cell viability was measurement by trypan blue assay. **A**, and **B,** cell viability (n=6) All values are expressed as mean ± SD. n = 3. *p< 0.05, ***p< 0.001 vs control. ***p < 0.001 vs control adipocytes (vehicle).

Effect of CCF plus PIO reduces fat accumulation and induces lipolysis in mature 3T3-L1 adipocytes

Total Oil red lipids, total cholesterol, triglycerides and glycerol release were measured to determine fat accumulation and lipolysis. CCF, at doses as of 15 and 50 µM, increased total lipid content in adipocytes after 24 hours (Table 1). PIO 5 µM plus CCF 5 to 50 µM reduced fataccumulation in mature adipocytes; this effect was dose dependent and reduced it at 24 hours (Table 1). In addition, PIO 10 µM plus CCF 5, 15 and 50 µM, did not show significant increases in fat content after 24 hours. In contrast, for both CCF, and its combination (Table 1) induces a significant increase in triglyceride and total cholesterol content after 24 hours compared to control adipocytes. At 24 hours of treatment, the combined PIO 1-15 µM with CCF 5-15 µMinduced a concentration-dependent decrease in the

quantity of triglycerides and total cholesterol at 24 hours of treatment, which was significant compared to control adipocytes.

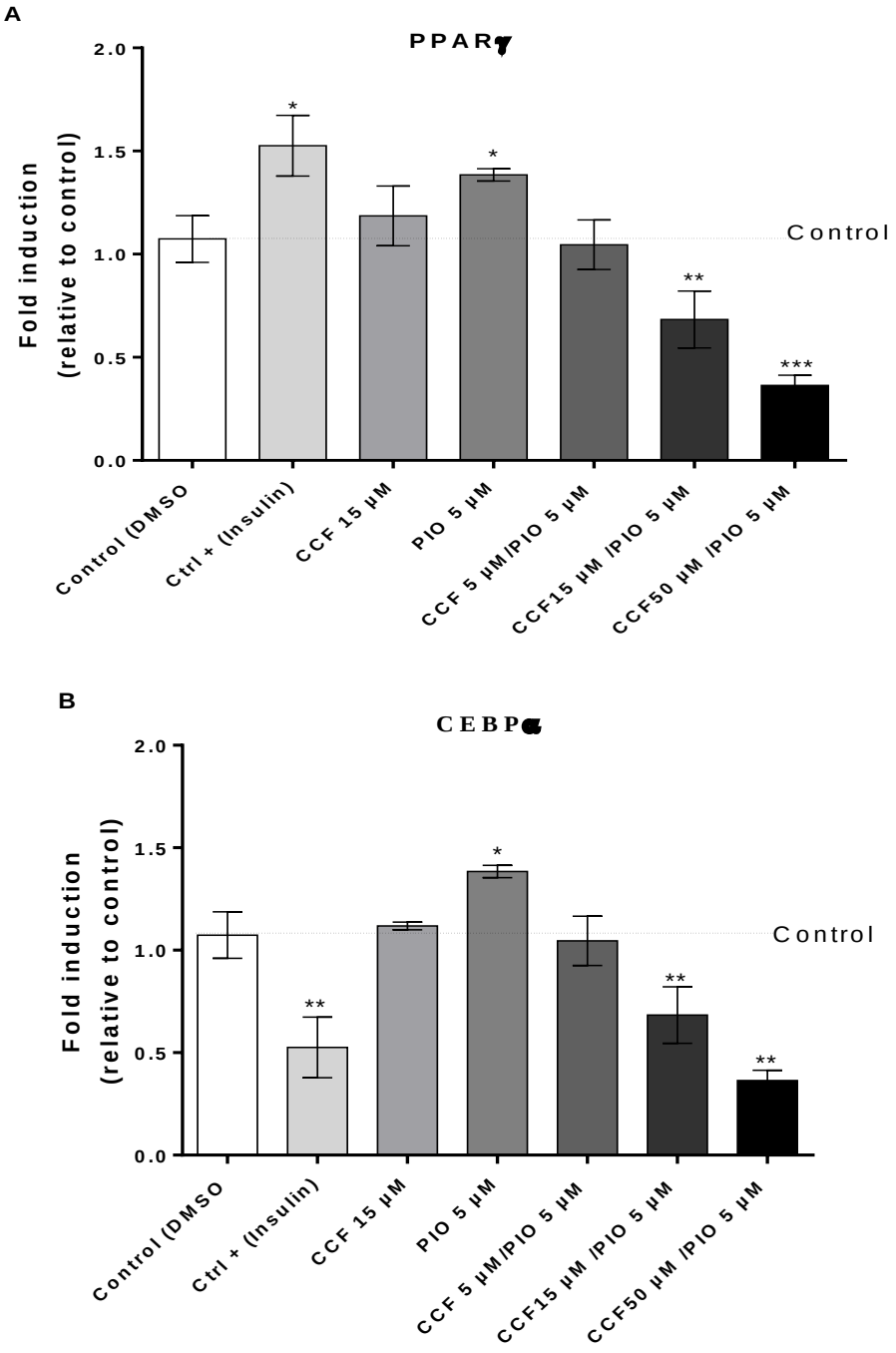
Table 1:- shows the effect of combining CCF and PIO on adipogenesis and lipolysis markers in mature 3T3-L1 adipocytes.

Assay	Total Lipids (oil red Abs 550nm)	P value	Cholesterol (µg/mL)	P Value	Triglycerides (µM/mg protein)	P value	Free glycerol (µg/mL)	P value
Vehicle	1.001±0.104	-	1.001±0.03	-	1.000±0.016	-	10.37±1.76	-
CCF 5µM	1.226±0.035	0.05	0.853±0.10	0.05	0.935±0.024	0.05	11.28±2.09	NS
CCF 15 µM	1.361±0.152	0.01	0.777±0.01	0.01	0.708±0.025	0.01	12.80±2.20	NS
CCF 50 µM	1.440±0.075	0.01	0.644±0.01	0.01	0.579±0.028	0.01	12.13±1.91	NS
CCF5 µM +PIO 1µM	1.012±.084	NS	0.701±0.01	0.01	0.863±0.017	0.05	13.29±0.98	NS
CCF5 µM +PIO 1µM	1.067±0.084	NS	0.616±0.03	0.01	0.862±0.017	0.05	11.19±2.33	NS
CCF5 µM +PIO 1µM	1.174±0.203	0.05	0.600±0.01	0.001	0.841±0.063	0.05	14.12±1.91	0.04
CCF15 µM +PIO 5µM	1.205±0.211	0.05	0.765±0.03	0.01	0.910±0.060	0.05	17.31±2.30	0.01
CCF15 µM +PIO 5µM	1.418±0.210	0.01	0.666±0.042	0.01	0.835±0.043	0.01	26.71±3.18	0.001
CCF15 µM +PIO 5µM	2.100±0.220	0.001	0.538±0.020	0.001	0.597±0.056	0.001	20.53±4.76	0.001
CCF50 µM +PIO 10µM	1.105±0.119	0.05	0.644±0.055	0.001	0.333±0.034	0.001	18.01±2.02	0.01
CCF50 µM +PIO 10µM	1.508±0.180	0.01	0.539±0.061	0.001	0.384±0.057	0.001	17.22±1.79	0.01
CCF50 µM +PIO 10 µM	1.830±0.207	0.001	0.440±0.042	0.001	0.287±0.071	0.001	23.09±1.23	0.001

The values are presented as the means ± SD. by duplicated. P<0.05 represents a significant difference vs Vehicle. (n=6), ND: not determined; NS: Not significant

In Figure 2 A, B and C, PIO-induced up-regulation of **PPARλ,C/EBP-α, and SREBP-1c mRNAs** expressions at 5µM. In contrast, both CCF and PIO causing down-regulation of mRNA of **C/EBP-α, SREBP-1c and PPARλ (Figure 2A, B and C).** Adipocytes treated with CCF plus PIO combination released significant free glycerol into the medium after 24 hours than control adipocytes, as shown in Table 1. This impact was more prominent with CCF 15 µM and PIO 5 µM combination. Figure 2 shows that CCF, and CCF plus PIO promoted upregulation of PPAR-α mRNA when compared to CCF and control treated adipocytes, this data show that CCF plus PIO induces lipolysis on mature adipocytes.

Figure 2:-


A
PPARγ
Fold induction (relative to control)
2.0
1.5
1.0
0.5
0.0
Control
*
*
**
***
Control (DMSO
Ctrl + (Insulin)
CCF 15 µM
PIO 5 µM
CCF 5 µM/PIO 5 µM
CCF15 µM /PIO 5 µM
CCF50 µM /PIO 5 µM
B
CEBPα
Fold induction (relative to control)
2.0
1.5
1.0
0.5
0.0
Control
**
*
**
**
Control (DMSO
Ctrl + (Insulin)
CCF 15 µM
PIO 5 µM
CCF 5 µM/PIO 5 µM
CCF15 µM /PIO 5 µM
CCF50 µM /PIO 5 µM

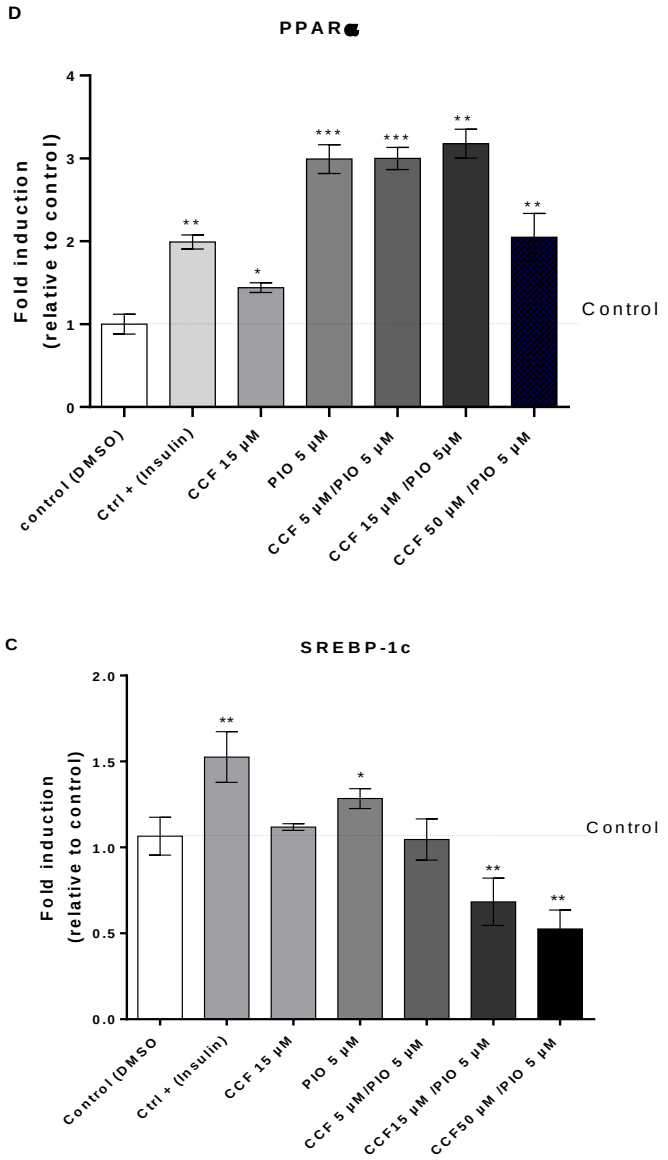


Figure 2:- Effects of CCF plus PIO on adipogenesis and lipolysis as well as PPAR-α, PPAR-ᵞ, CEBPα and SREBP-1C mRNA expression in mature 3T3-L1 adipocytes. Adipocytes were treated with CCF (5, 15 and 50µM) and PIO (5 µM) or both and after of 6 hours of treatment the amount of mRNAs of genes indicated above were quantified by real-time PCR method as described in methods. A, PPARᵞ expression; **B**, CEBPα; **C**, SRBP-1c and D, PPARα. The amounts of each gene are expressed relative to the control housekeeping gene **β-actin.** All values are expressed as mean ± SD. n = 3. **p*< 0.05, ****p*< 0.001 vs control.

Discussion:-

In this study CCF at 50 µM decreases adipocyte viability from 6 to 24 h post-treatment. In contrast, Kim and coworkers demonstrate that CCF reduces the percentage of viability 50 µM, after 24 hours [21]. The difference might be attributable to the fact that the methods used to achieve micro molar concentrations of CCF different. We underline that the combination of CCF plus PIO did not decrease cell viability at 6 hours, but only until 24 hours with CCF 5 plus PIO 10 µM. In this way, Spigoni et al, describe that PIO 10 µM improves cell viability in endothelial cells [22], which was demonstrated in this study, since cell viability recovered when CCF plus PIO were combined.Hyperphosphatemia is caused by vitamin D prophylaxis, with serum phosphate levels exceeding 5 mg/dl in adults and 6 mg/dl in children [23-25]; this causes inorganic phosphate to bind to calcium, inducing precipitation and deposition of calcium phosphate in soft tissues provoking organ calcification [26], as a result, preventing

hyperphosphatemia is critical. Hyperphosphatemia can also cause trigger autophagy in cultured endothelial cells, according to *in vitro* studies and decrease the number cells [17, 27, 28].

In this study, an increase of lipid content was observed, in contrast with the reported by Kim and collaborators, who reported a decrease in lipid accumulation. The difference between both studies were that their test was conducted on adipocytes in the process of differentiation rather than mature adipocytes, as is this study [21]. To assessment of triglyceride and cholesterol concentrations, in particular, demonstrated that the combination of CCF plus PIO generate a lipolytic effect. The described could have occurred because PPARα participates in beta oxidation of fatty acids and regulates the expression of several proteins involved in lipid (triglyceride) metabolism, such as fatty acid binding protein aP2, fatty acid transport protein (FATP), acyl-CoA synthetase (ACS), lipoprotein lipase (LPL) and malic enzyme (ME) [12, 29]. Likewise, our findings on the content of free glycerol in the culture medium support the existence of a lipolysis process, since the concentrations of free glycerol in the culture media with adipocytes treated with CCF plus PIO increased significantly, in accord, it is worth mentioning that calcitriol-bound VDR can inhibit or delay the adipogenesis process[30]. Similarly, PPARα mRNA expression was measured, and it increased with the combination of CCF and PIO. This result agrees with those obtained when determining cholesterol and triglyceride concentrations, because PPARα activation raises HDL cholesterol (high-density cholesterol) and free fatty acid levels, improving the lipid profile; in addition to having anti-inflammation and anti-insulin resistance effects [31, 32].

In our study, the expression of PPARγ remained steady at 6 hours with CCF plus PIO compared to the control; however, the expression of CEBPα, SREBP-1c and PPARα decreased, this transcription factors control adipogenesis and lipogenesis process in the second or late phase; in this study, its expression decreased significantly with CCF 15 µM plus PIO 5 µM and with CCF 50 µM plus PIO 5 µM. Although some study has indicated that vitamin D only intervenes by inhibiting early adipogenic factors, that is, whiting the first 48 hours of differentiation[33, 34]; when paired with pioglitazone, it can regulate second phase adipogenic factors such as CEBPα and PPARγ [35, 36]. Likewise, SREB-1c which is also involved in adipocyte differentiation decreases with CCF 15 µM plus PIO 5 µM and CCF 50 µM plus PIO 5 µM. This finding are important since previous research has not shown that vitamin D as CCF may decrease adipogenesis beyond 48 h of differentiation; nevertheless, our findings suggest that it is possible because it has a coadjuvant effect with PIO.

Conclusions:-

This study found that combining CCF plus PIO reduces its toxic effects in viability and induces lipolysis by down-regulating adipogenesis genes and up-regulating lipolytic PPAR-α gene; however, further investigation is needed to determine the efficacy of combined CCF plus PIO in the prevention or treatment of chronic diseases using animal models, as well as the molecular mechanism involved.

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Competing interests

The authors declare that they have no competing interest.

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