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Conjugated Linoleic Acid (CLA) co-treatment alleviates antidiabetic drug, rosiglitazone associated deterioration of bone remodeling.

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Abstract

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia due to decreased insulin secretion, defective action or both. The rosiglitazone (RSG) is one of the oral antidiabetic drug used in type 2 (T2) DM and has a unique insulin-sensitizing capacity. However, RSG has a negative side effect on the bone as it stimulates the differentiation of bone marrow-mesenchymal stromal cells (BM-MSCs) into adipocytes at the expense of osteoblasts in the bone marrow microenvironment, disturbing the normal balance of bone remodeling and causing BM adiposity. On the other hand, the trans-10,cis-12 conjugated linoleic acid (CLA), a fatty acid is known as anti-adipogenic, pro-osteogenic. Therefor, this study was designed to assess whether CLA can alleviate the negative effect of RSG on bone. We used adipose tissue derived-mesenchymal stem cells (AT-MSCs) as a human *in vitro* model to study the effect of CLA, RSG and combined treatment (RSG+CLA) on the osteoblastogenic and adipogenic differentiation of AT-MSCs. Osteoblastogenesis was assessed by Alizarin Red Staining and bone mineralization was assessed by OsteoImage™ assays, whereas adipogenesis was assessed by Oil Red O Staining and LipidTOX assays. Besides, the level of expression of osteogenic and adipogenic markers was measured on treated osteo- and adipo-differentiated MSCs using real time RT-PCR, immunohistochemistry (IHC) and western blot analysis. Compared to RSG group, the combined treatment group stimulates osteoblastogenesis, as evidenced by increased mineralization and upregulation of osteogenic markers OPN and RUNX2 and inhibits adipogenesis in osteogenic media as showed by decreased lipid content and downregulation of adipogenic markers FABP4, LPL and adipsin. In conclusion, the use of CLA as an adjunctive treatment reversed the effects of RSG on osteogenesis and adipogenesis. Further preclinical and clinical studies will be undertaken to establish this treatment regimen for the successful treatment of diabetic patients with rosiglitazone without adverse side effects on bone.

Background

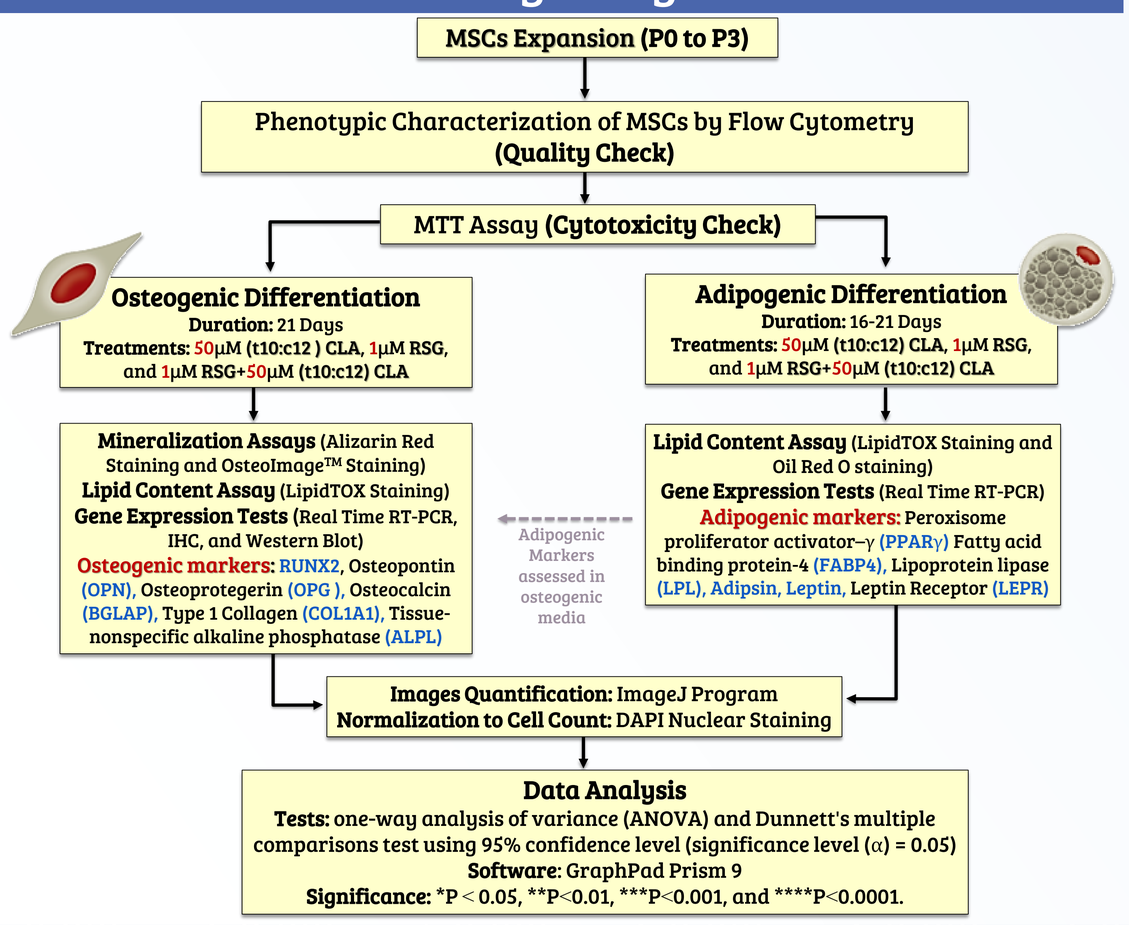
MSCs are adult and non-hematopoietic stem cells that have many distinct characteristics such as spindle shape, the capacity of multipotent differentiation *in vitro*, self-renewable ability, and plastic adherence. MSCs can be differentiated into osteocytes and adipocytes under appropriate conditions. MSCs were firstly isolated from the bone marrow (BM), but nowadays they can be isolated from almost all body tissues. Diabetes mellitus (DM) occurs due to insulin deficiency, resistance or both. It is associated with many complications and represents a global burden. RSG is an oral antidiabetic drug of the thiazolidinediones (TZDs) class which act by increasing insulin sensitivity in peripheral tissues through binding to the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) and affecting gene expression of glucose transporters to facilitate glucose uptake and used to treat T2DM . TZDs are the only class that specifically targets insulin resistance. PPAR-γ receptors are present on osteoblasts and adipocytes. RSG also directs the differentiation of MSCs toward the adipocyte lineage in BM, and since there is an inverse relationship between osteoblasts and adipocytes in the BM microenvironment (both derived from BM-MSCs), any stimulation of adipogenesis will reduce the number of osteoblasts leading to the formation of fatty BM, and imbalance bone remodeling eventually causing osteoporosis. The negative effects of RSG on bone metabolism including the significant reduction in bone mineral density (BMD) and the increase in fracture risk by twofold, are among the safety concerns that led to its withdrawal. The CLA, however, is a group of fatty acids that has many beneficial roles as anticarcinogenic, antiatherosclerosis and antiobesity agents. The (t10:c12) CLA isomer, in particular, is known for its pro-osteogenic and anti-adipogenic effects and has been shown to increase osteogenesis and decrease adipogenesis both *in vitro* and *in vivo* models.

Hypothesis and Objectives

Hypothesis: CLA co-treatment can restore the balance of osteogenesis and adipogenesis in bone marrow microenvironment and counterbalance the side effects of RSG on bone.

Objectives: Successful use of adipose-derived MSCs (AT-MSCs) to: **(1)** study the RSG-induced changes in the adipogenesis and osteogenesis *in vitro* and to **(2)** test the effect of CLA co-treatment in restoring the RSG-induced changes in the osteogenic and adipogenic setup by studying the gene expression modulation of linage specific markers and differentiations assays (i.e., OsteoImage™ mineralization assay, Alizarin Red, and LipidTOX staining).

Study Design



Results and Discussion

CLA co-treatment reverted the effects of RSG and restored the balance of osteogenesis and adipogenesis

Effect on osteogenesis - Mineralization

Calcium deposition (Alizarin Red Staining)

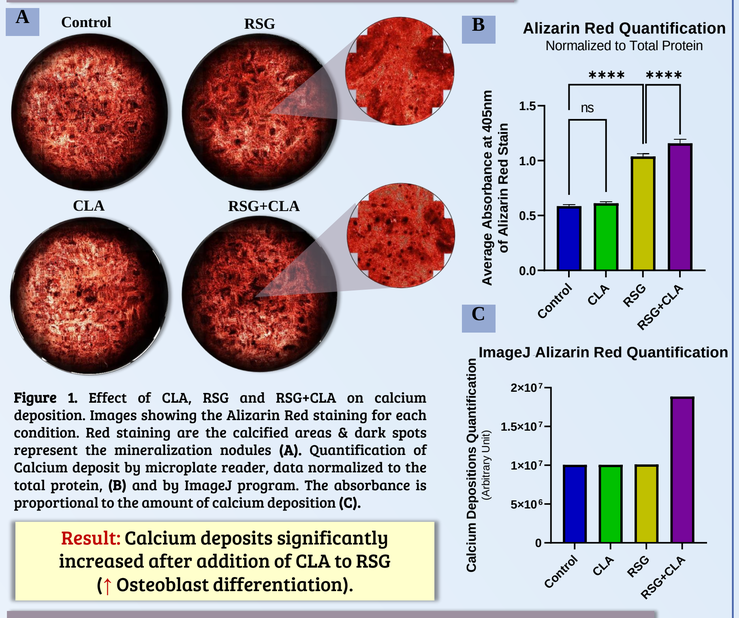


Figure 1. Effect of CLA, RSG and RSG+CLA on calcium deposition. Images showing the Alizarin Red staining for each condition. Red staining are the calcified areas & dark spots represent the mineralization nodules **(A)**. Quantification of Calcium deposit by microplate reader, data normalized to the total protein, **(B)** and by ImageJ program. The absorbance is proportional to the amount of calcium deposition **(C)**.

Result: Calcium deposits significantly increased after addition of CLA to RSG (↑ Osteoblast differentiation).

Hydroxyapatite (HA) deposition (OsteoImage™ Staining)

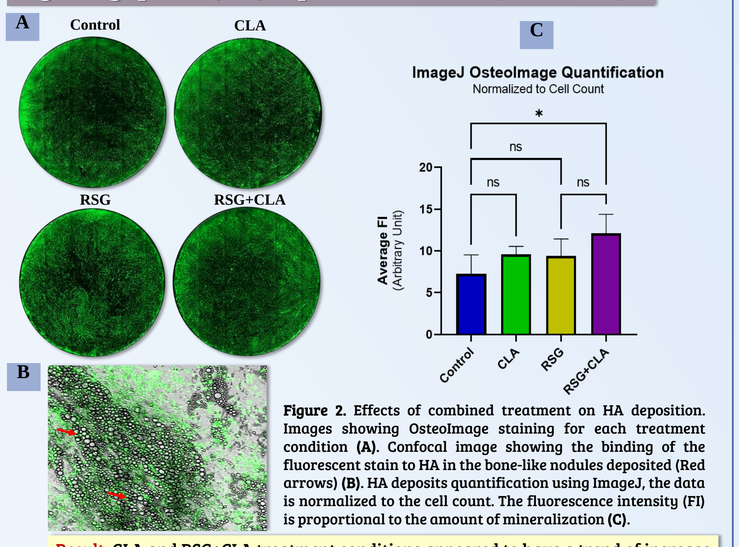


Figure 2. Effects of combined treatment on HA deposition. Images showing OsteoImage staining for each treatment condition **(A)**. Confocal image showing the binding of the fluorescent stain to HA in the bone-like nodules deposited (Red arrows) **(B)**. HA deposits quantification using ImageJ, the data is normalized to the cell count. The fluorescence intensity (FI) is proportional to the amount of mineralization **(C)**.

Result: CLA and RSG+CLA treatment conditions appeared to have a trend of increase in hydroxyapatite mineralization, though not statistically significant and the trend is more evident when normalized to cell count.

Effect on adipogenesis - Lipid Content

LipidTOX Staining

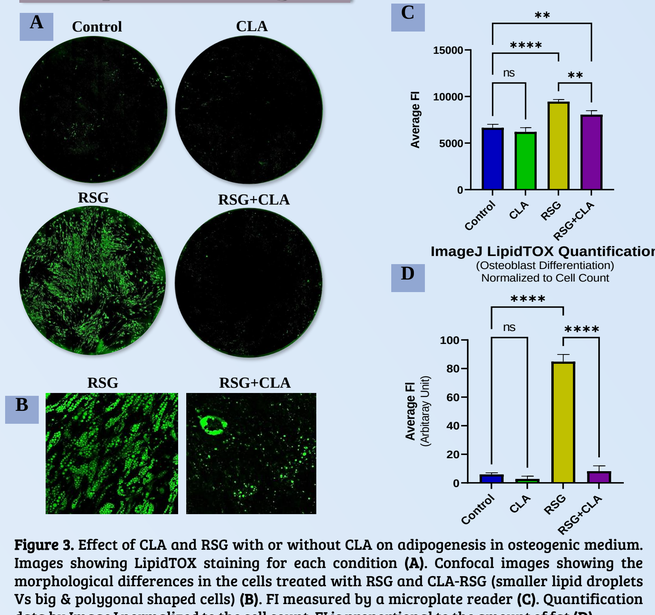


Figure 3. Effect of CLA and RSG with or without CLA on adipogenesis in osteogenic medium. Images showing LipidTOX staining for each condition **(A)**. Confocal images showing the morphological differences in the cells treated with RSG and CLA-RSG (smaller lipid droplets Vs big & polygonal shaped cells) **(B)**. FI measured by a microplate reader **(C)**. Quantification data by ImageJ normalized to the cell count. FI is proportional to the amount of fat **(D)**.

Result: RSG increased the lipid accumulation (intense fluorescence), whereas the addition of CLA is able to restore a normal lipidic content. When the image fluorescence was quantified using the ImageJ program, RSG alone was confirmed in its steep increase of lipids which was almost completely counterbalanced by CLA (↓Adipocyte differentiation).

RSG has a browning effect on adipocytes

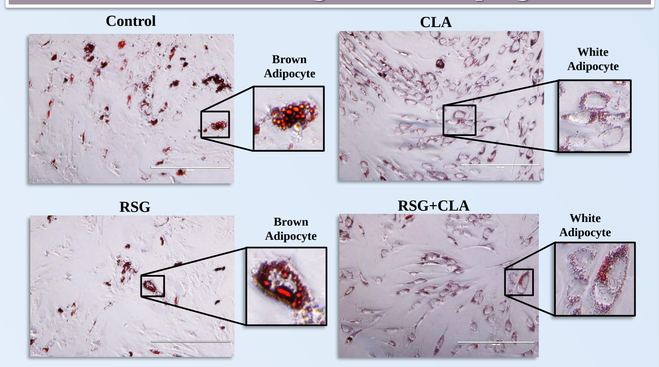


Figure 4. The appearance of the stained adipocytes (Oil Red O Staining). White adipocytes have large lipid droplets, surrounded by little cytoplasm and a decentralized nucleus. Brown adipocytes have a polygonal appearance with multiple small lipid droplets and a centralized nucleus surrounded by a clear cytoplasm.

CLA reverted both the inhibitory effect of RSG on the expression of the major osteogenic markers (i.e., RUNX2, COL1A1, and OPN) and the stimulatory effect of RSG on the expression of adipogenic markers (i.e., FABP4 & PPARγ).

Expression of Marker Proteins (IHC)

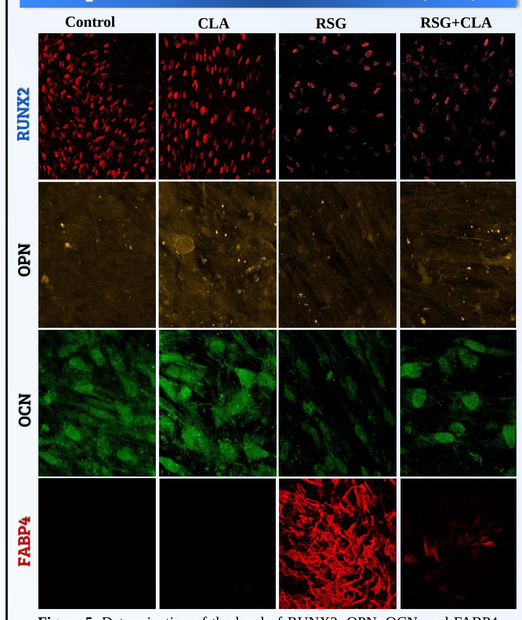


Figure 5. Determination of the level of RUNX2, OPN, OCN, and FABP4 by IHC after adding different treatments into osteogenic media (Day 21).

Result: CLA and RSG+CLA treatment conditions stimulate the expression of RUNX2, OPN, and OCN. However, RSG stimulate the expression of FABP4 which can be reverted by the addition of CLA.

Gene Expression of Osteogenic & Adipogenic Markers (Real Time RT-PCR)

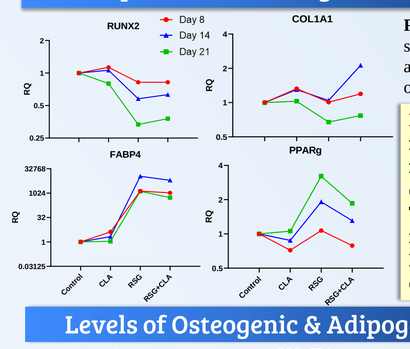


Figure 6. Effects of different treatments on the gene expression of some osteoblastogenic markers (RUNX2 & COL1A1) and adipogenic markers (FABP4 & PPARγ) on different days of the osteoblastogensis differentiation. **RQ:** Fold change.

Result: RSG increased the expression of all adipogenic markers (especially FABP4 and PPARγ which are the master regulators of adipogenesis) while the addition of CLA consistently reduced the RSG effect on them. The expression of the RUNX2, which is a major player in osteoblastogenesis, was significantly decreased by RSG and partially restored by CLA. Similar trend was observed for COL1A1.

Levels of Osteogenic & Adipogenic Markers (Western Blot Analysis)

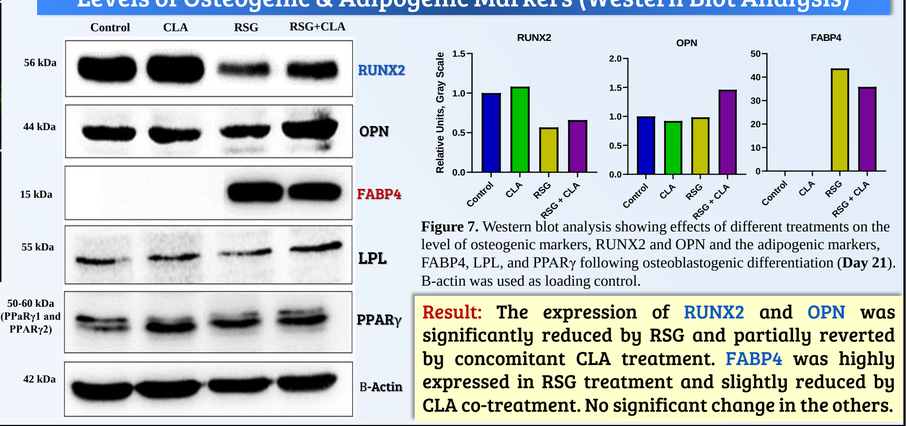


Figure 7. Western blot analysis showing effects of different treatments on the level of osteogenic markers, RUNX2 and OPN and the adipogenic markers, FABP4, LPL, and PPARγ following osteoblastogenic differentiation (**Day 21**). B-actin was used as loading control.

Result: The expression of RUNX2 and OPN was significantly reduced by RSG and partially reverted by concomitant CLA treatment. FABP4 was highly expressed in RSG treatment and slightly reduced by CLA co-treatment. No significant change in the others.

Conclusion

- AT-MSCs can be used successfully as a human *in vitro* model to study the effect of different treatments on the adipogenic and osteogenic differentiation of MSCs.
- CLA co-treatment can reverse the side effects of RSG on bone remodeling and restore the balance between osteogenesis and adipogenesis (Stimulate osteoblast differentiation and inhibit adipocyte differentiation of MSCs in osteogenic medium).
- (t10:c12) CLA might help alleviate the safety concerns regarding the deterioration of bone remodeling (BM adiposity and the increased fracture risk) associated with RSG treatment.
- The pre-clinical data regarding the *in vitro* effect of (t10:c12) CLA on restoring the RSG-disturbed bone remodeling have to be investigated further *in vivo* using animal models, and randomized clinical trials on T2DM patients.

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