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Metadichol®, Vitamin C and *GULO* Gene Expression in Mouse Adipocytes

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Abstract

Metadichol, a novel nano emulsion of lipid alcohols, up regulates the expression of *GULO* gene in mouse at picogram levels. 3T3-L1 pre adipocyte cells were differentiated using differentiating media. Post differentiation, the cells were treated with different concentrations of Metadichol for 24 hours and untreated cells served as control. Whole RNA was isolated after the treatment period and semi-quantitative reverse transcription PCR was run with *GULO* gene specific primer to obtain cDNA. Relative gene expression of *GULO* was determined by analysis of *GULO* gene amplicons using image J software. The GULO gene expression in the treated cells was up regulated fourfold relative to basal level of untreated cells.. Relative gene expression at concentrations 1 µg/ml, 100 ng/ml, 1 ng/ml, 100 pg/ml, 1 pg/ml was found to be 2.11, 2.64, 2.96, 3.96, 3.25 fold compared to control.

Keywords: GULO (Gulonolactone oxidase); Vitamin C; Ascorbic acid; Vitamin D receptor; Gene expression; Metadichol; Reverse transcription polymerase chain reaction; Inverse agonist; Aryl hydrocarbon agonist; Long chain alcohols; Nano formulation; *GULO* gene expression; Mouse 3T3-L1 adipocytes

Introduction

Metadichol® is a nanoformulation of long-chain alcohols that in Zucker diabetic rats and humans has been shown to increases Vitamin C levels above and beyond what is achieved orally [1-3]. Vitamin C is a potent antioxidant required for many biological functions [4]. Humans and a few other species are unable to synthesize their ascorbate, as we lack the final enzyme GULO (Gulonolactone oxidase) in the ascorbate biosynthesis pathway [5]. GULO converts L-gulono-1,4-lactone (Gulonolactone) to L-ascorbic acid in the liver of most animals. Plasma levels of ascorbate are maintained though renal processes, and cellular levels are regulated via ascorbate transporters, and ascorbate is labile and short-lived [6].

Ascorbic acid (Vitamin C) is necessary for proper folding and deposition of collagen proteins in the human body and has a substantial impact on the extracellular matrix (ECM) [7]. Human cells are unable to synthesize Vitamin C, and it is restored through the diet. When there is the shortage of Vitamin C human cells cannot maintain healthy tissues, and Vitamin C deficiency in humans causes scurvy, resulting in ECM dissolution and tissue disintegration.

Vitamin C has emerged as a critical regulator of stem cell behaviors by enhancing somatic cell reprogramming, leading to a generation of induced pluripotent stem cells (iPSCs), by modulating the cellular epigenetic profile [8-10].

This semi quantitative RT-PCR study was initiated to confirm if Metadichol enhances *GULO* in mouse 3T3-L1 adipocytes cells to confirm the *in-vivo* results in diabetic Zucker Rats.

Experimental

All work outsourced and carried out by Skanda Life Sciences, Bangalore, India. Primers synthesized at Eurofins Genomics, Bangalore, India Relative gene expression of *GULO* was determined by analysis of *GULO* gene amplicons using image J software.

Cell line

3T3-L1 obtained from ATCC.

Cell culture

3T3-L1 cells were cultured in DMEM complete medium with 10% heat inactivated fetal bovine Serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin at 37°C, 5% CO_2 incubator.

Procedure

Cell seeding

6×10^5 cells were seeded in 35 mm cell culture dishes in DMEM medium containing 10% FBS and 1% PenStrep. The cells were incubated for 24 hr in the dishes at 37°C, 5% CO_2 humidified incubator. After 24 hr the cells were differentiated into adipocytes.

Differentiation of 3T3 L1 cells into Adipocytes

3T3-L1 cells were obtained from the ATCC and grown to confluence in 12 well culture plates (60,000 cells/well). Two days post confluence, cell differentiation was initiated by incubation with 2 µg/ml insulin, 0.25 µM dexamethasone, and 0.5 mM isobutyl-methyl-xanthine (IBMX) in Dulbecco's modified eagle media (DMEM) containing 10% fetal bovine serum and 1% antibiotic antimycotic for 48 h. Medium was removed and replaced with standard medium containing 2 µg/ml insulin. After 2 more days, cells were replaced with standard medium (Table 1).

Sample	Concentration	Cell Line
Metadichol	Control (untreated)	3T3-L1
	1 µg/ml	
	100 ng/ml	
	1 ng/ml	
	100 pg/ml	
	1 pg/ml	

Table 1: Treatment protocol.

RNA isolation

Treated adipocytes were washed twice with PBS and to the adherent cells 1 ml of TRIzol (per P35 dish) was added and transferred to the tube and vortexed. Samples were allowed to stand for 5 minutes at room temperature. Added 0.2 ml of chloroform per 1 ml of TRIzol used. Closed the tube and shaken vigorously for 15 seconds. The tube was allowed to stand at room temperature for 5 minutes. Cells were centrifuged at 10,000 rpm for 15 min at 4°C. Transparent colorless upper aqueous phase was transferred to a new tube. 0.5 ml of isopropanol was added per 1 ml of TRIZOL used, mixed gently by inverting the sample 5 times and incubate at room temperature for 5 minutes. Contents were centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was discarded and the RNA pellet was washed by adding 1ml of 70% ethanol. Mix gently by inverting the sample a few times. Contents were centrifuged for 5 min at 14,000 rpm at 4°C. Supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5min at 55°C. The pellet was then re suspended in 25 µl of DEPC treated water.

RT-PCR

A semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels GULO and β-Actin mRNA expressions. DNase treatment was carried out before converting the RNA to cDNA. The cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit (Thermo-Fischer Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 µl and cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 85°C for 5 min (Table 2).

Gene	Primer pair	Sequence	Tm	Product size (bp)
β–actin	FP	TCCTCCTGAGCGCAAGTACTCT	62	153
	RP	GCTCAGTAACAGTCCGCCTAGAA	62	
GULO	FP	CTTTGTTCAACTTCCTGTGG	60	140
	RP	GGTAGTACATCTCTGGACTG	54	

Table 2: Primers details.

PCR

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1 µM of each complementary primer specific for *GULO* and *β-actin* (internal control) sequence. The samples were denatured at 94°C for 5 minutes, and amplified using 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute for *GULO*, for β-actin the renaturation was set to 55°C for 30 seconds followed by a final elongation at 72°C for 10 minutes. The optimal numbers of cycles have been selected for amplification of these two genes experimentally so that amplifications were in the exponential range and have not reached a plateau. 10 µl of the final amplification product were run on a 2% EtBr-stained agarose gel and photographed. Quantification of the results was accomplished by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-actin intensity levels.

Lane 1-DNA Ladder; Lane 2- Control (Untreated cells); Lane 3-1 µg/ml treated Cells; Lane 4-100 ng/ml treated cells; Lane 5-1 ng/ml treated cells; Lane 6-100 pg/ml treated cells; Lane 7-1 pg/ml treated cells (Figure 1).
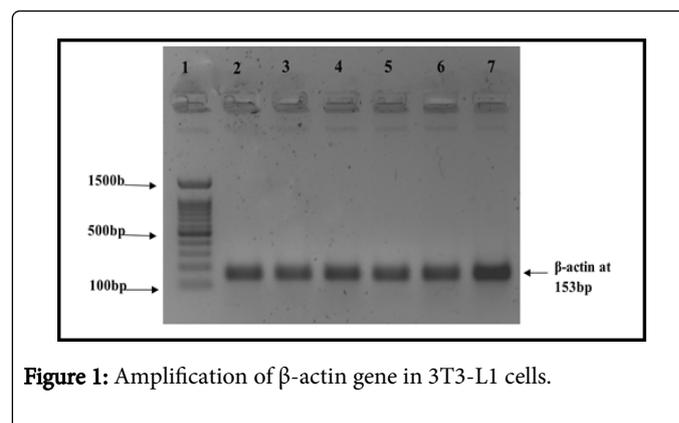

Figure 1: Amplification of β-actin gene in 3T3-L1 cells.

Lane 1- DNA Ladder; Lane 2- Control (Untreated cells); Lane 3-1 µg/ml treated Cells; Lane 4-100 ng/ml treated cells; Lane 5-1 ng/ml treated cells; Lane 6-100 pg/ml treated cells; Lane 7-1 pg/ml treated cells (Figure 2).
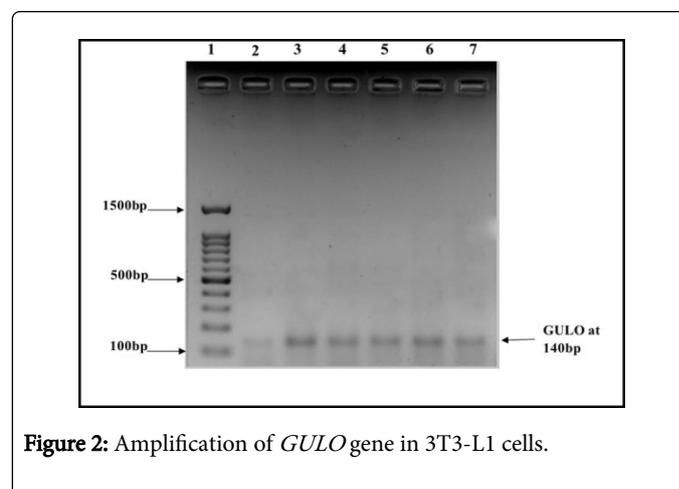

Figure 2: Amplification of *GULO* gene in 3T3-L1 cells.

Results and Discussion

As As discussed above that *in-vivo* study of Metadichol in Diabetic fed rats and humans increases Vitamin C even though no Vitamin C is administered. The present study is a semi-quantitative study *in-vitro* study that confirms results of the *in-vivo* study. Metadichol is the first known agonist of the *GULO* gene that amplifies the expression even at one picogram/ml. Researchers have shown that adding Vitamin C to the culture medium, results in high-quality iPSCs (Induced Pluripotent Stem Cells) from mouse and human cells routinely what has hampered progress is the low efficiency and slow kinetics of the reprogramming process. The activity of Metadichol at picogram levels compares favorably with known hormones like T3 (thyroid), Estrogen and Acetylcholine that are active at picogram levels. Vitamin C is known to enhance production of pluripotent stem cells [11,12]. It should be noted that one picogram corresponds to 12X in homeopathy concentration. Metadichol seems to follow the principles of hormesis, the biological and toxicological concept that low doses are more potent than larger doses [13]. Given these results, the effects of Metadichol on human-derived adipocyte cells, is ongoing and hopefully substantiate these findings and could be beneficial in the identification of biomarkers and development of PSCs will be reported in due course. Work is ongoing on how Metadichol affects human-derived adipocyte cells, which will substantiate the results of this study and its use in identification of biomarkers and development of PSCs and will be reported in due course (Table 3 and Figure 3).

3T3-L1				
Samples	**Band Intensity of PCR Amplicon of Genes**		**Normalized**	**Relative Gene Expression**
	β-actin	**GULO**		
Control	44477.3	11984.7	0.27	1
1 µg/ml	40980.5	23302.8	0.57	2.11
100 ng/ml	42919.9	30571.7	0.71	2.64
1 ng/ml	40756.2	32477.9	0.8	2.96
100 pg/ml	44945.3	49017.3	1.07	3.96
1 pg/ml	41650	36512.1	0.88	3.25

Table 3: Relative expression of *GULO* in treated and untreated 3T3-L1 cells.
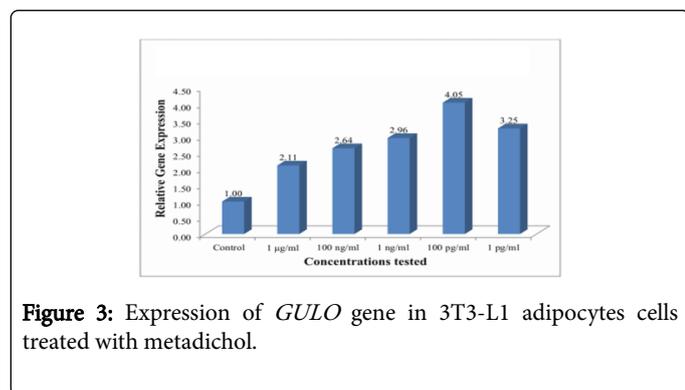

Figure 3: Expression of *GULO* gene in 3T3-L1 adipocytes cells treated with metadichol.

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