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Inhibition of Adipocyte Differentiation by Crocin in *in vitro* Model of Obesity

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Abstract: The present study was aimed to look for the effect of a natural compound i.e. Crocin on the viability of mature adipocytes and on inhibition of adipocyte differentiation. The 3T3-L1 cell line is one of the most well-characterized and reliable models for studying the conversion of pre-adipocytes into adipocytes. 3T3-L1 pre-adipocytic cell line was used and differentiated into mature adipocytes using standard adipogenic media. The MTT assay was used for the assessment of cell viability whereas effect on adipocyte differentiation was visualised by Oil Red O staining. The changes in relative lipid content were monitored spectrophotometrically at 510nm. Mature adipocytes when treated with Crocin decreased cell viability in a dose dependent manner. It also showed an enhanced inhibition of adipocyte differentiation as monitored by Oil Red O Staining. The western blot analysis revealed significant increase in the expression levels of AMP activated protein Kinase which is a key regulator of energy homeostasis. Thus, the crocin seems to affect multi-potent anti-obesity parameters and may have promising benefits for the treatment and prevention of obesity.

Keywords: Adipocyte differentiation, Crocin, 3T3-L1 pre-adipocytic cell line, Oil Red O Staining, Adipogenic media, Obesity

INTRODUCTION

Obesity is a condition characterised by either increase in number or size of fat cells which leads to adverse consequences [1]. It is a major risk factor for several medical disorders like Type 2 Diabetes, Hypertension, Cardiovascular diseases, liver abnormalities, respiratory problems and hormonal changes [2]. Obesity is increasing at an alarming scale and the risk factors associated with the disease pose a great threat to the entire world [3]. The disease is progressing not only in western countries but a marked rise has been observed in developing countries as well [4]. There is an urgent need to combat the disorder by screening the natural as well as synthetic compounds with anti-obesity potential. Currently, various natural anti-oxidants are the subjects of debate about their possible role in the treatment of metabolic disorders [5, 6].

Saffron is the dried stigma of the flowers of the saffron (*Crocus sativus*) and has numerous biological properties. Among the different constituents of saffron, Crocin has been found to be the most important chemical constituent. Crocin (C44H4O24) is a di-ester which is formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin and is considered as one of the few naturally occurring carotenoids easily soluble in water [7-9]. Crocin has shown various pharmacological activities such as anti-oxidant [10], antiplatelet [11], neuroprotective [12], anti-hyperlipidemic [13, 14], anti-diabetic [15-17], and anti-carcinogenic [18] properties due to its pharmacological effects. The anti-hyperglycaemic effects of saffron and its constituents have been observed in alloxan induced diabetic rats [19]. However, effect on adipocyte differentiation by treatment with Crocin is little known. Thus, our present study will play an important role in understanding the effect of a natural compound and the mechanisms through which a particular nutrient affects the differentiation to adipocytes. This study would eventually help to look for the compounds that prevent the initiation and progression of obesity.

MATERIALS

3T3-L1 mouse fibroblast cells were purchased National Centre for Cell Sciences (Pune, INDIA). Dulbecco's modified Eagle's medium (DMEM), Fetal Calf Serum (FCS), Penicilin Streptomycin mixed solution were obtained from GIBCO (St. Louis, USA). Insulin, Dexamethasone (DEX), Dimethyl sulfoxide (DMSO), Isobutylmethylxanthine (IBMX) and Oil Red O Stain and Crocin were purchased from Sigma Aldrich (St. Louis, MO USA). The antibodies for Phosphorylated AMPK and Tubulin were obtained from Santa Cruz Biotechnologies, (CA, USA) whereas secondary anti-rabbit and anti-mouse antibodies were purchased from Cell Signalling Technologies (Beverly, MA).

Cell Culture

3T3-L1 mouse embryonic fibroblasts were obtained from National Centre for Cell Science (Pune, India), maintained at 37°C in a humidified 5% CO_2 atmosphere and cultured as per standard protocol. Briefly, cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) Fetal Calf Serum (FCS), 100 U/ml of penicillin and 100 µg/ml of streptomycin, until confluent. Two days after confluence (Day 0), the cells were stimulated to differentiate with differentiation media (DI media) consisting of DMEM, 10% FCS, 167 ηM insulin, and 0.5 µM Isobutylmethylxanthine and 1 µM Dexamethasone for two days (Day 2). The differentiation media was replaced by DMEM+10% FCS+ 167 ηM insulin for next two days (Day 4), followed by culturing with DMEM+10% FCS for additional 4 days (Day 8), at which about 90% of cells were found to be mature adipocytes with fat droplets.

MTT cell viability assay

The assay was performed in 96 well flat bottomed plates. Almost 5000 cells/well were seeded and cultured as mentioned under section 2.1. Mature adipocytes were incubated with different doses of test compounds individually for 24 and 48 hours and in combination for 48 hours. This was followed by the addition of 100 µl of MTT reagent (5 mg/ml stock solution) in each well. Cells were then incubated for three hours with MTT and after this the MTT solution was removed carefully. Then, 100 µl of MTT solvent (4 mM HCl + 0.1% Nonidet P-40 in isopropanol) were added in each well. After this step, cells were again incubated for an hour. The absorbance was measured at the wavelength of 560 nm in a plate reader (Bio-Tek Instruments, USA) to determine the number of live cells.

Oil Red O staining

Crocin at different concentration along with DMSO control were added along with the differentiation media for six days to study their effect on the process of adipogenesis. On Day 6, the media was removed from the plates and the cells were washed twice with phosphate buffer saline (PBS), fixed in 3.7% formaldehyde for one hour and stained for 30 minutes with 0.2% (w/v) oil red O stain in 60% isopropanol. Cells were then washed with water and stained culture plates were placed at 32°C for evaporating excess water. The cells were visualised under phase contrast microscope at 10X magnification and then the images were captured. To quantify lipid content, about 200 µl of isopropanol was added to the each stained culture dish (12 well plates). The extracted dye was immediately removed by gentle pipetting and monitored spectrophotometrically at 510 nm [20].

Western blot Analysis

For immunoblotting, the Crocin at different doses were added along with the differentiation media and the differentiation of 2-day post confluent 3T3-L1 cells was carried out as described under "cell culture". On Day 6, cells were harvested, washed with phosphate-buffered saline (PBS) and suspended in NP-40 lysis buffer [20 mM Tris Cl (pH 8), 137 mM NaCl, 10% Glycerol, 1% Nonidet P-40, and 2mM EDTA] for one hour. The supernatant was separated by centrifugation (12,000 g for 10 min) and the protein concentration was estimated using Bradford's assay [25]. Protein extract, preheated at 100°C for 5 min in reducing SDS sample buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100 mM β-mercaptoethanol, was run on 10% SDS-polyacrylamide gel. After gel electrophoresis, separated proteins were transferred to PVDF membrane by semi-dry transfer method. The membranes were blocked with 5% bovine serum albumin (BSA) for 3 h, washed twice with PBS containing 0.2% Tween-20, and incubated with the respective primary antibodies AMPK (1:2000 dilution) and Tubulin (1:1000 dilutions) overnight at 4°C. The secondary detection was performed using anti-mouse IgG DyLight® 680 conjugate (1:10,000) or anti-rabbit IgG DyLight® 800 conjugate (1:10,000) secondary antibodies. The fluorescence was detected using Odyssey infrared detection system.

Statistical analysis

Results represent means ± SD of three independent experiments. Statistical analysis was performed using One Way ANNOVA by Dunnett's multiple comparison tests utilizing Graphpad prism 5 software. Statistically significant differences are defined at the 95% confidence interval (*P<0.05, **P<.01).

RESULTS

Crocin reduced the viability of mature adipocytes

To monitor the effect of different doses of Crocin on cell viability, 3T3-L1 mouse adipocytes were treated with different concentrations of Crocin (10-50 µM) for 24 and 48 hours as shown in Fig 1. There was just 4% decrease in cell viability with 10uM of Crocin whereas with increase in higher concentration of Crocin, there was decrease in cell viability. There was 47% decrease in cell viability with 50uM of Crocin. Thus, the cell viability data clearly indicate that Crocin decrease the cell viability of mature adipocytes.

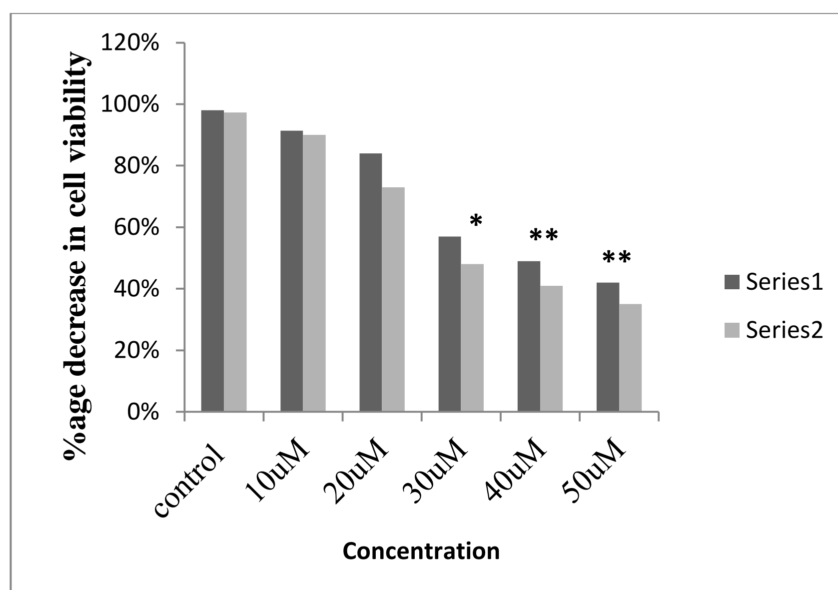

Fig-1: Effects of the test compound Crocin on the viability of mature adipocytes

The bar graphs represent the %age decrease in cell viability in response to different doses of Crocin. The series 1 represents the treatment with Crocin for 24 hours wheras series 2 represents treatment for 48 hours. Results are expressed as Mean $\pm$ SD of three independent experiments (*P<0.05, **P<.01).

Crocin inhibits the differentiation of pre-adipocytes into adipocytes

In order to understand the effect of Crocin on the differentiation of 3T3-L1 cells, the drugs were introduced along with the differentiation media and cultured as described in the "Material and Methods" section. The drug concentrations were selected in accordance with the decrease in cell viability data. The images captured showed a significant decrease in lipid droplets with the increase in the concentration of test compound as visualised from the stained images in Fig 2. The image 1 shows the undifferentiated cells, the image 2 represents the differentiated adipocytic cells whereas the images 3-6 represent the cells cultured in differentiated media along with the Crocin at different concentrations. The higher lipid content is visualised in differentiated cells and by increasing the concentration of Crocin, there is decrease in levels of lipid content as clearly visualised form the Oil Red O stained images. Furthermore, the observation was confirmed by the quantitative analysis of neutral lipid content. The lipids were gently extracted using isopropanol and measured spectrophotometrically at 510nm. The results showed significant decrease in the relative levels of lipid content with increase in concentration of Crocin as represented in Fig 3.

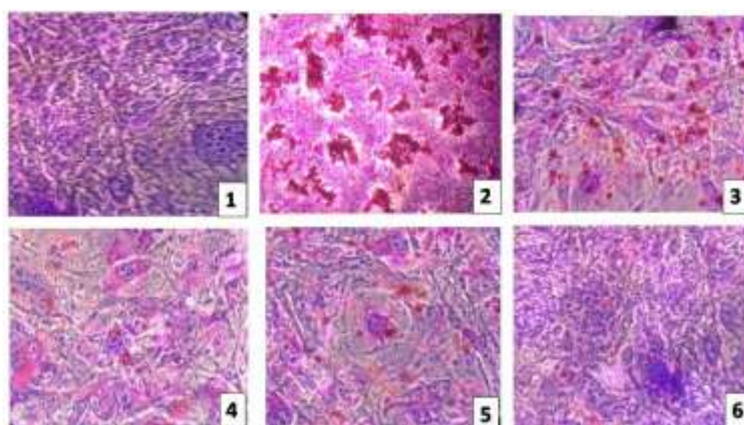

Fig-2: Effect of Crocin on the lipid accumulation during adipocyte differentiation

	1	2	3	4	5	6
DI Media	-	+	+	+	+	+
Crocin	-	-	10uM	20uM	30uM	40uM

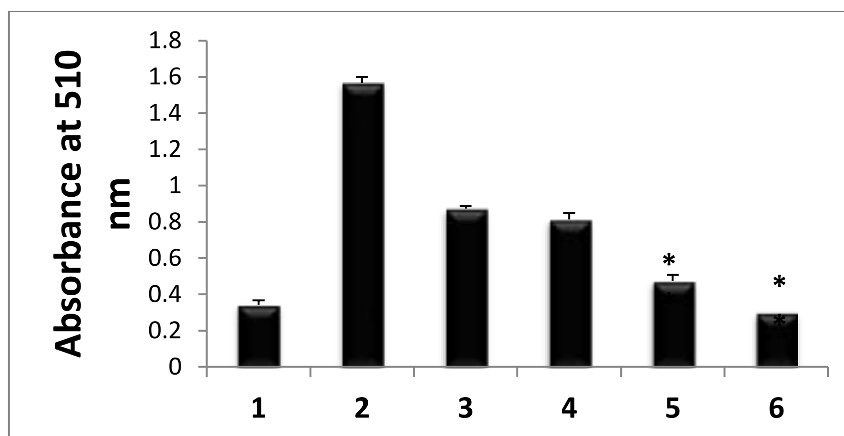

Fig-3: Effect of Crocin on the lipid accumulation during adipocyte differentiation

The representative images of Oil Red O staining in control vs treated cells are shown in Fig 2 whereas the bar graphs represent their relative levels of lipid content (Fig 3). Data expressed as Mean + SD of three independent experiments (**p<0.01)

Crocin promotes fatty acid oxidation by activating AMP-activated protein kinase (AMPK)

AMPK has been regarded as a "metabolic master switch" mediating the cellular adaptation to environmental or nutritional stress factors. The activation of AMPK by phosphorylation is known to play crucial role in fatty acid metabolism by controlling mitochondrial availability of fatty acids. Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways, such as fatty acid and sterol synthesis, and activation of ATP-producing catabolic pathways, such as fatty acid oxidation.

To know effect of crocin on the expression levels of phosho-AMPK, western blot analysis was done. 3T3-L1 pre-adipocytes were treated with test compound at different concentration during the differentiation process. Total cell lysates were subjected to immunoblotting using antibodies specific for phospho-AMPK (Thr172), and Tubulin. The immunoblotting results indicated a significant increase in the expression levels of AMPK. Tubulin was used as an internal control reference of sample loading (Fig 4).

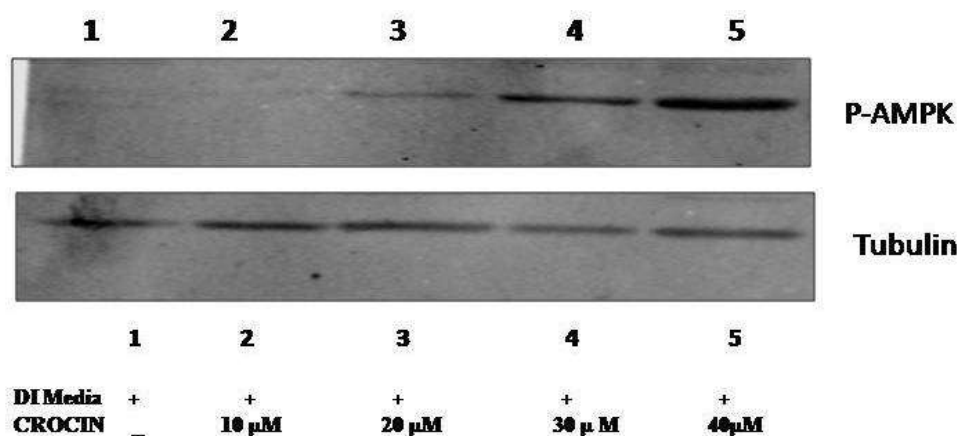

Fig 4: Effect of Crocin on the phospho-status of AMPK

Differentiated 3T3-L1 adipocytes were treated with test compound at different concentrations and cell lysates were prepared. The immunoblots for p-AMPK, in response to control versus treated cells reveals effect of test compounds in activating AMPK as seen from the immunoblots. Tubulin was used as an internal reference loading control.

DISCUSSION

The growth and proliferation of preadipocytes have a profound implication in the development of obesity therapeutics. The increase in adipose tissue mass is determined by the number and size of adipocytes which depends upon the proliferation and differentiation of preadipocytes into adipocytes [21]. To know the effect of test compounds on adipocyte differentiation, 3T3-L1 pre-adipocytic cell line was used as an *in vitro* model for the study. The pre-adipocytes were stimulated to differentiate into mature adipocytes by using a standard adipogenic medium that contains insulin, isobutylmethylxanthine and dexamethasone. Adipocytes precursor cells when

exposed to differentiation media were able to undergo full maturation into adipocytes.

The test compound showed a significant reduction in the viability of mature adipocytes with minimal effect of cell viability by 10uM and highest at 50uM. Moreover, Oil Red O Staining was carried out to visualise the effects of test compounds on the process of adipocyte differentiation. Crocin at different concentrations along with the differentiation media and the Oil Red O stain was used to monitor the accumulation of lipid droplets. The results clearly showed an enhanced inhibition of adipogenesis as visualised from decrease in lipid content when compared to the individual test compounds. The spectrophometric analysis also showed reduction in lipid accumulation ($p<0.01$).

Keeping in view that several natural and synthetic materials have shown anti-obesity effects via AMPK mediated mechanisms [22-24] and taking into consideration the role that AMPK plays in regulating whole body energy homeostasis, we looked to study the effect of our test compound on the phosphorylation status of AMPK. The increased in the expression level of P-AMPK with increase in drug concentration gave a notion that the activation of AMPK by Crocin might increase the energy expenditure by targeting processes like fatty acid oxidation, lipid synthesis, carbohydrate metabolism, etc. Consequently, this study shows that Crocin has an anti-adipogenic effect and speculate that Crocin be an active medication to treat obesity and related diseases. In a future study, we will further investigate the inhibition

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