Melatonin and lipid uptake by murine fibroblasts: Clinical implications

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Abstract

The current study was undertaken to uncover the role of melatonin in lipid metabolism in the murine fibroblasts. The results show melatonin in vitro enhances lipid accumulation and lipid droplet formation in this cell line. Using oil red O staining, it was found that when oleic acid was present in the culture media, melatonin at doses of 0.1–2 mM, significantly increased the lipid concentrations in the cells. However, low levels of melatonin, with or without oleic acid, did not influence lipid metabolism in the cultured fibroblasts. When a non-specific melatonin receptor antagonist, luzindole 10 μM was co-incubated with 1 mM melatonin, the stimulatory effects of melatonin on lipid accumulation in these cells was significantly reduced. It appears that the effects of melatonin on lipid metabolism in murine fibroblasts is mediated by melatonin membrane receptors.

ORIGINAL ARTICLE

INTRODUCTION

Adipogenesis is a complex process controlled by the interplay of intracellular factors and signals from the enviro nMent [1]. Obesity and cachexia in elderly patients are significant clinical problems. Both are associated with life-threatening diseases such as type 2 diabetes mellitus, coronary artery disease, hyperlipidemia [2, 3], cancer, AIDS and decubitus ulcers [2–4]. Therefore, an understanding of the detailed mechanisms of adipogenesis is essential for the development of a rational remedy for these disorders.

To understand of the process of adipogenesis and the formation of adipose tissue, several cell types can be induced to differentiate into adipocytes [1]. Murine fibroblast cell lines (NIH-3T3) are the fibroblastic phenotype of 3T3-L1, the most thoroughly characterized model for the study of adipogenesis in vitro [5].

Melatonin is a major secretory product of the pineal gland and is released into the cerebrospinal fluid and blood. Melatonin with its lipophilic characteristics allows it to pass all biological barriers with relative ease [6]. The nocturnal secretion of melatonin mediates photoperiodic entrai nMent of endogenous circadian rhythms and other physiologic functions [7]. Melatonin is also a potent free radical scavenger [8–11], an important fat metabolism regulator [12] and its role as an immuno-modulator is well established [13]. Recently, it was noted that melatonin acts directly on the adipose tissue through specific MT1 and MT2 receptors and it exerts some influence on the metabolism of these cells [14–16].

The aims of this study were to determine whether melatonin influences lipid accumulation in a fibroblast cell line. Secondly, we attempted to define the mechanisms by which melatonin has influence on lipid metabolism.

MATERIAL AND METHODS

Chemical reagents

Melatonin, oleic acid, luzindole, oil red O, dimethyl sulfoxide (DMSO) and ethanol were purchased from Sigma (St Louis, MO, USA). DMEM Dulbecco's modified Eagle's medium and penicillin-streptomycin-glutamine were obtained from Gibco BRL, USA.

1M Melatonin and 10 mM luzindole were dissolved in DMSO as the stock solutions and kept at –20 °C prior to used. Working solutions were prepared before use at room temperature by diluting the stock solutions with sterile DMEM to the desired concentrations. Final concentrations of solvents were less than 0.5%.

Oleic acid was first dissolved in absolute ethanol to a stock concentration of 1M and then diluted to working concentration of 50 μM. The final ethanol concentration was <0.01%.

Cell culture

The cells were grown and maintained in DMEM Dulbecco's modified Eagle's medium (Gibco BRL, USA) with 10% calf serum at 37 °C under 5% CO_2 in air. Fibroblasts were prepared in dishes and then counted with a hemocytometer (Neubauer Camera). Final suspensions were adjusted to concentration of $1x10^4$ cells/ml and cultured in 48/wells plates.

Experimental design

The fibroblast cell line, NIH-3T3, was used in this study. Two types of experiments were performed. The first was to examine whether melatonin at different concentrations influences lipid accumulation. The second was designed to examine the potential mechanisms of melatonin is action on lipid metabolism.

Experiment 1. When the cells were 80% confluent, the medium was changed to a medium which contained 4% calf serum; thereafter, different concentrations of melatonin (0.25 nM, 0.5 nM, 1 nM, 10 nM, 50 nM, 100 nM, 1 μM, 50 μM, 100 μM, 500 μM, 1 mM and 2 mM) were added to the cell cultures. Two hours later, 50 μM oleic acid was added to the media. The cells were then incubated for 24 hours. The cells were then fixed with 10% formalin in PBS. A control group of cells received no treatment. The vehicle groups contained either ethanol or DMSO at concentrations used for treatment groups.

Experiment 2. When the cells were 80% confluent, the medium was changed to a medium which contained 4% calf serum; thereafter, 10 μM luzindole was added. Two hours later 1 mM melatonin was added in to the same medium. After another two hours 50 μM oleic acid was added the cell cultures and cells were incubated for 24 hours. Cells were then washed three times with PBS and fixed in 10% formalin.

Lipid staining, extraction and analysis

The cell cultures were washed three times with deionized water and stained with oil red O to identify lipid droplets in mature fibroblasts [17]. The oil red O stock solutions was in 0.5% isopropanol. Working solution of oil red O was prepared before use by diluting a stock solution with deionized water (six parts oil red O stock and four parts H_2O). The samples were left overnight at 4 °C, and filtered twice. The cultured cells were complete immersed in the working solution of oil red O for two hours, and later exhaustively rinsed with water to remove excess free oil red O. The excess water was evaporated by placing the stained cultures at room temperature overnight. The stained plates were scanned for both experiments 1 and 2.

For determination of intracellular lipid content, cells were washed with PBS, and total lipids were extracted, well by well, with isopropyl alcohol; this extracted lipid remained bound to oil red O. Lipid content was measured using an colorimetric method in the spectrophotometer at 510 nM.

Determination of cell number

Relative cell numbers were determined using a colorimetric method in which the absorbance values obtained are proportional to the number of cells attached to the well. After removing the extracted oil red O from the wells, cells were washed three times with isopropanol, then air dried, and incubated with 200 μl/well cell staining solution (SuperArray) for 30 min. The staining solution was then discarded and attained cells were washed five times with distilled water to eliminate the unbound dye. Thereafter, the stain was extracted with 400 μl 1% SDS and the absorbance values of the wells were measured spectrophotometrically at 595 nM using a plate reader.

Data analysis

All data are presented as the mean ± standard error (S.E.M.). All statistical procedures were performed using GraphPad InStat statistical software package program 3.0 . Data were analysed using a one-way analysis of variance

(ANOVA) follow by Tukey test to compare differences between the groups. The differences were considered to be significant at $P<0.05$.

RESULTS

The results show that oleic acid at the concentration of 50 μM significantly enhances the lipid accumulation in the cultured murine fibroblast cells (Figs. 1,2,4 and 6). Low concentrations of melatonin (0.25 nM to 100 nM), without co-incubation with oleic acid, with cultured cells showed a small Gaussian distribution, but statistical analysis indicated the melatonin under these circumstance did not affect lipid accumulations significantly (Fig 3).

Results presented in the Figure 5 show that increasing concentrations of melatonin (1 μM, 50 μM, 100 μM, 500 μM, 1 mM and 2 mM) stimulate lipid accumulations in culture NIH-3T3 cells. The higher concentration of melatonin, the greater the lipid accumulation. Similar results have been obtained (Fig.6) when 50 μM oleic acid was added to the cultured NIH-3T3 cells containing the same concentrations of melatonin (Fig. 5).

The results indicate that high concentrations of melatonin (100 μM, 500 μM, 1 mM and 2 mM) induced enhancement of lipid accumulations, with or without oleic acid (Figs. 5 and 6; Table 2), while no significant changes in lipid accumulations, with or without oleic acid, were observed with low concentrations of melatonin (0.25 nM, 0.5 nM, 1 nM, 10 nM, 50 nM, 100 nM, 1 μM and 50 μM) (Figs. 3 and 4; Table 1).

The addition of 1 mM melatonin plus 10 μM luzindole caused a marked decrease of lipid accumulation versus 1 mM melatonin only (Fig 7). Similar results were obtained in cultured cells incubated in the presence of 50 μM oleic acid, the only difference was that the rate of lipid accumulation was about 35% higher with oleic acid than without it (Table 3 and Fig. 8).

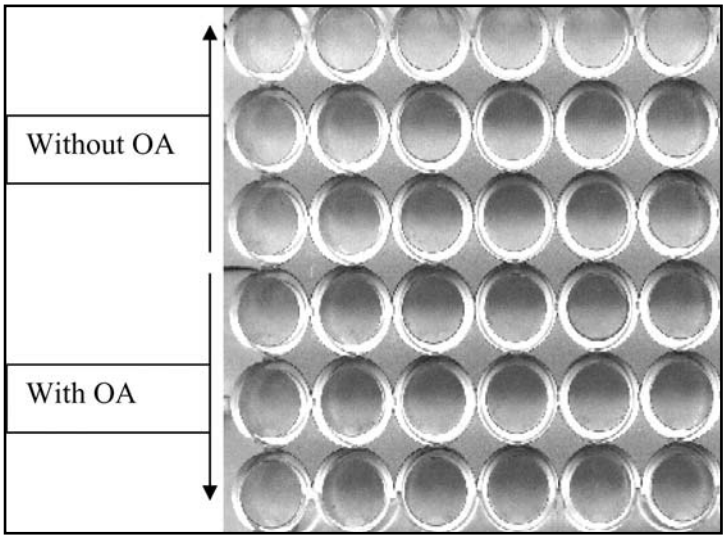

Fig. 1. Oil red O staining of cultured NIH-3T3 cells and treatment with different low doses of melatonin without and with 50 μM oleic acid. Data are representative of 4 independent experiments.

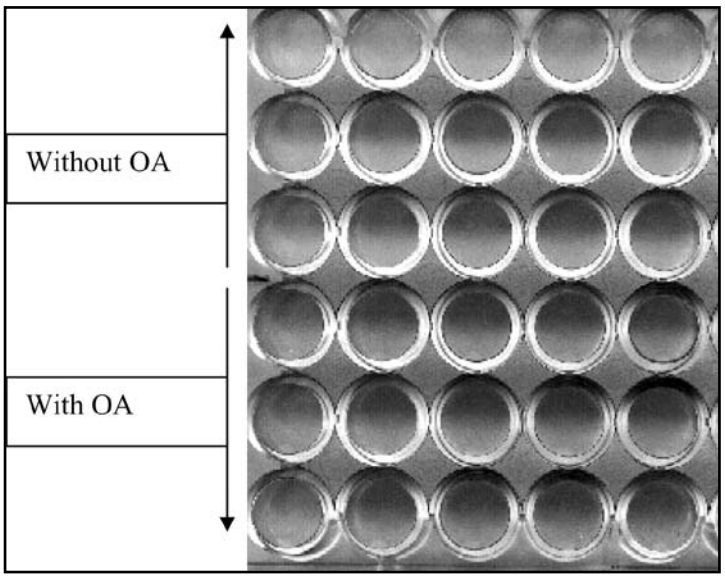

Fig. 2. Oil red O staining of cultured NIH-3T3 cells and treatment with different high doses of melatonin without and with 50 μM oleic acid. Data are representative of 4 independent experiments.

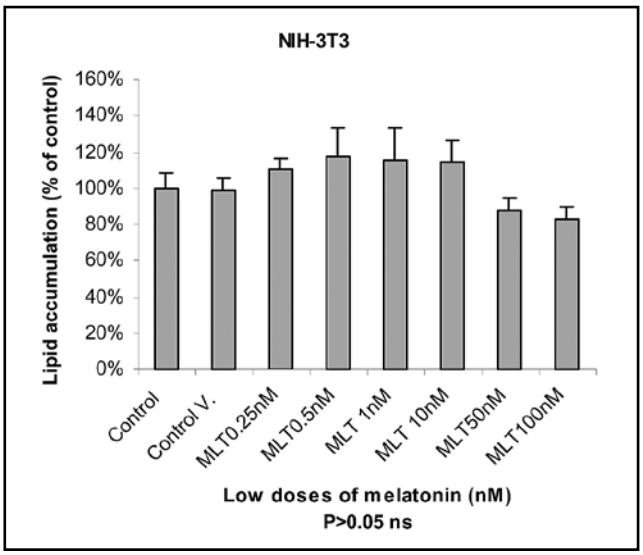

Fig. 3. Effects of low doses of melatonin (0.25 nM to 100 nM) on lipid accumulation in culture cells NIH-3T3. Each bar represents the means ± SEM for four separate experiments (n= 22 for control and vehicle control; and n= 11 for every dose of melatonin).
P>0.05 (ns) do not significantly affect lipid accumulations.

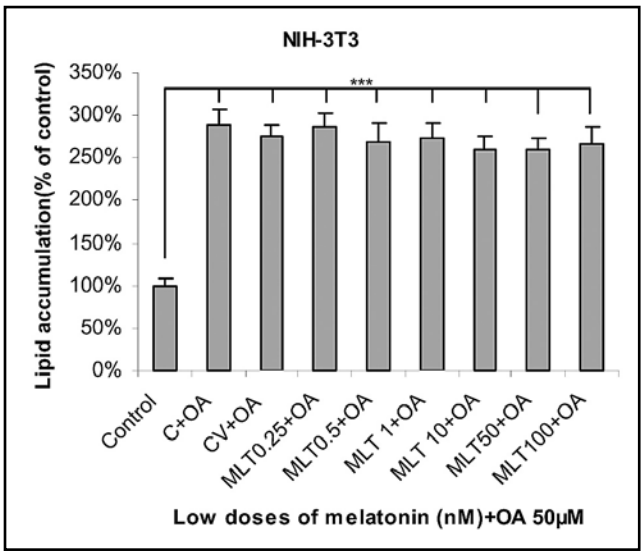

Fig. 4. Effects of low doses of melatonin (0.25 nM to 100 nM) plus oleic acid 50 µM on lipid accumulations in cultured NIH-3T3 cells. Each bar represents the means ± SEM for four separate experiments (n= 22 for control, control plus oleic acid or vehicle control plus oleic acid; and n= 11 for every low dose of melatonin plus 50 µM oleic acid).
*** P<0.001 versus control and ns P>0.05 versus control plus oleic acid.

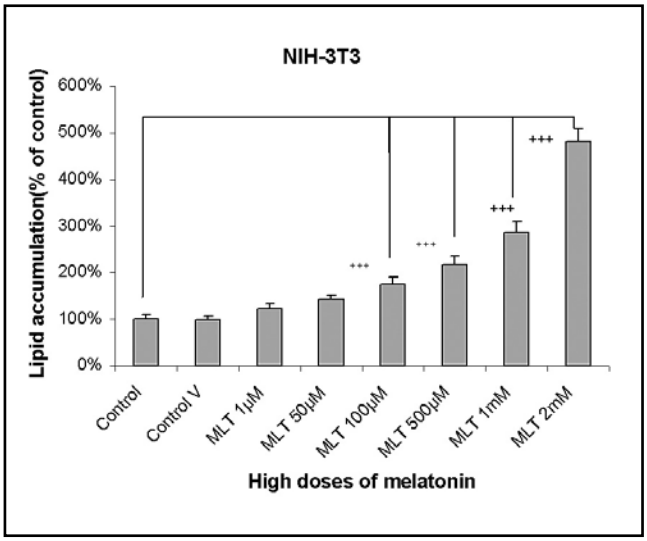

Fig. 5. Effects of high doses of melatonin (1 µM to 2 mM) on lipid accumulation in cultured NIH-3T3 cells. Each bar represents the mean ± SEM for four separate experiments (n= 22 for control and vehicle control; and n= 11 for each dose of melatonin).
ns P>0.05, $^{+++}$P<0.001 versus corresponding control without melatonin.

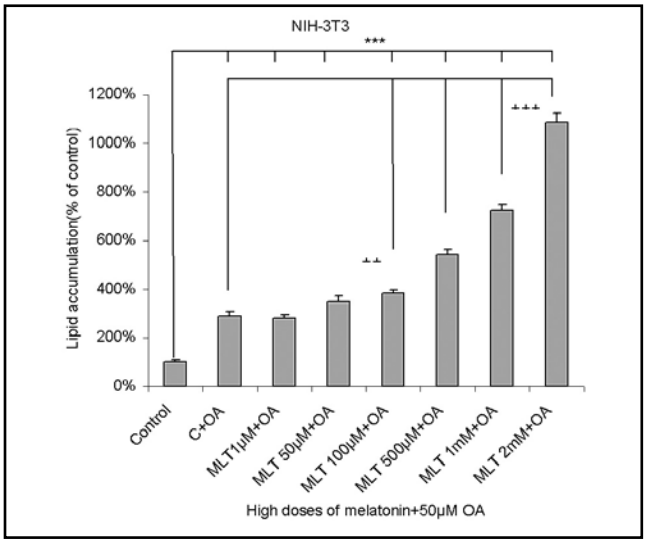

Fig. 6. Effects of high doses of melatonin (MLT) (1 µM to 2 mM) plus 50 µM oleic acid on lipid accumulation in culture cells NIH-3T3. Each bar represents the means ± SEM for four separate experiments (n = 11 for every high dose of melatonin plus 50 µM oleic acid).
ns P>0.05, $^{++}$ P<0.01, $^{+++}$ P<0.001 versus corresponding control plus oleic acid. ***P<0.001 versus corresponding controls without oleic acid.

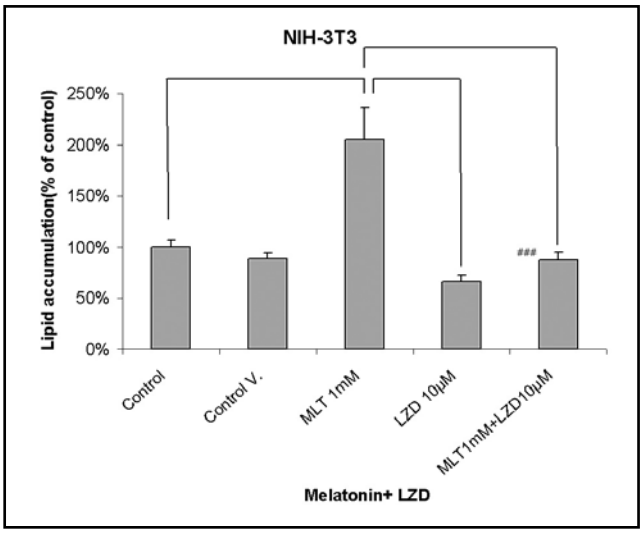

Fig. 7. The melatonin effects on lipid accumulation is blocked by the melatonin receptor antagonist luzindole. Each bar represents the mean ± SEM of lipid accumulation for four separate experiments (n= 33 for control; n= 44 for vehicle control; n= 22 for melatonin 1 mM and n= 11 for LZD 10 μM and 1 mM MLT + 10 μM LZD).
***P<0.001 statistical significance versus control.
+++ P<0.001 statistical significance versus 1 mM melatonin.
P<0.001 statistical significance versus 1 mM melatonin.

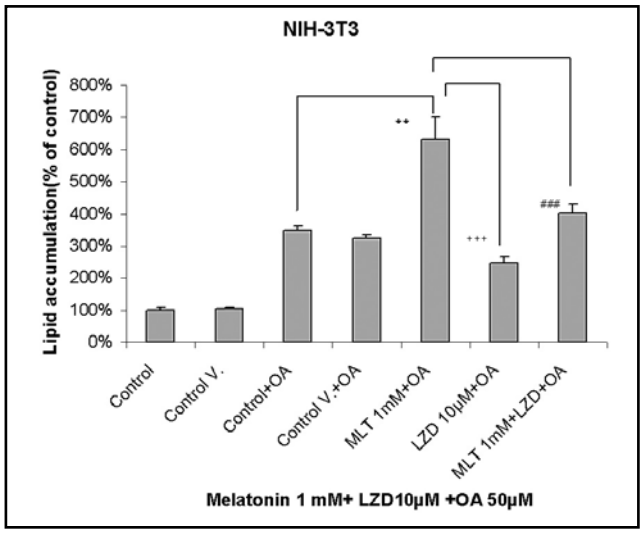

Fig. 8. The melatonin effects on lipid accumulation is blocked by the melatonin receptor antagonist luzindole. Each bar represents the mean ± SEM of lipid accumulation for four separate experiments (n= 33 for control and control + OA; n= 44 for vehicle control and vehicle control + OA; n= 22 for 1 mM MLT + OA and n= 11 for 10 μM LZD + OA and 1 mM MLT + LZD + OA) .
***P<0.001 statistical significance versus control + OA.
+++ P<0.001 statistical significance versus 1 mM MLT + OA.
P<0.001 statistical significance versus 1 mM MLT + OA.

Table 1. The effect of low doses of melatonin (0.25 nM to 100 nM) on lipid accumulation in culture NIH-3T3 cells in the absence (-OA) or presence of (+OA) 50 μM oleic acid.

		NIH-3T3	
	Size n	**–OA**	**+OA 50uM**
Control	22	1.29 ± 0.090	3.73 ± 0.175***
Control vehicles	22	1.27 ± 0.341	3.56 ± 0.130***
MLT 0.25 nM	11	1.43 ± 0.056	3.69 ± 0.159***
MLT 0.5 nM	11	1.52 ± 0.153	3.45 ± 0.224***
MLT 1 nM	11	1.48 ± 0.184	3.51 ± 0.183***
MLT 10 nM	11	1.47 ± 0.123	3.35 ± 0.153***
MLT 50 nM	11	1.13 ± 0.074	3.36 ± 0.132***
MLT 100 nM	11	1.07 ± 0.066	3.42 ± 0.202***

Results are expressed as mean ± SEM of lipid accumulations (DO/nº cells) for four separate experiments. Asterisks (***) indicate statistical significance (*P*<0.001) versus corresponding controls without OA.

Table 2. The effect of high doses of melatonin (1 μM to 2 mM) on lipid accumulations in culture NIH-3T3 cells in the absence (–OA) or presence of (+OA) 50 μM oleic acid.

		NIH-3T3	
	Size n	**–OA**	**+OA 50uM**
Control	22	1.29 ± 0.090	3.73 ± 0.175***
Control vehicles	22	1.27 ± 0.072	3.56 ± 0.130***
MLT 1 μM	11	1.56 ± 0.130	3.61 ± 0.225***
MLT 50 μM	11	1.81 ± 0.098	4.50 ± 0.145***
MLT 100 μM	11	2.24 ± 0.168^{+++}	4.93 ± 0.214***, $^{++}$
MLT 500 μM	11	2.77 ± 0.198^{+++}	6.99 ± 0.229***,$^{+++}$
MLT 1 mM	11	3.70 ± 0.220^{+++}	9.34 ± 0.390***,$^{+++}$
MLT 2 mM	11	6.20 ± 0.274^{+++}	13.99 ± 0.538***,$^{+++}$

Results are expressed as mean ± SEM of lipid accumulations (DO/nº cells) for four separate experiments. Asterisks(***) indicate statistical significance (*P*<0.001) versus corresponding controls without OA. Crosses ($^{+++}$) and ($^{++}$) indicate statistical significance (*P*<0.001) and (*P*<0.01) respectively versus corresponding controls w/wo OA.

Table 3. Melatonin effects on lipid accumulation is blocked by the melatonin receptor antagonist luzindole.

		NIH-3T3	
	Size n	**–OA**	**+OA 50uM**
Control	33	1.17 ± 0.071	3.48 ± 0.145
Control vehicles	44	1.03 ± 0.056	3.25 ± 0.097
MLT 1 mM	22	2.40 ± 0.307***	6.33 ± 0.689***
LZD 10 μM	11	0.77 ± 0.066^{+++}	2.47 ± 0.198^{+++}
MLT 1 mM+LZD	11	1.02 ± 0.081$^{\#\#\#}$	2.85 ± 0.196$^{\#\#\#}$

Data are expressed as mean ± SEM of lipid accumulation DO/nº cells for four separate experiments.
Asterisks (***) indicate significant increase (P<0.001), when the value obtained from 1 mM MLT is compared to control, both w/wo OA 50 μM.
Crosses ($^{+++}$) indicate significant decreases (P<0.001), when the value obtained from 10 μM LZD is compared to 1 mM MLT, both w/wo OA 50 μM. Cushion symbols ($^{\#\#\#}$) indicate significant (P<0.001) decreases below the value obtained with 1 mM melatonin, both w/wo 50 μM OA.

DISCUSSION

The results of the present study shows that melatonin influences lipid accumulation in the NIH-3T3 cell line and this effect is directly related to the concentration of melatonin. Thus, high concentrations of melatonin (100 μM, 500 μM, 1 mM and 2 mM), with or without oleic acid increased lipid accumulation in this cell line, while low doses of melatonin (0.25 nM, 0.5 nM, 1 nM, 10 nM, 50 nM, 100 nM, 1 μM and 50 μM), with or without oleic acid, did not significantly change lipid accumulation.

The effect of melatonin on lipid accumulation in NIH-3T3 murine fibroblast appears to involve its binding to membrane receptors as shown in Table 3 and Figs. 7 and 8, since luzindole, a non-selective competitive melatonin receptor antagonist [18], reduced stimulation of lipid accumulation by melatonin (1 mM).

Normally, especially in young subjects, there is an energy balance between food intake and body mass. This is achieved via endogenous controls (endocrine, immunology, psychology, metabolic and nervous systems) [19, 20]. As aging occurs, the control systems decline leading to clinical pathologies related to obesity and cachexia e.g., metabolic syndrome, cardiovascular diseases, infections and cancer [2–4]. Recent reports suggest that aging may be associated with a significant impairment in the ability to control food intake following overeating or undereating. This occurs routinely as part of the normal energy regulation pattern. These situations relating to control function loss may help to explain the vulnerability of older persons for unexplained weight gain or loss [21–23]. Therefore, for elderly patients to gain or lose fat can be positive or negative depending on each person's situation.

In any species, change in photoperiod induce major physiological adaptations (e.g., hibernation, migration and seasonal breeding). Melatonin plays an important role in regulating energy balance and fat distribution [24–27] in these situations. In these species, little is known about as melatonin influences energy homeostasis and less in non-hibernating animals, including humans.

The ability of the photoperiod and melatonin to manipulate energy balance in animals provides new opportunities to uncover the control mechanisms involved in both central and periphery energy homeostasis and body weight regulation [28, 29]. Thus, melatonin could act as a signal transduction molecule to notify the brain to adjust fat storage and body temperature according to the metabolic and fatty tissue requirements of the animals [12, 30].

Circulating melatonin levels drop with increasing age [31–34]. It has been hypothesized that the decreased circulating melatonin concentrations may lead to a variety of physiological changes associated with aging [35], including hyperisulinemia, increased visceral fat [36, 37], weight loss and geriatric wasting syndrome [21, 38].

The daily administration of melatonin may have some beneficial effects in patients who require fat adaptations due to their special conditions including cancer, infections and wasting syndrome. Energetically challenging conditions in these diseases can directly induce death via anorexia-cachexia. Thus, melatonin may affect body mass regulation, for example, by increasing lipid accumulation in tissues or enhancing food intake [24, 39].

In vitro, melatonin showed variable effects, in lipid metabolism, depending on cell type and culture conditions. Thus, melatonin was capable to suppress basal and insulin-stimulated lipogenesis in normal rat, rabbit and hamster adipocytes [40]. In human mononuclear leukocytes melatonin inhibited cholesterol synthesis [41]. In vivo, the melatonin supplementation on obese rats reduced body weight and on middle-aged male rats suppressed intraabdominal adiposity, plasma leptin and insulin [42, 43]. These results suggest that the decrease in endogenous melatonin with aging may alter lipid metabolism and exogenous melatonin administration can modulate lipid homeostatic actions.

Here, we focus on the role of melatonin in increasing lipid accumulation. Based on our observations with NIH-3T3 cells, melatonin membrane receptors may be

involve adipose tissue regulation [12, 15]. In mammals, two specific melatonin receptors subtypes, MT1 and MT2, have been cloned. Numerous groups showed that melatonin may have a direct effect on peripheral tissues involved in energy homeostasis such as pancreatic beta cell, hepatocytes and adipocytes [12, 44, 45]. The role of melatonin in increasing lipid accumulations in NIH-3T3 cells may be a direct effect on peripheral tissues which could help to enhance fat storage and weight gain in cachexic patients [46–48].

There are many clinical problems associated with anorexia-cachexia including fat and muscle tissue wasting. Cachexia arises from a complex interaction between the cancer and host [47], the HIV infection and AIDS patients [49, 50] or wasting disorders in elderly patients [4]. This process results from a failure of the adaptative feeding response seen in simple starvation and includes cytokine production, release of lipid-mobilizing and proteolysis-inducing factors, and alterations in intermediary metabolism. Cytokines play a pivotal role in long-term inhibition of feeding by mimicking the hypothalamic effect of excessive negative feedback signalling from leptin, a hormone secreted by adipose tissue, which is an integral component of the homeostatic loop of body weight regulation [51]. This could be done by persistent inhibition of feeding-stimulatory circuitry. Melatonin could act on the feeding-regulatory circuitry to increase appetite and inhibit disease-derived catabolic factors to antagonize tissue wasting and/or host cytokine release [38, 52, 53].

In summary, data presented in this investigation show that the stimulatory action of melatonin in lipid accumulation is dose dependent. The effects of melatonin on lipid metabolism, in the presence or absence of oleic acid, is likely due to its binding to specific MT1 and MT2 membrane receptors by means of G protein-coupled signal transduction pathway. These experimental findings suggest that melatonin supplementation may be beneficial in special situations such as anorexia-cachexia due to cancer, AIDS and geriatric wasting syndrome, and the indole may prevent or delay some of these devastating changes in older persons.

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