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Short communication

Chronic fluoxetine treatment induces lipid accumulation but does not alter the expression of Pref-1 in rat adipose tissue

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This study aims to investigate the effects of chronic fluoxetine (FLX) treatment on preadipocyte factor-1 (Pref-1) expression in subcutaneous, visceral and brown adipose tissues, and on the size of vacuoles in adipocytes obtained from the perirenal regions in rats. Twenty-eight Wistar rats were treated with FLX at two different doses and fourteen animals received vehicle. After 40 days of treatment, the subcutaneous, perirenal and interscapular adipose tissues were collected. Pref-1 expression was examined using an immunohistochemical method and the vacuolar area was measured in stained sections. In the low dose FLX group, the size of vacuoles increased both in male and female animals. The high dose of FLX also induced a significant increase of vacuole size, but only in male animals. Neither of the two doses of FLX has significantly affected the Pref-1 expression in any type of adipose tissue.

Keywords: fluoxetine, adipose tissue, preadipocyte factor-1, vacuole size

Major depression is one of the most important psychiatric disorders. Treatment of this illness often involves selective serotonin reuptake inhibitors (SSRIs), and a therapeutically important drug of this class is fluoxetine (FLX). Although the SSRIs are more or less devoid of cardiovascular toxicity, they show other side effects such as metabolic and weight changes or endocrine dysfunctions (*e.g.*, sexual disorders) (1). Regarding the metabolic effect of FLX medication, the initial observations were ambiguous; some evidence sustained an anorectic effect (2), others an orectic effect of this drug (3). FLX is currently considered to be an anorectic agent, since a large meta-analysis of 257 clinical studies performed by Domecq *et al*. (4) revealed weight loss associated with chronic administration of FLX.

While the central effects on metabolism, food intake and changes in body mass were extensively studied, the peripheral mechanisms underlying these changes remain unclear. Pref-1 is an adipokine, which influences the adipocyte function and maturation by inhibiting the differentiation process (5).

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The size of lipid vacuoles in adipocytes is an important parameter characterizing adipose tissue maturation since these vacuoles are triglyceride depots, the size of which reflects the energy storage and maturity of the cells (large vacuole size characterizes mature adipocytes, while smaller vacuoles can be seen in preadipocytes).

This study was aimed to evaluate the effect of chronic FLX treatment on vacuole size and Pref-1 expression in order to identify the peripheral processes of weight gain or loss, in which the studied drug may be involved.

EXPERIMENTAL

Chemicals

FLX was donated by Vim Spectrum S.R.L. (Romania) and produced by FIDIA S.p.A., Italy. Anti-Dlk/Pref-1 monoclonal antibody was obtained from MBL International Corporation, USA (clone 24-11).

Animals and experimental design

FLX was administered to 42 adult Wistar rats, divided into 6 groups of 7 animals each (6 mg kg^{-1} bm group, 12 mg kg^{-1} bm group, and a control group, male and female. Group codes used throughout for treated animals were: TM-6, TM-12 for males and TF-6, TF-12 for females). The animals were provided by the Biobase of the University of Medicine and Pharmacy of Tîrgu Mureş, Romania. All experimental procedures were conducted according to the institutional and national guidelines for animal experiments, respecting Directive 2010/63/EU, and have the approval of the Ethics Committee of University of Medicine and Pharmacy of Tîrgu Mureş. Food and water were available *ad libitum*, the light-dark cycle was assured by natural light. The animals were held at a temperature of 20 ± 3 °C. The drug was administered by oral gavage following a 7-day accommodation period as a 0.7 % aqueous solution. Control groups received vehicle (distilled water); group codes for control animals used throughout: CM for males and CF for females. Considering the half-life of FLX and of its active metabolite nor-FLX (5 hours and 15 hours, resp.), and high bioavailability at high doses (6), the drug was applied in two doses: 6 and 12 mg kg^{-1}, resp., once daily at 09:00. At the end of each week, body mass was measured, and the administered doses were adjusted according to the change in body mass. After a 40-day trial period, the animals were sacrificed by intraperitoneal injection of anesthetics (ketamine 300 mg kg^{-1} bm and xylazine 30 mg kg^{-1} bm, resp.). The perirenal white adipose tissues, interscapular brown tissues and subcutaneous fat tissues were collected.

Morphometric analysis and immunohistochemistry

Adipose tissue samples were fixed in a 4 % neutral formaldehyde solution and were later paraffin-embedded. Approximately 3–5 μm thick sections were deparaffinized by the xylene-ethanol sequence. Morphometric analyses of adipocytes consisting of vacuolar areas from the white perirenal adipose tissue were performed using sections after conventional hematoxyline-eosine staining. Immunohistochemical staining for Pref-1 expression was made for all types of adipose tissues, such as white, brown and subcutaneous. Anti-Dlk/Pref-1 (clone 24-11, MBL, USA) monoclonal antibody was used after a 1:100 dilution.

Application of the primary antibody was followed by the two-step peroxidase technique. The staining process was performed using diamino-benzidine (DAB) chromogen.

Digital image analysis

Digital slides were made with a Zeiss MiraxScan digital slide acquisition system (Carl Zeiss, Germany) mounted with a Marlin F-146C digital camera at a resolution of 1392×1040 pixels with a Sony ICX267 sensor (Allied Vision Technologies, Germany) through Zeiss Plan-Apochromat 20× magnification and 0.8 numerical aperture. The acquisition system was controlled by MiraxScan software (3DHistech, Hungary) installed on a Fujitsu-Siemens Celsius Workstation computer (4×2 GHz CPU, 2 GB RAM). For each tissue type, five images/specimens were saved from the digital slides both in TIFF and JPG formats. Thus, a total number of 300 digital images were prepared for the study. Digital images were not altered in any way (contrast, luminosity, levels, *etc.*) before morphometric analysis. Image analysis was carried out using ImageJ software, version 1.44c (Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

After a 7 % contrast enhancement, the images were transformed to 8-bit, black-and-white (inverted color). After transformation, the cell count was evaluated and vacuolar areas were measured for cells with circularity between 0.3 and 1.0, surface area ranging from 15 to 12,000 μm^2 (Fig. 1a).

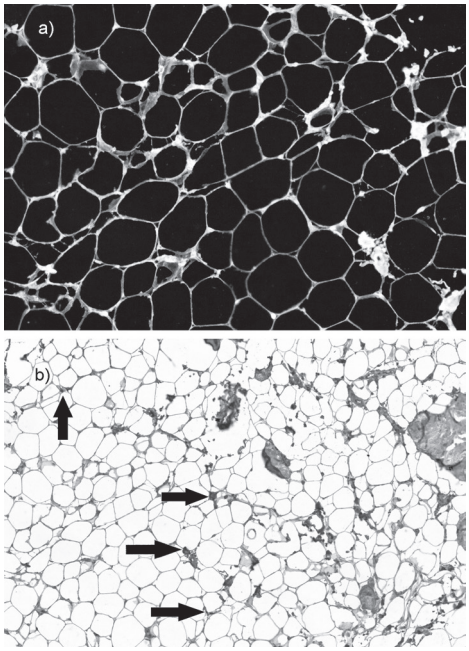

Fig. 1. a) The inverted 8-bit black-and-white image of adipose tissue, just before the particle size analysis. b) The membrane-linked form of the preadipocyte factor-1 (Pref-1) is located in the proximity of the membrane (arrows).

A color filter was applied to sections (R: 199/255, G: 60/255, B: 0/255). The areas in sections with the RGB values mentioned were brown colored, thus these were the high Pref-1 expressing tissue parts. After areas in sections with matching color values were selected, the total area was measured. The brown area was then taken as the percent of total area in sections (pure area, not counting other structures like capillary). As a result, we calculated the percentage of adipose tissue showing high Pref-1 expression since these can be seen as brown areas in sections (Fig. 1b).

Statistical analysis

The results were analyzed statistically using Graphpad Prism 5. The vacuolar dimensions and Pref-1 expression were compared between groups using the Kruskal-Wallis test and Dunn's multiple comparison test. The chosen statistical significance level was $\alpha = 0.05$.

RESULTS AND DISCUSSION

Vacuolar areas of adipocytes from perirenal adipose tissue

Comparing the groups of the same gender, it was found that FLX administration influenced the vacuolar areas significantly ($p < 0.001$). Male animals treated with the low dose (6 mg kg^{-1}) showed a significantly larger vacuolar area compared to the control group of the same gender (median: 298.99 μm^2 *vs.* 236.79 μm^2, $p < 0.0001$). A more evident increase was observed in the case of males treated with the higher dose (12 mg kg^{-1}). This group showed the largest vacuolar area of the three male groups (368.04 μm^2, $p < 0.0001$) (Fig. 2a). This suggests a pro-differentiating effect of FLX at both doses ($p < 0.05$ in both CM *vs.* TM-6 and CM *vs.* TM-12). In the case of female animals, the low dose produced a similar increase in area to that in males (286.13 μm^2 compared to 209.78 μm^2 seen in the control group, $p < 0.0001$) (Fig. 2b). Interestingly, the female group treated with the high dose had a substantially smaller

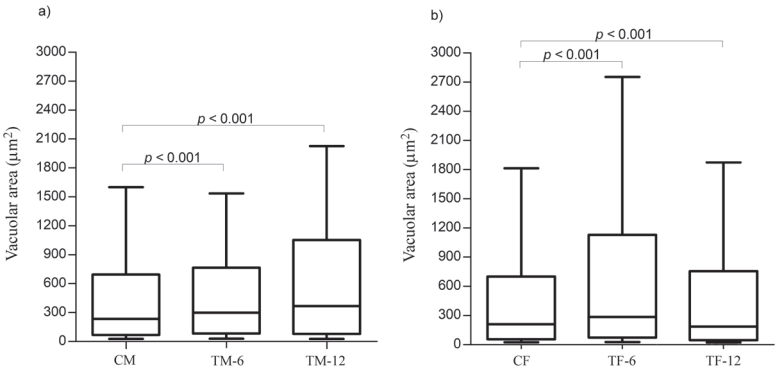

Fig. 2. Vacuolar areas of perirenal adipocytes in: a) male and b) female rats (line: median, boxes: interquartile range, whiskers: 10-90 percentile). CM – control males, TM-6 – males treated with 6 mg kg^{-1} bm FLX, TM-12 – males treated with 12 mg kg^{-1} bm FLX, CF – control females, TF-6 – females treated with 6 mg kg^{-1} bm FLX, TF-12 – females treated with 12 mg kg^{-1} bm FLX.

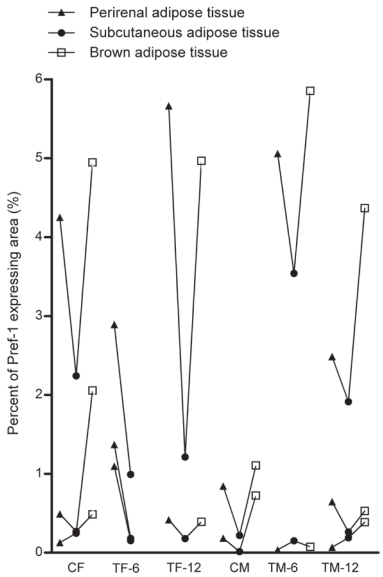

Fig. 3. The level of Pref-1 expression (percentage areas of sections) in different types of adipose tissues after FLX treatment compared to control groups. CM – control males, TM-6 – males treated with 6 mg kg^{-1} bm FLX, TM-12 – males treated with 12 mg kg^{-1} bm FLX, CF – control females, TF-6 – females treated with 6 mg kg^{-1} bm FLX, TF-12 – females treated with 12 mg kg^{-1} bm FLX. Note: No evaluable results were obtained from the brown interscapular adipose tissue of female animals treated with 6 mg kg^{-1} bm FLX.

vacuolar area (185.27 μm^2) compared to the female control group (209.78 μm^2, $p < 0.0001$) and to the group treated with the low dose of FLX (286.13 μm^2, $p < 0.0001$) (Fig. 2b).

There might be several reasons for the observed differences between the genders. For instance, female animals were not ovariectomized nor their estrous cycle was synchronized, and subsequently a hormonal heterogeneity appeared. At the same time, FLX inhibited the aromatase enzyme with a key role in estrogen synthesis, consequently disturbing the steroid metabolism (7). Therefore, it might be assumed that the observed results were influenced by the heterogeneity of steroid status.

Furthermore, Sun *et al.* (8) recently evidenced an anti-proliferative and inhibitory effect of FLX on the adipogenic differentiation in an *in vitro* model. In that study, lipid accumulation and PPARγ expression also decreased following FLX treatment. This observation is in accord with our previous results reporting mass loss tendency and decrease in visceral triglyceride content after FLX treatment, especially in female rats (9). Although the vacuole area is an important indicator of the adipocyte maturation process, it alone cannot characterize adipogenesis.

Pref-1 expression in different adipose tissues

Pref-1 has two forms, soluble and membrane-linked. The latter appears in the proximity of the cell membrane after immunostaining, as seen in Fig. 1b.

Analyses of the three types of fat tissue for each animal group showed no correlation between FLX administration and Pref-1 expression in any animal group for the three examined fat tissue types (Fig. 3). The Pref-1 expression level was lower in subcutaneous than in perirenal or brown adipose tissue in most of the analyzed sections. However, it must be highlighted that untreated males showed a somewhat lower level of Pref-1 expression than females. The treatment did not influence Pref-1 expression in female rats. Some male animals treated with 6 mg kg^{-1} FLX displayed intense expression of Pref-1, but the difference was not significant. It should be noted that dispersion of the data decreased the statistical significance of the results.

Explanation of these findings might include several mechanisms involved in the metabolic effects of fluoxetine. Stunes *et al.* (10) demonstrated that adipocytes have a complete functional system for 5-HT synthesis, reuptake and receptor activation. Adipocytes also exhibit elevated monoamine oxidase (MAO) levels, metabolizing the free circulating 5-HT to its metabolites, 5-hydroxy-indoleacetate and 5-methoxy-indoleacetate (11). Furthermore, these metabolites bind to the peroxisome proliferator-activated receptor γ (PPARγ) and enhance lipid accumulation in preadipocytes. The PPARγ activation results in altered gene expression and a subsequent increase in triglyceride accumulation and preadipocyte differentiation (12).

To the best of our knowledge, there is no other data in the literature about the relation between Pref-1 expression and FLX treatment in rats. Our study, however, suffers from some limitations, such as that, besides Pref-1, there are several other adipokines that govern the adipose tissue maturation and adipocyte function, which should be taken into account as well.

CONCLUSIONS

In conclusion, chronic FLX administration had some important effects on fat tissue maturation and adipocyte differentiation: treated male rats displayed increased vacuolar areas while female rats treated with the low dose of the drug had increased vacuolar areas whereas the high dose slightly lowered the vacuolar area compared to the control group. Chronic administration of FLX did not influence Pref-1 expression; a great dispersion of values occurred in all three types of fat tissues examined, white visceral (perirenal), white subcutaneous and interscapular brown adipose tissues. In light of this study, it can be stated that chronic FLX treatment seems to increase the adipocyte vacuolar area under *in vivo* conditions and thus might have a pro-differentiating effect on adipocytes, but it does not affect Pref-1 expression in a consistent way. In short, it was found that FLX did not exert any peripheral effect related to Pref-1 expression, but it increased the size of lipid vacuoles in perirenal adipocytes. To draw more accurate conclusions about the peripheral pro- or anti-adipogenic effect of FLX, evaluation of the expression of several other local factors is needed, and comparison of *in vitro* and *in vivo* data is urged for a deeper understanding of the entire phenomenon.

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REFERENCES

1. S. Adams, D. Heckard, J. Hassell and L. Uphouse, Factors influencing fluoxetine-induced sexual dysfunction in female rats, *Behav. Brain Res.* **235** (2012) 73–81; https://doi.org/10.1016/j.bbr.2012.07.029
2. M. Afkhami-Ardekani and H. Sedghi, Effect of fluoxetine on weight reduction in obese patients, *Indian J. Clin. Biochem.* **20** (2005) 135–138.
3. S. R. Blumenthal, V. M. Castro, C. C. Clements, H. R. Rosenfield, S. N. Murphy, M. Fava, J. B. Weilburg, J. L. Erb, S. E. Churchill, I. S. Kohane, J. W. Smoller and R. H. Perlis, An electronic health records study of long-term weight gain following antidepressant use, *JAMA Psychiatry* **71** (2014) 889–896; https://doi.org/10.1001/jamapsychiatry.2014.414
4. J. P. Domecq, G. Prutsky, A. Leppin, M. B. Sonbol, O. Altayar, C. Undavalli, Z. Wang, T. Elraiyah, J. P. Brito, K. F. Mauck, M. H. Lababidi, L. J Prokop, N. Asi, J. Wei, S. Fidahussein, V. M. Montori and M. H. Murad, Clinical review: Drugs commonly associated with weight change: a systematic review and meta-analysis, *J. Clin. Endocrinol. Metab.* **100** (2015) 363–370; https://doi.org/10.1210/jc.2014-3421
5. C. S. Hudak and H. S. Sul, Pref-1, a gatekeeper of adipogenesis, *Front. Endocrinol.* **4** (2013) 1–6; https://doi.org/10.3389/fendo.2013.00079
6. S. Caccia, M. Cappi, C. Fracasso and S. Garattini, Influence of dose and route of administration on the kinetics of fluoxetine and its metabolite norfluoxetine in the rat, *Psychopharmacology* (Berlin) **100** (1990) 509–514.
7. N. W. Jacobsen, C. H. Hansen, C. Nellemann, B. Styrishave and B. Halling-Sørensen, Effects of selective serotonin reuptake inhibitors on three sex steroids in two versions of the aromatase enzyme inhibition assay and in the H295R cell assay, *Toxicol. in Vitro* **29** (2015) 1729–1735; https://doi.org/10.1016/j.tiv.2015.07.005
8. B. K. Sun, J. H. Kim, J. S. Choi, S. J. Hwang and J. H. Sung, Fluoxetine decreases the proliferation and adipogenic differentiation of human adipose-derived stem cells, *Int. J. Mol. Sci.* **16** (2015) 16655–16668; https://doi.org/10.3390/ijms160716655
9. M. Kolcsár, Z. Gáll, I. L. Bíró, L. I. Bába, A. Imre, M. T. Dogaru and O. Martha, Fluoxetine influence on body weight, serum adiponectin level and adipose tissue triglycerides levels in rats, *Farmacia* **6** (2016) 599–604.
10. A. K. Stunes, J. E. Reseland, O. Hauso, M. Kidd, K. Tommeras, H. L. Waldum, U. Syversen and B. I. Gustafsson, Adipocytes express a functional system for serotonin synthesis, reuptake and receptor activation, *Diabetes Obes. Metab.* **13** (2011) 551–558; https://doi.org/10.1111/j.1463-1326.2011.01378.x
11. S. Grès, S. Canteiro, J. Mercader and C. Carpéné, Oxidation of high doses of serotonin favors lipid accumulation in mouse and human fat cells, *Mol. Nutr. Food Res.* **57** (2013) 1089–1099; https://doi.org/10.1002/mnfr.201200681
12. S. Grès, S. Gomez-Zorita, A Gomez-Ruiz and C. Carpene, 5-Hydroxytryptamine actions in adipocytes: involvement of monoamine oxidase-dependent oxidation and subsequent PPARγ activation, *J. Neural. Transm.* (Vienna) **120** (2013) 919–926; https://doi.org/10.1007/s00702-012-0959-8