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Research

Adipogenesis and reduction of lipid accumulation in retroperitoneal fat pad are stimulated by food deprivation.

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CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

ABSTRACT:
This study aimed to test the hypotheses that lipid drop density (d_L), lipocrite (Li) and mean diameter (Dm), volume (V) and mass (MAC) of adipocytes provide reliable values of the number of adipocytes in vivo (NA), and that fasting alters NA. Mean diameter (µm) and lipocrite were respectively measured by microscopy and by the ratio between relative volume occupied by isolated adipocytes in a fixed volume suspension obtained from fixed sampled mass of retroperitoneal fat pad (MAT) from rats maintained under normal feeding (NF) or submitted to food deprivation (FD) for 24 h or 72 h. $V(mL)=(\pi Dm^3/6)$, $MAC(g)=d_L x V$, and NA were calculated by (i) $(Li/V).10^{12}$ and (ii) MAT/MAC. Li was higher in FD-24h and FD-72h than in NF, while Dm was lower in FD-72h than in NF and FD-24h. NA, calculated by (i) and (ii), was higher in FD-72h than in NF and FD-24h. Increased Li in FD-72h should be due to hyperplasia, assuming that it does not occur through increased Dm (hypertrophy), since FD-72h had lower Dm than NF. Data suggested that in vivo adipogenesis occurs in retroperitoneal fat pad after 72 h of fasting as a homeostatic response to lipolysis and an adjustment to provide increased energy storage when there is new food supply.

KEY WORDS: fasting, starvation, adipocyte, adipogenesis, lipid droplet

INTRODUCTION

Food deprivation for periods ranging from 24 to 72 h has been applied experimentally for more than two decades to evaluate changes in various neuroendocrine factors that interact in nutritional and energy balance. In this way, this kind of methodology has its efficiency proved by the results reported in several publications (1-14).

The metabolic response to total food deprivation can be divided into three stages (15, 16). The first stage is a short period, in which occurs glycogenolysis (15, 16). The second stage is characterized by the preservation of protein content with the energy's needs supplied predominantly by lipid oxidation and the lipolytic rate increasing 2.5-times after 3-4 days (15, 16). The last stage is characterized by increased blood levels of urea and corticosterone (15). Among the endocrine changes known to occur during food deprivation are decreased plasma levels of insulin and leptin, increased glucocorticoids (17), GH (stimulation of lipolysis) (17, 18) and ghrelin (19), and decreased thyrotropin (TSH), triiodothyronine (T_3) and thyroxine (T_4), with consequent reduction of lipolysis and proteolysis [20, 21].Among these factors, glucocorticoids are also potent inducers of *in vitro*adipogenesis(22).

The differentiation process of mesenchymal stem cells in the stroma of adipose tissue into

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preadipocytes occurs when these multipotent cells lose the ability to differentiate in other mesenchymal lineages and become committed to the adipocyte lineage (21). After adipocyte lineage commitment, the preadipocytes become mature adipocytes, accumulating lipid droplets and showing the ability to respond to hormones (21). The knowledge about the process of adipogenesis has increased significantly (23-27), but with data obtained from *in vitro*studies. Meanwhile, *in vivo* adipogenesis has been hypothesized under several pathophysiological situations, but there is lack of demonstration due to absence of standardized, simple and efficient methodology to monitor this process (21). Results of experimental counting of adipocytes using Coulter Counter technology and submitted to a complex mathematical analysis, specifically developed for this purpose, allowed the development of a mathematical model of adipose tissue's growth, providing the most recent and consistent evidence for the existence of *in vivo* adipogenesis (28). However, this is an excessively laborious and expensive approach for routine calculations and/or for comparative purposes.

This study measured mean adipocyte diameter (Dm) in randomized adipocytes, as well as lipocrite (Li) of fixed volume suspension of isolated adipocytes obtained from fixed mass of retroperitoneal fat pad (MAT) from rats under normal feeding (NF) or food deprivation (FD) for 72h, and thus calculating adipocyte volume (V) and adipocyte mass (MAC) in order to test the hypotheses that these calculations provide approximate reliable values of the number of adipocytes *in vivo* (NA), as well as that FD alters NA.

MATERIALS AND METHODS

Animals, treatments and biometry

Male Wistar rats, 90 days old, 350-377 g, were housed in a polypropylene box (inside length × width × height, 56 cm × 35 cm × 19 cm), with commercially available food Nuvilab® CR-11, composed of 22% protein, 55% carbohydrate, 4% lipids, 9% fibers, 10% vitamins and minerals (total of 3kcal/g) and tap water *ad libitum*, in a ventilated container (AlescoInd Com, Monte Mor, SP, Brazil), with a controlled temperature (24±2°C), relative humidity (65±1%), and 12:12-hour light/dark photoperiod (lights on at 6:00 AM). These animals were divided into three groups: NF, animals that continued with food *ad libitum* for 72 hours, and FD-72h, animals submitted to food deprivation, which was performed by transferring pairs of animals, between 7:30-9:00 of the light period, into metabolic cages without food for 72 hours; and FD-24h, animals submitted to food deprivation, which was performed by transferring pairs of animals, between 7:30-9:00 of the light period, into metabolic cages without food for 24 hours, to confirm the accuracy of the methods showed in this study. During this period drinking water was provided *ad libitum* for both groups.

Body weight (BW) and nasoanal length (NAL) were measured and the Lee index ($BW[g]^{0.33}$ / NAL[cm]) of each animal was calculated (11-13).

The conducts and procedures involving animal experiments were approved by the Butantan Institute Committee for Ethics in Animal Experiments (License number CEUAIB 684/09) in compliance with the recommendations of the National Council for the Control of Animal Experimentation of Brazil (CONCEA). All efforts were made to minimize suffering.

Adipocyte isolation

After euthanasia by decapitation, retroperitoneal fat pad was removed through the manual dissection and washing with 0.9% NaCl solution. Total mass (g) of fat pad was measured and then MAT (3g) was separated and submitted to collagenase digestion (29, 30) as following: this fat pad mass was added to 9 mL DMEM (Cultilab, Brazil) containing 25 mM HEPES (pH 7.5), 4% bovine serum albumin (BSA) and 45 mg collagenase and incubated at 37°C, for 1 h, under gentle shaking. Subsequently, the incubate was washed with 8 volumes of buffered washing solution (pH 7.4, at 25°C) of 115 mMNaCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.3 mMKCl, 1.4 mM $CaCl_2 \cdot 2\ H_2O$, 0.89 mM $NaH_2PO_4 \cdot H_2O$, 25 mM HEPES, 1 mM Na pyruvate, 145 mM BSA. Then, the incubated was filtered through a nylon mesh and this filtrate was centrifuged at 200 rpm for 1 min, at 25°C. The pellet containing vascular stroma (capillary, endothelial cells, mast cells, macrophages and epithelial cells) was removed by suction and discarded, while the supernatant, containing the suspension of adipocytes, was washed and recentrifugated at the same conditions for 3 more times. The resultant suspension of isolated adipocytes was microscopically observed to verify the absence of vascular stroma.

Li measurement

The ultimate suspension of isolated adipocytes of each animal was homogenized individually by inversion and 0.05 mL was transferred to a microtube and then aspired to microhematocrit capillary tubes. After placed in a polystyrene tube of 15 mL these capillary tubes were centrifuged at 400 X *g* for 2 min, at 25°C. The adipocytes formed a phase at the top of the capillary. The column height of this phase containing the adipocytes and of the phase containing the total volume of solution were

measured by a ruler and the ratio between them was calculated in this order, according to Di Girolamo et al. (31).

Dm measurement

The ultimate suspension of isolated adipocytes of each animal was homogenized individually by inversion and 0.01 mL was applied over a histological slide. A plastic reinforcement was placed at both ends of the slide before it was coverslipped with coverglass in order to prevent cells' deformation. Under brightfield optical microscopy, the individual diameter of one hundred adipocytes randomly chosen was measured by the software Image-Pro ® Plus (Media Cybernetics) (Figure 1) in order to calculated Dm.

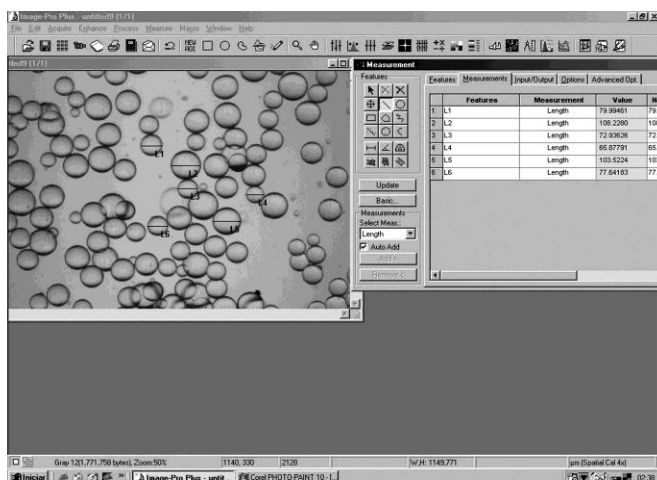


Figure 1. Illustration from computerized procedure for measuring the mean diameter of individual isolated adipocytes, using Image-Pro ® Plus software (Media Cybernetics).

Estimation of adipocytes number

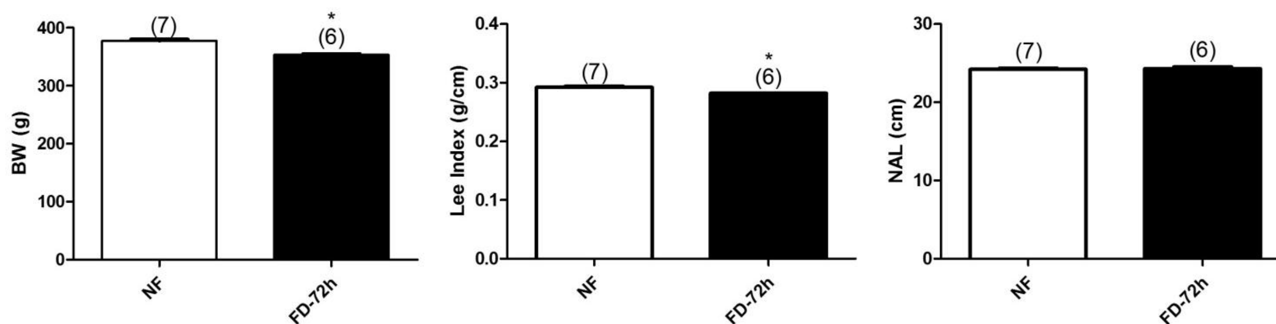
Given that MAT = 3g and lipid droplet density (d_L) = 0.91g/mL (32, 33), thus $V(mL)=(\pi Dm^3/6)$and $MAC(g)=d_L x V$ were calculated and the results used to obtain NA by calculation of (i) $(Li/V).10^{12}$ (number of isolated adipocyte per mL of MAT suspension)and (ii) MAT/MAC (number of isolated adipocyte in MAT).

Statistical analysis and presentation of results

Data were presented as mean ± S.E.M and statistically analyzed (FD-72h versus NF) by unpaired 2-side Student's *t* test ($p<0.05$) or ANOVA, followed by Multiple comparison Tukey test, when differences were detected, was used to compare the values among NF, FD-72h and FD-24h, using GraphPad Prism® software.

Percentual from starting body mass of food deprived rats after 72 h was 94.9±0.45(BW: NF, 377±2.8; FD-72h, 353±1.7), resulting in Lee index lower in FD-72h (0.282±0.0005) than in NF (0.292±0.002) with similar NAL between FD-72h (24.2±0.15) and NF (24.3±0.21) (Figure 2).No difference of total mass of periepididymal fat pad was show between NF and FD-72h (NF: 5.1±0.05; FD-72h: 5.2±0.1), but the total mass retroperitoneal fat pad was lower in FD-72h (2±0.09) than in NF (4.1±0.09) (Figure 3). As shown in Figure 4 Dm was lower in FD-72h (77.7±2) and FD-24h (98.8±4) than in NF (107.1±0.74), Li was higher in FD-72h (0.20±0.020) and FD-24h (0.18±0.009) than in NF(0.06±0.004), and Li/V and MAT/MAC were higher in FD-72h (Li/V: $7.48±0.52x10^6$; MAT/MAC: $13.74±1.13x10^6$) than in NF (Li/V: $0.95±0.05x10^6$; MAT/MAC: $5.13±0.1x10^6$) and FD-24h ((Li/V: $3.97±0.43x10^6$; MAT/MAC: $6.16±2.45x10^6$).

Figure 2. Comparison of body weight (BW), nasoanal length (NAL) and Lee index between rats maintained under normal feeding (NF) and food deprived for 72h (FD-72h). Mean values ± standard error of the means (S.E.M.) for n animals (over the bars), analyzed statistically using the GraphPadPrism^{tm} software package. *P<0.001, unpaired 2-side Student's *t* test.



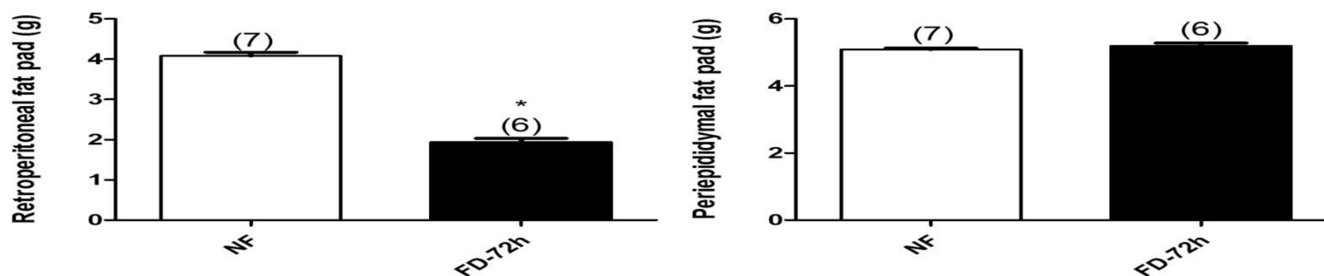


Figure 3. Comparison of retroperitoneal and periepididymal fat pad mass between rats maintained under normal feeding (NF) and food deprived for 72h (FD-72h). Mean values ± standard error of the means (S.E.M.) for n animals (over the bars), analyzed statistically using the GraphPadPrismtm software package. *P<0.001, unpaired 2-side Student's t test.

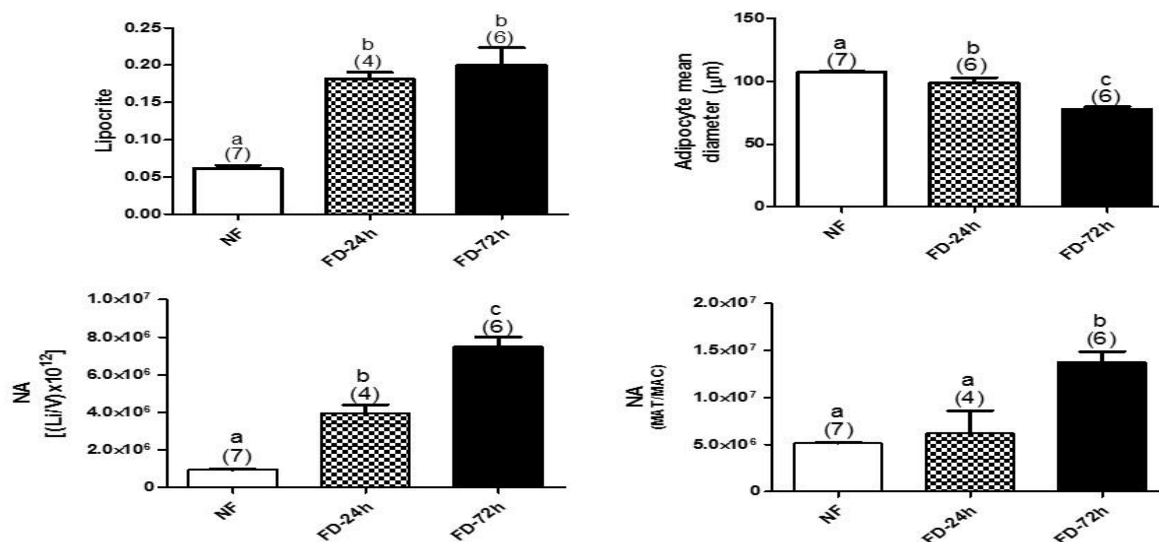


Figure 4.Lipocrite (Li), mean diameter of adipocytes (Dm) and number of adipocytes (NA) estimated by Li/V and by MAC in sampled 3 g of retroperitoneal fat pad (MAT) in rats maintained under normal feeding (NF), food deprived for 24h (Fd-24h) and food deprived for 72h (FD-72h). Mean values ± standard error of the means (S.E.M.) for n animals (over the bars), analyzed statistically using the GraphPadPrismtm software package. ANOVA, P<0.05; multiple comparison Tukey test, P<0.05, different letters (a,b,c) indicate significant differences among NF, FD-24h and FD-72h.

DISCUSSION

Two factors can explain whole body mass loss observed during 72-h starvation (5.1%): loss of muscle and/or adipose tissue. About 25% reduction in retroperitoneal fat pad mass shows the relevant contribution of this visceral adipose tissue depot to the loss of whole body mass.

The differences related to adipocytes morphology in subcutaneous and visceral depots are well known (34). On the other hand, the regional distribution of subcutaneous and visceral depots is related to metabolic differences, and the visceral adipose tissue is the major influence on metabolic balance (35, 36). In turn, the regional distribution of visceral adipose tissue depots is also related to metabolic differences (37). Therefore, the interpretation of our presented results may not be applicable to other kind of visceral fat pad and/or other fat type.

Decreased mass of retroperitoneal adipose tissue in food deprived for 72h should be attributed to decreased adipocyte size (diameter), due to increased lipolysis (16, 17,34) and/or to concomitant decreased number of adipocytes. In turn, increased lipocrite observed in food deprived for 72h should be due to increased cell diameter (hypertrophy) and/or to increased number of cells (hyperplasia). Assuming that increased lipocrite in food deprived for 72h was concomitant with decreased adipocyte diameter and decreased mass of retroperitoneal adipose tissue, then the adipocyte hyperplasia in this tissue induced by food deprivation for 72h can be clearly deduced. The adipocyte hyperplasia is

known to exert less significant contribution than the hypertrophy to increase the mass of adipose tissue (28). This fact could explain our finding of hyperplasia with concomitant reduction in retroperitoneal depot mass after food deprivation. Estimates the number of adipocytes by lipocrite or adipocyte mass calculations had the same order of magnitude. The differences observed can be easily explained, since the calculation using the adipocyte mass disregards the density of other intracellular components of the adipocyte besides the lipid, resulting in an overestimated mass. Furthermore, the existence of altered number of cells other than adipocytes in the retroperitoneal adipose tissue from food deprived is strongly suggested by this study. Values of Dmx(Li/V) for food deprived and controls should be similar if smaller mean diameter of adipocytes in the same mass of retroperitoneal fat pad would be concomitant with higher Li/V in food deprived than in controls. However, these values were 581.19 in food deprived and 101.74 in controls, suggesting a concomitant increase in the population of other cell types, in addition to adipocytes, in food deprived. The level of stress caused by starvation (38) could increase the number of immune cells in retroperitoneal adipose tissue, thus contributing to this difference between food deprived and controls.

Given these results, the association between the lipocrite and the mean volume of adipocytes was shown to provide a reliable estimate of the number of adipocytes in cell suspension from a fixed sampled mass of fat pad. Regarding the methodology designed by Jo et al. (28), our methodology is less accurate, but it is relatively more simple and can be considered enough efficient to estimates for routine and comparative purposes.

In the particular case of food deprivation, our methodology clearly demonstrated the occurrence of adipogenesis, which may be associated with several well-characterized hormonal stimuli (17, 23, 25-27). During starvation, glucocorticoids increases protein degradation, lipolysis and stimulates the gluconeogenesis, providing energy substrate required for homeostasis (17). Additionally, glucocorticoids are potent inducers of *in vitro*adipogenesis, since it activates the expression of two major initiators of adipogenesis, the PPAR gamma (peroxisome proliferator-activated receptor gamma) and family proteins C/EBP (CCAAT/enhancer-binding protein) (23, 25-27). Besides growth hormone (GH) also having an important role in energy supplying during food deprivation, stimulating lipolysis (17), its role in adipogenesis is still contradictory. In addition to biochemical factors, adipogenesis is also induced by biomechanical factors such as the rigidity of the extracellular matrix (39), and thus a lower stiffness could be hypothesized as a factor related to adipogenesis in food deprived animals.

In conclusion, the existence of the association between 72 h fasting and *in vivo*adipogenesis was demonstrated for the first time in the rodent retroperitoneal fat pad, being suggestive of a homeostatic response to lipolysis, probably as an adjustment to provide increased energy storage when there is a new food supply.

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AUTHOR CONTRIBUTIONS:

P.L.A. performed experiments, analyzed data, and interpreted results of experiments. P.F.S. analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised the manuscript and conceived and designed the experiments. R.F.A. performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised the manuscript and conceived and designed the experiments. All authors approved the final version of the manuscript.

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