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Research Article

A Stereological Estimation of Neuron Number, Density and Nucleus Volume of Median Eminence of Albino Wistar Rats Fed a High-fat Diet

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Obesity, Stereological method, Median eminence, High-fat diet, Neuron number, Nucleus volume

ABSTRACT

Objectives: Obesity is a complex medical condition that is caused by excess accumulation of body fat. It has a huge social and economic impact and it is the most stigmatized condition in the world. Understanding the Neuroscience of the disease will assist in understanding the devastating effect of the epidemic in the world.

Methods: Twenty young Wistar rats, aged 4 weeks, of varying weight (37.8g – 69.3g) were grouped into the control and a high-fat diet group. The control group were fed with standard rat chow and the experimental group with high-fat diet for 16 weeks. The animals were humanely sacrificed and the brain removed. The post-fixed brain tissue was sectioned with a microtome using systemic uniform random sampling (SURS) technique at a section thickness of 3µm and a section sampling fraction of 0.015. Sections were stained with toluidine blue. The neuron density, neuron number and nucleus volume of Median eminence were estimated using unbiased stereological method.

Result and Conclusion: Long-term consumption of high-fat diet (HFD) decreases the numerical density of neurons and increases the volume of median eminence (ME) in the hypothalamus. These findings may indicate that an increase in ME volume and a decrease in neuron density and number maybe due to inflammation and gliosis (as earlier reported) caused by HFD that leads to a reduction in leptin receptors found on neurons and subsequent obesity.

INTRODUCTION

Today there is no group that is more stigmatized than the morbidly obese. It has not changed appreciably from the time of Galen, who clearly held obese individuals responsible for their condition, or of Shakespeare, who, through Henry IV, hurled a barrage of insults at Falstaff, referring to him as "fat - witted", "chair- breaker" and "mare - crippler. Hippocrates wrote that the obese should "eat only once a day and take no baths and sleep on a hard bed and walk naked as long as possible.[1] Obesity is a complex medical condition caused by an accumulation of excess body fat (calorie intake exceeds calorie expenditure) leading to negative effects on health. It is the greatest public health problem in the western world (now arguably a problem of the developing world), and is associated with dreaded life-threatening co-morbidities, such as diabetes, cardiovascular diseases (mainly heart disease and stroke, hypertension).[2] Thus, having a huge social and economic impact, this has made it a devastating disease.

Obesity is diagnosed when body mass index (BMI) exceeds a defined threshold. People are said to be obese if their BMI exceeds 30kg/m^2. Above these BMIs (> 45kg/m^2, morbid obesity), more or less, the health risks of an increased weight become increasingly evident.[3] The risk for non-communicable diseases increases, with increases in BMI. Individuals with obesity (BMI > 40kg/m^2 or higher) have a higher risk of developing severe complication of Severe acute respiratory syndrome, corona virus 2 (SARS- CoV-2, COVID-19) (CDC, 2020). Obesity could shift severe COVID-19 disease to younger ages.[4]

Consumption of dietary fats is amongst the most important environmental factors that can lead to the development of obesity.[5] High-fat diets in rodents are generally considered to produce obesity, similar to that seen in humans. High-fat diets in the literatures are labeled such when fat composition is anywhere from 20-60%.[6,7]

Leptin hormone is a pleiotropic peptide hormone secreted mainly by but not exclusively by white adipose tissue cytokine [9,10], it functions as an afferent signal in a negative feedback loop, acting primarily on neurons in the hypothalamus and regulating feeding and many other functions.[11] Leptin acts on receptors in the hypothalamus where it inhibits appetite, counteracting the effects of neuropeptide Y (a potent feeding stimulant secreted by cells

in the gut and in the hypothalamus), and also promote the synthesis of alpha melanocyte stimulating hormone (α-MSH); an appetite suppressant.[12-14] The absence of leptin (or its receptor) leads to uncontrolled food intake and resulting obesity.[15] The leptin endocrine system serves a critical evolutionary function by maintaining the relative constancy of adipose tissue mass, thereby protecting individuals from the risks associated with being too thin (starvation and infertility) or too obese (predation).[16]

The median eminence (ME) is a circumventricular organ which is a specialised structure situated adjacent to the third ventricle, aqueduct of midbrain and the fourth ventricle. It has fenestrated capillaries and sinusoids.[17] The median eminence is located near the arcuate nucleus (ARCN), which is critical for the regulation of feeding and metabolism.[18] The ME facilitates transport of peripheral hormonal and nutrient signals and their sensing by the ARCN neurons.[19] The ARCN integrates hormonal and nutritional metabolic signals from the peripheral circulation as well as peripheral and central neuronal inputs to generate a coordinated feedback response.

The hypothalamus apart from its numerous functions is one of the most important centers that regulate body weight, appetite and energy balance,[20] many studies have been done regarding nutrition and hypothalamus. However, most of the studies concerning the effects of HFDs have been on molecular basis. As the hypothalamic nuclei are involved in the regulation of food intake, appetite control and energy balance[21,22] and HFD may affect some or all of these nuclei, therefore we decided to evaluate whether the consumption of high amounts of dietary fat can change the numerical density and neuron number, as well as the volume of the ME in the hypothalamus.

MATERIALS AND METHOD

This study was conducted to assess the effect of long-term (16 weeks) exposure of rats to a standard diet (control groups) and HFD (experimental groups). Twenty young Wistar rats of varying weight (37.8g – 69.3g) were obtained from the Laboratory Animal House of University of Jos at 4 weeks of age. Rats were housed in groups of five to acclimatize for two weeks prior to the study under 12-hour light/dark cycle and were fed food and water *ad libitum*. Standard protocol on animal use and care was adhered to. All procedures in this study were conducted based on the guidelines of the Ethical Committee of Our Lady of Apostles Hospital, Jos, Plateau State.

Experimental Animals

The rats were grouped into control (n = 6) and high-fat diet (n = 9) group. The animals were humanely sacrificed, and the brain removed. The post-fixed brain tissue was sectioned with a microtome using systemic uniform random sampling (SURS) technique at a section thickness of 3µm and a section sampling fraction of 0.015. Sections were stained with toluidine blue. The neuron density, neuron number and nucleus volume of Median Eminence were estimated using unbiased stereological method.

Body Mass Index (BMI) determination

BMI was calculated using the formula:

$$\text{Weight (g)} / \text{Length (cm)}^2$$

It was recorded Weight in grams (g) and the length (cm) - measured from the snout / nose to the beginning of the tail (tail root).

Experimental design

The two group of rats (control and experimental groups) were provided with Standard rat chow (control/normal diet; ND); MF nutrition plus (LF080119RF) and High-fat diet (Experimental diet, (HFD); MF nutrition plus (HF080119RF) from Grand Cereal Jos, Plateau State, Nigeria) with water *ad libitum* for a period of 16 weeks to induce obesity.[22]

Table 1: Constituent of HFD

Item	g/Kg of diet
Casein	164
Corn starch	303.1
Dextrose	115
Sucrose	89.9
Butter oil	190
Cellulose	58.6
Soyabean oil	10
Mineral mix	41
Vitamin mix	11.7
L-cysteine	2.1
Choline bitartate	2.9

Histological and stereological studies

Brain tissue: Dissection and removal

At the end of the experiment, the rats were anaesthetized with Isofluorine in a glass chamber and euthanized with Natrium Pentobarbital (0.5 ml/kg body weight) via the intra-muscular (im) route. The brains were carefully removed from the skull using careful dissection.

Histological studies

Fixation: Fixation was carried out for 24hrs. A critical step that preserves cell and tissue component in a life like manner. It maintains their structure and slows down tissue degradation. Microanatomical (general) fixation method was used - which aids the embedding media (Paraffin wax) to penetrate tissue. Formaldehyde – 10% formal saline, 90ml distilled water (dH_2O), 0.9g NaCl was used.

Dehydration: Different concentration of absolute alcohol was used to dehydrate the tissue; 50%, 70%, 80%, 90%, 95%, absolute I (100%), absolute II (100%)

Claring (De-alcoholization): Xylene was used as a claring agent to remove the alcohol.

Infiltration/Impregnation: Paraffin wax was used to remove Xylene, air and to fill micro holes or spaces in the tissue.

Embedding (encasing): Paraffin wax was used to embed the tissue on a plastic cassette. It involves supporting the tissue in media that have similar mechanical rigidity to the specimen itself. An embedding machine was used to pour a melted paraffin wax on the tissue that is in a rectangular steel mold, the plastic cassette was then laid on the mold and the paraffin wax was poured on it again. And allowed to stand and solidify.

Sectioning and Stereological studies: The brain tissue on the plastic cassette was mounted on a microtome holder and coronally sectioned using a Systemic Uniform Random

Sampling (SURS) technique at a section thickness of 3µm. The sections used for the estimation of the volume of the Nucleus of interest (NOI) and the total number of neurons were selected at regular intervals of 200 µm, from the coronal sections containing the NOI, by applying a systemic random sampling procedure.[24] The length of the Hypothalamus from the beginning of the NOI was estimated to be 3200µm.[25], the 15% shrinkage in length.[26], due to the Formalin Fixated Paraffin Embedded (FFPE) treatment was also considered and a length of 2800µm was used. This length (2800µm) was divided by our cutting interval (200µm) that can cover the smallest NOI and a value of 16 series was arrived at, the first section was randomly sampled and the subsequent sections were systematically sampled – the 2nd and 3rd sections, which is equivalent to 9µm * 2 = 18µm (including the 1st section). Two consecutive/serial sections (dissection) were sampled and mounted on the same slide. One hundred and eighty-two (182) sections were then discarded / wasted before the next series sampling is commenced. However, the number of discarded sections is increased if the sections to be sampled were missed in the initial point of sampling. Care was taking to ensure that the sections were sampled consecutively within the same series. The section sampling fraction was 3/200 = 0.015. All the estimations were performed by using Visio pharm pathology software (VIS 2019.02, 2019.02.1.6005, Visiopharm, DK-2970 Hoersholm, Agern Alle Denmark). The block tissue was intermittently place on a cold plate at -20^{0}c when it gets warm before cutting again, so as to a get a thin section (3µm). Cut sections were picked using a picker and placed into a warm bath (34^{0}C) guided by a paint brush. The section was strengthened out and picked using a glass slide (super frost – normal slide). It was then dried overnight in an oven at 360c.

Histochemistry Protocol

Toluidine blue was used as a nuclear staining agent after deparaffinization, dehydration and claring of the Formalin Fixated Paraffin Embedded (FFPE) tissue.

Light microscopy: A live modified research light microscope was used to view the sections on the slide at magnification ranging from 10x to 60x, the microscope, connected to a camera, which transmits the microscopic image to a computer monitor, and an electronic microcator with digital readout (ND 281, Heidenhain, D-83301, 2006, Traunreut, Germany).

Volume estimation: The volume was unbiasedly estimated by applying the principle of Cavalieri.[27] In all selected sections, the profiles of the NOI - Median eminence (ME) was delineated at magnifications ranging from 10x to 20x across the sections. In each section, the cross-sectional area of the ME was estimated by point counting with the use of a system of test points superimposed onto a monitor of a computer, in which the area per point (a/p) – equal to the area associated with one point in the grid, was calculated (a/p = *dx x dy* / magnification^2), the volume of the ME was calculated from the total points that fell on the it (recorded as sum of P = ∑P), and the distance between the systematically sampled sections (T).[28-30] This value was calculated taking into account the section sampling fraction (ssf) and the section thickness (t).

V = T * (a/p) * ∑P, V (mm^3) = volume, T = t * 1/1/ssf, t = 3µm (0.003mm), ssf = 3/200 = 0.015, T = 200µm (0.2mm), a/p (µm^2) – was automatically generated by the software using the formula (a/p = dx x dy / magnification^2)

The precision of the unbiased estimates of nucleus volume and neuron number was expressed by the coefficient of error (CE). The CE was calculated using the method by Gundersen.[30] An average of 5 (±1) sections were counted per brain. A total approximate of 200 points was counted per nucleus analysed. The mean CE of the estimates of the total volume and neuron number was 0.03 (3%) and 0.09 (9%) respectively. The Noise effect - the point counting variance, the Variance of ∑ area for Systemic Uniform Random Systemic (VAR_{SURS}) – a function of the number of sections and Coefficient of Variance is calculated using the formulas below:

Noise effect = $0.072 * (B/\sqrt{A}) * \sqrt{(n\sum P)}$

VAR_{SURS} (∑area) = $3(\sum(P_i * P_i) - \text{Noise}) - 4(\sum(P_i * P_i+1)) + \sum(P_i * P_i+_2) / 240$

Total variance of ∑P = Noise + VAR_{SURS}(∑area)

CE (∑P) = √(Total variance) / ∑P

Precision determination for Volume and Number

A. Coefficient of error (CE) calculation: For precision determination.

Table 2: Estimation of the Volume (V) of Median Eminence and the Coefficient of Error, CE (∑P) of the estimate in an Individual Rat[1]

Section	P_i	$P_i * P_i$	$P_i * P_{i+1}$	$P_i * P_{i+2}$
1	72	5184	7704	7632
2	107	11449	4708	4300
3	44	1936	748	0
4	17	289	697	0
5	41	1681	0	0
Sum (∑)	281	20539 (A)	13857 (B)	6791 (C)
a/p (mm^2)	0.000306			
t (mm)	0.003			

V (mm^3) = T * (a / p) * ∑P = 0.2 * 0.000306 * 281 = 0.017197

Noise effect = 0.017 * (B√A) * √(n∑P) = 0.0724 * 8 * 16.76 = 2.28[a]

VAR_{SURS} (∑area) = 3(A – Noise) – 4B + C / 240 = 3(20539 – 2.28) – 4 * 13857 + 6791 / 240 = 54.1[b]

Total variance of ∑P = Noise + VAR_{SURS} (∑area) = 2.28 + 54.1 = 56.4

CE (∑P) = √Total variance / ∑P = √56.4 / 281 = 0.03 (3%)

[1]Five sections were used in the analysis. Pi is the number of points counted on each of the 5 sections (i = 1 → 5). a/p is the area associated with each point.

[a]The variance of the point counting for each section was calculated according to Gundersen [30] and the boundary/area ratio B/√A) was estimated to be 8.[24]

[b]The Systematic Uniform Random Sampling Variance (VAR_{SURS}) was calculated with the quadratic approximation formula,[30] taking into account the noise effect.

* Table 2 showed the coefficient of error (CE) to determine the precision for the calculations of the nucleus volume (0.03), the calculated CE is adequate for the sample slide used.

Table 3: Estimation of the Total number, N_v of Median Eminence (ME) and the Coefficient of error, CE (∑P), of the estimate in an Individual[1]

Section	$\bar{Q}_i$	$\bar{Q}_i * \bar{Q}_i$	$\bar{Q}_i * Q_{i+1-}$	$Q_i * Q_{i+2-}$
1	32	1296	936	1296
2	26	676	936	260
3	36	1296	360	0
4	10	100	0	0
5	0	0	0	0
Sum (∑)	108	3368 (A)	2232 (B)	1556 (C)
∑P	72			
a/p = 14300/4	3575			
t (mm)	0.003			

$V\ (mm^3) = T * (a / p) * \sum P = 200 * 868.94 * 253 = 43968364$
$\mu m / 10^9 = 0.043968364\ mm^3$
$Nv\ (mm^3) = \sum \bar{Q} / t * (a/p) * \sum P = 108/3 * 3575 * 72 = 9266400$
$N = V * Nv = 0.043968364 * 9266400 = 407721.6$
Noise effect = $\sum \bar{Q}i = 108$
$Var(SURS, \bar{Q}i) = 3(A - Noise) - 4B + C / 240 = 3(3368 - 108) - 4 * 2232 + 1556 / 240 = 10.03$[a]
Total variance of $\sum \bar{Q}$ = Noise + VAR_{SURS} (∑area) = 108 + 10.03 = 118.03
$CE\ (\sum \bar{Q}) = \sqrt{}$Total variance / $\sum \bar{Q} = \sqrt{118.03 / 108} = 0.09\ (9\%)$

[1]Five sections were used in the analysis. Qi is the number of neurons counted in the dissector samples each of the 5 sections (i = 1 → 5).
[a]The Systematic Uniform Random Sampling Variance (VAR_{SURS}) was calculated with the quadratic approximation formula [26,30] taking into account the noise effect.
* Table 3.0 showed the coefficient of error (CE) to determine the precision for the calculations of the total neuron number (0.09), the calculated CE is adequate for the sample slide used.

Our calculated Coefficient of error (CE) to determine the precision of our estimates - nucleus volume (CE, 0.03), neuron number (CE, 0.09) - was adequate (Tables 2 & 3 respectively).

Statistical Analysis

Data obtained were expressed as Mean ± SEM. Kruskal-Wallis test, Independent student t test and One-way analysis of variance (ANOVA) were used to analyse the data and to evaluate the significant difference within and between the groups, a p-value < 0.05 was considered significant. All analysis was performed using IBM SPSS, version 26.0.

The precision of the individual estimates was evaluated as the coefficient of error (CE). The CE of the estimates of Nucleus volume and Neuron number were evaluated as a function of two independent factors: the noise effect and the variance due to sampling between systematically random sampled sections.

RESULTS

Below are the results obtained from the study.

A. BMI:

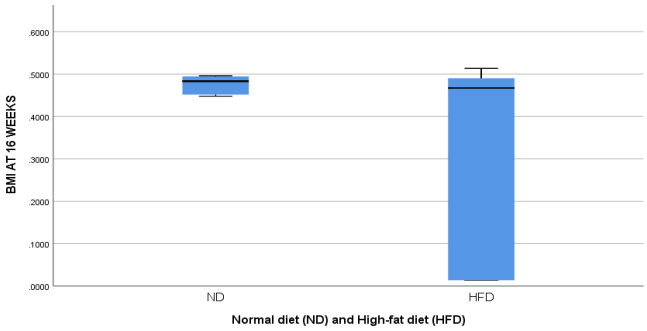

Figure 1: BMI in Normal and High fat diet
* There was no significant difference P > 0.05 (Asymp. Sig. = 0.41)
* t (13) = - 0.817, P = 0.43, d = 0.001, 95% CI [- 0.03, 0.01]
* ND – normal diet, HFD – high-fat diet, d = Standard Error Difference, CI = Confidence interval

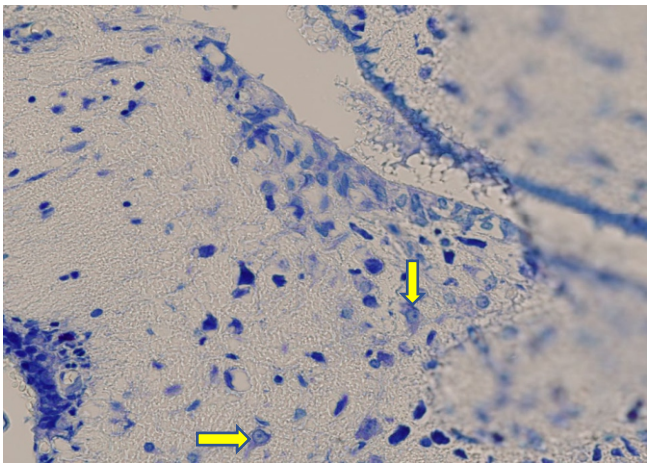

Figure 2: A histological photomicrograph showing neurons (inset: Yellow coloured arrows)

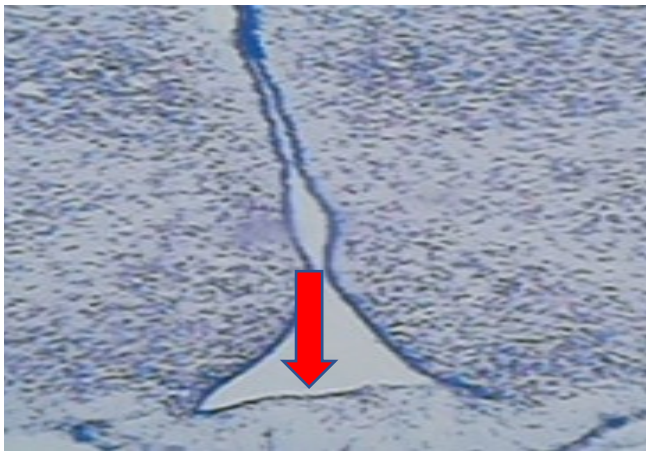

Figure 3: The median eminence (red arrow) [32]

B. RESULTS FOR NUCLEUS VOLUME, NEURON DENSITY AND NEURON NUMBER IN THE MEDIAN EMINENCE OF THE HYPOTHALAMUS

Median Eminence (ME):

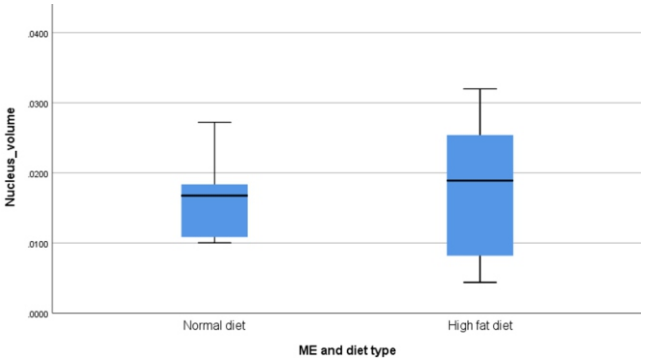

Figure 4: The Nucleus Volume in Normal and High-fat diet, it showed an increase in Nucleus volume with HFD.
* Nucleus volume was not statistically significantly different, P > 0.05 (P = 0.87)
* Nucleus volume, t (11) = - 0.17, P = 0.87, d = 0.01, 95% CI [-0.01, 0.01]
* d = Standard Error Difference, CI = Confidence interval

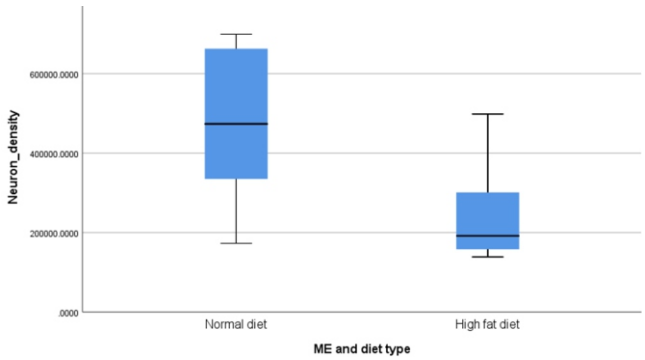

Figure 5: The Neuron density in Normal and High-fat diet. It showed a decrease in Nucleus volume with HFD
* Neuron density was statistically significantly different, P < 0.05 (P = 0.04)
* Neuron density, t (11) = 2.35, P = 0.04, d = 220090.45, 95% CI [13853.56, 426327.33]
* d = Standard Error Difference, CI = Confidence interval

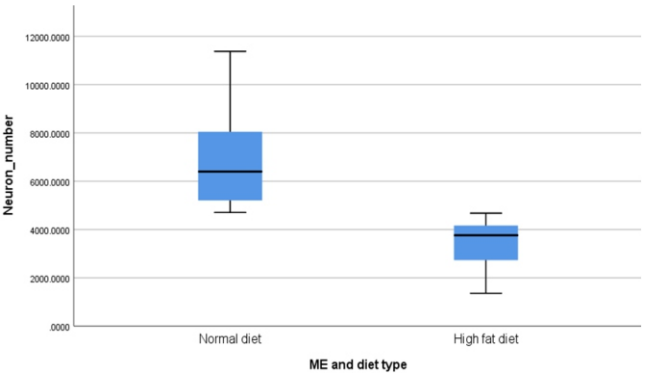

Figure 6: The Neuron number in Normal and High-fat diet, it showed a decrease in Nucleus volume with HFD

* Neuron number was statistically significantly different, P ≤ 0.05 (P = 0.05)
* Neuron number, t (11) = 3.52, P = 0.05, d = 1038.05, 95% CI [1369.31, 5938.76]
* d = Standard Error Difference, CI = Confidence interval

DISCUSSION

This is amongst the few studies to our knowledge and based on our literature search evaluating the effects of high-fat diet on the nucleus volume, neuron density and neuron number of the Median eminence in the hypothalamus. By using unbiased stereological technique, we showed that long-term feeding (16 weeks) of high-fat diet increases nucleus volume and decreases neuron density and neuron number in the Median eminence (ME) of the hypothalamus.

The leptin hormone receptor is a peptide hormone that is located on the surface of the neuron soma. Cells were identified as neurons if they had a nucleolus, dendritic processes, euchromatin material within the nucleus, and nuclei surrounded by cytoplasm.[31,32] A morphologically normal neurons in quality and quantity in the nuclei of interest of the hypothalamus can indirectly infer a normal level of the leptin hormone.

The median eminence is a specialised circumventricular organ that facilitates transport of peripheral hormonal and nutrient signals and their sensing by the arcuate nucleus (ARCN).[19] A study has shown that ablation of neurons (NG2-Glial cells) in the median eminence (ME) leads to selective degeneration of LepR dendrites in the arcuate nucleus, causing primary leptin resistance and obesity. These effects are reproduced with ME-directed X-irradiation, possibly explaining the obesity risk associated with cranial radiation therapy. [33]

In this study, the long-term use of high-fat diet insignificantly increases the BMI. The higher BMI in rat on HFD could be as a result of the cleavage of the leptin receptor (LR) found on the surfaces of neurons by activated matrix metalloproteinase-2 [22] or as a result of expression of pro-inflammatory cytokines and inflammatory responsive proteins in the brain and hypothalamus.[34] High-fat diet gives rise to reduced brain-derived neurotrophic factors that have direct anti-inflammatory effects in the brain, [35] as was earlier reported in the cited literatures. This deletion in the hypothalamus can result in hyperphagia and obesity. [21,36] These factors lead to leptin resistance resulting from inactivation of the Janus kinase/signal transduction and activation of transcription (JAK/STAT) signalling and subsequent increase in BMI and obesity. Simply put a reduction in neuronal cells will indirectly lead to reduction in leptin receptors and hence an increased risk of developing obesity.

The ME volume was insignificantly (P > 0.05) increased in rats fed high-fat diet. However, the neuron density and neuron number were significantly (P < 0.05) decreased. The finding supports the idea that high-fat diet has an effect on neuron density and neuron number.

Some studies have reported that volume of brain regions gives rather unspecific information about the function of that region, but structural changes may indicate a functional dysfunction of relevance taking place in that specific area.[37] The increase in the volume of ME in the hypothalamus in our study may be due to an increase in inflammation by high-fat diet, and an increase in the extracellular space. However further studies need to be carried out to ascertain this possibility. The reduction in neuron density is explainable by the fact that it is the number

of neurons per unit volume and when the volume of the ME in the hypothalamus is increased, neuron density decreases (since the number of neurons has not increased). However, the neuron density and to some extend the neuron number in the median eminence in high-fat diet fed rats was significantly decreased. These increase in volume and decrease in neuronal density and neuron number following a high-fat diet may suggest an increased intercellular space by induction of inflammation or gliosis as has been earlier reported,[38] neurogenesis in the hypothalamus[34] and increase apoptosis in the hypothalamus.[39] The studies cited above showed a non-significant reduction in neuronal cells. The result of our study showed an increase in nucleus volume (statistically insignificant) and a decreased in neuron density and number (statistically significant) in rats fed HFD.

CONCLUSION

Long-term consumption of HFD decreases the numerical density of neurons and increases the volume of the ME of the hypothalamus. These findings may indicate that the increase in the ME volume and decrease in neuronal density and number maybe due to inflammation or gliosis as earlier reported caused by HFD that leads to a reduction in leptin receptors found on neurons.

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Conflict of interest

There is no conflict of interest

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