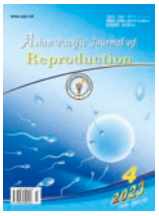

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Oxidized palm oil impairs reproductive functions and architectures in female rats

Wankeu-Nya Modeste[1]✉, Kengne Inès T[1], Ateba Benjamin S[1], Bend Fortune E[2], Djeumeni Ornela N[1], Hatho Dominique T[1], Ngadjui E[4], Moundipa Paul F[3], Massoma Dieudonné L[1], Dongmo Alain B[1], Watcho Pierre[4]

[1]*Department of Animal Organisms Biology, Faculty of Science, University of Douala, Douala, Cameroon*

[2]*Department of Animal Production, Faculty of Agronomy and Agricultural Sciences, University of Dschang, Dschang, Cameroon*

[3]*Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon*

[4]*Department of Animal Biology, Faculty of Science, University of Dschang, Dschang, Cameroon*

ABSTRACT

Objective: To evaluate the effects of three oxidized palm oil diets (OPD) on female rat reproductive function.

Methods: Forty-four female Wistar rats presenting five consecutive and regular estrous cycles were divided into 4 groups. The rats were fed with: a standard diet, 70% of standard diet+30% oxidized palm oil diet (OPD1), OPD1+5 g of boiled yolk egg (OPD2) and OPD1+10% sucrose (OPD3) for 125 days, respectively. During the feeding period, morphometric, estrous cycle, sexual behavior, gestation, biochemical and histomorphometric parameters were evaluated.

Results: All OPDs significantly increased abdominal circumference, body mass index and Lee index coupled to an irregularity and lengthening of the estrous cycle. They significantly decreased appetite and consumption behaviours, quantic pregnancy index, fertility rate, implantation sites and index, serum progesterone and high-density lipoprotein levels, increased pre-implantation losses, anti-implantation activities, serum estradiol, triglycerides, total and low-density lipoprotein-cholesterol levels, and impaired brain and ovaries oxidative status. Histomorphometric examinations revealed increases in the number of atresic and primary follicles and decreases in secondary, tertiary, Degraaf, total and corpus luteum follicles in ovaries coupled to a neurodegeneration of hypothalamic anteroventral periventricular neurons in the OPD groups compared to the standard diet group.

Conclusions: The three OPDs induce obesity and impair the female reproductive function, especially OPD2 and OPD3. These findings contribute to a better understanding of the adverse effects of palm oil bleaching on the reproductive function in female rats, which could be useful in the management of women with obesity-related sexual dysfunction.

KEYWORDS: Bleached palm oil; Reproductive function; Anteroventral periventricular nucleus; Ovary; Estrogen; Progesterone; Female rat

1. Introduction

Numerous nutrition-related behaviours are well-known to be major risk factors of overweight and obesity strongly associated with both mental and physical health problems. A study on 117 dishes conducted by Kouebou *et al*[1] showed that Cameroonian food is mainly based on a high consumption of high-fat foods, up to nearly 77% of lipids mainly from vegetable origin such as palm oil. Palm oil is consumed by about 80% of Cameroonian[2], and the consumption per capita is regularly increasing. Despite the influence of processed fruit types and process parameters[3], crude red palm oil is rich with polyunsaturated fatty acids (PUFAs), and

Significance

Studies reported that high-fat diets contribute to obesity-induced female sexual dysfunctions. However, a reliable animal model using repeated heated palm oil close to what is seen in human of many low-income settings is not yet characterized. This study revealed that oxidized palm oil diets induce obesity, dyslipidemia, anteroventral periventricular neurodegeneration, and alterations of estrous cycle, ovarian structure/function and gestation parameters in female rats. This model provides innovative options for treating obesity-caused female infertility.

✉To whom correspondance may be addressed. E-mail: modewans@yahoo.com

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minor components such carotenoids (pro-vitamin A), tocopherols and tocotrienols (vitamin E), sterols, phospholipids (lecithin), coenzyme Q10 and squalene. Studies indicate the health-promoting effects of these constituents[4]. Due to the high pro-vitamin A carotenoid content, crude red palm oil can prevent or relieve vitamin A deficiency disease, commonly faced by public in developing countries[5]. Carotenoids, vitamin E and coenzyme Q10 are known as powerful lipid antioxidants that can prevent or minimize oxidative stress and thus chronic degenerative diseases associated with free radicals such as atherosclerosis, cancer, premature ageing and neurodegenerative diseases[6]. PUFAs are known as important versatile mediators with potential health benefits in several human pathologies, such as inflammation, obesity, cancer, type 2 diabetes mellitus as well as cardiovascular system disorders[7,8]. However, the red palm oil is very often refined or bleached in order to improve its purity, appearance, taste and odour and obtain dishes with a better texture[9]. However, during this process, especially at high temperatures (deep frying or thermal oxydation), most of the carotenoids and vitamin E (tocopherols and tocotrienols) are degraded and lost[10], while numerous free radicals and toxic substances are accumulated leading to the oxidation of lipids, including the PUFAs of cell membranes, and thus generate lethal repercussions on cardiovascular, haematological, renal, liver and reproductive functions[10,11].

In many low-income settings such as Cameroon there is a high tendency to reuse deep frying oils to reduce the cost of food preparation[12]. This practice aggravates the detrimental effects of the thermal oxidation[13] by increasing the level of free radicals to which the body is exposed. Moreover, the thermal oxidation of oil changes the configuration of fatty acids from cis to trans isomers, strongly correlated to the obesity, cardiovascular diseases and insulin resistance[14].

Obesity is a complex disease involving an excessive amount of body fat[15]. Mostly represented in women because of their high percentage of body fat, obesity affects several systems and increases the risk of other diseases such as diabetes mellitus, hypertension, coronary heart disease and female sexual dysfunctions[15,16]. Indeed, in obese women because of the excess of adipose tissue, the high rate of aromatization of androgens into estrogens affects the gonadotropin secretion leading to menstrual disorders, lower implantation and pregnancy rates which lead to female infertility[16]. In Cameroon, infertility is a real problem of public health and social integration and 30% of infertility causes in couples is attributed to women[17]. This prevalence of female infertility could be correlated to the over increasing prevalence of obesity in this society. Indeed, many studies reported the involvement of dietary changes or food intake on the onset of obesity[18]. The consumption of repeatedly heated palm oil coupled with the ingestion of sweetened drinks and proteins (meat, egg) in the Cameroonian society could mostly contribute to the high prevalence of obesity (15.1%) observed in this population[18].

Several studies have explored the effects of the consumption of high fat diets-induced obesity on the sexual function[14,19] with some discrepancies depending on the model. However, no study has evaluated the effect of the supplementation of these high fat diets with some carbohydrates and proteins on the female sexual function in a comparative manner. Therefore, this study adopts a reliable high fat diet-induced obesity model negatively affecting the female sexual function to investigate the impact of three oxidised palm oil diets on some female reproductive function parameters. Because of the ethical considerations, female rats instead of women are used in this study.

2. Materials and methods

2.1. Preparation of diets

2.1.1. Preparation of standard diet

For 100 g, the standard diet was made of corn meal (57%), soy meal (10%), fish meal (18%), wheat bran meal (7%), bone meal (7%) and salt (1%).

2.1.2. Thermoxydation of palm oil

Twenty liters of palm oil purchased in the Nkongsamba market (Littoral region of Cameroon) were used in this study. Four liters of this oil were submitted to five time-repeated thermo-oxidizing/bleaching (30 min at 143 ℃ on a hot plate)-cooling (5 h) process using an aluminium container, according to the modified protocol described by Elemi *et al*[20]. The resulting thermo-oxidized palm oil was used for the preparation of three oxidized palm oil diets (OPD).

2.1.3. Preparation of oxidized palm oil diets

The first oxidized palm oil diet (OPD1) was prepared with modifications[10]. Briefly, 30% of thermo-oxidized palm oil was added to 70% of standard diet and the mixture was grilled (in order to facilitate the consumption by the rat) for 7 min in an aluminium container on a hot plate at 143℃. The second oxidized palm oil diet (OPD2) was made up by adding to OPD1, 5 g of boiled yolk egg, using a modified protocol[21]. The third oxidized palm oil diet (OPD3) was constituted of OPD1 plus 10% of sucrose solution, based on the modified methodology described by Ngueguim *et al*[22].

2.2. Animals care, selection and housing

A total of 66 healthy adult albino Wistar rats, obtained from the animal house of the Department of Animal Organisms Biology (University of Douala, Cameroon), were used in this study. These rats (located in group of 5 and 6 in plastic cage using wood shavings as bedding material) were maintained at room temperature with a natural light: dark cycle (12 h: 12 h). Forty-four young adult (three

months old), nulliparous and non-pregnant female Wistar rats [(180±3) g] displaying five-six consecutive and regular estrous cycles were selected following a 30-day vaginal smear examination[23]. Twenty-two robust adult male Wistar rats of proved fertility were also selected and used in the sexual behavior and gestational tests.

2.3. Animals repartition and treatment

The 44 selected female Wistar rats were divided into four groups of eleven animals each and fed for 125 days either with standard diet (group 1, the control group receiving the standard diet), OPD1 (group 2, treated with 70% of standard diet supplemented with 30% of oxidized palm oil), OPD2 (group 3, administered OPD1 supplemented with 5 g of boiled yolk egg), or OPD3 (group 4, fed with OPD1 supplemented with 10% of sucrose solution).

The sample size was chosen in order to obtain reliable data for better statistical analysis. To avoid confusion of cages during treatment, the different cages were numbered, arranged in order and kept in the same position throughout the treatment period and the treatment provider was not aware of the codifications of the groups and even the treatments. The selected animals were acclimated for one week in the experimental room before the treatment and they were fed every morning (8:00 a.m., local time) during the treatment period.

2.4. Evaluation of morphometric parameters

For 98 days, body mass (g) and nasoanal lengh (cm) were measured using Kern Emb (600-2) balance and measure tape, respectively. Data obtained were used to calculate the body mass index (BMI) (BMI=body mass/nasoanal lengh^2)[24] and the Lee index[25]. In addition, abdominal circumference (cm) was also determined using a measure tape.

2.5. Evaluation of relative masses of organs

The body and absolute masses of abdominal and perigonadal adipose tissues, ovary and brain of all rats were measured with an electronic balance [Kern Emb (600-2)]. These absolute masses were used to calculate the relative masses of the above organs according to the following formula:

Relative mass (%) = (Absolute mass/Body mass) ×100

2.6. Evaluation of the estrous cycle parameters

Eight weeks (56 days) after the beginning of rat's feeding with the different diets, vaginal smears of all female rats were examined daily (11:00 a.m.) for 25 consecutive days in order to observe the frequency of occurrence of proestrus, estrus, metestrus and diestrus phases. The coefficient of proestrus (Cp) and metestrus/diestrus (Cmd) were then calculated in each group as previously defined[26]. In this study, the coefficients of proestrus/estrus (Cpe) was determined by dividing the number of times that proestrus phase was followed by the estrus phase, by the number of estrous cycles corresponding to the observation period. Likewise, the coefficient of estrus/metestrus/diestrus (Cemd) was evaluated by dividing the number of times that estrus phase was followed by metestrus/diestrus phases, by the number of estrous cycles corresponding to the observation period.

2.7. Determination of female sexual behaviour parameters

During the late proestrus phase, each virgin female rat was mated (between 19:00 and 22:00 p.m.) with a robust and sexually experienced male rat in an experimental setup (lenght 70 cm, width 54 cm, height 46 cm) constituted of two equal compartments separated by a transparent plexiglas wall (lenght 44.4 cm, width 50 cm), containing two modulated openings through which only the female can pass to control the sexual interaction. A robust male rat was gently introduced in the operating compartment (male compartment) and a virgin female in the no-operating compartment and after 5 min of acclimatisation, their sexual interactions were videotaped during 15 min following the protocol described by Sagae *et al*[27]. The videos recorded were analysed to determine female sexual behaviour parameters such as the compartment change latency and frequency, the number of visits to the operating compartment, the time spent by the female with the male, the number of male's solicitations by female, the latency and frequency of darting, the latency and frequency of hoping, the latency, frequency, time and score of lordosis as described by Heijkoop *et al*[28].

2.8. Determination of the gestational parameters

The gestational parameters were evaluated during 10 days, starting from day 98 of diets' administration. During this period, each female mated with a pair of male rats of proved fertility until a sperm positive smear. The vaginal smear of each female was examined daily (11:00 a.m.). Sperm positive females were removed from the mating cage, followed up for 17 days of pregnancy (mid-pregnancy) before being sacrificed by decapitation under diazepam (10 mg/kg)/ketamine (50 mg/kg) anaesthesia. The number of mated, sperm positive and pregnant females, implantation sites (uterine cords) and corpus luteum (ovaries) were counted and results were used to calculate some gestation parameters according to the methodologies described by Yakubu and Fakai[29], and Wankeu-Nya *et al*[30]:

Quantic pregnancy index = (number of pregnant females/number of sperm positive females) × 100

Fertility rate = (number of pregnant females/total number of mated females) × 100

Implantation index = (total number of implantation sites/number

of corpus lutea) × 100 pre-implantation loss = (number of corpus lutea–number of implantation sites)/number of corpus lutea) × 100

Anti-implantation activity = (number of female without implantation sites/total number of females mated) × 100

2.9. Samples collection

Sacrifice was performed in all animals at the 17th day of pregnancy (mid-pregnancy) at the anestrous phase and blood was collected in dry tubes, kept at room temperature for sedimentation before being centrifuged at 1 118×*g* for 15 min. Serum collected was stored at -20 ℃ for biochemical analyses. Ovaries, abdominal and perigonadal adipose tissues and brain were immediately removed, cleaned, washed in saline solution (0.9%) and weighed for further biochemical and histological analyses.

2.10. Biochemical analysis

The serum estradiol (ErbaLisa kits, Erba Mannheim, IME00014, Calbiotech Inc, Cordell Court, El Cajon, CA 92020 USA, sensitivity: 8.7 pg/mL) and progesterone (ErbaLisa kits, Erba Mannheim, IME00017, Calbiotech Inc, Cordell Court, El Cajon, CA 92020 USA, sensitivity: 0.112 ng/mL) levels were measured using enzyme-linked immunosorbent assay (ELISA) technique and a Microreader V-320 microplate reader according to the manufacturer's instructions (ErbaLisa kits). Serum triglycerides (TG), total cholesterol (TC) and high-density lipoprotein-cholesterol (HDL-c) levels were evaluated using commercial kits (SGM Italia), while low-density lipoprotein-cholesterol (LDL-c) concentration was calculated using Friedewald formula[31]. Ovaries and brain were crushed and homogenized in Tris-HCl (50 mM; pH=7.4). The homogenates were then centrifuged at 1 118×*g* for 15 min and used to determine oxidative stress markers such as malondialdehyde (MDA) superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and nitric oxyde (NO)[32,33].

2.11. Histomorphometric examination

Histological investigations on ovaries and brain were performed using the methodologies described by Wankeu-Nya *et al*[30]. Briefly, the different organs were respectively fixed in Bouin's solution, deshydrated in a series of increasing concentrated alcohol solutions, cleared in xylene, embedded in liquid paraffin (60 ℃), cut at 5 µm thick (using Reichness jung 2030 microtome), defrosted (gelatin bath 40 ℃), dried in an oven (45 ℃/24 h), deparaffined, rehydrated and finally the slides were stained using hematoxylin and eosin. Slides were observed and examined using light microscope (Leitz wetzlar Germany 513) equipped with a digital camera set-up (Celestron 44421) connected to a computer. Using imageJ software (version 1.50), microphotographs of tissues were used to count the the number of follicles in ovaries and the number of neurons in the anteroventral periventricular nucleus of the hypothalamus.

2.12. Statistical analysis

GraphPaD Prism software version 5.03 (GraphPad Software, Inc. San Diego, California) was used for data analyses. The normal distribution of data has been checked using D'Agostino & Pearson omnibus normality test. Data from percentage of relative mass of organs, sexual behaviour parameters, gestational and biochemical parameters as well as the number of anteroventral periventricular neurons in the brain and ovarian follicles and corpus luteum were analysed using one-way analysis of variance (ANOVA) followed by Bonferonni *post–test*. Two-way ANOVA followed by Bonferonni *post–test* was used to evaluate the effects of time and different OPD on morphometric and oestrous cycle parameters (repeated measures). Data were expressed as mean±standard deviation (mean±SD). $P<0.05$ was considered statistically significant.

2.13. Ethics statement

All these experimentations were carried out in accordance with the guidelines established by the institutional ethical committee of the University of Douala through a delivered ethical clearance number 2907 CEI-Udo/08/2021/M.

3. Results

3.1. Effects of OPD on morphometric parameters

Compared to the standard diet group, significant and progressive increases in the BMI, Lee index and abdominal circumference were observed from day 14 until day 98 in all female rats receiving the OPD ($P<0.05$). Furthermore, BMI and Lee index values were significantly greater than 0.50 and 300 respectively starting from the day 56. These significant increases in BMI, Lee index and abdominal circumference were more important after 98 days of feeding in the female rats receiving OPD2 [BMI: (0.63±0.04) g/cm^2; Lee index: 318.87±6.45; abdominal circumference: (16.58±0.26) cm] compared to the standard diet, OPD1 and OPD3 groups, respectively (Figure 1).

3.2. Effects of OPD on the relative mass of organs

Compared to the standard diet group, significant increases in abdominal (56.7%; 50%) and perigonadal (57.81%; 44.13%) relative masses were noticed in females fed with OPD2 and OPD3, respectively ($P<0.01$). No significant differences in the relative masses of ovaries were obtained between the OPD groups and the standard diet group; however, compared to the standard diet group, significant decreased in the brain relative mass was observed in females fed with OPD2 ($P<0.05$) (Table 1).

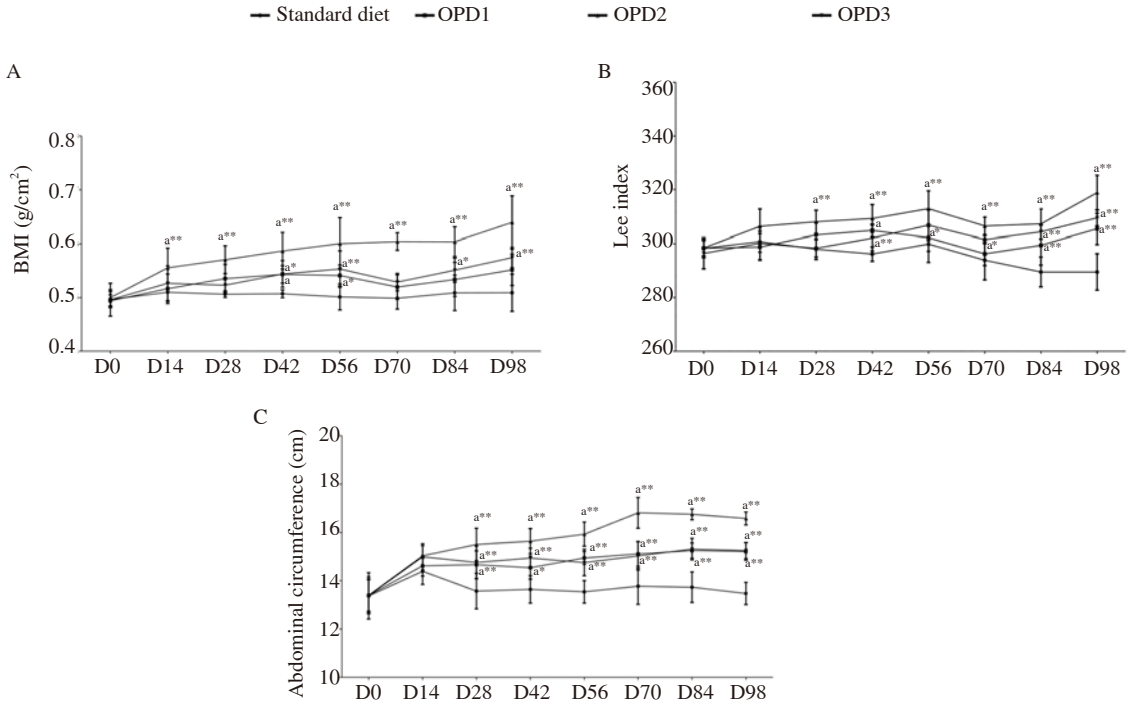

Figure 1. Variation of morphometric parameters: body mass index (BMI) (A), Lee index (B) and abdominal circumference (C) in female rats fed with oxidized palm oil diets (OPD). Each point represents mean±SD; *n*=11; two-way ANOVA (repeated measures) is used, followed by Bonferonni *post-test*. $^{a}P<0.05$.$^{a*}P<0.01$, $^{a**}P<0.001$: compared to the standard diet group. OPD1: oxidized palm oil diet 1; OPD2: oxidized palm oil diet 2; OPD3: oxidized palm oil diet 3. D: day.

Table 1. Variation of relative masses of abdominal and perigonadal adipose tissues, ovaries and brain in female rats fed with oxidized palm oil diets.

Groups	Relative mass (g/100 g of body weight)			
	Abdominal adipose tissue	Perigonadal adipose tissue	Ovaries	Brain
Standard diet	1.00±0.43	1.62±0.83	0.02±0.01	0.70±0.05
OPD1	1.68±0.61	2.16±0.92	0.02±0.01	$0.75\pm0.05^{c**}$
OPD2	$2.31\pm0.68^{a**b**d}$	$3.84\pm0.65^{a**b**d}$	0.02±0.01	0.63 ± 0.05^{a}
OPD3	$2.00\pm0.84^{a**}$	$2.90\pm0.62^{a*}$	0.02±0.01	$0.73\pm0.07^{c*}$

Data are expressed as mean±SD, *n*=11; one-way ANOVA is used, followed by Bonferonni *post-test*. $^{a}P<0.05$, $^{a*}P<0.01$, $^{a**}P<0.001$: compared to the standard diet group; $^{b**}P<0.001$: compared to OPD1; $^{c*}P<0.01$, $^{c**}P<0.001$: compared to OPD2; $^{d}P<0.05$: compared to OPD3. OPD1: oxidized palm oil diet 1; OPD2: oxidized palm oil diet 2; OPD3: oxidized palm oil diet 3. OPD: oxidized palm oil diets.

3.3. Effects of OPD on estrous cycle parameters

No significant difference was observed in all estrous cycle parameters between groups before the feeding period. However, compared to the standard diet and to the period before feeding, the appearance of the proestrous phase (Cp) was significantly higher in the OPD2 group ($P<0.001$; 17.24%) and significantly lower in the OPD3 group ($P<0.001$; 37.5%) (Figure 2A), while significant decreases in the succession from proestrus to estrous phases (Cpe) in all OPD groups compared to the standard diet group (Figure 2B). Moreover, the appearance of the metestrous/diestrous phases (Cmd) was higher in the OPD1 ($P<0.05$; 8.9%), OPD3 ($P<0.001$; 17.74%) groups and nonsignificantly lower in the OPD2 group ($P>0.05$; 9.8%) (Figure 2C), while significant decreases in the succession from estrous to metestrous/diestrous phases (Cemd) were recorded in all females receiving the OPD ($P<0.05$), and especially in the OPD3 group (63.79%; 36.84%), compared to the standard diet group and their initial values, respectively (Figure 2D).

3.4. Effects of OPD on sexual behaviour parameters

Concerning the appetite behaviour parameters and compared to females receiving standard diet, increases in the compartment change latency, latencies of darting and hoping coupled to decreases in the time spent with male, compartment change frequency, visit of operating compartment, females solicitations and number of darts and hops, were recorded in female receiving the OPD. Moreover, for consumption behaviour parameters, significant increases in the latency of lordosis ($P<0.001$) associated to significant decreases in score ($P<0.001$), number ($P<0.001$) and time ($P<0.05$) of lordosis were observed in females fed with the OPD. The most significant variance in these behaviours was obtained in the OPD3 group, especially for the compartment change latency (85.86% of increase; $P<0.001$), compartment change frequency (89.25% of decrease; $P<0.001$) and the number of lordosis (91.67% of decrease; $P<0.001$) (Table 2).

3.5. Effects of OPD on gestation parameters

Compared to the standard diet and OPD3 groups where all females were sperm positive (90.91%), decreases in quantic pregnancy index were noticed in OPD1 and OPD2 groups (75% and 62.5%, respectively). Moreover, in all the OPD groups, insignificant decreases in fertility rate (P>0.05) and number of pregnant females (P>0.05) were recorded, and were associated to significant decreases in implantation index (P<0.001, OPD1: 62.18%, OPD2: 48.72%, OPD3: 52.29%) and implantation sites (P<0.001, OPD1: 66.67%). Compared to the standard diet group, increases in pre-implantation losses (P<0.001, OPD1, OPD2 and OPD3) and anti-implantation activities (P>0.05, OPD1, OPD2) were recorded (Table 3).

3.6. Effects of OPD on biochemical parameters

In the ovary, compared to the standard diet group, females receiving OPD presented significant (P<0.05) increases in MDA and GSH levels, SOD activities in the OPD1, OPD2 and OPD3 groups and catalase in the OPD3 group, associated to a decrease in NO levels in the OPD2 and OPD3 groups. No significant variations of these parameters in the brain were observed in the OPD groups compared to the standard diet group.

Compared to the standard diet group, increases in TG, TC, LDL-c levels coupled to a decrease in HDL-c level were observed in the female rats of the OPD2 and OPD3 groups, with the most significant values obtained in the OPD3 group for TG, TC, LDL-c (P<0.001; 60.11%, 28.07%, 26.21% of increases, respectively) and HDL-c (P<0.001; 26.41% of decrease).

Compared to the standard diet group, a significant increase in estradiol level in rats receiving different OPD (P<0.001), with the most significant value obtained in the OPD1 group (P<0.001, 49.33% of increase). Compared to the standard diet group, a decrease in progesterone level in the different OPD groups (OPD1, P<0.01; OPD2, P>0.05; OPD3, P<0.001, 13.63% of decrease) (Table 4).

3.7. Effects of OPD on ovaries and anteroventral periventricular nucleus structures

Alterations of ovaries and anteroventral periventricular neurons were observed in female rats fed with OPD compared to those receiving the standard diet. These alterations were characterized by the presence of many atresic follicles in ovaries coupled to the presence of hyperchromatic nucleus in pyknotic neurons in the anteroventral periventricular neurons of hypothalamus (Figure 3).

Table 2. Variation of appetite and consumption behaviour parameters in female rats fed with oxidized palm oil diets.

Parameters	Standard diet	OPD1	OPD2	OPD3
Appetite behaviour				
Compartment change latency, s	35.3±9.8	73.6±34.2	120.3±67.1^{a**}	249.6±73.5^{a**b**c**}
Time spent with male, s	563.2±204.1	429.1±85.7	465.4±53.1	312.8±78.2^{a**c}
Compartment change frequency	9.3±1.2	5.0±2.9^{a**c*}	8.6±3.2	1.0±0.1^{a**b**c**}
Visit of operating comparment	5.0±1.4	3.0±1.4^{a**c*}	4.0±1.6	1.0±0.9^{a**b**}
Female sollicitations	29.0±12.1	11.0±3.0^{a**}	13.0±5.4^{a**}	3.0±2.5^{a**bc}
Latency of darting, s	368.5±237.1	523.9±158.1	537.7±139.6^{a}	503.3±173.1
Latency of hoping, s	26.2±22.9	311.0±279.5^{a**}	478.2±54.4^{a**b}	471.3±41.3^{a**}
Number of darts	27.0±8.9	1.0±1.0^{a**}	3.0±1.6^{a**}	2.0±1.3^{a**}
Number of hops	69.0±17.0	3.0±0.9^{a**}	6.0±0.1^{a**}	3.0±0.1^{a**}
Consumption behaviour				
Latency of lordosis, s	44.5±25.8	600.0±0.0^{a**}	600.0±0.0^{a**}	600.0±0.0^{a**}
Score of lordosis	2.0±0.1	1.0±0.7^{a**}	1.0±0.5^{a**}	1.0±0.6^{a**}
Number of lordosis	12.0±4.1	6.0±0.2^{a**}	1.0±0.4^{a**b**}	1.0±0.6^{a**b**}
Time of lordosis, s	1.0±0.0	0.6±0.3^{a}	0.3±0.2^{a**}	0.5±0.3^{a**}

Data are expressed as mean±SD, n=11; one-way ANOVA is used, followed by Bonferonni *post–test*. ^{a}P<0.05, $^{a*}P$<0.01, $^{a**}P$<0.001: compared to the standard diet group; ^{b}P<0.05, $^{b*}P$<0.01, $^{b**}P$<0.001: compared to OPD1; ^{c}P<0.05, $^{c*}P$<0.01, $^{c**}P$<0.001: compared to OPD2. s: time in second.

Table 3. Evolution of gestation parameters in female rats fed with oxidized palm oil diets.

Parameters	Standard diet	OPD1	OPD2	OPD3
Total number of mated females	11	11	11	11
Number of sperm positive females	11	8	8	11
Number of pregnant females	11	6	5	10
Quantic pregnancy index	100	75	62.50	90.91
Fertility rate, %	100	54.5	45.5	90.9
Implantation sites$^{\#}$	9.0±1.4	3.0±0.2^{a**cd}	7.0±4.5	7.0±3.5
Implantation index$^{\#}$, %	98.1±16.1	37.1±13.4^{a**}	50.3±15.4^{a**}	46.8±17.4^{a**}
Pre-implantation loss$^{\#}$, %	1.1±0.4	79.7±23.6^{a**d}	66.0±27.6^{a**}	53.1±17.4^{a**}
Anti-implantation activity, %	9.09	45.45	54.54	9.09

$^{\#}$Data are expressed as mean±SD, n=11; one-way ANOVA is used, followed by Bonferonni *post–test*. $^{a**}P$<0.001: compared to the standard diet group; ^{c}P<0.05: compared to OPD2. ^{d}P<0.05: compared to OPD3.

Table 4. Variation of biochemical parameters in female rats fed with the oxidized palm oil diets.

Parameters	Standard diet	OPD1	OPD2	OPD3
MDA				
Ovaries	1.9±0.3	4.8±0.6^{a**}	4.9±0.6^{a}	6.7±1.3^{a**b**c**}
Brain	0.5±0.1	0.5±0.2	0.8±0.0	0.9±0.1
SOD				
Ovaries	35.2±3.5	120.3±61.5^{a*}	110.9±37.5^{a}	141.1±78.5^{a**}
Brain	27.2±2.2	28.0±6.0	34.2±10.9	34.2±10.1
Catalase				
Ovaries	81.1±53.8	176.0±60.9	146.2±21.2	211.4±135.3^{a*}
Brain	23.1±10.6	36.0±7.2	24.0±8.4	28.5±5.4
GSH				
Ovaries	52.5±24.1	157.0±7.1^{a**d**}	152.0±17.4^{a**d*}	122.1±4.5^{a**}
Brain	31.3±11.8	54.1±33.1	25.1±3.5^{b}	28.7±12.8^{b}
Nitric oxide (NO)				
Ovaries	208.1±53.8	178.5±7.9	67.3±23.2^{a**b**}	7.4±1.3^{a**b**c**}
Brain	59.1±8.9	75.2±17.1	57.2±19.0	56.8±17.2
Serum triglyceride (TG)	450.1±54.4	378.3±44.8	544.3±122.9^{b*}	1128.5±193.5^{a**b**c**}
Total serum cholesterol (TC)	1015.1±42.6	962.8±95.7	1057.6±47.8	1411.4±52.3^{a**b**c**}
Serum HDL-c	80.3±7.6	113.4±12.5^{a**}	78.4±0.7^{b**}	59.1±8.6^{a**b**}
Serum LDL-c	831.6±33.4	768.7±31.8	887.9±48.0^{b*}	1127.0±87.0^{a**b**c**}
Serum estradiol	155.7±11.1	307.3±19.9^{a**b**d**}	207.5±18.2^{a**}	250.3±16.8^{a**c**}
Serum progesterone	69.7±2.0	60.6±8.2^{a*}	66.2±1.1	60.2±2.2^{a**}

Data are expressed as mean±SD, n=11; one-way ANOVA is used, followed by Bonferonni *post-test*. ^{a}P<0.05, $^{a*}P$<0.01, $^{a**}P$<0.001: compared to the standard diet group; $^{b*}P$<0.01, $^{b**}P$<0.001: compared to OPD1; $^{c**}P$<0.001: compared to OPD2; ^{d}P<0.05, $^{d*}P$<0.01, $^{d**}P$<0.001: compared to OPD3. MDA: malondialdehyde, SOD: superoxyde dismutase, GSH: glutathione, HDL-c: high density lipoprotein cholesterol, LDL-c: low density lipoprotein cholesterol.

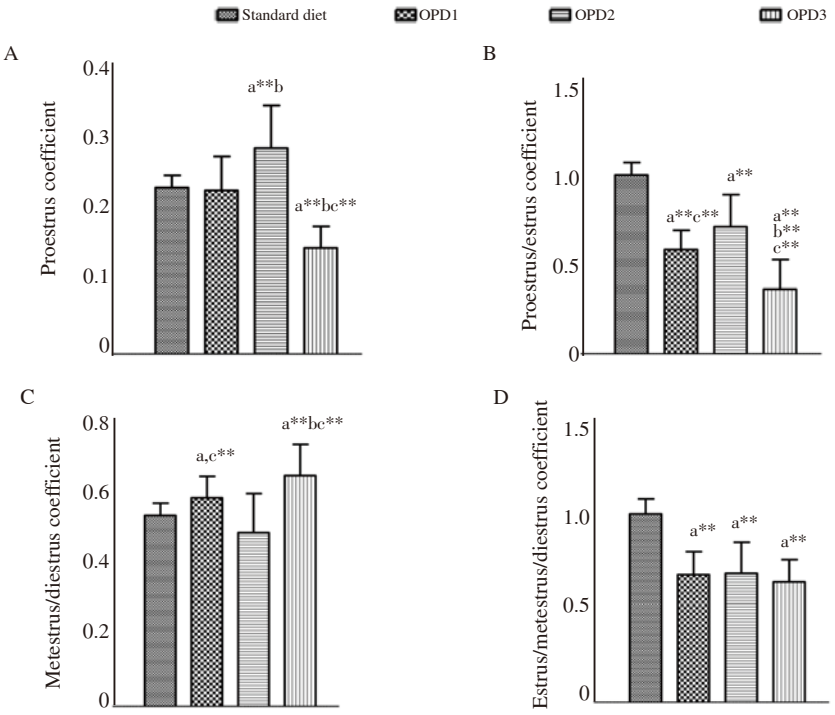

Figure 2. Variation of proestrus (A), proestrus/estrus (B), metestrus/diestrus (C) and estrus/metestrus/diestrus (D) coefficients in female rats fed with oxidized palm oil diets (OPD). Each bar represents mean±SD; n=11; two-way ANOVA (repeated measures) is used, followed by Bonferonni *post-test*.; $^{a**}P$<0.001: compared to the standard diet group. ^{b}P<0.05, $^{b**}P$<0.001: compared to OPD1; $^{c**}P$<0.001: compared to OPD2. OPD1: oxidized palm oil diet 1; OPD2: oxidized palm oil diet 2; OPD3: oxidized palm oil diet 3.

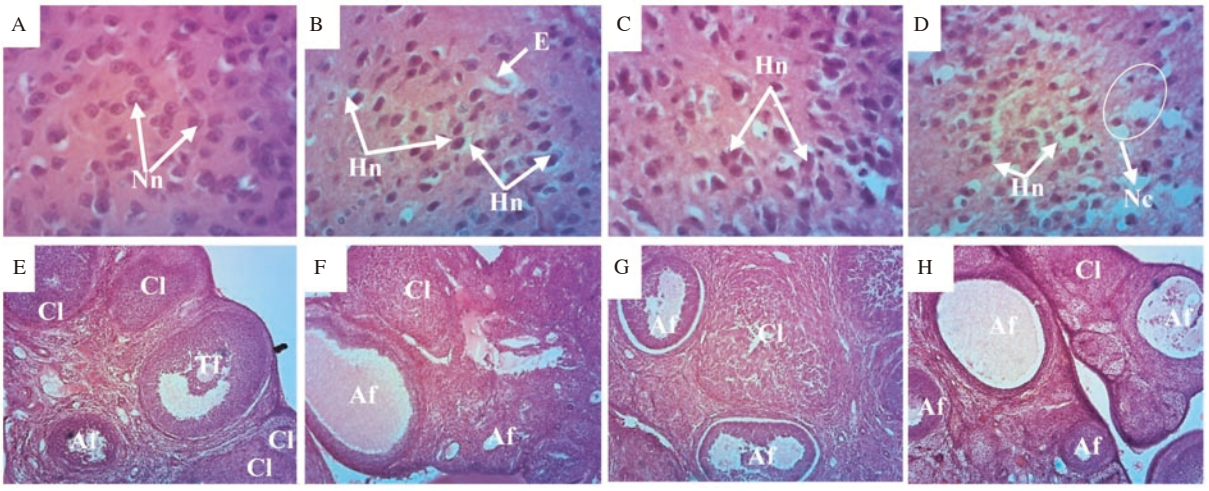

Figure 3 A–D. Photomicrographs of anteroventral periventricular neuron structures in female rats fed with oxidized palm oil diets (OPD). Alterations of anteroventral periventricular neurons are observed in the female rats of the OPD1 (B), OPD2 (C), OPD3 (D) groups, compared to the standard diet group (A). Nn: normal neurons, Hn: hyperchromatic neurons, E: edema, Nc: neuronal cytolysis. **E–H.** Photomicrographs of ovary structures in female rats fed with OPD. Alterations of ovaries are observed in the female rats of the OPD1 (F), OPD2 (G), OPD3 (H) groups, compared to the standard diet group (E). Af: atresic follicles, Cl: corpus luteum, Tf: tertiairy follicles.

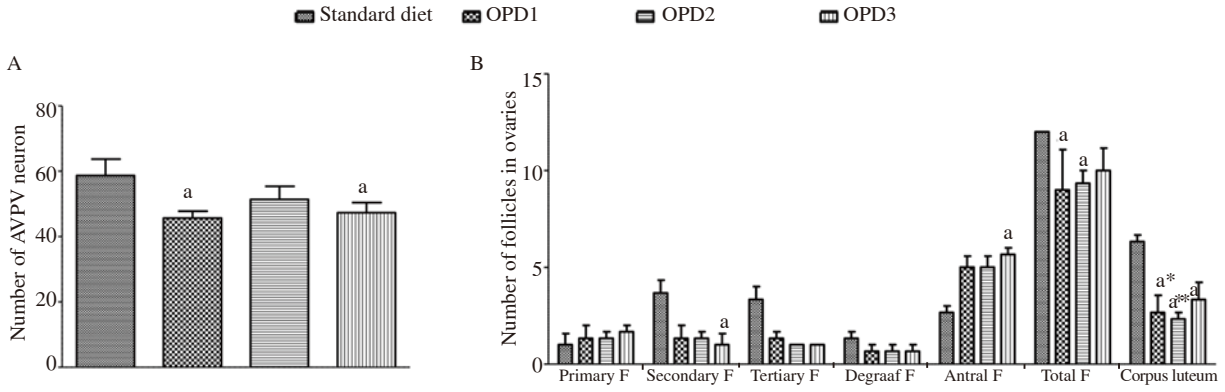

Figure 4. Variation in hypothalamus anteroventral periventricular neurons (A) and follicle number in ovaries (B) of female rats fed with oxidized palm oil diets. Each bar represents mean±SD; n=11; one-way ANOVA is used, followed by Bonferonni *post–test.* $^{a}P<0.05$, $^{a*}P<0.01$, $^{a**}P<0.001$: compared to the standard diet group; OPD1: oxidized palm oil diet 1; OPD2: oxidized palm oil diet 2; OPD3: oxidized palm oil diet 3. AVPV: anteroventral periventricular nucleus; F: follicles.

In anteroventral periventricular nucleus of hypothalamus, general decreases in the total number of neurons was also obtained in all females fed with the OPD compared to the standard diet but these decreases were significant ($P<0.05$) in the OPD1 (22.18%) and OPD3 (13.78%) groups (Figure 4A).

Moreover, histomorphometric evaluations indicate increases in primary follicles ($P>0.05$), antral follicles (OPD3: $P<0.05$; 53%) associated to decreases in secondary follicles (OPD3: $P<0.05$; 72.67%), tertiary follicles ($P>0.05$), Degraaf follicles ($P>0.05$), corpus luteum ($P<0.05$; OPD1, OPD2 and OPD3) and total follicles ($P<0.05$; OPD1 and OPD2) in the ovaries of females fed with the different OPD compared to those receiving the standard diet (Figure 4B).

4. Discussion

The study evaluated the effects of three repeated OPD on the estrous cycle, sexual behaviour and fertility/gestation parameters in female rat. In the present study, the administration of the three OPDs to female rats induced obesity and dislipidemia, impaired estrous cycle, sexual behavior and fertility, and altered brain and ovarian structures and functions.

The consumption of three OPDs in the present study led to important changes in morphometric parameters of female rats, characterized by significant increases in abdominal circumference, BMI and Lee index, especially between day 56 and day 98, where BMI and Lee index values were greater than 0.50 and 300,

respectively. Such increases in morphometric parameters are indicators of the onset of obesity[24,25,34], a condition associated with intensive processes of hypertrophy and/or hyperplasia of adipocytes[35]. Our results show increases in relative mass of abdominal adipose tissue, particularly in animals receiving OPD2. Obesity is usually associated with dislipidemia[14]. In this study, increases in lipid profiles involving rise in triglyceridemia, total and LDL cholesterol levels associated to a decrease in HDL cholesterol level were recorded in females receiving OPD2 and OPD3. These results corroborate those obtained by many other authors who reported that obesity is characterised by a local and systemic chronic low-grade inflammation that induces adipose tissue dysfunction, overproduction of free fatty acids and their ectopic deposition, particularly in the liver. This accumulation promotes the overproduction of triglyceride-rich lipoproteins, and increased activity of cholesterol ester transfer protein and hepatic lipase that reduces the levels of HDL particles while increasing the levels of small dense LDL particles[36]. In this study, increases in abdominal circumference, BMI, Lee index and abdominal adipose tissue relative mass were more obvious in the OPD2 group compared to the OPD1 and OPD3 groups, while dyslipidemia was significantly pronounced in the OPD3. These differences in the effects could be correlated to the composition of the different OPD used in this study. Indeed, the boiled yolk egg present in OPD2, contains proteins, cholesterol, saturated and unsaturated fatty acids, which promote cell development in female rats receiving this diet[37]. In contrast, sucrose present in OPD3 contains fructose whose overconsumption leads to increased liver lipid deposition and dyslipidemia[38].

Concerning the estrous cycle, decreases in the succession from proestrus to estrous phases (Cpe) and from estrous to metestrous/diestrous phases (Cemd) in the three groups receiving the OPD were coupled to increases in the appearance of the metestrous/diestrous phase (Cmd) (OPD1 and OPD3) and the appearance of proestrous phase (Cp) (OPD2). These results illustrated an irregularity in the estrous cycle marked by the lengthening of luteal (OPD1, OPD3) and follicular phases (OPD2) respectively in these females. Several studies indicated such irregular estrous cycles in diet-induced obesity situations[39]. The mechanisms of obesity-induced irregular estrous cycle remain unclear. However, some studies hypothetically linked obesity-associated hyperleptinemia to the abnormal hypothalamus-pituitary-gonadal function, and thus irregular estrus cyclicity[40]. On the other hand, studies suggested the follicular apoptosis induced by the periovarian fat accumulation[41] or the reduction of the ovarian nitric oxide levels[42]. Interestingly, significant increases in the relative mass of periovarian adipose tissue (more obvious in OPD2) and decreases in ovarian nitric oxide (more obvious in OPD3) in females receiving the OPD respectively were recorded in this study. Female obesity is associated with intensified inflammation and oxidative stress in follicular fluid, and apoptotic ovarian follicle, which are detrimental to oocyte growth[43]. Moreover, increases in lipids peroxidation marker (MDA) and antioxidant defences (SOD, catalase and GSH) observed in the ovaries of females fed with OPD could also justified the negative effects of these diets on the estrous cycle parameters. These data are in harmony with those obtained previously approving the association between the alteration of ovarian cells with the high production of reactive oxygen species[44].

The second aspect of the female reproductive function evaluated in this study was the sexual behaviour parameters. Using a rhythmic mating paradigm, the sexual behaviour parameters were evaluated during the receptive period of the females[28]. Decreases in appetite and consumption behaviours were observed in OPD groups. Receptivity in female rodents is limited by threshold levels of estradiol after progesterone secretion, corresponding to the estrus phase (ovulation period)[45]. Accordingly, the observed alterations could be justified by the decrease in proestrus/estrus coefficient. Sexual behavior in female rats is tighly and finely controlled by kisspeptin neurons in the anteroventral periventricular nucleus as well as the hypothalamic levels of NO[46–48]. Therefore, alterations of these anteroventral periventricular neurons' structure (characterized by the presence of hyperchromatic nuclei in pyknotic neurons) coupled to a decrease in the total number of these neurons in animals fed with the OPD explain this sexual behaviour impairment. Normal NO level is indispensable for the vascular smooth muscle relaxation in the female genital area leading to an optimal sexual response[46]. On the other hand, the modifications in the oxidative stress markers (low NO concentration coupled to the high levels of MDA and SOD and catalase activities) in the brain recorded in this study could elucidated this neurodegeneration[46–48]. All these negative effects in the sexual behaviour parameters were more pronounced in females treated with OPD3 which corroborate data obtained behind concerning estrous cycle parameters. These results are in harmony with those obtained previously[49] and in opposition to those recorded by Atuadu *et al*[47].

In normal conditions, mating between male and receptive female results in pregnancy. In this study, gestation parameter evaluation indicates decreases in the number of sperm positive and pregnant females, quantic pregnancy index, fertility rate, implantation site and index associated to increases in pre-implantation losses and anti-implantation activities in female rats receiving the different OPD compared to their counterparts in the control group. These decreases in the quantic pregnancy index and fertility rate could be attributed to the observed decrease in proestrus/estrus coefficient, an indicator of the ovulation and the female receptivity[28]. Decreases in implantation sites and index coupled to increases in pre-implantation losses and anti-implantation activities suggest unfavourable environment for the nidation and/or the development of an embryo[50]. Moreover, this result could also be a consequence of the ovarian dysfunction (folliculogenesis and steroidogenesis)

induced by the alimentation. Indeed, increases in the number of atresic and primary follicles associated to decreases in secondary, tertiary, Degraaf and total follicles were recorded in the ovaries of females fed with OPD. These alterations of folliculogenesis could be attributed to the dislipidemia in the different OPD groups as confirmed behind[51] or to a dysregulation of some transcription factors involved in follicular growth[52]. These results are in opposition with those obtained by some authors[53], probably because of the type of fat in different diets, the duration to the high-fat diet exposure and the type of animals used. In addition, decreases in serum progesterone coupled to increases in serum estradiol levels were obtained in mid-pregnancy females fed with the OPD. This decrease in progesterone level could be explained by a decreased number of corpus luteum recorded in these female groups[54] as shown on the ovarian microphotographs. It is well known that during mid-pregnancy in female rat, the corpus luteum secretes progesterone which inhibits the secretion of estradiol by the granulosa cells[54]. The increase in estradiol level could be explained by the high rate of aromatization of androgens into estrogens because of the excess of adipose tissue due to the consumption of OPD by female rats, which affects the gonadotropin secretion leading to disrupted estrous cycle, lower implantation and pregnancy rates and ultimately to female infertility as previously reported[19,55].

Our study shed light on the previously misunderstood mechanism involving anteroventral periventricular neurons by which repeated oxidized palm oil diet and its supplementation with boiled yolk egg or sucrose solution-induced obesity caused detrimental effects on female rat's reproductive function. Moreover, these models of high fat diet-induced obesity resemble the most what is seen in human of many low-income settings, where people have tendency to reuse deep frying palm oil to reduce the cost of food preparation, and where there is a high consumption of sugar and proteins. This diet could be useful for the treatment options of female fertility problems in these societies. However, the non-evaluation of the effects of high-fat diets in the present work on some biomarkers involved in obesity-induced reproductive dysfunctions such as the levels of leptin, insulin, kisspeptin, pro-inflammatory cytokines (tumor necrosis factor α, interleukin-1, interleukin-6), peroxisome proliferator-activated receptor γ and nuclear factor kappa B as well as on the offspring quality could be considered as the limitations of this study.

In conclusion, this study indicates that the three oxidized palm oil diets used are capable to induce obesity at different degree and to alter estrous cycle, sexual behavior and fertility/gestation parameters in female rats through dyslipidemia, oxidative stress, neurodegeneration of anteroventral periventricular neurons and sexual hormones disturbances. Concerning the morphometric parameters, the OPD2 is the most efficient followed by OPD3 and OPD1. Regarding the alteration of the estrous cycle and sexual behavior and gestation parameters, the OPD3 is the most harmful followed by the OPD2 and OPD1. These findings show that the repeated bleaching practice of palm oil for cooking represents a danger for women fertility and could be useful for the management of women suffering from sexual dysfunction pertaining to obesity.

Conflict of interest statement

The authors of this paper declare no conflict of interest.

Aknowlegments

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Data availablility statement

The datasets used in this study are available from the corresponding author upon request.

Authors' contributions

Wankeu-Nya M, Kengne IT and Ateba BS did the study design and wrote the manuscript. Kengne IT, Bend FE, Djeumeni ON and Hatho DT collected the data; Wankeu-Nya M, Kengne IT, Ateba BS and Ngadjui E defined the intellectual content. Moundipa PF, Massoma DL, Dongmo AB and Watcho P did the manuscript review.

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