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Universidade Estadual Paulista "Júlio de Mesquita Filho"
Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas
Associação ampla UFSCar/UNESP

TAIS VARANDA

ALTERAÇÕES NA INGESTÃO DE LÍQUIDOS EM RATAS OVARIECTOMIZADAS ALIMENTADAS COM DIETA HIPERLIPÍDICA COM OU SEM REPOSIÇÃO DE ESTRÓGENO

Araraquara, 2022

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Dissertação apresentada ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas PIPGCF UFSCar/UNESP, como parte dos requisitos para obtenção do Título de Mestre em Ciências Fisiológicas.
Orientadora: Débora Simões de Almeida Colombari.

Araraquara, 2022

To my dear parents and brother,

and to my beloved husband and son.

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Há coisas que são boas por alguns instantes, outras por algum tempo.
Só algumas são para sempre.

Olavo de Carvalho

RESUMO

Nas últimas décadas a obesidade, uma doença não-comunicável, se tornou uma epidemia mundial. Durante a obesidade, tem sido descrito em humanos e animais, reduções na ingestão de água. Além da obesidade, a desidratação acomete uma parcela da população brasileira, principalmente nas épocas de clima mais quente. Experimentalmente, a desidratação pode ser induzida pela privação hídrica (PH), e induz sede, decorrente da ativação dos osmorreceptores e da ação da ANG II nos receptores angiotensinérgicos do tipo 1 (AT1) no sistema nervoso central (SNC) e ingestão de solução hipertônica de sódio, pela ação da ANG II em receptores AT1 no SNC. Durante a menopausa, comumente são observadas desordens também, na ingestão de água e sódio, devido à ausência do hormônio estrógeno. Experimentalmente, o tratamento com estrógeno em ratas ovariectomizadas (OVX) tem se mostrado eficaz em reduzir estas alterações. Desta forma, nosso objetivo foi estudar a ingestão diária de água e sódio em fêmeas intactas, ou OVX, com ausência de 17-β-estradiol (E2) ou reposição de E2 que foram alimentadas com dieta hiperlipídica. As possíveis alterações na ingestão de água e sódio induzidas pela PH de 24 horas também foram estudadas. Para isso, fêmeas Holtzman (260-300 g) foram alocadas em gaiolas individuais, com livre acesso à ração, água, e solução de NaCl 0,3 M. Durante uma semana foi realizado registro diário de ingestão de água e NaCl 0,3 M, enquanto todas as ratas foram mantidas intactas e alimentadas com DP, possibilitando a verificação de valores de ingestão basal nestes animais (semana basal). Passada esta semana, as ratas foram submetidas a OVX ou cirurgia fictícia (SHAM), e foram alimentadas com DP (11% calorias de gordura) ou DH (46% calorias de gordura) por 6 semanas. Após 3 semanas de dieta, parte das ratas OVX recebeu tratamento com E2 (10μg/dia/rata) pelas próximas 4 semanas. A ingestão espontânea de água e NaCl 0,3 M foi registrada diariamente durante 6 semanas, e o peso corporal foi avaliado semanalmente. Ao final das 6 semanas para indução de sede e apetite ao sódio, foi utilizado o protocolo de PH-Reidratação Parial (RP). Brevemente: as ratas foram mantidas apenas com ração por 24 h e, depois, foi ofertada uma bureta de água por 2 h (RP – teste da sede), e em seguida foi ofertada uma bureta adicional contendo NaCl 0,3 M por mais 2 h (teste do apetite ao sódio). Ao final dos experimentos, foi feita a análise de valores plasmáticos de insulina, leptina e E2, e coleta de tecido adiposo e útero. Os principais achados foram: 1) ratas DH, em comparação com ratas DP, tiveram aumento de ganho peso corporal, tecido adiposo visceral e leptina plasmática e, todos estes parâmetros foram potencializados em ratas DH/OVX e revertidos pela reposição com E2 (DH/OVX+E2); 2) Uma redução na ingestão diária de água foi observada em ratas DH/SHAM ou DH/OVX, em comparação com ratas DP com os mesmos tratamentos, nas ratas DH/OVX+E2 os valores de ingestão espontânea de água retornaram para valores semelhantes aos observados na semana basal; 3) a ingestão espontânea de sódio foi aumentada em ratas DH/SHAM quando comparadas com ratas DP/SHAM; 4) em ratas DH/OVX, o tratamento com E2 (DH/OVX+E2) restaurou a ingestão aumentada de sódio; 5) Comparado com ratas DP/SHAM, ratas DH/SHAM ingeriram menos água e mais sódio quando submetidas a 24 h de PH; 6) Em ratas DP/OVX, houve uma menor ingestão de água induzida pela PH, que não foi revertida pelo tratamento com E2; 7) A OVX não

alterou a ingestão de sódio induzida pela PH em ratas DP, porém na presença de estrógeno houve uma redução na ingestão de sódio induzida pela PH, 8) Por outro lado, a ingestão de água à PH em ratas DH/OVX foi menor do que em ratas DH/SHAM, bem como a de sódio; 9) No caso de ratas DH, o tratamento com E2 restaurou a ingestão tanto de água, quanto de sódio. Portanto, enquanto nas ratas DP o E2 parece ter um efeito inibitório sobre a ingestão de sódio durante apenas a PH, nas ratas DH o efeito parece ser excitatório tanto para a ingestão de água como para a ingestão sódio, seja na ingestão espontânea ou na induzida pela privação hídrica.

Palavras-chave: obesidade, privação hídrica, desidratação, sede, apetite ao sódio, estradiol, menopausa

ABSTRACT

In recent decades, obesity, a non-communicable disease, has become a worldwide epidemic. During obesity, reductions in water intake have been described in humans and animals. In addition to obesity, dehydration affects a portion of the Brazilian population, especially in times of warmer weather. Experimentally, dehydration can be induced by water deprivation (WD), which induces thirst, due to the activation of osmoreceptors and the action of ANG II on angiotensin II receptors type 1 (AT1) receptors in the central nervous system (CNS), and ingestion of hypertonic sodium solution, by the action of ANG II on AT1 receptors in the CNS. During menopause, disorders in the water and sodium intake are commonly observed, due to the absence of the hormone estrogen. Experimentally, estrogen treatment in ovariectomized (OVX) rats has been shown to be effective in reducing these changes. Thus, we were aimed to study the daily water and sodium intake in intact females, and in OVX rats lacking 17-β-estradiol (E2) or E2 replacement that were fed a high-fat diet. Possible changes in water and sodium intake induced by 24-hour PH were also studied. In order to study that, Holtzman females (260-300 g) were placed in individual cages, with free access to food, water, and 0.3 M NaCl solution. During one week daily water and sodium intake was registered, while all rats were kept intact and fed with SD, allowing the verification of basal intake values in these animals (baseline week). Last week, the rats underwent OVX or sham surgery (SHAM) and were fed either SD (11% calories from fat) or HFD (46% calories from fat) for 6 weeks. After 3 weeks of diet, part of the OVX rats received treatment with E2 (10μg/day/rat) for the next 4 weeks. Spontaneous ingestion of water and 0.3 M NaCl was recorded daily for 6 weeks, and body weight was assessed weekly. At the end of 6 weeks, for induction of thirst and sodium appetite, the WD-Partial Rehydration (PR) protocol was used. Briefly: the rats were maintained with only chow for 24 h and then, a water burette was offered for 2 h (PR – thirst test), and then an additional burette containing 0.3 M NaCl was offered for another 2 h (sodium appetite test). At the end of the experiments, the analysis of plasma values of insulin, leptin and E2 were performed, and visceral adipose tissue and uterus were collected and weighted. The main findings were: 1) HFD rats, compared to SD rats, had increased body weight gain, visceral adipose tissue and plasma leptin, and all these parameters were potentiated in HFD/OVX rats and reversed by E2 replacement (HFD/ OVX+E2); 2) A reduction in daily water intake was observed in HFD/SHAM or HFD/OVX rats, compared to SD rats with the same treatment, in HFD/OVX+E2 rats the values of spontaneous water intake returned to values similar to those observed at baseline; 3) spontaneous sodium intake was increased in HFD/SHAM rats when compared to SD/SHAM rats; 4) in HFD/OVX rats, E2 treatment (HFD/OVX+E2) restored the increased sodium intake; 5) Compared with SD/SHAM rats, HFD/SHAM rats ingested less water and more sodium when submitted to 24 h of WD; 6) In SD/OVX rats, there was a lower water intake induced by WD, which was not reversed by the E2 treatment; 7) OVX did not change the sodium intake induced by WD in SD rats, but with E2 treatment there was a reduction in sodium intake induced by WD, 8) On the other hand, water intake after WD in HFD/OVX rats was lower than in HFD/SHAM rats, as well as the sodium intake; 9) In HFD rats, E2 treatment restored both

water and sodium intake. Therefore, while in SD rats E2 appears to have an inhibitory effect on sodium intake only during WD, in HFD rats the effect appears to be excitatory for both water and sodium intake in spontaneous or WD-induced ingestion.

Keywords: Obesity, Water-deprivation, Dehydration, Thirst, Sodium appetite, Estradiol, Menopause.

FIGURE LIST

ABBREVIATION LIST

ACE – Angiotensin-converting enzyme
ANG I – Angiotensin I
ANG II – Angiotensin II
AT1 – Angiotensin II receptor type 1
CNS – Central nervous system
E2 - 17-β-estradiol
ER – Estrogen receptor
HFD – High-fat diet
MnPO - Median preoptic nucleus
OVX – ovariectomy/ovariectomized
PR – Partial Rehydration
PVN - Paraventricular nucleus
RAS – Renin-angiotesin system
SD – Standard diet
SFO – Subfornical organ
SON - Supraoptic nucleus
VOLT –Vascular organ of the lamina terminalis
VP - Vasopressin
WD – Water deprivation

SUMARY

1. INTRODUCTION

1.1.EPIDEMIOLOGY OF OBESITY

Obesity may be defined by the excessive accumulation of fat that harms the health of the individual, and individuals with a body mass index greater than or equal to 30 kg/m² are considered obese. Obesity affects a large part of the world population and the prevalence of obesity (BMI ≥30 kg/m2) in woman was about 377 million and in men about 266 million in 2014, a significant increase compared to the levels observed in 1975 when around 71 million women and 34 million men were obese. In 2016, it was estimated that more than 650 million adults worldwide suffer with obesity, and it is estimated that by 2025 around 700 million adults will be obese [1]These increase in the number of obese individuals in recent decades may be the result of the lifestyle that has been adopted by the world population, based upon high consumption of foods rich in carbohydrates and fats, associated with low energy expenditure, and also, genetic factors may contribute to this situation [2,3]Data from the Surveillance of Risk and Protection Factors for Chronic Diseases by Telephone Survey-Vigitel [4], created by the Ministry of Health, to minimize chronic non-communicable diseases in Brazilian states, demonstrated by the body mass index (BMI) that in 2006, 11.8% of the Brazilian population was obese, but in 2019 this number increased to 20.3% of the population. Also, in the same year, about 18.7% of the obese population was male and 20.7% was female.

The obesity epidemic is responsible for promoting increased spending by public health systems such as the Unified Health System, where the Brazilian population is supported to obtain medical care. In addition, the other social sectors may be affected, since the excess of weight can lead to a decrease in quality of life, generating loss of productivity and early retirement[5,6]. This decrease in quality of life may occur due to the emergence of comorbidities associated with obesity, such as: dyslipidemia, diabetes type II, respiratory disorders, hepatic steatosis, and cardiovascular disorders.

In rodents, obesity can be induced through a palatable high-fat diet (HFD), which resembles the diet adopted by western society, rich in fats and carbohydrates[7–9]. Data from literature and from our laboratory, show that rats fed with this HFD develop a series of metabolic and cardiovascular changes, such as: dyslipidemia, increases in serum leptin

and insulin, increase in glycaemia, and increase in blood pressure, due to a decrease in baroreflex activity and a greater activity of the renin-angiotensin system (RAS) in the central nervous system (CNS) [8–10]

1.2.OBESITY AND HYDROMINERAL BALANCE

The renin-angiotensin system (RAS) is an important hormonal system for maintaining blood pressure and fluid-electrolyte balance. The activation of this system occurs in situations of hypovolemia, hypotension, or hyponatremia, which is sensed by juxtaglomerular cells in the kidney, leading to renin release, which cleaves angiotensinogen, a globulin secreted by the liver, producing the decapeptide angiotensin I (ANG I). This decapeptide suffer the action of the angiotensin-converting enzyme (ACE), located major in the lungs, which cleaves ANG I into an octapeptide, the ANG II, which have activity in several target organs and in the brain. The ANG II induces thirst and sodium apettite by acting mainly on its AT1 receptors, found in circumventricular organs (CVO) in the forebrain, located outside the blood-brain barrier, such as the subfornical organ (SFO) and vascular organ of the lamina terminalis (VOLT) (review in [11]).

Furthermore, ANG II can act by stimulating the release of vasopressin (VP) by the neurohypophysis, a hormone that acts in the kidneys to increase water reabsorption, and in the blood vessels, generating vasoconstriction Peripherally, ANG II acts by regulating the secretion of aldosterone by the adrenal gland, which has the function of stimulating sodium reabsorption in the renal collecting ducts [12,13].

RAS is also found in adipose tissue, and during obesity, due to the increase in adipose tissue, there is an increase in the formation of ANG II by adipose tissue, increasing plasma levels of this hormone [14,15]Disorders in water intake were found in obese individuals. Studies by Rosinger et al [16] demonstrated that the relationship between water intake and urinary osmolarity, a biomarker of hydration, is modified by weight gain in US adults. The same study showed that, although water consumption is increased in obese individuals, they also have an increase in urine osmolarity, thus suggesting, that obese adults are more likely to be in a dehydrated state. In addition, we and other have demonstrated that HFD fed rats had a reduced daily water intake, probably associated with an increase in salivary secretion during food intake [8] or a difference in the pattern of drinking [17], although the formation of metabolic water in HFD-fed rats cannot be completely ruled out, since it has been seen in another rodent species[18].

However, most studies that analyzed the fluid intake are carried out in male subjects, whether animals or humans. Thus, there is a lack of studies in obese female subjects and, mainly, in the different stages of sexual maturation.

1.3.MENOPAUSE, OBESITY AND RENIN-ANGIOTENSIN SYSTEM

The menopause is characterized by the absence of menstruation for at least 1 year, and it occurs in the female body with natural aging, due to the absence of ovarian follicles, which are depleted throughout the menstrual cycles. This phenomenon of menopause occurs in women between the ages of 45 and 55 years old, and as a result, the ovaries no longer produce progesterone and estrogens in significant amounts. The hormone estradiol is secreted by the internal theca cells by the mature follicle during the menstrual cycle, and it enables ovulation to occur[19]. Estradiol is also associated with other functions in the female body, such as: acting on bone metabolism, contributing to cognitive health, participating in the regulation of energy metabolism. Regarding this last matter, a study of Razmjou et al [20]showed that after going through menopause, women had increased waist circumference, as well as increased fat mass, when compared to themselves in a premenopausal period[20]

It is also known that gonadal hormones may influence the control of fluid and electrolyte balance. Since the 1960's, studies made with female intact rodents have been showing that during the estrus period, which is the phase of the reproductive cycle where there is a higher plasma concentration of estrogen, the animals showed a decrease in both water and sodium intake, and on the other hand, during the diestrus, when there are low levels of plasma estrogen, the animals increased their fluid intake [21–24] Recent study by Santollo et al [25]showed similar data. Hormone replacement studies made in ovariectomized (OVX) rodents showed that estradiol therapy decreases both spontaneous fluid intake and stimulated fluid intake [23,26–28]Water and sodium intake were decreased in OVX rats treated with estradiol after intracerebroventricular injections of ANG II, compared to the ones that did not receive estradiol treatment[27]. It was also demonstrated that estradiol replacement can reduce water intake induced by 24 h of water deprivation (WD), and sodium intake induced by sodium depletion by Furosemide[28]. Similar data was found in Scheidler et al [29], where OVX rats treated with estradiol showed a reduced sodium intake after sodium deprivation after 8 days.

All these facts can be explained by the fact that estradiol receptors (ER) are present in brain regions also responsible for controlling the maintenance of hydrosaline balance, such as: paraventricular nucleus (PVN), supraoptic nucleus (SON), median preoptic nucleus (MnPO), OVLT and SFO [30]. Regarding these last two regions, ER were found colocalized with angiotensin II receptors type 1 (AT1) receptors, and other studies have already shown that estrogen exerts effects on the RAS. A study made by Mecawi et al [28], demonstrated that central blockade of AT1 with Losartan inhibited the dipsogenic response induced by water deprivation, and inhibited sodium intake induced by sodium depletion in ovariectomized rats. Also, other study has shown estrogen directly interacts with the RAS by decreasing the expression of AT1 receptors in brain areas involved in the regulation of sodium appetite and thirst [31].

Furthermore, studies by Omouessi et al [32]demonstrated that female obese Zucker rats have an increased basal sodium appetite when compared to female lean rats or to males obese or lean rats. But, in the literature, there is a lack of studies evaluating the hydrosaline balance in obese females, and no studies were found in which OVX was associated with HFD, with or without hormone replacement. Therefore, it is interesting to study the *ad libitum* fluid intake in intact or ovariectomized female obese rats, with or without estrogen replacement.

1.4. DEHYDRATION AND ESTRADIOL INFLUENCE ON RAS

Water deprivation (WD), which cause double dehydration, is a common disturbance seen in wildlife and in human [33–36]. The dehydration of intracellular compartment results in increased plasma osmolarity, which activates osmoreceptors found in the SFO and OVLT, causing thirst as a compensatory response. Regarding the dehydration of extracellular compartment, it leads to volume loss, which is sensed by the juxtaglomerular cells in the kidneys, leading to renin release, activating RAS, and therefore, neural circuits modulated by AT1 receptors, facilitating the dehydrated organism to ingest water and sodium intake [37–39]

It is well known that after WD, animals prefer to drink water, but when hypertonic saline solution is also available, there are a considerable amount of ingestion [38,39]. In order to separate the ingestion of water (thirst) from the ingestion of hypertonic saline solution (sodium appetite) after WD, a protocol was developed by Sato et al [38]. During this protocol, after WD, the animals first have access only to water for 2 h (Partial Rehydration - PR), and after this period, they had access to both water and hypertonic

sodium solution for an additional 2 h. The 2 h interval between the access to only water and hypertonic sodium solution lead to a greater preference for salt intake rather than water during the 2 additional hours. Studies made by de Luca Jr. et al [40] investigated the plasma volume and osmolarity in 24 h-WD rats. It was found that after PR, animals had the plasma osmolarity corrected, but not the plasma volume. After sodium intake, animals showed plasma volume corrected. In the same study it was shown that after 24 h of WD, there are increased FOS expression in in the OVLT, MnPO, SON, and to a lesser extent, in the SFO. After PR, the FOS expression was reduced in the OVLT, MnPO, and SON, but it was not altered in the SFO, suggesting that this last brain region is involved more to sodium than to water intake.

Several studies have demonstrated that female OVX rats treated with estradiol ingest less water when compared to OVX rats that receive vehicle either ad libitum or when submitted to different protocols to induce water intake such as WD or central ANG II [28,41–43]. This attenuation of water intake by estradiol appears to involve central angiotensinergic pathways, which seems to be mediated by neurons present in CVOs, mainly in the SFO [44,45]. Data from literature demonstrated that SFO neurons express estrogen receptor (ER) [30]. Regarding estradiol modulating sodium intake, some studies have shown no differences in sodium intake after WD in OVX rats treated with estradiol or not [28,46]. However, it was found that OVX rats treated with estradiol when exposed to sodium depletion, a situation that also causes plasma volume loss, and thereafter activates RAS, ingested a smaller amount of sodium when compared to OVX rats that received vehicle [28,29]. All these data together suggest the participation of estradiol on mechanisms related to fluid intake control, especially in those involved with RAS activation.

Thus, we hypothesized that OVX rats when fed a HFD would exhibit disorders in RAS, which would be noted by a further increase in sodium appetite, and that estrogen replacement would be responsible for decrease this ingestion. We also aimed to study the water intake in these animals. In order to verify that, we evaluated the spontaneous fluid intake and the fluid intake after 24-h of WD, using a protocol known for causing thirst and sodium appetite dependent of ANG II [24].

2. OBJECTIVES

We aimed to study in female rats fed with standard diet or high-fat diet, intact or ovariectomized, with or without estrogen replacement, if they have a change in both spontaneous and induced water and sodium intake. Furthermore, we also aimed evaluate the effects on metabolic disorders caused by the association between a high-fat diet and ovariectomy in these same rats.

3. MATERIAL AND METHODS

3.1.Animals

Adult female Holtzman rats (260–300 g) from the colony of UNESP Araraquara were maintained in individual cages, with food (composition bellow), water and 0.3 M NaCl solution, provided ad libitum, in a room with controlled temperature (23 ± 2 °C) and humidity (55 ± 10%). Lights were on from 7:00 am to 7:00 pm. Ethics Committee for Animal Care and Use of the Dental School of Araraquara, UNESP, approved the experimental protocols used in the present study (protocol number CEUA 18/2020), which were performed according to the principles of the National Institutes of Health guide for care and use of laboratory animals (NIH Publications no. 8023, revised 1978).

3.2.Diets

Two diets were used in the present study, standard rat chow (SD) and high fat diet (HFD). The SD was composed of a balanced diet of the SOCIL brand (Neovia LTDA, Descalvado, SP, Brazil) containing 23 g of protein, 49 g of carbohydrate, 4 g of fat, 5 g of fiber and 270 mg of sodium (per 100 g of diet), as supplied by the manufacturer and 9.9% of humidity as bromatological analysis. The HFD was composed of a concentrated standard rat chow with a mix of mineral and vitamins (PragSoluções Biociências, Jaú, SP, Brasil) plus peanuts, milk chocolate, and sweet biscuits in a proportion of 3:2:2:1 as previously described [8]. For the HFD, all the components were grinded, mixed and prepared as pellets. The final content of the HFD was 22.3 g of protein, 40.8 g of carbohydrate, 24.5 g of total fat, 5.9 g of fiber and 253 mg of sodium per 100 g of diet and 22% of humidity. The caloric values of the diets were approximately 3.24 kcal/g for the SD (11% calories from fat) and 4.73 kcal/g for HFD (45% calories from fat).

3.3.Anesthesia and euthanasia

Female rats were anesthetized with ketamine [80 mg/kg of body weight (b. wt.)] combined with xylazine (7 mg/kg of b. wt.) or with isoflurane (5% in 100% O2; Cristália, Itapira, SP, Brazil) depending on the surgery (see below). During the surgeries/procedures, the level of anesthesia was monitored by checking the eye blink reflex and a reaction to paw pinch and was adjusted if necessary. Following the surgeries animals received a prophylactic dose of penicillin (50,000 IU, intramuscularly) and a dose of the anti-inflammatory ketoprofen (1 mg/kg of b. wt., subcutaneously). At the end of the experiments, rats were euthanized by placing them under deep anesthesia with sodium thiopental (100 mg/kg of b. wt, i.p.).

3.4.Ovariectomy

The ovaries were bilaterally removed through two small lateral abdominal surgical incisions. A group of rats underwent sham surgery, where the ovaries were exposed, but were kept intact.

3.5.Estrogen Replacement

A small skin incision was made in the scruff of the neck and a subcutaneous pocket was made to accommodate an osmotic pump (Alzet osmotic pump, model 2ML4, Alzet, USA) filled with 17β-Estradiol (E2) with a10 μg/rat/day delivery rate, dissolved in 99.5% propylene glycol and 0.5 % ethanol solution (vehicle). Rats that did not receive E2 replacement had vehicle (0.5 ml/rat/week) administered once a week by subcutaneous injection. The dose of E2 was based in previous study [26].

3.6.Blood Collection

After 12 h fasting, trunk blood was collected in non-coated tubes for insulin, leptin and 17-β estradiol analysis. All hormones serum levels were analyzed using commercially available ELISA kits, following the instructions of the manufacturer (MILLIPORE, Billerica, MA, USA for leptin, ALPCO for insulin Salem, NH, USA and ABCAM, USA for 17-beta-Estradiol).

3.7.Adipose Tissue Collection

Visceral (retroperitoneal, mesenteric, and ovarian) white adipose tissue was removed and weighed according to previous studies [5-7, 27].

3.8.Uterine Index

To verify the efficiency of the OVX and E2 treatment, the uterus was removed and immediately weighted for calculation of the uterine index (uterus weight/body weight, expressed as milligrams per 100 g body weight).

3.9.Water and 0.3 M NaCl intake measurements

Daily water and 0.3 M NaCl intake were recorded using polypropylene bottles with 1- ml divisions.

3.10. Water Deprivation

To evaluate thirst and sodium appetite separated we used the WD-partial rehydration (PR) protocol, WD-PR, developed by Sato et al [24]. Briefly, female rats were deprived of water and 0.3 M NaCl for 24-h, with free access to food, consisting in the WD period. After this period, food was removed from the cages and a glass burette with 0.1-ml divisions fitted with a metal drinking spout containing water was offered for, consisting in the PR period. The non-cumulative water intake was measured every 30 min for 120 min (thirst Test). At the end of the PR, a glass burette containing 0.3 M NaCl was added to the cage and the non-cumulative 0.3 M NaCl and water intake was measured every 30 mins for 120 min (sodium appetite test).

3.11. Statistical Analysis

All data are expressed as means ± SEM. Results of intake, renal excretion and adipose and uterine tissues were expressed as means ± SEM/100 g of b. wt. One or two-way ANOVA followed by Fisher test were used for the comparisons, as indicated. Differences were considered significant at $p < 0.05$.

3.12. Experimental Groups and protocols

Animals were randomly divided into 6 groups: SD/SHAM (rats fed with SD, sham surgery for OVX and no E2 replacement); SD /OVX (rats fed with SD, OVX and no E2 replacement); SD /OVX+E2 (rats fed with SD, OVX and E2 replacement); HFD/SHAM (rats fed with HFD, sham surgery for OVX and no E2 replacement); HFD /OVX (rats fed with HFD, OVX and no E2 replacement); HFD/OVX+E2 (rats fed with HFD, OVX and E2 replacement).

All animals were placed in individual cages with water, 0.3 M NaCl and food (SD or HFD) ad libitum. In order to measure basal parameters all rats were fed with SD for one week (Week 0). At the end of the Week 0, female rats were anesthetized with xylazine and ketamine (see above) and underwent OVX or sham surgery. After two days of recovery, at the beginning of Week 1, part of the animals started been fed with HFD or were kept with SD, according to their groups. Three weeks after starting the diets (at the end of Week 3), part of the female OVX rats were anesthetized with isoflurane (see above) and the Alzet mini-pump was implanted subcutaneously. They were treated with E2 or vehicle for 28 days (weeks 4 to 7). The WD-PR protocol (see above) was carried during the 7th week, thus 6 weeks of OVX and diet treatment (SD or HFD), and 3 weeks of E2 replacement.

For ad libitum analyzes, fluid intake was measured five days a week and were averaged by the week starting at Week 0 throughout Week 6. During the 7th week, rats underwent 24-h WD and WD-PR protocol was performed. After 2 days of recovery from WD, animals were fasted for 12 hours, and were euthanized for tissue and blood collection.

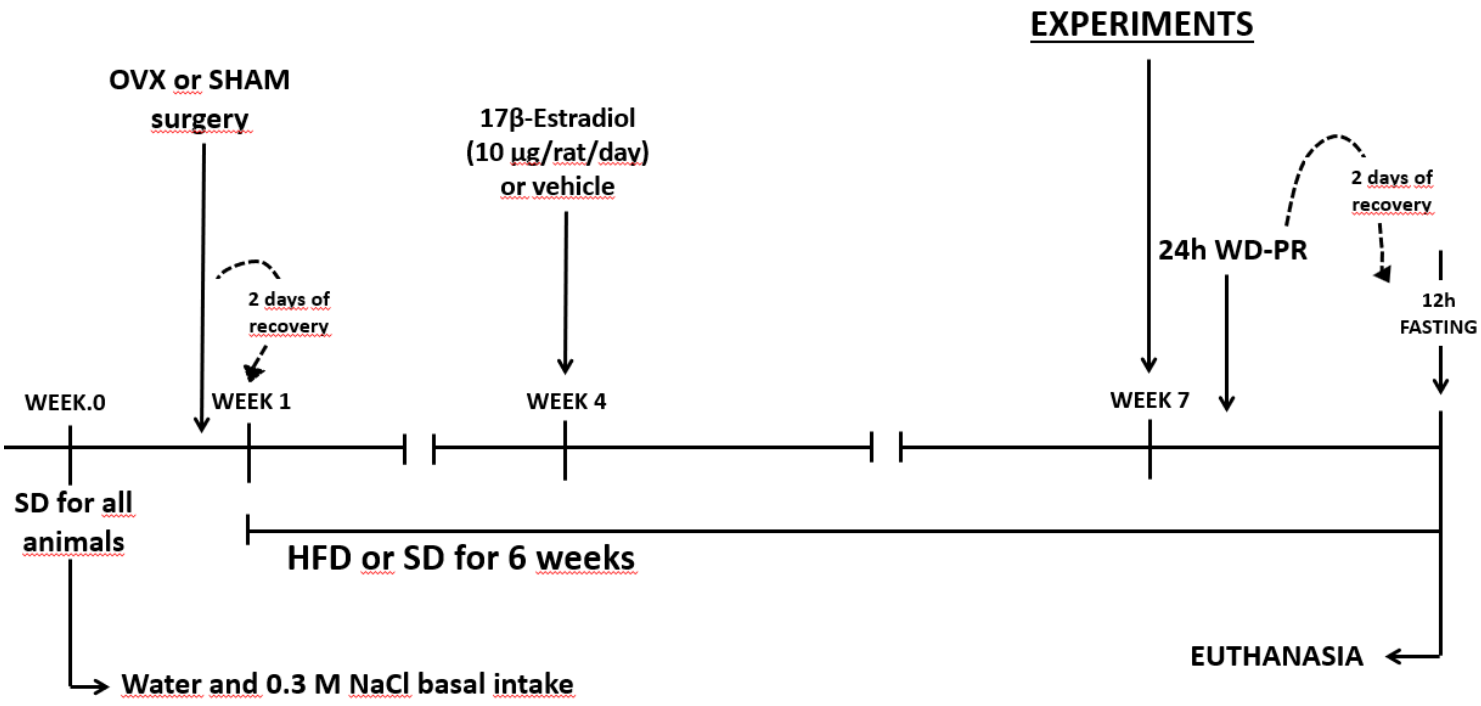

Figure 1: Experimental protocol design.

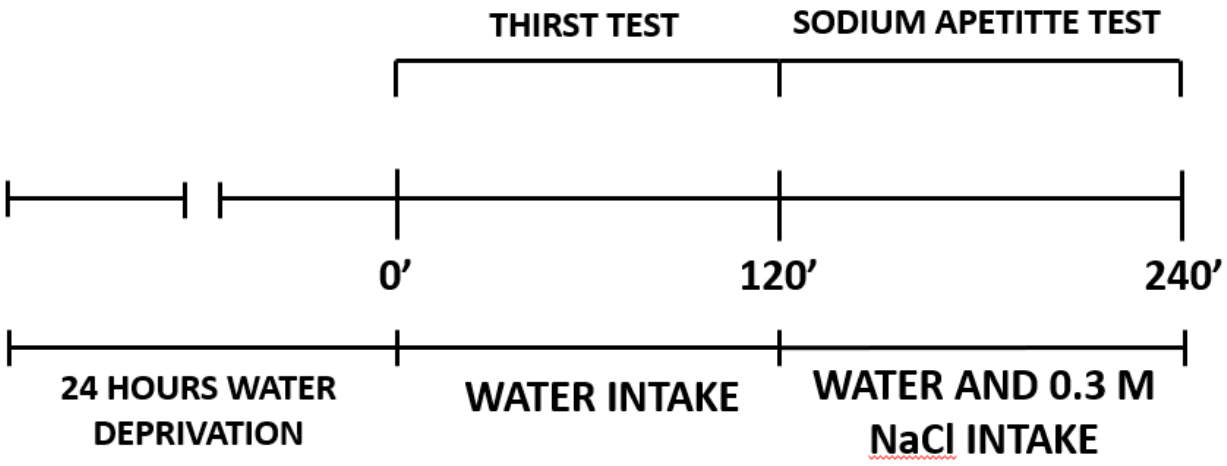

Figure 2: Experimental 24h - Water Deprivation – Partial Rehydration Protocol design.

4. RESULTS

4.1. Effect of OVX and E2 replacement in anthropometric and plasma hormone values in SD and HFD-fed female rats

At week 0, all animals were fed with SD diet and were without any treatment (OVX or E2). At this week, body weight was comparable between all groups (Figure 3A). In SD rats, OVX group presented an increase in body weight over the weeks compared to SD/SHAM rats (362.9 ± 5.3 vs. SD/SHAM: 302.8 ± 4.1 g at week 6; $p < 0.05$), which was reduced after treatment with E2 (SD/OVX+E2: 333.7 ± 5.9 vs. SD/OVX: 362.9 ± 5.3 g at week 6; $p < 0.05$), but still greater than the body weight in SD/SHAM at the end of the treatment [$F(5,952) = 118.549$; $p < 0.05$], Figure 3A. Similarly, in HFD rats, OVX group presented an increase in body weight over the weeks compared to HFD/SHAM rats (at week 6; $p < 0.05$), which was completed reverted after treatment with E2 (at week 6; $p < 0.05$), [$F(5,952) = 118.549$; $P < 0.05$], Figure 3A. HFD/SHAM rats had a greater body weight than SD/SHAM from the 3rd week after starting the diet, throughout the entire treatment (at week 6; $p < 0.05$), as well as HFD/OVX group (at week 6; $p < 0.05$), however, E2 treatment reverted the increase in body weight in HFD/OVX-E2 group to values comparable to SD/OVX-E2 group (Figure 3A).

Visceral adipose tissue was increased in OVX rats in SD and HFD fed rats, and E2 treatment reduced these levels to the ones observed at intact female SD or HFD rats (Figure 3B). Nonetheless, HFD rats always presented a greater visceral adiposity than SD rats in all treatments [$F(5,96) = 28,736$; $p < 0.05$].

Treatment with E2 proved to be effective in simulating physiological doses in SD and HFD rats compared to their respective OVX groups [$F(3,13) = 10.103$; $p < 0.05$], Figures 4A and 4B. In addition, HFD/SHAM had increased leptin levels compared to SD/SHAM; [$F(5,31) = 28,694 = p < 0.05$], Figure 4C. OVX rats presented a greater leptin levels in both SD and HFD groups (Figure 4C), however, only in HFD/OVX rats, E2 treatment was effective in completely reverted the greater increase in leptin levels observed in HFD/OVX, and the values were similar to the HFD/SHAM and SD/SHAM rats [$F(5,31) = 28,694 = p < 0.05$], Figure 4C. Insulin levels were only increased in HFD/OVX rats and were blocked by E2 treatment (Figure 4D).

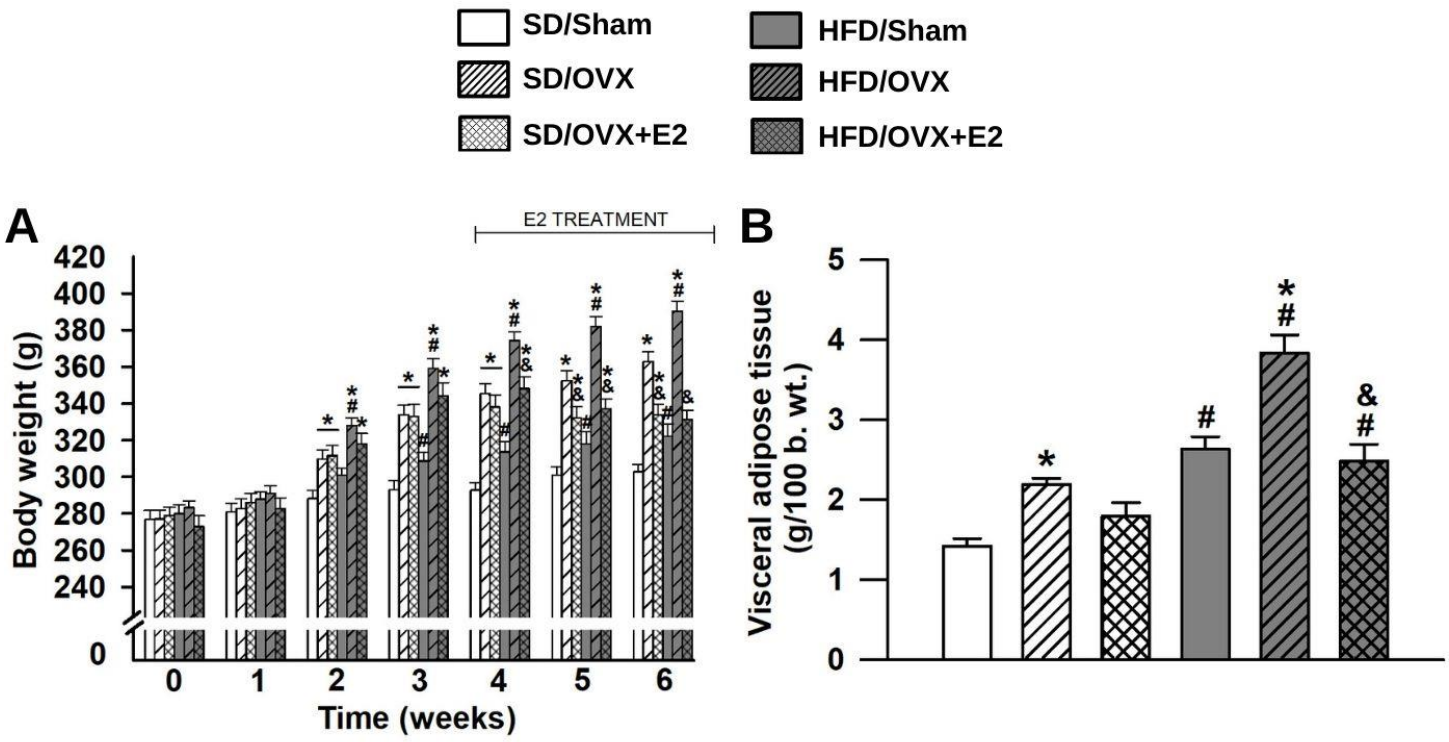

Figure 3: Effect of OVX and E2 replacement in anthropometric values in standard diet (SD) and high-fat diet (HFD) fed female rats. (A) Body weight (g; n = 16 – 28), (B) Visceral adipose tissue (g/100 g of body weight, b.wt; n = 11 – 23, in SD or HFD-fed female sham, ovariectomized (OVX) or OVX treated for 3 weeks with 17β-Estradiol (E2). Data are expressed as means ± SEM; two-way ANOVA (body weight) or one-way ANOVA (other variables) followed by the Fisher Exact Test, $p < 0.05$; ***** different from SHAM with same diet; **#** different from SD with same treatment; **&** different from OVX group with same diet.

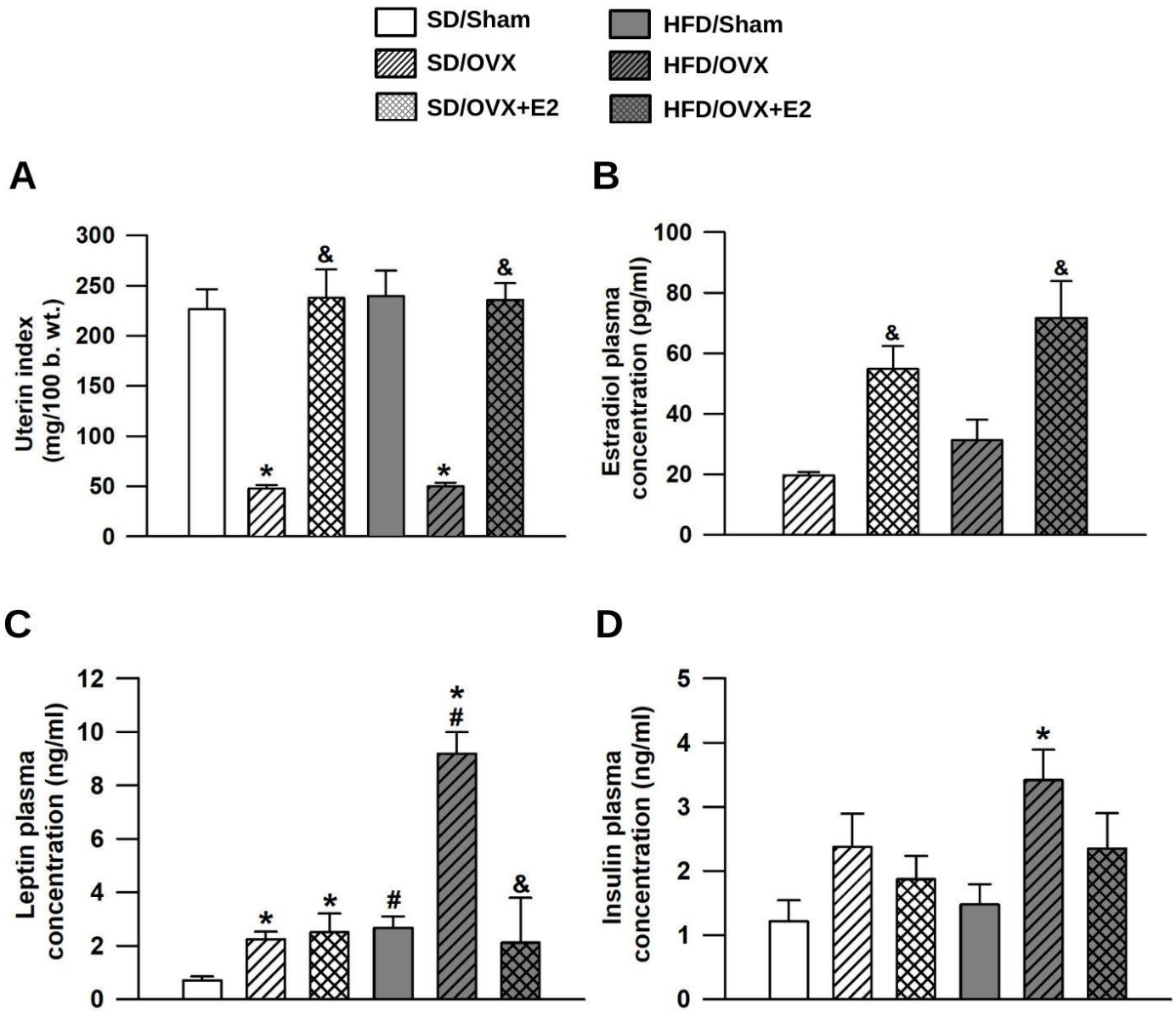

Figure 4: Effect of OVX and E2 replacement in uterin index and plasma hormone values in standard diet (SD) and high-fat diet (HFD) fed female rats. (A) Uterin index (mg/ 100 g of b. wt; n = 11 – 17) and (B) 17β-Estradiol (n = 4 – 8), (C) Leptin (n = 3 – 13) and (D) Insulin (n = 5 – 13) plasma concentration in SD or HFD-fed female sham, ovariectomized (OVX) or OVX treated for 3 weeks with 17β-Estradiol (E2). Data are expressed as means ± SEM; two-way ANOVA (body weight) or one-way ANOVA (other variables) followed by the Fisher Exact Test, $p < 0.05$; ***** different from SHAM with same diet; **#** different from SD with same treatment; **&** different from OVX treated group with same diet.

4.2. Effect of OVX and E2 replacement in daily fluid intake in SD and HFD-fed female rats

At week 0, all animals were fed with SD diet and were without any treatment (OVX or E2). At this week fluid intake were comparable between all groups. In SD rats, daily water intake was transiently (1-2nd week) increased by OVX compared to week 0 ($p < 0.05$), Figure 5A. Moreover, E2 treatment in SD-OVX rats did not promote change in daily water intake (Figure 5A). Conversely, daily water intake was reduced in HFD from the 1st week of the HFD, throughout the experimental period (6 weeks), except for the HFD/OVX-E2 group, which recovered the water intake to the one observed at week 0 (Figure 5A).

While in SD group daily sodium intake was not altered by OVX nor E2 treatment, in HFD rats a robust need-free sodium intake was observed starting in the firsts 2 weeks after HFD in all groups (Figure 5B). In HFD-OVX, a reduction in sodium intake compared to HFD/SHAM group was observed at 3rd week, however, after E2 replacement, a further decrease in 0.3 M NaCl intake was observed, although it was still greater than its baseline levels (week 0) or to the one observed in HFD/SHAM group (Figure 5B), [$F(5,529) = 25.973$; $p < 0.05$].

When we analyzed total fluid intake (Figure 5C), we observed that it was comparable between groups from week 0 to week 4. After that, HFD/OVX and HFD/OVX+E2 rats presented a decrease in total fluid intake, whereas no change at all was observed in SD rats [$F)2,528) = 6.846$; $p < 0.05$]. However, if analyzing the 0.3 M NaCl/water ratio there was a shift from water to sodium intake in HFD rats from the 1st week of HFD (Figure 5D) with or without OVX. After E2 treatment, from week 4, HFD/OVX+E2 rats decreased 0.3 M NaCl/water ratio compared to HFD/SHAM reaching values comparable to SD rats, regardless the treatment (Figure 5D). HFD/OVX rats also decreased 0.3 M NaCl/water ratio 6 weeks after HFD and OVX (Figure 5D); [$F(5,518) = 40.329$; $p < 0.05$].

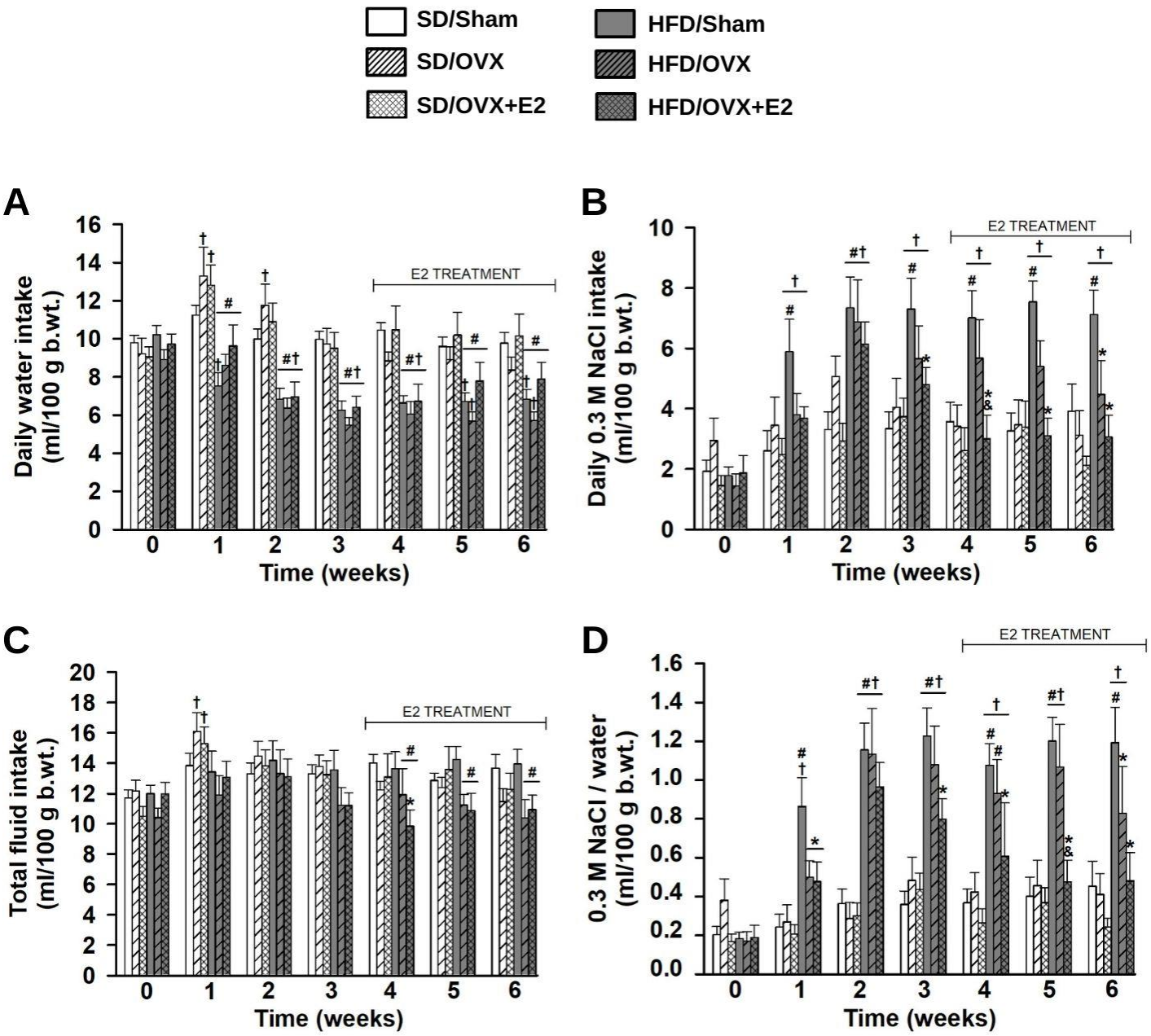

Figure 5: Effect of OVX and E2 replacement in daily fluid intake in standard diet (SD) and high-fat diet (HFD) fed female rats. (A) daily water intake (ml/100 g of body weight, b.wt/day; n = 10 – 22), (B) daily sodium intake (ml/100 g of body weight, b.wt/day; n = 10 – 22), (C) daily total fluid intake (ml/100 g of body weight, b.wt/day; n = 10 – 22), and (D) ratio of NaCl 0.3 M / water daily intake (ml/100 g of body weight, b.wt/day; n = 10 – 22) in SD or HFD-fed female sham, ovariectomized (OVX) or OVX treated for 3 weeks with 17β-Estradiol (E2). Data are expressed as means ± SEM; two-way ANOVA followed by the Fisher Exact Test, $p < 0.05$; ***** different from SHAM with same diet; # different from SD with same treatment; **&** different from OVX treated group with same diet, **t** different from time 0.

4.3.Water and sodium intake after 24 hours of water deprivation

During the thirst test induced by water deprivation, the water intake was reduced in SD/OVX and SD/OVX+E2 in the first 30 min (Figure 6A). In contrast, in HFD female rats, the reduction of water deprivation-induced water intake was only observed in HFD/OVX rats compared to HFD/SHAM rats (Figure 6A). It should also be pointed out that the water intake observed in HFD/SHAM and OVX rats were lesser to the one observed in SD rats with the same treatment (Figure 6A); [$F(5,264) = 11.747$; $p < 0.05$]. In the sodium appetite test, in SD rats, a smaller increase in 0.3 M NaCl intake was observed in SD/OVX female rats in the first 30 min of the test (Figure 6B). On the contrary, in HFD rats, the OVX inhibited the full appearance of sodium intake, which was reverted in the female OVX group with E2 replacement (Figure 6B) in the first 30 min of the test. Opposite to what was observed in the thirst test, HFD/SHAM and HFD/OVX+E2 female rats displayed an enhanced sodium intake compared to SD rats with the same treatment (Figure 6B); [$F(5,264) = 6.884$; $p < 0.05$]. No significant changes in water intake associate with sodium intake in SD group were observed, however in the HFD group, there was a lesser water intake in HFD/OVX group for first 30 min out of the 120 min of the recording (time 150 min of the graph) as can be observed in Figure 6C.

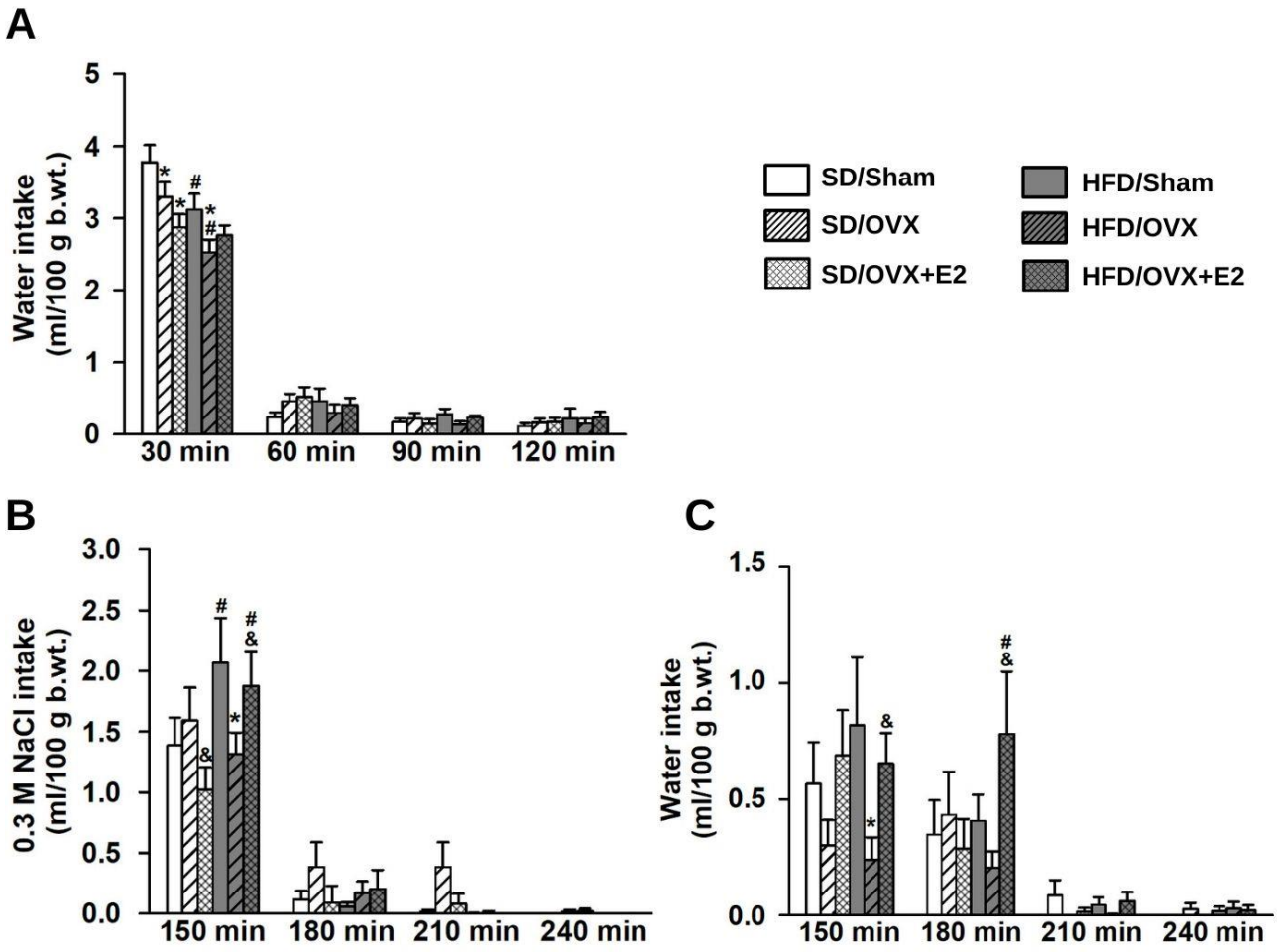

Figure 6: Water intake in the thirst test (A), and 0.3 M NaCl (B) and water – associate sodium intake (C) in the sodium appetite test in 24-h water-deprived (WD) rats fed with SD or HFD, intact, OVX and OVX treated E2; n = 8 – 16. Data are expressed as means ± SEM; two-way ANOVA followed by the Fisher Exact Test, $p < 0.05$; ***** different from SHAM with same diet; **#** different from SD with same treatment; **&** different from OVX treated group with same diet.

5. DISCUSSION

This is the first study to analyze the *ad libitum* and WD-induced water and 0.3 M intake in intact or ovariectomized female rats fed with HFD and the role of E2 modulating these responses. In addition, anthropometric and hormonal profiles were also studied. All the data are discussed as follows.

First, as expected HFD sham rats presented a greater body weight, adipose tissue and leptin levels compared to sham SD at the end of 6 weeks. An increase in these variables in SD/OVX and a greater increase in HFD/OVX rats was also expected. Ovariectomy experimentally simulates pos-menopause, and it is well known that at this period there is a body weight gain, body fat accumulation, increase in leptin and insulin levels and insulin resistance [47–49]. In both groups, SD/OVX and HFD/OVX, E2 restore or almost restore to levels of SD/OVX-E2 group, demonstrating that the absence of E2 is the responsible for these responses in HFD/OVX rats and that E2 replacement had a beneficial effect in these variables. In fact, the effect of E2 controlling the metabolic rate is well known (reviewed in [47]). Leptin levels increase in both OVX groups and further increase in HFD/OVX rats, probably related to the respective increase in the body weight in these groups. In menopause woman treated at least for 1 year with hormonal therapy, the increase in leptin levels was decreased [49] as well as OVX rats treated for 7 weeks with E2 [50]. In the present study we only observed a reduction in leptin levels by E2 in HFD/OVX rats. Maybe a longer E2 treatment would be need to the inhibitory effect of E2 in leptin levels appears. In humans and animal models have been shown that estrogen has an anti-diabetic effect (reviewed in[51]). In rats it has been reported that only OVX does not change insulin levels in rats fed with a standard diet [52], similarly to what we have seen in the present study. We did not see a significant increase in insulin levels in HFD female rats, as we observed in male rats in other studies from our lab[8,10]. However, we observed an increase in insulin levels in HFD/OVX rats, which was restored by E2 treatment. Similar data, but analyzing insulin sensitivity instead of insulin levels, also demonstrated that in HFD/OVX rats the decreased insulin sensitivity was restored with E2 treatment[48]. Finally, to be sure of the effectiveness of E2, the uterine index of sham SD or HFD rats were reduced to the same extent in OVX rats and were restored after E2 treatment. In fact, OVX rats had a very low levels of E2, which was restored to levels found in intact female rats found in the literature [53].

In the present study, a transient increase in daily water intake was observed in rats fed SD after OVX (weeks 1 and 2), when compared with themselves before OVX surgery, and this increase was not maintained until the end of six weeks. This result is according to other studies [22,23]in which they suggested that this increase was dependent of increased body weight and increased food intake. However, recent studies made by Santollo et al [25], reported in OVX rats that water intake is not correlated with body weight nor with food intake .However, it is important mentioning that there are few studies available in the literature that compare groups of OVX rats with intact rats, as well as those that analyze the effects on fluid intake for a long period of time after OVX (such as 6 weeks), and no studies were found in which it was waited for such a long period to start hormone replacement after OVX.

Recent studies from our laboratory and others [8,17] demonstrated that HFD-fed male rats presented a reduction in daily water intake. Sa et al [8] suggested that an increase in salivary secretion in HFD rats may be responsible for at least part of the reduced water intake. Volcko et al[17], in another study, suggested that the decrease in water intake seems to be at least partially dependent of a decrease in licking bouts in HFD fed rats. Thus, it is possible that in HFD intact female rats the same mechanisms are occurring to decrease the *ad libitum* water intake. In addition, other mechanisms not yet studied, such as neuroinflammation could also account for that. For instance, it has been shown that an increase in pro-inflammatory cytokines in HFD fed rats, not only in peripheral, but also in central areas [10,54,55], and inflammation has been shown to decrease fluid intake (reviewed by [56]). However, it should be noted that all these experiments were done in male rats. In HFD/OVX female rats there was no change in the decreased water intake, but if in these females the E2 treatment was performed, compared to the pre-OVX values, they were similar, demonstrating that in HFD fed rats E2 was facilitating water intake.

An expected effect was the daily increase in *ad libitum* sodium intake in HFD intact female rats. We have hypothesized that in female rats fed with a HFD, OVX would further potentiate the sodium intake. But, in this group, sodium intake was not increased, in the opposite, there was a reduction in the sodium intake after 3 weeks post OVX, which were sustained until the end of the 6 weeks, independent of hormonal replacement, since both OVX and OVX+E2 rats have shown a decreased *ad libitum* sodium intake when compared to HFD intact rats, and no difference were observed between these groups and SD-fed rats, while HFD intact rats have shown a greater sodium intake during all the weeks.

Regarding the total fluid intake, it was not seen major differences between all groups, but we can clearly see that it is much related to the sodium intake in HFD OVX and OVX+E2. Moreover, in HFD intact rats there was a shift from water to sodium intake. In other words, after 6 weeks, HFD intact rats ingested more sodium than water, which may be observed by the fact that 0.3 M NaCl/Water presented values greater than 1.0 in this group. Surprisingly, HFD/OVX and HFD/OVX+E2 rats had a decrease in the ratio of 0.3 M NaCl/Water, but this ratio became less than 1.0 only in the E2 treated group, despite of the lack of difference between the OVX and OVX+E2 groups in week 6.

The mechanisms involved with the increase in *ad libitum* sodium intake in HFD fed female rats is not known. We may speculate that female HFD fed female rats display an increase in sodium palatability, which was seen in an experiment with female rats fed with high sucrose liquid diet for 40 days[57]. At least in humans, more recently it has been demonstrated that obese subjects had a greater salt consumption, with greater preference and a lesser sensitivity [58]. If this is the same in HFD female rats, it still must be studied.

After 24-hour water deprivation, water intake was reduced by estradiol treatment in SD/OVX, which was different from data from the literature demonstrating that estradiol has an inhibitory effect on water intake after water deprivation in OVX fed a SD chow[28,41] . However, other study, like ours, did not find differences in water intake after 24 h WD between OVX Holtzman rats with or without estradiol replacement [46]. WD also induces sodium appetite[38,59], and the sodium intake induced by WD observed in SD/SHAM rats was not changed by OVX in SD/OVX, however, E2 treatment reduced the sodium intake as observed in SD/OVX+E2 group, similarly to what was seen in other studies [28]. In intact HFD/SHAM fed rats, WD induced a greater sodium intake than intact SD/SHAM rats, but different from SD/OVX rats, in HFD/OVX rats there was a decrease in sodium intake which was reversed by E2 treatment. Thus, in WD HFD fed rats, E2 seems to have an excitatory effect on sodium intake.

We do not know the mechanism by way of water and sodium intake were facilitated in HFD/OVX+E2 rats. Studies by Liu et al [60] suggested that the HFD diet alters the serum composition of fatty acids, leading to a higher proportion of saturated fatty acids and serum fatty acids may cause alterations in the signaling and expression pathways of the E2 receptor Erα [61,62]. In addition, estrogen receptors are found in colocalization with several regions that control water and sodium ingestive behaviors

[63]. It is also known that WD stimulates an increase in mRNA Erα expression in the lamina terminalis region [64].

6. CONCLUSIONS

In conclusion, while in SD female rats E2 appears to have an inhibitory effect on sodium intake only during WD, in HFD female rats the effect appears to be excitatory in both ad libitum or water deprivation-induced water and sodium intake. Knowing the mechanisms involved can lead us to better treat the hydroelectrolytic changes in menopause obese woman.

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