



Research reports

Effects of prenatal exposure to WIFI signal (2.45 GHz) on postnatal development and behavior in rat: Influence of maternal restraint

Haifa Othman^a, Mohamed Ammari^{a,b,*}, Mohsen Sakly^a, Hafedh Abdelmelek^a^a University of Carthage, Faculty of Sciences of Bizerte, Laboratory of Integrative Physiology, Jarzouna 7021, Tunisia^b University of Tunis El Manar, Higher Institute of Applied Biological Sciences of Tunis, 9, Rue Zouhair Essafi, 1006 Tunis, Tunisia

HIGHLIGHTS

- Effects of gestational exposure to WiFi signal and restraint along gestation period on the offspring were studied.
- The pups were evaluated for physical development and neuromotor maturation.
- Gestational WiFi exposure and restraint, adversely affected offspring neurodevelopment and behavior at adulthood.
- Progeny brain oxidative balance and serum biochemistry were disrupted.

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ABSTRACT

The present study was carried out to investigate the potential combined influence of maternal restraint stress and 2.45 GHz WiFi signal exposure on postnatal development and behavior in the offspring of exposed rats. 24 pregnant albino Wistar rats were randomly assigned to four groups: Control, WiFi-exposed, restrained and both WiFi-exposed and restrained groups. Each of WiFi exposure and restraint occurred 2 h/day along gestation till parturition. The pups were evaluated for physical development and neuromotor maturation. Moreover, elevated plus maze test, open field activity and stationary beam test were also determined on postnatal days 28, 30 and 31, respectively. After behavioral tests, the rats were anesthetized and their brains were removed for biochemical analysis. Our main findings showed no detrimental effects on gestation progress and outcomes at delivery in all groups. Subsequently, WiFi and restraint, *per se* and mainly *in concert* altered physical development of pups with slight differences between genders. Behaviorally, the gestational WiFi irradiation, restraint and especially the associated treatment affected the neuromotor maturation mainly in male progeny. At adult age, we noticed anxiety, motor deficit and exploratory behavior impairment in male offspring co-exposed to WiFi radiation and restraint, and in female progeny subjected to three treatments. The biochemical investigation showed that, all three treatments produced global oxidative stress in brain of both sexes. As for serum biochemistry, phosphorus, magnesium, glucose, triglycerides and calcium levels were disrupted. Taken together, prenatal WiFi radiation and restraint, alone and combined, provoked several behavioral and biochemical impairments at both juvenile and adult age of the offspring.

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Abbreviations: AP, access point; μ L, microlitre; ALAT, alanine aminotransferase; ANOVA, analysis of variance; ASAT, aspartate aminotransferase; CAT, catalase; CG, control group; GSH, glutathione; GSH-Px, glutathione peroxidase; GSM, global system of mobile; H_2O_2 , hydrogen peroxide; LDH, lactate dehydrogenase; MDA, malondialdehyde level; mg, milligramme; MHz, megahertz; MW, microwave; PBS, phosphate buffered saline; pSAPs, protected stretched attend postures; RF, radiofrequency fields; RG, restrained group; ROS, reactive oxygen species; rpm, revolution per minute; S.E.M, standard error of the mean; SAR, specific absorption rate; -SH, sulfhydryl; SOD, superoxide dismutase; WG, WiFi-exposed group; WIAD, wireless internet access device; WiFi, wireless fidelity; WPA, Wi-Fi protected access; WRG, WiFi-exposed and restrained group.

* Corresponding author at: Laboratoire de Physiologie Intégrée, Faculté des Sciences de Bizerte, Jarzouna 7021, Tunisia.

E-mail address: mohamed.ammari@fsb.rnu.tn (M. Ammari).

1. Introduction

The world-wide and rapidly growing use of Wireless Fidelity (WiFi) technology using radio for communication, operating typically at 2.45 GHz frequency, thus, it has raised serious concerns about the biological and health-related effects of radio frequency (RF) radiations, particularly upon the nervous system. Of note, WiFi refers to every system operating at 802.11 .g standards and which was developed by the Institute of Electrical and Electronics Engineers (IEEE). It has in general higher frequency ranges and longer exposure times than wireless phones [1]. Research shows that con-

stant exposure to RF radiations can affect human health such as causing headaches, anaemia, cancer and other health hazards [1–4]. These concerns exist because WiFi devices are placed near to public area and placed in many locations to increase the coverage area.

Potentially harmful effects of radiofrequency (RF)/microwave (MW) exposure have been investigated on different body systems and tissues. For instance, Saili et al. [5] proved that acute exposure to WiFi signal (2.45 GHz) affected heart rhythm, blood pressure, and catecholamines efficacy on cardiovascular system in adult male rabbits. Shokri et al. [6] showed that 2.45 GHz WiFi radiation, induced a decrease in sperm parameters along with an increase in apoptosis-positive cells in the seminiferous tubules of rats. According to Atasoy et al. [7], continuous long-term WiFi exposure oxidatively affected the testes in growing rats.

A special focus was accorded to the nervous system given its great cellular diversity, organizational complexity and electrical nature. However, most of studies dealing with neurological impact of 2.45 GHz WiFi radiations were conducted on adult animals [8–12] while reports on such effects following WiFi exposure during gestation or early in life are scarce.

In this regard, Pouletier de Gannes et al. [13] showed that, in utero exposure to a 2450 MHz WiFi signal (2 h/day, 6 days/week for 18 days, specific absorption rate (SAR = 4W/Kg)) did not induce significant signs of teratology during pre- or postnatal development. Aït-Aïssa et al. [14] reported that repeated exposure to WiFi during gestation and early life (2 h/day; SAR = 0.08, 0.4, and 4 W/kg) has no deleterious effects on the brains in case of young rats. In the other hand, pregnant women are particularly highly sensitive to a wide range of daily stresses including depression, anxiety, anger, day-to-day challenges, sudden environment change, social isolation and pathological conditions [15,16].

Studies in rodents have demonstrated that maternal stress during pregnancy may be associated with adverse effects on embryo/fetal [17] and neural [18] development, cognitive function [19] as well as social [20] and reproductive [21] behaviors. Interestingly, some researches held that male and female offspring responded differentially to maternal stress such as for gestation outcomes (preterm births, birth weight. . .)[22], emotional behavior and learning abilities of neonatal rats [23]. Noteworthy, some studies have shown that maternal stress during gestation can enhance the adverse effects on the development and behavior of fetuses and neonates induced by some elements like aluminium, arsenic, mercury or manganese [24–29].

Since electromagnetic fields sources (power lines, radio and TV broadcasting stations, cellular phones and their base stations, computer monitors), including WiFi devices, are nowadays ubiquitous, pregnant women exposure to RF is unavoidable. Likewise, they can be concurrently subjected to various types of stress, either at work or at home. Hence, the purpose of the present study was to evaluate the effects of maternal concurrent exposure to stress and WiFi signal on the postnatal development and behavior of rat offspring.

2. Material and methods

2.1. Animals and husbandry

Adult female Wistar rats (230–250 g) were obtained from SIPHAT (Tunisia) and quarantined for at least 7 days to acclimatize with the new housing conditions and coordinate their estrous cycle. Animals were group-housed (2 rats per cage) in an animal room (Faculty of Sciences of Bizerte, Tunisia), under a 12:12 h light/dark cycle and controlled temperature ($23 \pm 2^\circ\text{C}$) with *ad libitum* access to food and tap water throughout the experiments (except during WiFi exposure and restraint stress sessions). Once acclimatized, female rats were mated with healthy Wistar male

rats (2:1) overnight and were examined the following morning for copulatory plugs. A positive vaginal smear was defined as day 1 of gestation. Animals were cared for in compliance with the Tunisian code of practice for the Care and Use of Animals for Scientific Purposes. The experimental protocols were approved by the Faculty Ethics Committee (Faculty of Sciences of Bizerte, Tunisia).

2.2. Experimental design

2.2.1. Group assignment

Plug-positive female rats were randomly allocated to four groups ($n=6$): control group (CG), WiFi-exposed group (WG), restrained group (RG) and WiFi-exposed and restrained group (WRG).

Control dams were left undisturbed in the home cage and isolated from WiFi sources and environmental stressors (e.g., noise) as much as possible throughout the experimental period.

WiFi exposure and restraint stress were performed once daily for 2 h per day each, along the gestation period in an experimental room and the animal room respectively. If combined, restraint followed WiFi exposure session.

2.2.2. WiFi exposure system

The freely moving animals were whole-body exposed to an access point (AP) from WiFi device (D-Link DWL-3200 AP with 802.11g mode and WPA2 net-work protection) as previously described in Saili et al. [5]. WIFI integrated two omnidirectional antennas that were setup for internet broadcast via wireless at 2.45 GHz. Antennas of WIFI were placed at 25 cm from cages of exposed groups.

2.2.3. Restraint stress procedure

Restraint device consisted of a plexiglas cylindrical holder with air holes for breathing, the plexiglas disk was placed at the back to prevent the rat from backing out of the restraint device and minimize its mobility. The length could be adjusted to fit tightly animal body size. Once individually enclosed into the restrainer, rats were held in a prone position for two hours and periodically checked by observation [25].

2.3. Gestational outcomes and offspring physical development

Pregnant rats were allowed to deliver and wean their offspring. At birth, the following data were recorded: length of gestation, number of live and dead pups and sex. The day after, pups were identified with tail-marks and weighed each 4 days till sacrifice. The day of occurrence for pinna detachment, incisor eruption and eye opening was also monitored.

2.4. Neuromotor maturation

Tests of the offspring functional development were carried out included response to negative geotaxis and suspension test. The details of these tests have been previously described [30].

The pups underwent the negative geotaxis test on postnatal days 10 and 12. The time needed by the animal to achieve the test was noted to be 180 s during a trial. Suspension test was conducted on postnatal days 10–13. At the beginning of the test, the front paws of the rat pup were placed on the horizontal wire, the time taken before animal fall was measured (cut-off: 60s).

2.5. Post-weaning tests

2.5.1. Elevated plus-maze

Anxiety-like behavior of 28-day old rat offspring was assessed using elevated plus maze test according to the previously described

procedure [31,32]. The elevated plus maze is a four arm apparatus standing 60 cm above the floor, consisted of two open and two closed arms linked by a common central platform. The arms were 50 cm long and 10 cm width. Closed arms were surrounded by a 50 cm wall while open arms had 0.5 cm edges in order to maximize open arms entries [33]. The animals were allowed to explore the maze for 5 min. The measured variables were Time (in s), number of entries in the different parts of the maze and numbers of rears were recorded as standard measures. Protected (i.e. closed arm) stretched attend postures (pSAPs), closed arm returns (the rat enters the central part with two paws and then goes back into the same closed arm). The Ratio of latencies on open arms was relatively evaluated to a total time spent in opening and closed arms. The maze was cleaned with cotton and 10% alcohol solution after each animal trial.

2.5.2. Open field test

In the present study, open field test was conducted after 30 postnatal days. The apparatus is constructed from a clear plastic circular area (100 cm in diameter), surrounded by a 50 cm wall and divided into 1 central and 6 peripheral parts with equal surface [31]. Three identical glass-made rectangular prisms (8.5*7*4 cm), heavy enough to not be easily displaced by the rat, served as objects located in three fixed points of the peripheral part of the arena. During the test, rats were individually placed in the same corner (departure point) of the peripheral part of the open field, facing the wall, and were allowed to move freely around the open-field and to explore environment and objects for a single session of 5 min. Number and time of entries into the central part of the field were measured and considered as an index of anxiety [34]. Locomotion in the peripheral part (i.e. number of peripheral quadrants crossings) and time were measured. Frequency and time of contact with an object and total number of faecal boli (defecation) were also recorded. The apparatus and the objects were wiped with 10% alcohol solution between trials to eliminate the use of local odor cues.

2.5.3. Stationary beam

On 31st postnatal day, offspring dynamic equilibrium was tested on a wooden beam (100 × 2 × 2 cm) which was divided into 10 segments of 10 cm each [35]. Both ends of the beam were limited by cardboard. During the test, the covered distance and walking time were recorded. The trial was stopped after 3 min or according to the convenience of the animal [36].

2.6. Biochemical determinations

2.6.1. Serum isolation and brain homogenization

The day after the stationary beam test, rats were sacrificed by decapitation in the morning (between 08:00 and 12:00 h) in order to keep the circadian rhythm for all the groups. Blood was collected then centrifuged at 3000 rpm for 15 min at 4 °C to yield serum in supernatant fraction.

For tissue processing, brains were rapidly removed and rinsed with ice-cold sterile physiological saline solution. Only 0.4 g of each brain were homogenized in 4 ml of 50 mM phosphate buffered saline (pH 7.4) and centrifuged at 10000 rpm for 10 min at 4 °C. Then, supernatant was aliquoted and stored in frozen, as well as serum, at –80 °C until subsequent biochemical measurements.

2.6.2. Biochemical determinations in brain

Lipid peroxidation was determined by malondialdehyde (MDA) measurement according to the double heating method [37]. The activity of superoxide dismutase (SOD) was assessed using modified epinephrine assays [38]. The catalase (CAT) activity was assayed by measuring the initial rate of H₂O₂ disappearance at

240 nm [39]. Thiol groups (–SH) concentration was measured according to Hu et Dillar method [40]. Finally, the protein content in brain supernatant was determined by the method of Bradford [41] using bovine serum albumin (BSA) as standard.

2.6.3. Serum analyses

Animals serum was analyzed, using an automated biochemical analyzer EliTech[®], for glucose [42,43], triglycerides [44], lactate dehydrogenase LDH [45], alanine aminotransferase ALAT [46], aspartate aminotransferase ASAT [46], phosphorus [47] and calcium [48].

2.7. Statistical analysis

Statistical analysis of the obtained data was performed by Statview[®] software using analysis of variance (ANOVA) for comparison between groups, followed by a Fisher's Protected Least Significant Difference (PLSD) post-hoc test for multiple comparisons between all groups. Values for $p < 0.05$ and $p < 0.001$ were considered statistically significant. The data are shown as a mean ± standard error of the mean (SEM). Histograms were done by GraphPad Prism 5[®] software.

3. Results

3.1. Gestation outcomes and offspring physical maturation

There were no significant differences among groups in the length of gestation, viability (live pups on postnatal day 4/live pups at birth) and lactation index (live pups on postnatal day 21/live pups on postnatal day 4) as well as general health status of pups for both genders. However, female offspring displayed that in utero WiFi exposure significantly ($p < 0.05$) decreased the mean number of pups per litter compared to control and restrained groups.

For developmental landmarks, pinna detachment was prematurely induced by prenatal restraint stress in both male and female pups compared to control group ($p < 0.05$) and compared to WiFi group for female pups ($p < 0.001$). When WiFi radiations and restraint were associated, pinna detachment in female progeny was significantly ($p < 0.05$) premature than WiFi group and delayed than restraint group. WiFi signal and restraint, either ($p < 0.05$) or both ($p = 0.053$), induced delayed eye opening compared to control group in female pups (Table 1).

3.2. Neuromotor maturation

3.2.1. Suspension test

Male offspring prenatally treated with WiFi, only or combined with restraint, fell earlier than control group ($p < 0.05$) at postnatal day 10. Further, in utero restraint and WiFi association decreased suspension time at postnatal day 13 when compared to control, prenatally WiFi-exposed and restrained groups ($p < 0.05$). Suspension test was not affected in female offspring (Fig. 1).

3.2.2. Negative geotaxis reflex

As shown in Fig. 2, male pups of restrained dams need significantly ($p < 0.05$) more time to succeed the test compared to the control group on the postnatal day 10. Gestational co-exposure to WiFi and restraint extended significantly ($p < 0.05$) the time needed to fulfill negative geotaxis reflex at both postnatal days 10 and 12 when compared to the control group of male pups then to control and WiFi and restraint (acting alone) groups for female progeny (Fig. 2).

Table 1
Effects of WiFi, restraint, and combined WiFi and restraint on gestation outcomes and offspring physical maturation.

Prenatal treatment	None (Control offspring)	WiFi exposure	Restraint stress	WiFi exposure + restraint stress
Gestation length	20.17 ± 0.6	20.17 ± 0.7	24.6 ± 3.06	21.33 ± 1.28
No. of litters	6	6	6	6
No. fetuses per litter				
Males	5.67 ± 0.99	4.33 ± 1.28	3.4 ± 0.51	5.83 ± 1.2
Females	4 ± 0.93	1.83 ± 0.31 a	5 ± 0.55	3.33 ± 0.49
Viability index of pups (%)				
Males	97.22 ± 2.78	100 ± 0	100 ± 0	95.83 ± 4.17
Females	100 ± 0	100 ± 0	100 ± 0	94.44 ± 5.56
Lactation index of pups (%)				
Males	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Females	100 ± 0	100 ± 0	100 ± 0	100 ± 0
No. of days at pinna detachment				
Males	2.75 ± 0.14	2.67 ± 0.14	2.29 ± 0.11 [†]	2.47 ± 0.09
Females	2.54 ± 0.12	2.83 ± 0.12 b	2.16 ± 0.08 ab	2.47 ± 0.12
No. of days at incisor eruption				
Males	10.41 ± 0.13	10.28 ± 0.2	10.41 ± 0.15	10.24 ± 0.09
Females	10.29 ± 0.17	10.44 ± 0.25	10.24 ± 0.15	9.95 ± 0.16
No. of days at eye opening				
Males	15.38 ± 0.15	15.22 ± 0.1	15.59 ± 0.15	15.35 ± 0.08
Females	15.08 ± 0.15	15.67 ± 0.16 a	15.64 ± 0.15 a	15.53 ± 0.18

Data are given as mean ± S.E.M. Number of pups per group (developmental landmarks) is as follow: CG: 32, WG: 18, RG: 17 and WRG: 34 for male offspring and CG: 24, WG: 18, RG: 25 and WRG: 19 for female offspring. CG: Control group; WG: WiFi group; RG: Restraint group; WRG: WiFi + Restraint group.

[†](males) and **a** (females): $p < 0.05$ WiFi and Stress groups compared to control group.

[#](males) and **b** (females): $p < 0.05$ WiFi and Stress groups compared to WiFi + Stress group.

^{##}(males) and **bb** (females): $p < 0.001$ WiFi and Stress groups compared to WiFi + Stress group.

a(females): $p < 0.05$ WiFi and stress groups compared to control group.

b(females): $p < 0.05$ WiFi and stress groups compared to WiFi + Stress group.

bb(females): $p < 0.001$ WiFi and stress groups compared to WiFi + Stress group.

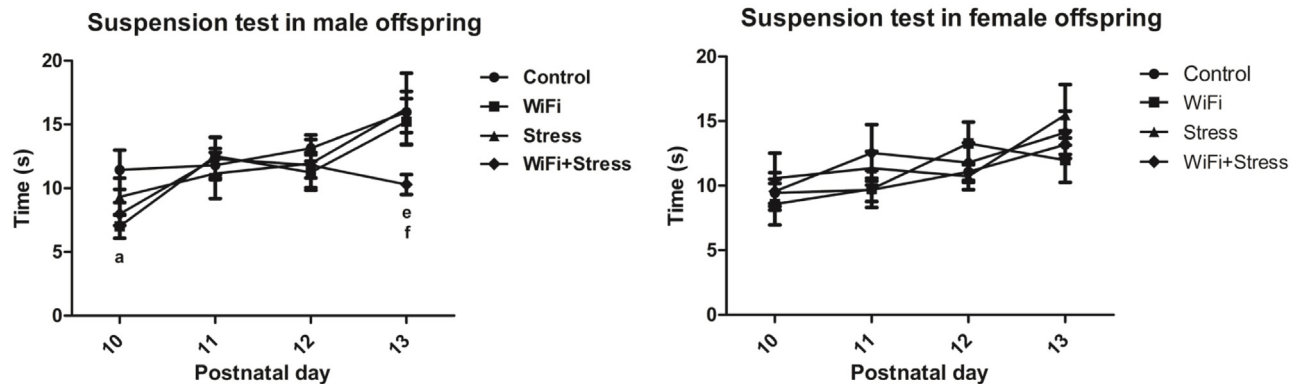


Fig. 1. Suspension test in male and female offspring. Animals were prenatally subjected to one of the following treatment: none, 2.4 GHz WiFi signal exposure, restraint stress and both WiFi and restraint, for 2 h/day along gestation period. Data are given as mean ± S.E.M. Number of pups per group is as follow: CG: 32, WG: 18, RG: 14 and WRG: 34 for male offspring and CG: 23, WG: 18, RG: 20 and WRG: 19 for female offspring. CG: Control group; WG: WiFi group; RG: Restraint group; WRG: WiFi + Restraint group. $p < 0.05$ a: WG versus CG; e: WRG versus WG; f: WRG versus RG.

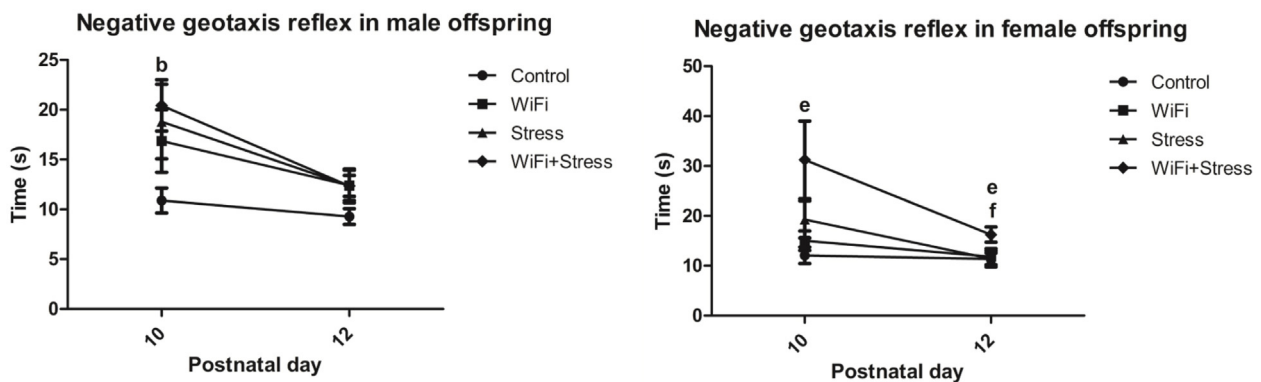


Fig. 2. Negative geotaxis reflex test in male and female offspring. Animals were prenatally subjected to one of the following treatment: none, 2.4 GHz WiFi signal exposure, restraint stress and both WiFi and restraint, for 2 h/day along gestation period. Data are given as mean ± S.E.M. Number of pups per group is as follow: CG: 32, WG: 18, RG: 14 and WRG: 34 for male offspring and CG: 23, WG: 18, RG: 20 and WRG: 19 for female offspring. CG: Control group; WG: WiFi group; RG: Restraint group; WRG: WiFi + Restraint group. $p < 0.05$ a: WG versus CG; b: RG versus CG; e: WRG versus WG; f: WRG versus RG.

3.3. Post-weaning tests

3.3.1. Elevated plus maze test

As shown in Table 2, both genders of the offspring, tested on postnatal day 28 in elevated plus maze apparatus, did not display anxiety effects due to the *in-utero* exposure to WiFi, restraint and combined WiFi-restraint. Indeed, no significant inter-group differences ($p > 0.05$) were found in entries number or time spent in open arms and their ratio (%) for both male and female offspring.

3.3.2. Open field test

As seen in Table 3. Open field testing was essentially affected by the association of WiFi exposure and restraint treatment in case of male offspring at Postnatal Day 30. However, for female progeny, the behavior in this test was altered by WiFi signal, restraint acting separately and in combined treatment. As behavior impairments, both male and female progeny showed an anxiety-like behavior (decrease in center entries and time), motor defect (decrease in locomotion time and peripheral crossings number and increase in immobility time) and a decline in exploratory behavior (diminishment of object contact time and frequency).

Indeed, in male offspring, prenatal restraint alone and combined with WiFi radiations elicited a highly significant ($p < 0.001$) decrease in locomotion time and increase in motionless time in peripheral parts of the open field compared to control group. In gestational restraint and WiFi irradiation treatment also in combined, the centre time, the frequency and the time spent for exploring objects, decrease significantly compared to control group. Besides, the co-exposure to restraint and WiFi radiations during pregnancy decreased significantly ($p < 0.05$) the number of traveled peripheral quadrants compared to restraint and WiFi signal acting separately. Restraint and WiFi radiations in concert, decreased significantly ($p < 0.05$) the centre entries number compared to the stressed group and exploring object frequency compared to WiFi group.

Regarding female offspring, *in utero* WiFi exposure decreased significantly ($p < 0.05$) the centre entries and time, locomotion in peripheral arena parts and time spent in exploring objects. Restraint alone decreased significantly ($p < 0.001$) the locomotion time and increased ($p < 0.05$) rearing. Combined exposure to restraint and WiFi radiations affected all testing parameters (except defecation) compared to control, WiFi and restraint groups.

3.3.3. Stationary beam test

Offspring motor performance on the stationary beam test (crossed distance and walking time on the beam) was unaffected in the restrained and/or WiFi-exposed groups on postnatal day 31 (data not shown). Consequently, the motor deficit already detected in open field test was due to neurological rather than physical disorder (muscles).

3.4. Brain oxidative stress response

Gestational WiFi exposure and restraint, when acting separately, induced cerebral stress response in gender-dependent manner. In male offspring, the *in utero* WiFi exposure increased significantly ($p < 0.05$) the MDA level, CAT and SOD activities compared to control group ($p < 0.001$). In addition, MDA concentration and SOD activity were also increased ($p < 0.05$ and $p < 0.001$ respectively) in WiFi group than restrained group (Fig. 3). As for female offspring, maternal restraint induced highly significant increase in MDA and SH content compared to control and WiFi group ($p < 0.001$).

The association between WiFi radiations and restraint emphasized a stress response in both genders. Indeed, MDA level was significantly increased ($p < 0.001$) compared to the control as well as WiFi and restraint acting separately in case of male and female

progeny. Concerning antioxidant system, SH level was significantly increased ($p < 0.05$) relatively to control, WiFi and restraint groups in only male offspring. Both SOD and CAT activities were decreased ($p < 0.05$) compared to male offspring subjected to prenatal WiFi exposure. Only CAT activity seems to be decreased in comparison to the WiFi group in female offspring case.

3.5. Serum biochemical assays

The LDH level in Prenatal WiFi exposure case decreased significantly ($p < 0.05$) and the same treatment increased the magnesium level at $p < 0.001$ compared to control and restrained groups in male offspring and diminished the phosphorus level at $p < 0.05$ compared to other groups in female offspring (Table 4).

Concerning male progeny, gestational restraint caused a significant increase in phosphorus level ($p < 0.05$) when compared to control and WiFi exposed groups. In female offspring, the *in utero* restraint increased significantly the level of glucose ($p < 0.05$) compared to the other experimental groups. Furthermore, *in utero* restraint increased the triglyceride level compared to WiFi ($p < 0.05$) and both WiFi and restraint treated ($p = 0.073$) groups and decreased LDH level compared to control group. Besides, restraint, alone and associated with WiFi exposure, caused non significant trend to increase calcium level as compared to control ($p = 0.066$ and $p = 0.068$) and only WiFi exposed ($p = 0.052$ and $p = 0.054$) groups.

For males, WiFi signal and restraint, once combined, induced highly significant decrease in magnesium level ($p < 0.001$) compared to WiFi-exposed group and a no-significant trend to decline the LDH level ($p = 0.051$) compared to the control group.

The treatment failed to alter the seric ASAT, ALAT and alkaline phosphatase activities in both genders, also in case of glucose, triglycerid and calcium levels in male offspring and only magnesium level in female progeny.

4. Discussion

In the present study, we investigated the effects of 2.45 GHz WiFi signal and restraint, acting in two different modes, separately and combined, on gestational outcomes and offspring especially at neurological level, at juvenile and adult age. The main findings showed that there are no deleterious effects of prenatal WiFi exposure and/or restraint on gestational outcomes and offspring general health status except that WiFi, *per se*, lowered the mean number of female pups per litter. Later, WiFi and restraint, separately and mainly when combined, altered physical and neuromotor maturation of the specimens at juvenile age and induced anxiety-like behavior, motor deficit and exploratory behavior impairment at adult age for both male and female offspring. WiFi exposure and restraint, acting separately, induced brain oxidative stress response in male and female progeny, respectively. However, the stress response was emphasized in both sexes by WiFi and restraint association. Finally, *in utero* WiFi exposure and restraint affected the offspring serum biochemistry which suggesting some diseases (diabetes, bone anomalies, motor defects...). Overall, the treatment had sex-dependent effects on the offspring for most of tests.

For gestational outcomes, albeit we don't found major defects, since there were neither macroscopic abnormalities nor viability and lactation index alteration and the *in utero* WiFi exposure induce attenuation in female litter size. The present results don't match well with the literature since previous document related to gestational WiFi and restraint impacts are contradictory.

Indeed, while many previous studies linked the prenatal restraint with alterations of various gestation outcome endpoints (including gestation length, implantation, teratology signs, litter

Table 2
Elevated plus maze test in male and female offspring. Animals were prenatally subjected to one of the following treatment: none, 2.4 GHz WiFi signal exposure, restraint stress and both WiFi and restraint, for 2 h/day along gestation period. Data are given as mean $\pm$ S.E.M.

Prenatal treatment/Parameter	None (Control)	WiFi exposure	Restraint stress	WiFi exposure and restraint
Open arms entries				
Males	2,85 $\pm$ 0,4	3,06 $\pm$ 0,42	2,87 $\pm$ 0,83	3,07 $\pm$ 0,46
Females	3,07 $\pm$ 0,58	2,77 $\pm$ 0,62	4,11 $\pm$ 0,6	2,56 $\pm$ 0,63
Closed arms entries				
Males	7,26 $\pm$ 0,6	8,22 $\pm$ 0,92	7,53 $\pm$ 1,3	7,3 $\pm$ 0,63
Females	7 $\pm$ 0,77	8,23 $\pm$ 0,81	9,53 $\pm$ 0,84 ab	6,89 $\pm$ 0,68
Open arms time				
Males	31,62 $\pm$ 5,19	33,12 $\pm$ 5,39	27,65 $\pm$ 8,57	31,73 $\pm$ 5,29
Females	29,32 $\pm$ 6,14	32,34 $\pm$ 8,73	40,96 $\pm$ 6,5	28,85 $\pm$ 8,85
Closed arms time				
Males	206,74 $\pm$ 8,33	213,52 $\pm$ 8,14	210,45 $\pm$ 13,32	216 $\pm$ 9,24
Females	221,55 $\pm$ 9,83	210,64 $\pm$ 12,68	188,05 $\pm$ 10,39 a	220,41 $\pm$ 12,66
Center time				
Males	61,65 $\pm$ 5,36	53,36 $\pm$ 4,62	61,9 $\pm$ 8,63	52,27 $\pm$ 5,06
Females	49,13 $\pm$ 5,56	57,03 $\pm$ 5,8	70,99 $\pm$ 5,94 ab	50,74 $\pm$ 6,2
Rears				
Males	12,82 $\pm$ 0,68	17,61 $\pm$ 1,62 [*]	15,33 $\pm$ 1,37	14,26 $\pm$ 1,3
Females	14,33 $\pm$ 2	13,23 $\pm$ 1,13	20,21 $\pm$ 1,4 a	16,11 $\pm$ 2,17
Stretched attend postures				
Males	2,44 $\pm$ 0,39	1,61 $\pm$ 0,29 ^{##}	7,33 $\pm$ 1,11 ^{*,#}	4,82 $\pm$ 0,72
Females	2,73 $\pm$ 0,8	3,15 $\pm$ 0,85	5,32 $\pm$ 0,63 a	4,33 $\pm$ 1,62
Closed arms reentries				
Males	0,59 $\pm$ 0,15	0,89 $\pm$ 0,23	1,2 $\pm$ 0,24 [*]	0,74 $\pm$ 0,15
Females	0,6 $\pm$ 0,16	1 $\pm$ 0,38	0,84 $\pm$ 0,28	1 $\pm$ 0,33
Fecal boli number				
Males	0,78 $\pm$ 0,32	1,17 $\pm$ 0,45	1,73 $\pm$ 0,47	1,74 $\pm$ 0,45
Females	0,93 $\pm$ 0,46	0,85 $\pm$ 0,39	2,05 $\pm$ 0,47	1,56 $\pm$ 0,78
% open arms time				
Males	13,82 $\pm$ 2,26	13,74 $\pm$ 2,23	12,1 $\pm$ 3,63	13,65 $\pm$ 2,39
Females	12,07 $\pm$ 2,57	13,96 $\pm$ 3,98	18,63 $\pm$ 3,01	11,9 $\pm$ 3,64
% open arms entries				
Males	27,3 $\pm$ 2,78	27,21 $\pm$ 2,76	20,87 $\pm$ 4,24	26,79 $\pm$ 3,04
Females	28,01 $\pm$ 2,41	23,7 $\pm$ 3,52	26,75 $\pm$ 3,42	24,6 $\pm$ 4,41

a(females) : $p < 0.05$ WiFi and Stress groups compared to control group.

aa(females): $p < 0.001$ WiFi and Stress groups compared to control group.

b(females): $p < 0.05$ WiFi and Stress groups compared to WiFi + Stress group.

bb(females) : $p < 0.001$ WiFi and Stress groups compared to WiFi + Stress group.

^{*} (males) and **a** (females): $p < 0.05$.

^{**} (males) and **aa** (females): $p < 0.001$ WiFi and Stress groups compared to control group.

[#] (males) and **b** (females): $p < 0.05$.

^{##} (males) and **bb** (females): $p < 0.001$ WiFi and Stress groups compared to WiFi + Stress group.

size, pups birth weight and body weight gain) in rodents [22,49,50] and humans [51], other reports denied such effects [52–54]. For the *in utero* WiFi exposure, the negative effects on gestational parameters mentioned above were detected in different animal species (rat, mice, rabbit and chicken) in some investigations [55–57] but not in rat in other reports [58–60].

In term of pups physical and neurodevelopment, the current results are in line with some previous works reporting adverse gestational restraint effects on offspring development at both physical and neurological levels [49,50,61–63].

Among the mechanisms mediating such stress-induced impairments, Laloux et al. [64] suggested the alteration of leptin secretion in progeny. Feeding-related hormones, as leptin, are prominently involved in neurodevelopment regulation during early postnatal life [65].

Conversely, according to other investigators, gestational WiFi exposure failed to elicit developmental impairments [13,66,67]. Likewise, Lee et al. [68] did not notice any signs of teratogenicity (mortality, growth retardation, changes in head size and other morphological abnormalities) in mouse fetuses after combined exposure to Code division multiple access (CDMA) and Wideband Code Division Multiple Access (WCDMA) radiofrequency electromagnetic fields during pregnancy. These discrepancies in results may be due to various factors such as experimental animal species and strains, gestational timing and duration of restraint and WiFi

exposure, restraint procedures and WiFi exposure mode (head only/whole body exposure).

Behaviorally, our data revealed that WiFi and restraint, separately and especially if combined, induced anxiety-like behavior, motor deficit and exploratory behavior impairment at adult age for both male and female offspring. These results are in agreement with other studies in which prenatal stress has been shown to yield anxiety-like behavior in offspring in elevated plus maze and open field tests during early postnatal life [64] as well as at adult age [69–74]. Yet, prenatal stress did not evoke anxiety in various testing tasks [75–77].

Many molecular, cellular, biochemical and neuroendocrine basis are involved in the link between prenatal stress and behavioral disorders. Firstly, adult prenatally restrained animals showed modified expression of “anxiety related receptors” namely GABA A and metabotropic glutamate mGlu receptors (mGlu2/3 and mGlu5 receptor subtypes in particular) in the hippocampus and amygdala [64,74,78–82].

Moreover, feeding-related hormones have a pivotal role in psychiatric disorders [83]. Indeed, many neuronal circuits responsible for emotion and anxiety are leptin-dependent [84]. In this respect, Laloux et al. [64] demonstrated that gestational restraint suppressed the peak of blood leptin levels normally seen around the second week of postnatal life [85,86].

Additionally, neuroendocrine functioning is likely to be a key factor in mediating association between prenatal stress and off-

Table 3

Open field test in male and female offspring. Animals were prenatally subjected to one of the following treatment: none, 2.4 GHz WiFi signal exposure, restraint stress and both WiFi and restraint, for 2 h/day along gestation period. Data are given as mean $\pm$ S.E.M.

Prenatal treatment	None (Control offspring)	WiFi exposure	Restraint stress	WiFi exposure + restraint stress
Centre time				
Males	15.83 $\pm$ 2.24	12.79 $\pm$ 2.67	15 $\pm$ 2.36	10.04 $\pm$ 1.83
Females	21.18 $\pm$ 2.77	7.94 $\pm$ 1.65 a	17.85 $\pm$ 3.05	10.76 $\pm$ 3.47
Number of centre entries				
Males	5.26 $\pm$ 0.69	4.33 $\pm$ 0.77	6.67 $\pm$ 0.85 [#]	3.78 $\pm$ 0.68
Females	6.87 $\pm$ 1.01	3.23 $\pm$ 0.62 a	7.32 $\pm$ 0.96 b	3.22 $\pm$ 0.93
Number of crossed peripheral quadrants				
Males	45.7 $\pm$ 2.91	54.33 $\pm$ 4.15 [#]	50.87 $\pm$ 2.94 [#]	39.89 $\pm$ 2.65
Females	51.07 $\pm$ 2.68	54.46 $\pm$ 2.35 b	53.68 $\pm$ 3.23 b	44 $\pm$ 2.75
Locomotion time in peripheral part				
Males	237.52 $\pm$ 5.64	219.82 $\pm$ 7.14	190.27 $\pm$ 9.18 ^{**}	198.44 $\pm$ 8.36
Females	245.22 $\pm$ 4.98	235.19 $\pm$ 3.17 abb	206.14 $\pm$ 5.77 aa	194.5 $\pm$ 9.29 ^{##}
Immobility time in peripheral area				
Males	46.65 $\pm$ 6.7	67.39 $\pm$ 8.35	94.73 $\pm$ 10.77 ^{**}	91.52 $\pm$ 9.13
Females	33.61 $\pm$ 5.98	56.86 $\pm$ 3.68 abb	76.01 $\pm$ 6.78 aa	94.74 $\pm$ 9.46
Rearing number				
Males	12.22 $\pm$ 1.3	14.72 $\pm$ 1.72 [#]	16.2 $\pm$ 2.01 [#]	9.7 $\pm$ 1.01
Females	15.13 $\pm$ 1.28	13.15 $\pm$ 1.28 [#]	19.53 $\pm$ 1.33 abb	11.33 $\pm$ 1.89
Exploring objects frequency				
Males	19.04 $\pm$ 1.32	18.06 $\pm$ 1.34 [#]	15.8 $\pm$ 1.67	13.56 $\pm$ 1.31
Females	20.8 $\pm$ 1.63	17.46 $\pm$ 1.64 b	19.21 $\pm$ 1.64 b	11 $\pm$ 1.65
Time spent in exploring objects				
Males	54.055 $\pm$ 13	40.48 $\pm$ 4.29	46.26 $\pm$ 5.89	34.91 $\pm$ 4.15
Females	63.99 $\pm$ 8.73	42.08 $\pm$ 6.29 a	58.11 $\pm$ 5.58 b	29.02 $\pm$ 5.9
Fecal boli number				
Males	1.78 $\pm$ 0.32	2.56 $\pm$ 0.61	3.27 $\pm$ 0.66	2.89 $\pm$ 0.56
Females	2 $\pm$ 0.61	2.62 $\pm$ 0.49	2.74 $\pm$ 0.59	2.44 $\pm$ 0.63

Number of rats per group is as follow: CG: 27, WG: 18, RG: 15 and WRG: 27 for male offspring and CG: 15, WG: 13, RG: 19 and WRG: 9 for female offspring. CG: Control group; WG: WiFi group; RG: Restraint group; WRG: WiFi + Restraint group.

a(females): $p < 0.05$ WiFi and Stress groups compared to control group.

aa(females): $p < 0.001$ WiFi and Stress groups compared to control group.

b(females): $p < 0.05$ WiFi and Stress groups compared to WiFi + Stress group.

bb(females): $p < 0.001$ WiFi and Stress groups compared to WiFi + Stress group.

[#](males) and **a** (females): $p < 0.05$ WiFi and Stress groups compared to control group.

^{**}(males) and **aa** (females): $p < 0.001$ WiFi and Stress groups compared to control group.

[#](males) and **b** (females): $p < 0.05$ WiFi and Stress groups compared to WiFi + Stress group.

^{##}(males) and **bb** (females): $p < 0.001$ WiFi and Stress groups compared to WiFi + Stress group.

spring behavioral outcomes (emotional and cognitive disorders) in both animal and human offspring [87]. Indeed, fetal life stressful conditions induced lifelong programming of the hypothalamic-pituitary-adrenal (HPA) axis function leading to emotional (anxiety and depression) and cognitive disorders in animals [75,88–90] and humans [91–94].

Furthermore, the serotonergic (5-HT) system is well known to play an important role in the pathophysiology of anxiety and depressive disorders [95,96]. Prenatal restraint disrupted serotonergic metabolism causing emotional disorders as anxiety and depression [90,97].

In addition, prenatal stress has been reported to cause neurogenesis down-regulation by impairing maternal behavior [98,99] and increasing exposure to glucocorticoids in young rats [100,101].

As for emotional effects of the *in utero* irradiation, prenatal exposure to 9.417 GHz microwaves increased anxiety in adult offspring in both genders [102]. Furthermore, gestational radiofrequencies (including CW, GSM 900 MHz signals) exposure induced losses of dentate gyrus granule cells [103] and hippocampal pyramidal cells [104] in rats as well as Purkinje cells in mice cerebellum [105,106]. Neurogenesis dysregulation has been suggested to be involved in depression- and anxiety-like behaviors [107,108], cognitive impairments [109] and addiction [110]. By contrast, mouse fetal exposure to GSM 800–1900 MHz radiations led to anxiety decrease that persist into adulthood [111].

In our study, open field test revealed a motor deficit in offspring of dams undergoing WiFi exposure and restraint, *per se* and especially combined, along gestation period. This effect can

be accounted by the decrease in the expression of acetylcholine receptors (namely alpha-7 nicotinic receptor) in frontal cortex and hippocampus which was seen in adult prenatally stressed rat offspring [52].

The current data are although in contrast with the findings of Golubeva et al. [75] who reported hyperlocomotion in open field test in prenatally stressed offspring, suggested to be compensatory response to restraint movement restriction. In this vein, Aldad et al. [111] reported the hyperactivity in adult mouse offspring due to fetal exposure to GSM 800–1900 MHz radiations.

Biochemically, the results showed that the treatment induced a brain oxidative stress with a sex-dependent manner. Indeed, MDA and SH level as well as SOD and CAT activities were increased in prenatally WiFi-treated male offspring and restrained female progeny. The elevated level of MDA reflects lipid peroxidation whereas the activities of enzymatic (CAT and SOD) and non enzymatic (SH) antioxidant system were increased to counteract ROS over production. Once associated, WiFi signal and restraint established critical phase of oxidative stress response marked by higher production of MDA and depletion of SOD and CAT activities in both offspring genders. We suggest that while SH continued defending brain tissue, antioxidant system was overwhelmed by ROS and MDA, leading to its depletion.

It is well known that the brain is the most sensitive target of oxidative stress due to its high oxygen consumption, elevated polyunsaturated fatty acid [112] and redox-active metals contents [113], deficient activity of enzymatic antioxidant system [114] as well as high sensitivity of blood brain barrier [115]. Of note,

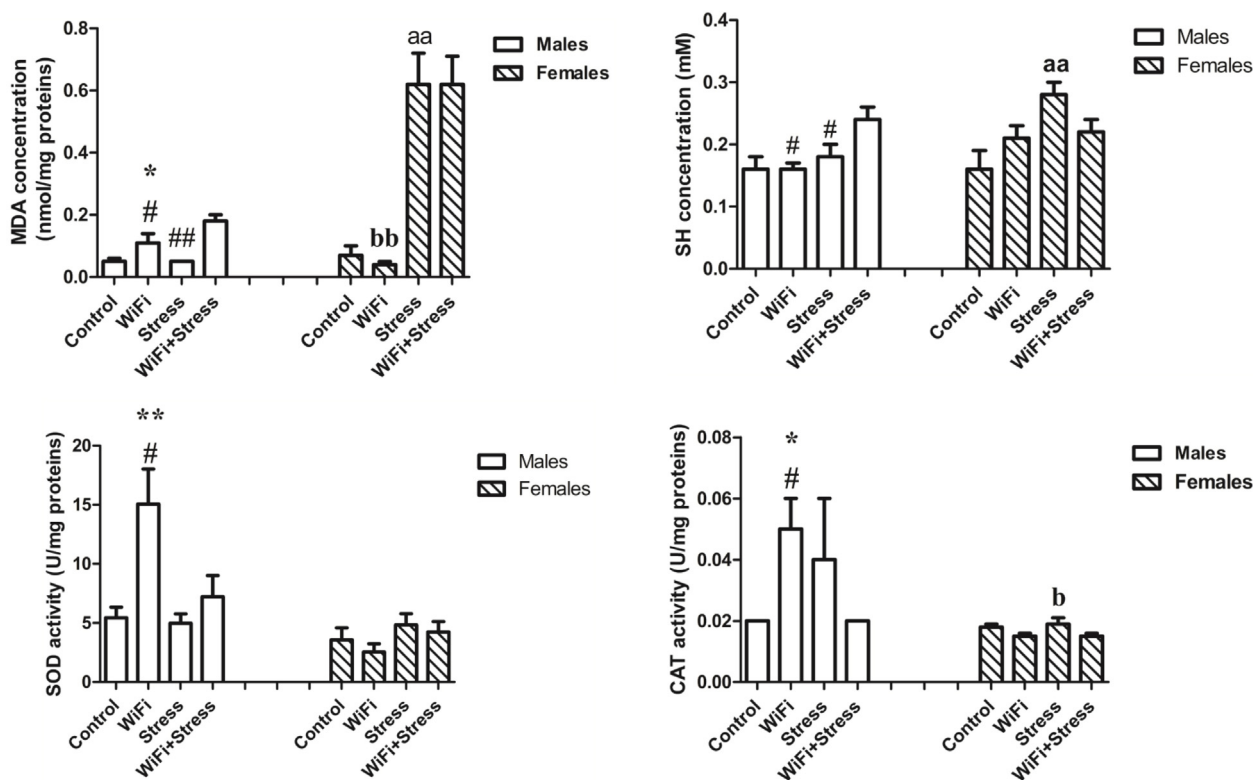


Fig. 3. Oxidative stress response in offspring brain. Animals were prenatally subjected to one of the following treatment: none, 2.4 GHz WiFi signal exposure, restraint stress and both WiFi and restraint, for 2 h/day along gestation period. Data are given as mean $\pm$ S.E.M. $n = 15$ for male offspring and 8 for female offspring. * (males) and a (females): $p < 0.05$ and ** (males) and aa (females): $p < 0.001$ WiFi and Stress groups compared to control group; # (males) and b (females): $p < 0.05$ and ## (males) and bb (females): $p < 0.001$ WiFi and Stress groups compared to WiFi + Stress group.

oxidative stress at brain level is an underlying basic etiology of neurodegenerative, psychiatric and cognitive disorders [116–119].

Recent reviews shed light on the link between wireless technologies radiofrequencies and oxidative stress in the developing brain [120,121]. Çelik et al. [122] reported that 2.45 GHz WiFi irradiation from gestation till three weeks old induced oxidative stress in the brain of developing rats by increasing lipid peroxidation levels and decreasing glutathione peroxidase, glutathione activity and antioxidant vitamin concentrations.

However, according to Ait-Aïssa et al. [58], in utero WiFi exposure, even with extended postnatal exposure to a WiFi signal, triggered neither astroglia nor apoptosis in the brain of young rats. In addition to that, studies on gestational exposure to other radiofrequencies types gave similar results. Indeed, GSM radiations led to lipid peroxidation and DNA molecules damage in brains of pregnant dams but not in those of the offspring in rabbit following acute mid-gestation exposure [123] and failed to induce stress response (c-fos expression) in fetal mouse brain after full gestational exposure [124]. Furthermore, DNA oxidative damage in offspring brain was reported in several previous works following prenatal exposure to 2.45 GHz WiFi signal in mice [56,57] and to restraint in rats [125].

Prenatal WiFi exposure and restraint, either or both, did not damage liver since ASAT, ALAT and alkaline phosphatase levels were unchanged. Low seric LDH levels observed in offspring undergoing gestational WiFi exposure and restraint, separately or in association, do not indicate a specific disorder. (Only high LDH levels are pathological signs of liver, heart and muscle disorders [126] and low LDH concentrations are sometimes due to ingestion of large amounts of vitamin C, which is not the case herein).

Female offspring of restrained dams exhibited high seric glucose and triglyceride levels probably due to lipid and glucide metabolism disorders which is indicative of diabetes status [127] and high risk of atherosclerosis [128]. In this vein, Barker [129] proposed “the fetal origins of adult disease hypothesis”, stating that challenging stimuli during intrauterine life is susceptible to (re)program fetal development leading to pathological consequences in later life, including diabetes and heart diseases.

In utero restraint stress *per se* increased phosphorus seric level in male progeny and induced hypercalcemia in female offspring when acting alone and combined with WiFi signal. Besides, gestational WiFi exposure raised seric magnesium level in male offspring. Substantial evidence suggests that phosphorus, magnesium and calcium are the most important mineral constituents of the bone [130]. In addition, calcium and magnesium are involved in synaptic transmission especially at neuromuscular junction level. Consequently, high seric level of these elements is likely to reflect bone defects and synaptic transmission disorder which may explain, at least in part, motor deficit detected in open field test.

In agreement with our results, gestational exposure to radiofrequencies (GSM 900 MHz) was reported to be harmful for ossification [131]. Overall, we found that prenatal exposure to WiFi radiations and/or restraint often triggered sex dependent effects on the offspring which is consistent with previous reports [88,132,133].

5. Conclusion

In summary, the current study point out that prenatal WiFi exposure and restraint, separately and especially in association, adversely affected offspring physical and neurodevelopment as

Table 4

Biochemical indices in the blood serum of male and female offspring. Animals were prenatally subjected to one of the following treatment: none, 2.4 GHz WiFi signal exposure, restraint stress and both WiFi and restraint, for 2 h/day along gestation period. Data are given as mean $\pm$ S.E.M. n = 15 for male offspring and n = 8 for female offspring.

	Control offspring	In utero WiFi exposed offspring	In utero restrained offspring	In utero WiFi and restraint exposed offspring
Glucose(g/L)				
Males	1.21 $\pm$ 0.06	1.36 $\pm$ 0.04	1.33 $\pm$ 0.07	1.22 $\pm$ 0.08
Females	1.16 $\pm$ 0.08	1.19 $\pm$ 0.04	1.48 $\pm$ 0.04 ab	1.2 $\pm$ 0.1
Triglycerides (g/L)				
Males	1.19 $\pm$ 0.15	1.22 $\pm$ 0.112	1.31 $\pm$ 0.22	1.19 $\pm$ 0.13
Females	1.09 $\pm$ 0.14	0.92 $\pm$ 0.16	1.48 $\pm$ 0.18	1.02 $\pm$ 0.21
ASAT(U/L)				
Males	136.53 $\pm$ 11.26	128.23 $\pm$ 8.59	148.5 $\pm$ 7.48	132.4 $\pm$ 11.76
Females	174.29 $\pm$ 10.45	142.88 $\pm$ 15.54	154.75 $\pm$ 18.66	169.29 $\pm$ 19.44
ALAT(U/L)				
Males	32.53 $\pm$ 1.97	31.92 $\pm$ 1.57	33.57 $\pm$ 1.57	31.67 $\pm$ 2.26
Females	35.43 $\pm$ 2.52	32.13 $\pm$ 3.44	30.5 $\pm$ 3.19	36.29 $\pm$ 3.73
LDH(U/L)				
Males	1052.8 $\pm$ 81.72	745.71 $\pm$ 63.23 [*]	1007.71 $\pm$ 49.93	855.67 $\pm$ 80.33
Females	1281.5 $\pm$ 103.96	1211.25 $\pm$ 94.68	967.63 $\pm$ 142.54 a	1022.17 $\pm$ 70.94
Calcium (mg/L)				
Males	106.33 $\pm$ 2.21	107.14 $\pm$ 2.91	112.36 $\pm$ 2.26	111.38 $\pm$ 3.68
Females	110.18 $\pm$ 3.87	109.75 $\pm$ 3.14	117.45 $\pm$ 1.16	117.66 $\pm$ 1.53
Phosphorus (mg/L)				
Males	90.62 $\pm$ 2.45	91.63 $\pm$ 2.92	99.68 $\pm$ 2.55 [*]	96.76 $\pm$ 3.32
Females	93.8 $\pm$ 4.49	77.13 $\pm$ 9.17 ab	101.31 $\pm$ 2.96	102.85 $\pm$ 4.2
Magnesium (mg/L)				
Males	21.96 $\pm$ 0.4	25.69 $\pm$ 0.82 ^{**} , ^{##}	21.65 $\pm$ 0.55	20.8 $\pm$ 0.91
Females	23.6 $\pm$ 1.29	23.64 $\pm$ 1.16	21.93 $\pm$ 0.13	22.76 $\pm$ 0.2
Alkaline phosphatase (U/L)				
Males	915.2 $\pm$ 43.44	815.71 $\pm$ 26.89	891.2 $\pm$ 40	832 $\pm$ 74.39
Females	802.14 $\pm$ 61.14	759 $\pm$ 52.83	732.13 $\pm$ 29.95	781.71 $\pm$ 45.94

a(females) : p < 0.05 WiFi and Stress groups compared to control group.

aa(females): p < 0.001 WiFi and Stress groups compared to control group.

b(females): p < 0.05 WiFi and Stress groups compared to WiFi + Stress group.

bb(females) : p < 0.001 WiFi and Stress groups compared to WiFi + Stress group.

(males) and **b** (females): p < 0.05.

^{*} (males) and **a** (females): p < 0.05.

^{**} (males) and **aa** (females): p < 0.001 WiFi and Stress groups compared to control group.

^{##} (males) and **bb** (females): p < 0.001 WiFi and Stress groups compared to WiFi + Stress group.

well as emotional responses, motor and exploratory behavior at adulthood. Progeny brain oxidative equilibrium and serum biochemistry were also altered. Most of these impacts depended on offspring gender. Further investigations are obviously needed to test for neuro molecular and biochemical mechanisms mediating such detrimental effects.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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