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Endocrine and behavioral effects in male rats after birth with their pregnant mother taking ibuprofen

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Ibuprofen, a non-selective inhibitor of cyclooxygenase, is one of the most used non-steroidal anti-inflammatory drugs (NSAID) and pain relievers. Its effect on developing fetal neuroendocrine system when taken by a pregnant mother does not well established. The aim of this study was to evaluate the long-term consequences of the administration of ibuprofen to pregnant rats with regard to hormonal profile and sexual differentiation of the brain in male offspring. Pregnant rats were given ibuprofen per os in a dose of 30 mg/kg bw twice a day during days 15-21 of gestation. On the 2 and 10 postnatal days (PND), anogenital distance (AGD) was measured in male pups. In adulthood, sexual behavior, hormone levels, and hypothalamic-pituitary-adrenal axis (HPAA) response to acute immobilization stress were studied. Prenatal ibuprofen increased AGD on PND 2 and caused incomplete masculinization of copulative behavior in adult offspring. Significant increases in the latent periods of the first mount, the first intromission, and a decrease in the number of intromissions were observed. Basal blood plasma levels of testosterone, estradiol, and corticosterone, as well as HPAA response to immobilization stress, did not change. Orchidectomized and primed with estradiol and progesterone males did not exhibit lordosis response to the presence of a sexually experienced male. Conclusion: Administration of ibuprofen to female Wistar rats during the last week of gestation disrupts partially neuroendocrine programming of male-type copulative behavior in male offspring with no changes in female-type sexual behavior and HPAA function. It is hypothesized that incomplete masculinization of the developing fetal brain with ibuprofen is due to two co-operative mechanisms: inhibition of synthesis of testosterone in the fetal gonads and prostaglandin E2 in the preoptic area of the hypothalamus.

Keywords: ibuprofen; prenatal effect; sexual behavior; testosterone; estradiol; corticosterone; stress response; male rats.

INTRODUCTION

A number of *in-utero* environmental influences, including pharmaceuticals, may result in reproductive abnormalities in the offspring in later life [1-4]. Among these influences, analgesics, antipyretic and anti-inflammatory drugs are the focus of developmental physiology and pathophysiology. Analysis of the non-clinical NSAID literature demonstrated a possible association between exposure to NSAIDs and developmental anomalies.

Acetaminophen, ibuprofen, and acetylsalicylic acid, non-selective cyclooxygenase (COX) inhibitors, are widely used as over-the-counter pain-relief medications [5]. Despite the fact that their intake during pregnancy should be strictly limited in a real-life this cannot always be avoided. In the Western world, the majority of pregnant women report an intake of mild analgesics, and some of them exhibit antiandrogenic properties [6]. In Great Britain, ibuprofen is one of the most frequently used pharmaceutical compounds by pregnant women with up to 28% reporting use [7]. In the Norwegian population, 5.9% of pregnant women used to take ibuprofen [8]. In the USA, 46.1% of mothers used acetaminophen ≥10 times and 18.4% used ibuprofen during pregnancy [9]. Exposed children demonstrated worse executive functioning and neurobehavioral problems. A number of studies

of the developmental toxicity of NSAIDs point to an association between their use during pregnancy and negative developmental outcomes in humans and animals [10-18]. Most of them describe external malformations, changes in the skeleton, arterial, respiratory, and immune systems, etc.

Various reproductive disruptions were found in human fetuses, newborns, children, and adults in association with maternal exposure to ibuprofen [13, 15, 19]. They include cryptorchidism, reduced AGD in sons, and low semen quality. In the culture and xenograft systems, therapeutically relevant doses of ibuprofen cause direct endocrine disturbances in the human fetal testis and alteration of the germ cell biology [7, 20]. After *in utero* exposure to ibuprofen, adult mice exhibited a reduced production of testosterone and epididymal sperm parameter defects, namely, a reduced sperm count and motility [21] However, many long-term reproductive outcomes of prenatal exposure to non-steroid analgesics drugs, in particular ibuprofen, were not fully established.

So far, one study has investigated the perinatal effect of ibuprofen on steroid testosterone profile and sexual behavior in male rats [22]. The authors introduced ibuprofen to mothers during the last week of pregnancy. They reported the persistence of copulative behavior in the animals, however, in the presence of another male, they also presented a female-type behavior. Taking into consideration the current view on the role of prostaglandins in the brain sexual differentiation [23, 24], we supposed that some components of male sexual behavior could be changed in male offspring prenatally exposed to ibuprofen during sexual brain differentiation. Exploration of this hypothesis was the aim of this study. In addition, we evaluated the functional state of the hypothalamic-pituitary-adrenal axis (HPAA), because this aspect has not been studied before.

METODS

Ethical approval. The experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 18 March 1986), and Recommendations of the First National Congress on Bioethics Issues (Kyiv, Ukraine, 20 September 2001). The experimental design and procedures were approved by the Bioethics Commission of the Institute (Protocol No. 28/5-KE from 12.04.2019).

Materials. Ibuprofen tablets was from Pharmaceutical firm "Darnitsa" (Ukraine). Estradiol-17β diacetate was purchased from "Sigma" (USA). Progesterone, 1% olive oil solution, was from "Biopharma" (Ukraine). Corticosterone was from "Sigma" (USA). Immunoassay kits Estradiol ELISA and Testosterone ELISA were purchased from "DRG" (Germany).

Design of the experiments. Female Wistar rats of the local Institute vivarium breeding with a regular 4-5 estrous cycle that has been estimated previously by everyday microscopic examination of the vaginal smears during 2 weeks were selected for fertilization. They were placed in the cage together with a sexually active male, and the day of the appearance of spermatozoa in the vaginal smear was considered the first day of conception.

Pregnant dams have received intragastrically through feeding tube ibuprofen tablet powder suspended in Dorfman's gel (carboxymethyl cellulose gel in 0.9% sodium chloride solution, containing Tween-80 and benzyl alcohol) in a dose of 30 mg/kg bw twice a day for gestation days 15-21. Control pregnant females were given an appropriate volume of Dorfman's gel. Experimental male offspring groups were formed by randomization and allowed to be kept in the institutional vivarium until 5-8.5 months of age. The animals were housed under standard conditions.

AGD was measured on PND 2 and 10, and calculated in relation to body weight. The day

of the testicle descent into the scrotum was recorded. At 5 months of age, experimental and control males were euthanized, and the trunk blood samples were taken for hormone assay. At the same time, other experimental and control animals were immobilized strictly for 1 h followed by euthanasia with quick decapitation and taking of blood samples. At 8 months of age, the males were tested for the exhibition of male-type sexual behavior, and at 8.5 months for female-type sexual behaviors.

Male-type sexual behavior. Male-type sexual behavior was tested at the standard condition in the presence of sexually receptive female [25, 26]. 5 males delivered by different dams were recruited in ibuprofen or control groups. Before testing, the male was kept in darkness and then moved to an empty cage for a 5-minute adaptation. One week before testing, the partner female was ovariectomized and injected intramuscularly with 0.1 mg estradiol diacetate 48 h before the test. 0.5 mg progesterone oil solution was introduced 4 h prior to the test. Then the female was placed for 15 min under dim red light in the cage with the male. The following indices of copulative behavior were recorded: duration of latent periods of the first mounting and the first intromission, the first ejaculation, post-ejaculatory refractory period, the numbers of ejaculations, mountings without intromissions, and the total number of intromissions. All procedures were carried out twice at one-week interval taking into consideration that by the time of the second test they gained some sexual experience.

Female-type sexual behavior. Male descendants were orchidectomized one week before testing and treated with steroid hormones as mentioned above for preparing receptive females. The males were introduced to sexually experienced males that were kept in the test cage under red dim light for at least 5 min. Testing lasted 10 min or up to 10 mounts of an active male. The number of lordosis reactions to approaches and mounts of the normal male was recorded.

Hormone assay. After decapitation, the trunk blood samples from control, ibuprofen and corresponding stressed control and ibuprofen animal groups were collected in heparinized tubes. Plasma was obtained by centrifugation and kept at –20°C prior be analyzed for testosterone, estradiol or corticosterone levels. Hormone immunoassays were performed by Testosterone ELISA and Estradiol ELISA kits (“DRG”, Germany) followed by measuring at immunoenzyme analyzer “Stat Fax” (USA). Blood plasma corticosterone concentrations were measured by spectrofluorimetric microassay using a spectrofluorimeter Hitachi MPF-4 (Japan) [27].

Stress-test procedure. The rats were undergone to 1 h strict restriction followed by quick decapitation. Non-stressed animals were used for the determination of basal corticosterone levels.

Sperm count. Using Goryaev’s chamber, a number of spermatozoa were counted in epididymis after a dosed washout with 2 ml saline [28].

Statistical analysis. The results were averaged and compared with those of appropriate controls. They were presented as mean ($M \pm m$) and processed with an Excel computer program by one-way analysis of independent experiments using the Student’s t criterion in a case of normal distribution of variants which was tested according to the Shapiro-Wilk criterion. In the absence of a normal statistical distribution, the Wilcoxon-Mann-Whitney non-parametric U criterion was used. The difference was set as significant at $P \leq 0.05$.

RESULTS

Pregnancy outcome and the offspring development. Experimental and control females gave birth to the same number of pups at term, on average, 8.6 and 9.0 per litter, respectively. The overall ratio of males to females was 1.5 in the experimental and control groups. On the PND 2 and 10, the weights of exposed to mater-

nal ibuprofen newborns were the same as those of controls. At PND 2, an average AGD in the ibuprofen group was greater, compared to that of the control group ($P < 0.05$). The statistical difference between the groups disappeared by PND 10 (Table 1). Other visible teratogenic effects of prenatal ibuprofen were not found. The testicle omission into the scrotum in the ibuprofen group slightly accelerated and occurred at average on PND 35.00 ± 0.24 *vs* 35.65 ± 0.17 in the controls ($P < 0.05$). During the postnatal observation period, there were no changes in the male body weights, general appearance, social and eating behaviors, or physical activity as compared to controls.

Male-type sexual behavior. Control 8-month-old males were tested at the end of November and the beginning of December, and demonstrated low sexual activity, which is typical for the winter season. In particular, they did not ejaculate for 15 min of observation. The low sexual activity may be caused by the seasonal fall in testosterone secretion [29]. After the animals have gained sexual experience, in the second test they become more sexually active. That manifested in the appearance of ejaculation, significantly shorter latent periods of the first mount and the first intromission, and an increase in the number of mounts with intromission.

In contrast to the sexual behavior in normal males, in the ibuprofen group of 8-month-old rats, in both test sessions, there was found significantly weakened sexual capacity. That was evidenced by the dramatic decrease in sexual motivation and the ability of males to mate with females. This applies to all studied indicators, which characterize the central and peripheral components of sexual behavior. As the result of the prenatal exposition of ibuprofen, the duration of latent periods of the first mount in the first test increased by an average of 4 times, the first intromission - by more than 3 times, and the number of mounts with intromission halved. These differences were even more dramatic in the second test. Prenatally exposed to ibuprofen rats did not show ejaculation. The duration of the latent periods of the first mount increased 23 times, the latent period of the first intromission lengthened 14 times, and the number of the mounts with intromission decreased 6.5 times (Table 2).

Female-type sexual behavior. The data that have been obtained when studying female-type behavior did not indicate feminization of their neuroendocrine reproductive phenotype compared to control. The number of approaches of experimental males to normal ones did not differ statistically from controls; no one of those animals demonstrated lordosis responses in the presence of a normal male (on average 4.0 ± 0.4 *vs* 4.6 ± 0.5 relatively, 6 rats in each group).

Basal hormone levels. At rest, testosterone, estradiol (Table 3) and corticosterone (Figure) levels in blood plasma of 5-month-old males were the same in the experimental and control groups ($P > 0.05$, Student *t* criterion for testosterone and estradiol, Wilcoxon-Mann-Whitney U criterion for corticosterone). The ratio of concentrations of testosterone and estradiol slightly increased in the ibuprofen group, but

Table 1. Body weight and anogenital distance (AGD) in the rat male offspring prenatally exposed to ibuprofen (M±m)

Animal group	n	Body weight, g	AGD, mm	AGD/g bw
PND 2				
Control	27	7.39 ± 0.08	4,11 ± 0.09	0.56 ± 0.01
Ibuprofen	26	7.17 ± 0.13	4.64 ± 0.16*	0.65 ± 0.03*
PND 10				
Control	26	18.59 ± 0.54	7.53 ± 0.16	0.41 ± 0.01
Ibuprofen	26	18.51 ± 0.67	7.15 ± 0.13	0.40 ± 0.01

Note: * P<0,01 compared to control (Student test).

Table 2. Effect of prenatal ibuprofen exposure on male-type sexual behavior in male rats of 8-month age

Feature	First test		Second test	
	Control	Ibuprofen	Control	Ibuprofen
Latency period, sec:				
first mount	64.0 (9-201)	184.0 (114-590) *	7.0 (0-21)	60.0 (24-577) *
first intromission	73.0 (11-390)	324.0 (243-695)*	19.0 (2-57) **	303.0 (73-601) *
first ejaculation	-	-	543.0 (193-717) **	-
Number				
mounts without intromission	4.0 (2-6)	7.0 (3-8)	4.0 (3-11)	5.0 (2-7)
mounts with intromission	6.0 (5-16)	2.0 (2-7) *	15.0 (14-20) **	2.0 (1-5) *
ejaculations	0	0	1.0 (1 - 1) **	0*

Notes: 5 rats in each group. The test lasted 15 min. The data are presented as medians and minimum and maximum values (in the brackets). Statistical analysis by Wilcoxon-Mann-Whitney U criterion.
* $P \leq 0.05$ compared to controls; ** $P \leq 0.05$ compared to the first test.

did not reach statistical significance

Stress-test. A pronounced HPAA response to acute stress was observed in both groups of animals: the level of corticosterone increased from 2.6 to 3.4 times at average after 1-hour immobilization ($P < 0.05$ by Wilcoxon-Mann-Whitney U criterion (Figure). No statistical difference of stress reactivity between control and experimental groups was found.

Sperm count. Prenatal ibuprofen had no effect on the content of sperm in epididymis: ibuprofen - $50.75 \pm 1.10\ 10^6$/ml ($n = 6$), control - $47.08 \pm 1.20\ 10^6$/ml ($n = 6$).

DISCUSSION

The developing fetal brain is very vulnerable to environmental influences including drugs [3]. Concerns exist regarding the harmful effects of analgesics used during pregnancy on the health of offspring. It has been established that even mild analgesics *in utero* exposure during fetal development are associated with antiandrogenic effects and congenital malformations in adult men and rats. In particular, mRNA expression of steroidogenic genes which are involved in testosterone synthesis is significantly suppressed *in vitro* with ibuprofen, a non-selective reversible cyclooxygenase inhibitor [30].

Ibuprofen is one of the most widely used among pregnant women, a non-steroidal anti-inflammatory medication as an analgesic drug. Its ability to cross the placenta and enter the fetal blood circulation followed by inhibition of cyclooxygenase and, therefore, the synthesis of prostaglandins, which are one of the mediators of the formation of sex-specific neuroendocrine regulation of sexual behavior in the fetus [23, 24], was the basis for suggesting the presence of behavioral anomalies in male offspring.

We have found only one research work regarding the effect of ibuprofen administered *in utero* and during lactation on sexual behavior and some other characteristics of the reproductive system in adult male rat offspring [22]. Similarly, we introduced ibuprofen to pregnant dams

Table 3. Testosterone (T) and estradiol (E2) levels in the blood plasma of the 5-month old rat male offspring prenatally exposed to ibuprofen (M±m)

Animal group	n	T, nmol / L	E2, pmol / L	T / E2
Control	6	51.92 ± 4.55	77.23 ± 7.71	707 ± 99
Ibuprofen	6	48.02 ± 6.53	52.74 ± 11.42	1190 ± 345

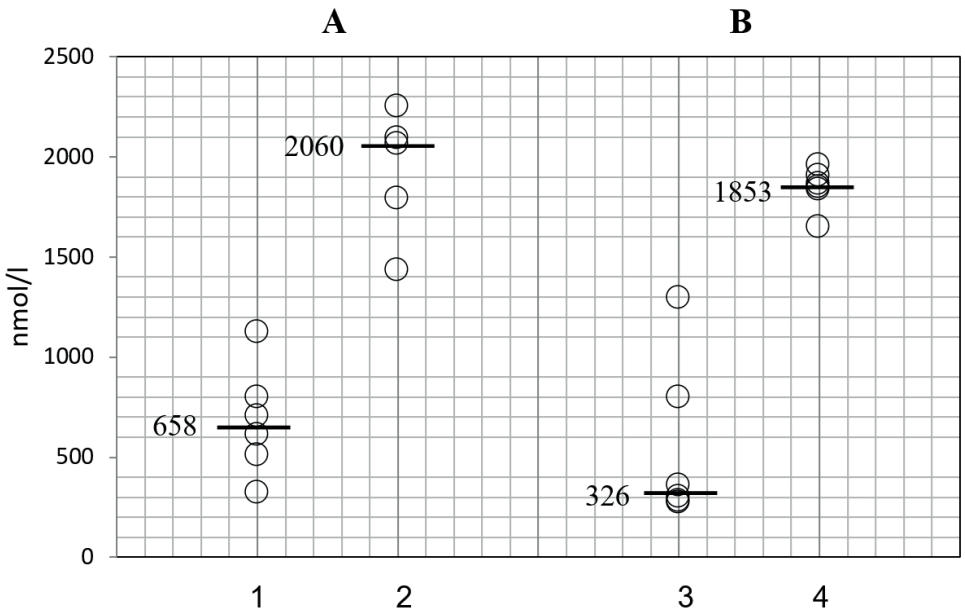

Fig. Corticosterone blood plasma levels (nmol / L) in rats before and after 1-hour immobilization stress (data are presented as individual values and median). A – control; B – ibuprofen; 1 and 3 - basal levels; 2 and 4 – stress.

during the narrow time window of fetal brain sexual development. The dose of the drug used was comparable to that normally used by an adult human.

All adult male rats exposed to prenatal ibuprofen presented male-type sexual behavior in the presence of receptive females. However, in contrast to results by Balin et al. [22], their copulative potential was affected significantly with ibuprofen. When compared with the concurrent controls, experimental rats exhibited very low sexual activity, especially in the second testing session, despite the acquired sexual experience. Ibuprofen-treated offspring demonstrated a dramatic decrease in sexual motivation and the ability of males to copulate with females. Multiple increases in the duration of the latent periods of the first mount and the first intromission, a decrease in the numbers of intromissions, and no ejaculations in both tests indicate a violation of the central and peripheral components of sexual behavior.

Thus, the developing male fetal brain of the rats was partially demasculinized with prenatal ibuprofen. However, testosterone and estradiol blood plasma levels in adult offspring were not disturbed with prenatal ibuprofen. Therefore, an association between disruption of male-type sexual behavior and sex steroid hormone levels has not been found under the experimental design that we used in this study, whereas under other experimental conditions, some researchers have observed a decrease in testosterone levels in adult males [21, 22].

Further, we did not confirm the data by Balin et al. [22] about the feminization of the male brain due to prenatal exposure to ibuprofen, because no lordosis response of castrated experimental males primed with estradiol and progesterone was observed were contact with normal, sexually experienced male.

There are at least two possible mechanisms underlying the phenomenon of incomplete masculinization of the fetal brain under the influence of prenatal ibuprofen. One of them is the deficiency of testosterone, which is synthesized by the testicles of the intrauterine fetus, due to the inhibition of steroidogenesis enzymes induced by ibuprofen [7, 30]. Testicular testosterone is a hormonal factor that initiates the sexual differentiation of the brain in a male fetus. In rodents, this process is going on in

the preoptic area of the hypothalamus. Fetal testosterone is converted to estradiol, which in turn is converted to 4-hydroxyestradiol. This catechol estrogen inhibits catechol-O-methyltransferase, resulting in the accumulation of norepinephrine in the hypothalamus. Sexual differentiation of the brain according to the male type is carried out by the joint action of catechol estrogen and norepinephrine, which acts as an inducer of neuroblast differentiation [1, 3]. Confirmation of the antiandrogenic effect of ibuprofen in male fetuses is a decrease in the AGD of male rat fetuses [16, 22].

Another possible mechanism is ibuprofen-induced repression of prostaglandin synthesis. It was shown that the signaling molecule prostaglandin E2 is induced by estradiol in the microglia of the neonatal preoptic area, and estrogen in cooperation with prostaglandin E2 realizes the masculinization of neuroanatomical architectonic in the male brain [24, 31]. Obviously, inhibition of prostaglandin E2 synthesis with ibuprofen underlies unfinished sexual differentiation of the male fetal brain. It seems that both mentioned mechanisms are working.

Taking into consideration that prenatal ibuprofen did not change either basal corticosterone level or its response to acute stress in adult males, we suggest that this endocrine disruptor selectively affects the neuroendocrine system of reproduction.

CONCLUSION

Administration of ibuprofen to female Wistar rats during the last week of gestation disrupts partially neuroendocrine programming of male-type copulative behavior in male offspring with no changes in female-type sexual behavior. Basal blood plasma levels of testosterone, estradiol, and corticosterone, as well as HPAA response to immobilization stress, did not change. It is hypothesized that incomplete masculinization of the developing fetal brain with ibuprofen is due to two co-operative mechanisms: inhibition of synthesis of testosterone and prostaglandin E2 in the preoptic area of the hypothalamus.

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ЕНДОКРИННІ ТА ПОВЕДІНКОВІ ЕФЕКТИ У САМЦІВ ЩУРІВ ПІСЛЯ НАРОДЖЕННЯ ПРИ ПРИЙОМІ ЇХНЬОЮ ВАГІТНОЮ МАТІР'Ю ІБУПРОФЕНУ

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Ібупрофен, неселективний інгібітор циклооксигенази, є одним з найчастіше використовуваних нестероїдних протизапальних та знеболювальних засобів. Його вплив на нейроендокринну систему плода, що розвивається, при прийомі вагітною матір›ю точно не встановлено. Метою нашого дослідження була оцінка віддалених наслідків введення ібупрофену вагітним самицям щурів щодо гормонального профілю та статевої диференціації головного мозку у чоловічого потомства. Їм перорально вводили ібупрофен у дозі 30 мг/кг маси тіла двічі на день протягом 15–21 дня. На 2-й та 10-й постнатальні дні (ПНД) у самців вимірювали аногенітальну відстань (АГВ). У дорослому віці вивчали статеву поведінку, вміст гормонів та реакцію гіпоталамо-гіпофізарно-адреналової системи (ГГАС) на гострий іммобілізаційний стрес. Пренатальна дія ібупрофену збільшувала аногенітальну відстань на ПНД 2 і викликала неповну маскулінізацію копулятивної поведінки у дорослих нащадків. Відзначено достовірне збільшення латентних періодів першої садки, першої інтромісії та зменшення кількості інтромісій. Базальний вміст тестостерону, естрадіолу та кортикостерону у плазмі крові, а також реакція ГГАС на іммобілізаційний стрес не змінилися. Самці після орхідектомії та обробки естрадіолом і прогестероном не виявляли лордозної реакції на присутність сексуально досвідченого самця. Припускається, що неповна маскулінізація головного мозку плоду, що розвивається, під дією ібупрофену

зумовлена двома кооперативними механізмами: інгібуванням синтезу тестостерону в гонадах плоду і простагландину Е2 у преоптичній ділянці гіпоталамуса. Таким чином, введення ібупрофену самицям щурів лінії Вістар впродовж останнього тижня вагітності частково порушує нейроендокринне програмування копулятивної поведінки у чоловічого потомства без зміни статевої поведінки за жіночим типом і функції ГГАС.

Ключові слова: ібупрофен; пренатальні ефекти; статева поведінка; тестостерон; естрадіол; кортикостерон; стрес-реактивність самці щурів.

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