

Subchronic perinatal asphyxia increased anxiety- and depression-like behaviors in the rat offspring

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Abstract

OBJECTIVES: Perinatal asphyxia is one of the major cause of mortality in newborns and cause of neurological disorders in adulthood. Brain damage is of the most concern due to high sensitivity of nervous system to suboptimal intrauterine oxygen condition. The aim of this study was to assess effect of subchronic prenatal asphyxia (SPA) during sensitive stages of brain maturation on behavioral changes in rats, as a method of prenatal programming of anxiety and depression-like behavior.

METHODS: Pregnant Wistar/DV females were exposed to environment containing lower oxygen (10.5% O₂) during sensitive stages of brain maturation (day 19–20 of gestation) for 4h a day and anxiety- and depression-like behaviors in offspring were assessed using battery of behavioral tests – Open field (OF), Elevated plus maze (EPM), Light/dark test (L/D), Forced swim test (FST), and Stress induced hyperthermia (SIH).

RESULTS: OF did not induced changes of locomotor and exploration activities. The anxiety-like behavior was induced by SPA in EPM and L/D. These results were significant in males SPA group only. The higher response to the stress stimulus in SIH was recorded in both males and females SPA group. The intensity of climbing on the walls of cylinder in FST in males SPA group was significantly decreased indicating depression-like behavior in adulthood.

CONCLUSIONS: In conclusion, we found out that perinatal asphyxia on 19th and 20th day of gestation caused anxiety- and depression-like behaviors in the rat offspring. Our model of SPA has proved to be useful to study the conditions of asphyxia during pregnancy, and could be suitable model for studies uncovering the mechanisms of prenatal programming of psychiatric diseases.

INTRODUCTION

Environmental exposure encountered in early life influences the health and risk of disease in adulthood. The greatest risks to life are in its beginning. A general mechanism by which early environmental exposure could be linked to adult phenotypic changes is the alteration of epigenetic signatures, which are crucial in orchestrating the output of genomic information. Understanding of disease susceptibility will require consideration of both genetic and epigenetic information that indicates environmental interactions integrating with the genome. Indeed, it has been reported that prenatal and early postnatal environment factors, such as maternal hypoxia (Gonzalez-Rodriguez *et al.* 2014; Zucchi *et al.* 2013, Fan *et al.* 2014), maternal nutritional supplements (Waterland *et al.* 2006), and xenobiotic chemicals (Ho 2006), are responsible for altered reprogramming of epigenetic marks and subsequent changes of disease susceptibility in the developing fetus.

Prematurity and congenital anomalies account for more than one third of newborn deaths, and these often occur in the first week of life. A further quarter of neonatal deaths are attributable to asphyxia – also mainly in the first week of life (WHO 2005). Intrauterine hypoxia can be related to various complications occurring in the mother, placenta, or fetus, which can manifest differently and lead to different consequences. There are three types of hypoxic condition during pregnancy: preplacental hypoxia, in which both the mother and her fetus are hypoxic; uteroplacental hypoxia, in which the maternal oxygenation is normal but the fetus is hypoxic because of impairment of uteroplacental circulation; and postplacental hypoxia, in which only the fetus is hypoxic (Kingdom & Kaufmann 1997).

Asphyxia during pregnancy, delivery, or in the early stages of life is a major cause of neurological disorders and newborn mortality. It has been estimated that the incidence of perinatal asphyxia vary depending on the definitions used. In resource-rich countries, the incidence of severe perinatal asphyxia (causing death or severe neurological impairment) is about 1/1000 live births (Thornberg *et al.* 1995). In resource-poor countries, perinatal asphyxia is probably much more common. Data from hospital-based studies in such settings suggest an incidence of 5–10/1000 live births (Jones *et al.* 2003) However, this probably represents an underestimate of the true community incidence of perinatal asphyxia in resource-poor countries (McGuire 2007). Birth asphyxia, defined as the failure to establish breathing at birth, accounts for an estimated 900 000 deaths each year and is one of the primary causes of early neonatal mortality (Lawn *et al.* 2007). While the burden of neonatal deaths and stillbirths is very substantial, it is in many ways only part of the problem, as the same conditions that contribute to it also cause severe and often lifelong disability. For example, over a million children who survive birth asphyxia each year

develop problems such as cerebral palsy, learning difficulties and other disabilities including psychiatric disorders (Golan *et al.* 2004; Bhat *et al.* 2005).

Hypoxia-induced brain injury occurs in both white matter and gray matter, which has been confirmed in animal models showing that severe acute hypoxia causes an equal injury in subcortical white matter and gray matter region in preterm fetal sheep at 0.6 and 0.7 of gestation (full term is approximately 147 days) (Dean *et al.* 2006; Bennet *et al.* 2007). Brain injury caused by fetal hypoxia consequently leads to abnormal behavioral presentations. The aim of this study was to assess neurobehavioral changes of the rat offspring after perinatal asphyxia using new non-invasive model of subchronic perinatal asphyxia (Ujhazy *et al.* 2013).

MATERIAL AND METHODS

Animals

Virgin female Wistar/DV rats (weight 200–220 g, age 3–4 months, n = 24) of monitored conventional breeding were obtained from the breeding station Dobrá Voda (Slovak Republic, reg. No. SK CH 24011). These animals were housed in transparent plastic cages under standard laboratory conditions (12/12 h light-dark cycle, $23 \pm 1^\circ\text{C}$, 50–70% humidity, food and water *ad libitum*). After 7 days of adaptation, the females were mated with males in the ratio 1 male: 3 females. The presence of spermatozoa in vaginal smear was considered day 0 of gestation. The pregnant rats were allowed to deliver spontaneously. The day of delivery was designated as day 0 after birth. The experiments were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, and the experimental design was approved by the State Veterinary and Food Administration of the Slovak Republic.

Subchronic perinatal asphyxia (SPA)

Pregnant rats were exposed to a lowered oxygen containing environment (reference calibrated gas Class I: 10.5% O₂ in 89.5% N₂ obtained from Linde Gas, Slovakia) for 4 h/day in hermetically sealed hypoxic chambers during sensitive stage of brain development (days 19–20 of gestation). The generated CO₂ in the chamber was replaced every 30 min by reflow of the calibrated gas for 60 s. Animals were returned to their home cage after SPA.

Behavioral analysis

Elevated plus maze (EPM)

Animals (n=24) were tested in EPM at postnatal day 60. All parts of the apparatus were made of dark polyvinyl plastic. The arms of the maze were 50 cm above the floor, 50 cm long and 10 cm wide. The movements of the rats were tracked with digital camera and analyzed by computer software ANYMAZE™ (Stoelting Europe,

Ireland). Each session was started by placing the rat in the central area facing the open arms of the maze and lasted 5 min. Between trials, the maze was wiped with a mild detergent. All testing was carried out between 8:00 a.m. to 12:00 a.m.

Stress induced hyperthermia (SIH)

Animals (n=24) were tested in SIH at postnatal day 65. Basal rectal body temperature of the animals was measured using a digital rectal thermometer BAT-12 (Physitemp Clifton, NJ, USA). Lubricated thermistor probe RET-2 was inserted into the rectum and was left there until a constant reading was obtained (± 10 s). Handling of the animals together with temperature measurement represents a stress response resulting in an increase of rectal temperature. Therefore, the rectal temperature was taken also 30 min after the first measurement. The difference between the second (T_1) and first measurement (T_0) was calculated for individual animals ($\Delta T = T_1 - T_0$).

Light/Dark Box (L/D)

Animals (n=24) were tested in L/D at postnatal day 70. The test apparatus consisted of a white plastic arena (60×40 cm) with a black plastic box (40×20×40 cm, opening 10×8 cm) inserted in the arena. An incandescent light bulb fixed 1 m above the arena provided the illumination. At the beginning of the experiment, the rat was placed in the light part of the box, facing the opening. The movement of the rat was recorded using digital camera and further analyzed by computer software ANYMAZE™ (Stoelting Europe, Ireland). All testing was carried out between 8:00 a.m. to 12:00 a.m.

Forced swim test (FST)

Animals (n=24) were tested in FST at postnatal day 85. The apparatus consisted of a vertical cylindrical glass container (height 45 cm×diameter 25 cm) filled with tap water at 26 ± 1 °C. The depth was sufficient to ensure that the animals could not touch the bottom of the container with their hind paws. The forced swim test is a two-day test where on the first day rats were introduced to the cylindrical glass tank filled with water for 15 min, patted dry, placed in a home cage, allowed to rest under heating lamp until dry and then returned to the colony. Twenty-four hours later, the animals were exposed to the same experimental conditions for 5 min, dried and returned to their home cage. Sessions were videorecorded and scored using the software ANYMAZE™ (StoeltingEuropa,Co., Ireland). All testing was carried out between 8:00 a.m. to 12:00 p.m.

Open field test (OF)

Animals (n=24) were tested in OF at postnatal day 90–94. Locomotor activity was evaluated in apparatus consisted of a white plastic arena (60×40 cm) and the movement of the rat was recorded using digital camera and analyzed by computer software ANYMAZE™

(Stoelting Europe, Ireland). The animals were placed in the center of the open-field arena and let freely explore daily 5 minutes for five consecutive days. After each session, the number of fecal pellets (defecation) was noted for assessment of emotional reactivity and the open-field arena was cleaned with a mild detergent and allowed to dry. All testings were carried out between 8:00 a.m. to 12:00 a.m.

Statistical examination

The data were analyzed by means of multifactor analyses of variance (ANOVA) followed by Fisher's LSD post hoc test to assess statistical significance. For the open field data, ANOVA for repeated measures was conducted with the condition (SPA vs. Control) and gender (males vs. females) as the between-subject factors, and day (repeated measure) as the within-subject factor. These tests were run using the statistics program STATISTICA 7.0 (StatSoft, Tulsa, OK, USA). The results are presented as means \pm S.E.M. The confidence limit of $p < 0.05$ was considered statistically significant.

RESULTS

Elevated plus maze (EPM)

Although we observed decreased time spent in the open arms, these results failed to be significant ($F_{1,20}=2.48$; $p=0.13$; Figure 1C). However, SPA rats spent significantly less time head dipping compared to control ($F_{1,20}=6.8$; $p=0.017$; Figure 1A,B). Head dipping was recorded when the animal stopped at the platform edge of the open arm, head bent down, showing downward visual screening (eye level below the platform surface) with intense vibrissa twitching and sniffing.

Stress induced hyperthermia (SIH)

The stress response to a first measurement of basal temperature was significantly higher in SPA rats compared to control ($F_{1,20}=10.7396$; $p=0.0038$; Fig. 2B). Although the response was increased in both males and females, after post-hoc comparison the statistical significance reached significance in females only (males: $p=0.08$; females: $p=0.011$; Fig. 2A).

Light/Dark Box (L/D)

SPA induced anxiety-like behavior in L/D test. SPA rats spent significantly less time in bright lit arena compared to control ($F_{1,20}=5.77$; $p=0.026$; Fig. 3B). Following post-hoc comparison revealed significant effect of SPA in males only ($p=0.0064$, Fig. 3A).

Forced swim test (FST)

Occurrence of behavioral despair behavior was assessed using FST. The swim test involved the scoring of active (climbing) and passive (immobility) behavior when rats were forced to swim in a cylinder from which there is no escape. Active behavior (climbing) was significantly suppressed by SPA ($F_{1,20}=6.33$; $p=0.02$; Fig. 4B)

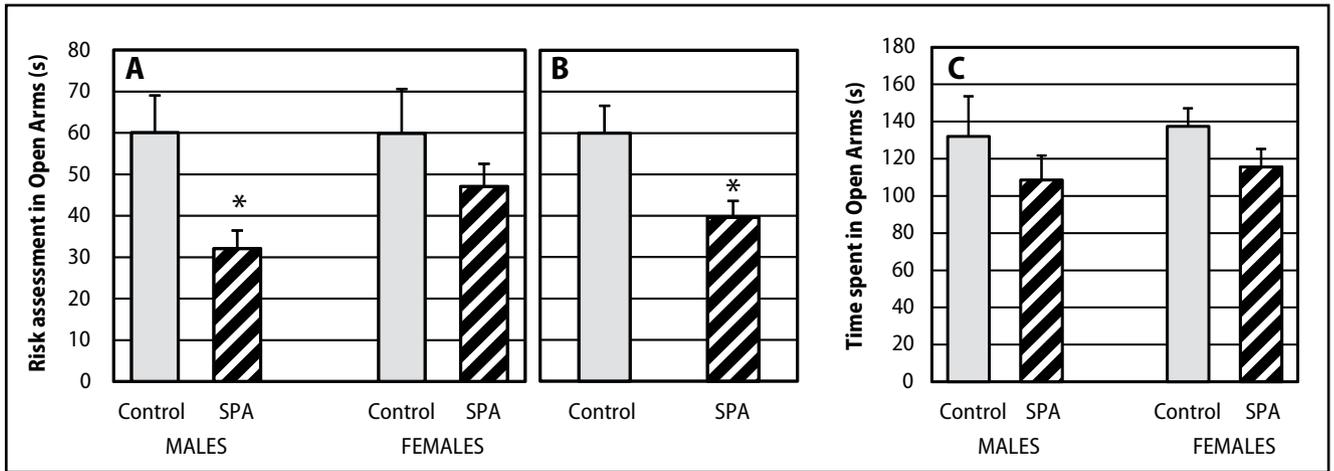


Fig. 1. Effect of subchronic perinatal asphyxia (SPA) on offspring performance in elevated plus maze test. * $p < 0.05$ compared to control group.

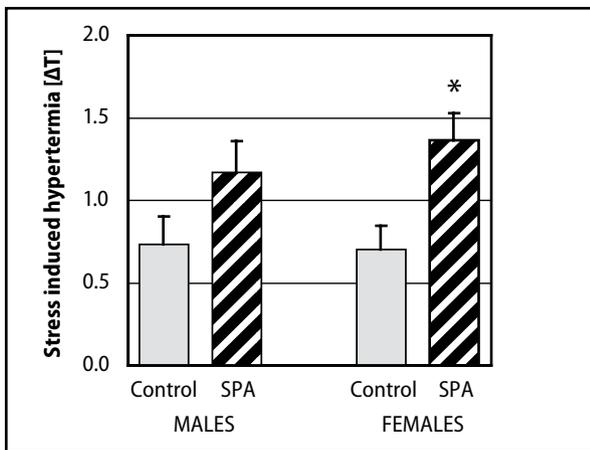


Fig. 2. Effect of subchronic perinatal asphyxia (SPA) on the ability to cope with stress situation (Stress induced hyperthermia). * $p < 0.05$ compared to control group.

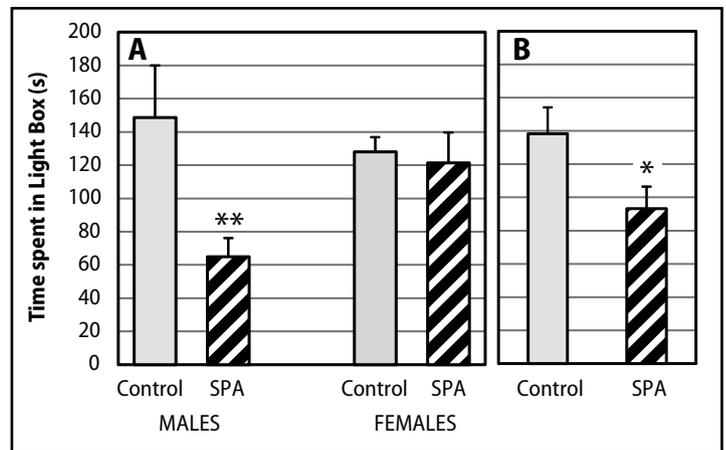


Fig. 3. Effect of subchronic perinatal asphyxia (SPA) on offspring performance in Light/Dark box test. * $p < 0.05$; ** $p < 0.01$ compared to control group.

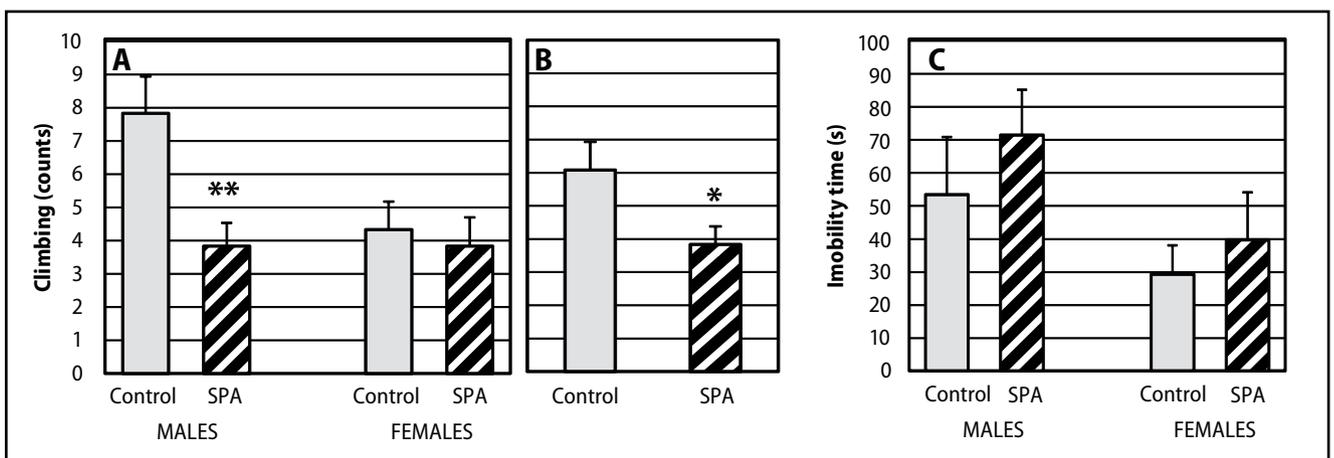


Fig. 4. Effect of subchronic perinatal asphyxia (SPA) on offspring performance in forced swim test. * $p < 0.05$; ** $p < 0.01$ compared to control group.

and post-hoc analysis revealed that SPA males were affected most ($p = 0.004$; Fig. 4A). SPA did not significantly increase passive behavior – immobility time

($F_{1,20} = 1.19$; $p = 0.28$) however, post-hoc comparison revealed significant increase in immobility time in SPA males ($p = 0.05$).

Open field test (OF)

SPA did not affect the locomotor (distance traveled) and exploratory (rearing) behavior tested in OF. We found only gender differences in activity and exploration with no effect of SPA. Females were more active in both variables (distance: $F_{1,20}=9.26$; $p=0.006$; rearing: ($F_{1,20}=7.65$; $p=0.01$; data not shown).

DISCUSSION

In this study we used non-invasive model of subchronic perinatal asphyxia (SPA) and examined the impact of asphyxia during sensitive stages of brain maturation on the possible development of depression and anxiety-like behavior using well-established ethological approaches.

Incidence of mental diseases in the developed countries has an increasing trend. The most frequent psychiatric disorders are anxiety disorders, depressive, somatoform and substance dependence disorders. The prevalence of the anxiety and mood disorders is estimated about 21.1%, what equals amazing 64 million of the adult EU population, 18–65 of age (Wittchen & Jacobi 2011).

A growing body of data indicates that human susceptibility to mood disorders such as depression and anxiety can be determined early in life. These data support the view that early developmental mechanisms can set the lifelong tendency of an organism to express anxiety in response to threatening stimuli. Such developmental mechanisms are under both genetic and environmental control.

Fetal programming occurs during a critical period of intrauterine development. Disturbances of homeostasis (endogenous or exogenous stimuli) may have long-term and life-long consequences. Intrauterine conditions in which the embryo develops play an important role in the regulation of functions of the physiological systems in adulthood (Godfrey and Barker 2001). The critical period, duration, severity and type of insult during development determines the specific physiological (functional) changes (Fowden *et al.* 2006).

According to the authors Jänicke and Coper (1994), exposure to hypoxia induces a characteristic pattern of damage during different life stages. Main characteristics (as a negative geotaxia, regulation of body temperature) have changed only slightly, or not changed at all compared to the control group. However, in more complex functions (locomotor activity – running, rotarod) indicated significant deterioration. In some cases, they observed developmental delays in functional tests of motor function, e.g. swimming and chimney test.

In our case SPA induced anxiety-like behavioral patterns observed in EPM. Several research studies exist in which the authors observed changes in behavior in the elevated plus maze after prenatal or perinatal hypoxia/ischemia. The authors Fan *et al.* (2009) found that intermittent hypoxia during pregnancy increase the anxiety like behavior. They observed a reduced percentage of

entries to the EPM open arms (up to 20%). Time spent in the open arms was dramatically reduced (by up to 49.2%) compared to control. Perinatal asphyxia was the cause of anxiety-like behavior manifested by an increased number of entries into the closed arms in the work of Weitzdoerfer *et al.* (2004). In contrast to the above mentioned authors Ikeda *et al.* (2005) revealed no significant changes in the activity of hypoxic-ischemic group and recorded no change in weight. Buwalda *et al.* (1995) also fail to detect a significant difference in the time spent in the open or closed arms between anoxic group and controls. These facts point out that a type time and severity of insult are important factors of prenatal programming of anxiety-like behavior. Although our results did not revealed statistically significant decrease in time spent in the open arms of EPM, an obvious trend of reduction of stay in the open arms were recorded in SPA group. We also observed significantly reduced exploratory activity (head dips) in the open arms which corresponds with the results of other authors. This behavior is a pattern considered to have a risk assessment or an exploratory role and is used as a complementary parameter to evaluate anxiety in experimental animals (Hoshino *et al.* 2004). Fan *et al.* (2009) observed changes in the elevated maze after exposure of pregnant mothers to intermittent hypoxia during the entire course of pregnancy. In our case, we used a model of SPA in the 19th and 20th DG, which we think is relevant time in case of a possible effect on brain development and prenatal programming and induction of anxiety-like behavior.

The stress-induced hyperthermia (SIH) response is a relatively short-lasting rise in body temperature in response to stress which has been reported in rodents, baboons, sheep, impalas and chimpanzees (for review see Bouwknecht *et al.* 2007). Our results confirmed increased responsiveness of SPA rats to stress. Shiloh *et al.* (2007) suggested that dysfunctional thermoregulation in psychiatric patients corresponds to symptom severity of disorder. Although the SIH response does not model any specific psychiatric condition, this response may be useful as a read-out parameter of stress. It can be studied at the preclinical and clinical level in different stressful experimental setups and may therefore serve as an animal-to-human stress-related parameter (Winkers *et al.* 2008).

Light/dark (L/D) box exploration test revealed anxiety-like behavior in SPA group. We also observed a longer latency time to rearing, indicating inhibited exploratory activity. To our knowledge similar approach was not studied. We could not find papers studying impact of perinatal asphyxia on changes in anxiety and depression-like behavior tested in light/dark box test. The impact of perinatal asphyxia on the performance L/D box is therefore unique and original result.

Rodents forced to swim in a narrow space from which there is no escape adopt, after an initial period of vigorous activity, a characteristic immobile posture.

It was hypothesized that these animals had learned that escape is not an option and given up hope and immobility was given name “behavioral despair” (Porsolt *et al.* 1977; Gerfen *et al.* 2007). FST revealed behavioral despair behavior of SPA rats manifesting as a lower frequency of climbing on the wall as well as increased immobility time in male SPA rats. Our results corresponds with the research done by authors Mikhailenko *et al.* (2009), who found that hypoxia is a major cause of a depression-like behavior manifested by significantly increased immobility in FST. Similar results were also observed by authors Mueller and Bale (2008), who found a significant effect of prenatal stress on the immobility time in the forced swimming test.

Open-field test did not revealed any effect of SPA on observed behavior such as locomotor activity, exploration, rearing, time spent in the central zone, etc. Similarly other authors did not find a significant differences in the OF between asphyxial and normoxic control group (Hoeger *et al.* 2000; Balduini *et al.* 2000) suggesting less involvement of prenatal asphyxia in locomotor behavior tested in OF.

Our study of behavioral changes after SPA revealed an induction of anxiety- and depression-like behavior, especially in male rats. These suggest that males are more sensitive during critical periods of development. Several authors found that estrogen has a protective effect in neurodegenerative diseases such as stroke, Parkinson’s disease and Alzheimer’s (Biggsby *et al.*, 1999; Dhandapani and Brann 2002) and therefore females might be more resistant to adverse stimuli during development.

In conclusion we confirmed that the end of pregnancy is very sensitive to the effect of exogenous factors such as reduction of oxygen supply. Such disturbances can affect the development of the brain and cause functional changes. The hippocampus and amygdala are the one most sensitive to oxygen deficiency and asphyxiation probably affected the development of these structures involved in the anxiety and depression patterns. The presented model of SPA proved to be useful for the study of asphyxial conditions during pregnancy. It’s non-invasive approach allow us to study effect of asphyxia on prenatal programming and fetal origins of adult diseases. It could be also a suitable model for screening and investigating indicators of asphyxia in the mother and fetus.

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Conflict of Interest

All authors declare no conflict of interest.

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