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Original article

The effects of lack of movement in sows during pregnancy period on cortisol, acute phase proteins and lymphocytes proliferation level in piglets in early postnatal period

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Abstract

Prolonged exposure to stress may cause adverse effects on animal physiology. It is especially important during the gestation period as female physiology can affect the unborn offspring in the form of prenatal stress. Intensive pig farming industry developed gestation crates that enable to keep sows during gestation period in small stalls which do not allow animals to move freely for a maximum of 4 weeks after successful insemination (Council Directive 2008/120/EC**)**. Although these crates have production advantages, many health and welfare issues have been raised recently. In this study we tested to what extent the lack of movement of sows kept in the gestation crates had an impact on some blood and saliva constituents of new-born piglets. In total, the samples were collected from 80 piglets when they were 3, 7 and 21 days of age and tested for cortisol levels in blood and saliva, acute phase proteins (amyloid A, C-reactive protein, haptoglobin) and lymphocytes proliferation index (in response to ConA, PHA and PWM). 40 piglets were from sows kept in free movement housing (FM group) from day 1 to day 100 of pregnancy and forty piglets were from sows in the movement restriction group (MR), in which the sows were kept in crates just allowing them to stand up and lie down from day 1 to day 100 of the pregnancy (research was conducted before the implementation Directive 2008/120/EC i.e. January 1,2013). The results of the study showed that the piglets delivered by sows kept under movement restriction conditions exhibited higher cortisol and acute phase protein levels as well as a lower lymphocytes proliferation index. This suggests that lack of movement in sows during the gestation period influences piglets' physiology and indicates that the piglets are suffering from prenatal stress caused by insufficient housing conditions of their mothers potentially leading to poor health and welfare of their offspring.

Key words: lack of movement, gestation crates, sows, stress, piglets, cortisol, acute phase protein, immunity

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Introduction

The individual stall for keeping dry sows during gestation period is one of the most controversial issues facing swine industry. For 4 weeks after insemination the sows are kept in crates that allow them to stand up and lie down "undisturbed" as the law provides (Council Directive 2008/120/EC). The animals cannot turn or move around. This system has many advantages from the breeder's point of view. It gives high pregnancy rates, enables individual feeding and prevents aggression among rivalling sows. However, it is widely questioned in terms of animal welfare. Limitation of living space is the main cause of body injuries related to living conditions in pigs (McGlone et al. 2004). The reason for that is a constant contact of animal's body with steel elements of the crate while standing up or lying down (Baxter et al. 2011). In sows kept in gestation crates an increased number of urinary tract infections occur due to the fact that animals are often forced to lie in their own feces. In addition, animals that are devoid of movement possibility do not drink enough water and urinate irregularly (Sanders et al. 2004, Moura et al. 2017). Urinary tract infections may even contribute to increased mortality (Irvin at al. 2000, Sanz et al. 2002, Sanz et al. 2007).

Movement restriction results in inability to express natural behaviour which can lead to the occurrence of mental disorders such as stereotypies. Most common stereotypic behaviours observed in sows kept in gestation crates are: biting of crate elements, head wagging (weaving), starting the watering devices without the need for drinking and exercise of chewing movements with an empty mouth indicating severe stress in the animals (Chapina et al. 2010, Zhang et al. 2017, Tatemoto et al. 2019).

Movement restriction can also influence the immune system. Kulok et al. (2018) reported that movement restriction of pregnant sows in gestation crates stimulated several stress responses indicating compromised welfare and impaired immune response. They also found that plasma cortisol in sows kept in movement restriction conditions was significantly higher than in sows kept in group pens. At the same time, lymphocyte proliferation indexes (in response to ConA, PHA and PWM) and neutrophils chemotaxis were significantly lower in the sows kept in individual gestation crates as compared to those kept in group pens.

In addition to physical consequences of crating during farrowing and lactation, also stress responses, such as increased heart rate (Damm et al. 2003) and increased plasma concentration of cortisol (the stress hormone) were observed in crated sows as compared to loose housed sows before (Jarvis et al. 2002, 2006) and after farrowing (Oliviero et al. 2008).

There is evidence that the gestation period is especially important, as stressors affecting

pregnant sows also affect the unborn piglets, which is referred to as prenatal stress (Hausmann et al. 2000, Tuscherer et al. 2002), which can have detrimental effects on growth (Fowden et al. 2006) and immune functions (Merlot et al., 2008) in the offspring after birth. The stressor acts on the fetus through the mother's body and may compromise the ability of the newborn to cope with pressure situations in life. This may explain inadequate reactions of both young and mature offspring in stressful situations (Krankendonk et al. 2006).

Although the present regulations do not permit keeping pregnant sows (after 28 days of pregnancy) in individual crates, this is still allowed by law (EU Directive 2001/88/CE) for the sows in the last week of pregnancy and during the weaning period, which may consequently result in pre- and neonatal stress in piglets.

The purpose of the present study was to find out whether the stress of sows caused by restricted mobilization in crates during pregnancy and piglets rearing under such conditions caused prenatal stress in the offspring measured by cortisol levels in their blood and saliva as well as acute phase protein levels and some immune-based indexes as compared to the piglets of sows kept in group crates during pregnancy and in free movement groups during and after farrowing.

Materials and Methods

The experiment was conducted on two farms using both individual and group housing systems. The experiment was divided into two groups:

Free movement group (FM): 10 pregnant sows (3 gilts, 4 sows in second pregnancy and 3 in third pregnancy) were kept together in one group pen at an area of 2.25 m^2 per animal from day 1 to day 100 of pregnancy. The sows in this group had the possibility to move freely. On day 100 the sows were moved to loose-housed farrowing pens.

Movement restriction group (MR): 10 pregnant sows (2 gilts, 4 sows in second pregnancy, 3 sows in third pregnancy and 1 sow in fourth pregnancy) in this group were kept in conventional individual crates at an area of 1.3 m^2 from day 1 to day 100 of pregnancy. Movement of these animals was limited to the possibility of getting up and lying down. On day 100 the sows were moved to farrowing crates.

The piglets in the two groups (FM and MR) were weaned on day 28 after farrowing.

The sows in the two experimental groups were randomly selected from the sow herd of the farm. All ani-

mals came from PIC production line (gilts of Camborough 22 line were inseminated with semen of PIC 410 line boars).

In order to eliminate as many additional differentiating factors as possible for the time of the experiment the pigs in both groups were fed the same commercial feed (Sauen-gold Trag, Sano International).

The levels of metabolic energy, total protein, exogenous amino acids, minerals and vitamins in full--portion feed for pregnant and feeding sows were adjusted to the levels recommended by Polish standards for swine feeding (2014). A daily portion for sows until 90th day of pregnancy was 2.9 kg/head and 3.0 kg/head was a daily portion for sows from 91st day of pregnancy. The portions were consumed completely. As a result, a daily intake of standardized lysine (digestible to the end of small intestine) by the sows in early pregnancy was 11.2 g and 11.5 g in late pregnancy. Lactating sows were fed *semi ad-libitum* and the feed mixture contained 13.50 MJ of metabolic energy and 0.81% of standardized lysine, digestible to the end of the small intestine.

The sows kept in pens were fed by hand and those in group pens were fed with automatic feeding stations. The animals on both farms had constant access to water provided by nipple drinkers installed in the pens. The farm buildings were provided with mechanical negative pressure ventilation systems regulated with an automatic thermostat system adjusted to maintain the same temperature ranges from 19°C to 21°C during the entire experiment. Both buildings were equipped with an artificial lighting system programmed to provide light from 6 a.m. till 6 p.m. Environmental parameters such as air temperature and relative humidity, ammonia and dust concentrations as well as noise level and light intensity were monitored continuously.

Blood samples were collected from 4 randomly selected and labeled piglets in a litter (only females - boars were excluded because of castration. Body weight of the piglets on the day of birth ranged from 1.3 to 1.6 kg. In total, the blood samples were collected from *vena jugularis externa* of 40 piglets (3, 7 and 21 days of age) in each group to heparinised tubes (Vacuette®, Greiner Bio-One). The samples were always collected between 5 p.m. and 7 p.m. Saliva samples were collected 30 minutes before blood collection. Environmental parameters such as air temperature and relative humidity, ammonia and dust concentrations as well as noise level and light intensity were monitored continuously.

Laboratory tests:

a) Cortisol level was determined by commercial ELISA kit, Diagnostic Systems Laboratories, Inc. USA.

b) Acute phase protein (APPs), haptoglobin (Hp), serum amyloid A and C-reactive protein (CRP) level was determined by commercial ELISA kit, Tridelta Company Ltd. Ireland. For determination of both cortisol and acute phase proteins Bio Tek Power Wave analyser was used.

c) Cell proliferation assay was taken according to the method described by Tuchscherer et al. (1998). Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by centrifugation onto Histopaque 1.077 (Sigma, USA). The cells were counted in Bürker chamber and their viability was determined with Trypan Blue (Stem Cell Technologies, UK) staining. PBMCs proliferation was determined using cells that were cultured in plastic vials at a density of 1 x 10^6 viable cells/ml medium (RPMI 1640 containing 10% fetal bovine serum, 2mM L-glutamine and 1% of antibiotic-antimycotic solution) at 37°C and in a humidified 5% carbon dioxide atmosphere in the presence of ConA, PHA and PWM and without stimulation (control). The cultures were pulsed with 0.5 µCi [3H]-thymidine (MP Biomedicals, USA). In the next step the cells were harvested on the glass microfiber filters (GF/C Whatman®, Whatman International Ltd, England). Filters were transferred into counting vials containing 10 ml of scintillation liquid (ICN, USA). The incorporated radioactivity was measured in a Liquid scintillation counter (Tri-Carb 2500TR, Packard, USA). Proliferation was expressed as the lymphocytes proliferation index (LPI). The LPI was calculated as the number of counts per minute (cpm) of mitogen stimulated PBMC divide by the number of cpm of the non-stimulated cells (in each cases taking mean of triplicate vials).

d) Neutrophil chemotaxis assay was performed according to the method described by Smith et al. (1985).

For chemotaxis assay the erythrocytes were allowed to sediment in an equal volume of 3% dextran (Life Sciences Group, USA) in PBS. The supernatant was collected, washed and resuspended in 5ml of PBS. This suspension was layered over 3 ml of Ficoll and Histopaque 1.077 (Sigma, USA) and centrifuged at 1100 g for 15 minutes. The supernatant was decanted and the pellet washed with RPMI medium (Gibco, USA). The pellet was resuspended (total white blood cell and differential counts performed -over 80% neutrophils), and the sample diluted so as to contain 5x10^5 neutrophils/µl. For the measurement of chemotaxis the centre well of each three-well series received a 10 µl volume of the cell suspension containing 5x10^6 neutro-

Table 1. Cortisol level in piglets' plasma [ng/ml] and saliva [ng/ml][n=40].

Groups		Days of sample colletcion					
		3		7		21	
		Plasma	Saliva	Plasma	Saliva	Plasma	Saliva
FM	x	130[a]	10.4[a]	27.6[A]	2.15	32.3	2.20
	SD	20.1	2.17	3.85	0.34	6.28	0.34
MR	x	135[b]	11.3[b]	35. 2[B]	2.29	33.9	2.06
	SD	13.9	1.76	4.59	0.29	8.84	0.26
p- value		0.045	0.038	0.006	0,072	0.281	0,103

a, b – values within the same column with different superscript letters differ significantly at $p<0.05$.
A, B – values within the same column with different superscript letters differ significantly at $p\leq0.01$.

Table 2. Level of acute phase proteins (APPs);Haptoglobin(Hp) [mg/ml], C-reactive protein(CRP) [µg/ml], serum amyloid A (SAA) [mg/ml] in piglets' plasma [n=40] .

Groups		Days of sample collection								
		3			7			21		
		Hp	CRP	SAA	Hp	CRP	SAA	Hp	CRP	SAA
FM	x		0.51[A]	14.6[A]	149	0.57[a]	14.1	48.5	0.68	13.5	53.8
	SD		0.07	1.68	21.4	0.093	1.97	6.68	0.132	1.75	10.0
MR	x		0.56[B]	15.8[B]	147	0.61[b]	13.8	48.9	0.70	13.0	55.9
	SD		0.09	1.78	28.7	0.09	1.15	7.01	0.13	2.02	9.80
p-value			0.008	0.003	0,218	0.043	0,721	0,097	0,185	1,103	0,162

a, b – values within the same column with different superscript letters differ significantly at $p<0.05$.
A, B – values within the same column with different superscript letters differ significantly at $p\leq0.01$.

phils. The outer well received 10 µl of the chemoattractant (zymosan activated serum, ZAS), while the inner well received 10 µl of nonchemotactic M199 medium. The plates were incubated at 37°C in a humified atmosphere containing 5% CO2 in air. After incubation cells were fixed by flooding the plates with absolute methanol for 30 minutes. The methanol was then poured off and the plates filled with 47% buffered formalin for 30 minutes. The hardened agarose gel was removed. The neutrophils remaining on the plate were stained using 20% Giemsa (Giemsa Stain, Sigma, USA). Neutrophil migration patterns were measured by microscopy (measuring the linear distance the neutrophils had moved from the margin of the well towards the chemotactic factor and the linear distance (in mm) the cell shad moved towards the control).

The study protocol was approved by the II Local Ethics Commission in Wrocław, Poland (No. 4/05).

Statistics

The results of the study were analyzed statistically using a two-factor analysis of variance, where one factor was a housing system and the other one was timing of sample collection. Significant differences between the groups were analyzed using Duncan's multiple range test and STATGRAPHICS 5.0. software. The results were presented as arithmetic means and standard deviations.

All animal experimental procedures in this study were performed with the approval of the 2nd Local Ethics Committee on Animal Experimentation in Wroclaw (permission No. 4/05).

Results

Plasma cortisol levels were higher at all three sampling times in MR as compared to FM, but a significant difference at $p\leq0.05$ was found in the piglets 3 days after birth while a significant difference at $p\leq0.01$ was observed only on day 7 after birth (Table 1). On average, the cortisol level of saliva was significantly higher ($p\leq0.05$) only on day 3 after birth in MR group (Table 1). Cortisol concentrations in both saliva and blood were the highest on day 3 and they decreased with piglets' age. This was likely due to gradual adaptation of piglets to new environment (Katesch et al. 1990,

Table 3. Lymphocyte proliferation index (LPI) in response to ConA [n=40], PHA [n=8], PWM [n=8] in piglets and neutrophil chemotaxis (NCh) [n=8].

Group		Days of sample collection											
		3				7				21			
		ConA	PHA	PWM	NCh	ConA	PHA	PWM	NCh	ConA	PHA	PWM	NCh
FM	x	1.21^{A}	1.6^{A}	1.45^{A}	1.34	2.19^{a}	2.97	3.13^{a}	1.23	3.28^{a}	3,24	3.75	1.28
	SD	0.08	0.13	0.04	0.15	0.12	0.2	0.33	0.08	0.23	0.24	0.46	0.08
MR	x	1.09^{B}	1.41^{B}	1.28^{B}	1.23	2.02^{b}	2.72	2.72^{b}	1.21	3.07^{b}	3,07	3.42	1.29
	SD	0.04	0.06	0.08	0.04	0.15	0.22	0.38	0.06	0.10	0,23	0.25	0.06
p- value		0.006	0.007	0,009	0,081	0,035	0,092	0,030	0.117	0,026	0,059	0,091	0,911

a, b – values within the same column with different superscript letters differ significantly at $p<0.05$
A, B – values within the same column with different superscript letters differ significantly at $p\leq0.01$

Otten et al. 2001, Moya et al. 2007). Hp levels were significantly higher on days 3 and 7 in MR group. No significant difference was observed on day 21 (Table 2). Haptoglobin is one of the acute-phase reactants that increase in blood serum after stress reaction. Increased levels of Hp can indicate that stress reaction occurred in MR group. Also the level of CRP in MR group was highly significant ($p\leq0.01$) on day 3 after birth (Table 2). Piglets' plasma amyloid-a (SAA) values decreased with age of the piglets, but no significant differences were found between the groups (Table 2). The lymphocytes proliferation index (LPI) was generally higher in FM than in MR group. Significant differences were observed in the LPI in response to ConA with all three sampling times (Table 3). LPI in response to PHA was significantly higher in FM group only on day 3 (Table 3), while LPI in response to PWM was significantly higher in FM group on days 3 and 7 (Table 3). Insignificant differences in neutrophil c hemotaxis (Nch) were observed between the two groups (Table 3). Increased plasma and saliva cortisol as well as Hp and CRP protein in MR group indicate that animals in this group were exposed to higher level of stress than those in FM group. Additionally, a decreased LPI index in response to ConA, PHA and PWM suggests that the animals in MR group will have a weaker immunity barrier as compared to the piglets in FM group.

Discussion

The occurrence of prenatal stress in piglets is undesired because of economic as well as ethical reasons (animal welfare). Stress, especially immediately after birth, can induce numerous reactions of the immune system. It reduces postvaccinal response (Cirulli 2001), exacerbates the course of infections (de Groot et al. 2001, Otten et al. 2001), impairs wound healing (Haussmann et al. 2000), changes the course of autoimmune diseases, inhibits the migration of neutrophils, NK, T and B cells, reduces the production of pro-inflammatory cytokines and chemokines and also the production of cytokines required to operate a specific immune system as well as the function of effector macrophages and NK cells (Tuchscherer 2000, Padgett and Glaser 2003, Sutherland et al. 2006, Couret et al. 2009).

Cortisol level is a well-known indicator of the occurrence of stress in animals. In the present study, we measured the level of cortisol in saliva and blood. The measurements showed significantly elevated levels of cortisol in the group of piglets born from sows kept in crates which do not allow much movement (Table 1). This may suggest that movement restriction of sows during gestation results in the occurrence of stress indicators in newborn piglets.

Acute-phase reaction is a non-specific reaction to the disruption of homeostasis induced by infections, trauma, inflammation or cancer (Murata et al. 2004, Petersen et al. 2004, Gutierrez et al. 2009, Pomorska-Mól et al. 2012, 2014, 2015). It begins with activation of leucocytes (mainly macrophages and monocytes) and as a consequence leads to the secretion of cytokines IL-1, IL-6, TNF-α (Kołacz and Bodak 2000, Kostro and Gliński 2000). Cytokines as mediators of receptors of hepatocytes start a set of reactions which lead to secretion of proteins known as acute phase proteins. Acute phase proteins can be divided into two groups (Moya et al. 2007, Pomorska-Mól 2010, Pomorska-Mól et al. 2012): a positive group (haptoglobin, serum amyloid A, C-reactive protein) and a negative group (albumin, transferrin, α-lipoprotein). The data in literature reported to the date have shown that the level of acute phase proteins is not affected by sex, breed and feed (Pomorska-Mól et al. 2012). The age of animals may only affect the acute phase proteins not longer than one month after birth. Many authors reported that the concentration of acute phase proteins changes as a result of stress (Petersen et al 2005, Pineiro et al. 2007, Sorrells et al. 2007). The results obtained in the present study show that the levels of haptoglobin and C-reactive protein (Table 2) were higher in the movement

restriction group (RM) than in the free movement (FM) group.

Prenatal stress may affect various components of the immune system (Merlot et al. 2007). Hausmann et al. (2000) reported that stress of sows during farrowing impaired wound healing in piglets, while Otten et al. (2001) observed that prenatal stress increased mortality and disease incidence rates. In contrast, Kanitz et al. (2003) investigated the effect of movement restriction of pregnant sows and found that it had no consequences in the physiology of piglets after birth. In our study we observed that in general, a higher lymphocyte proliferation index was observed in the FM group. The level of lymphocyte proliferation in response to ConA was lower throughout the whole experiment, lymphocyte proliferation in response to PHA was significantly lower for the first three days after birth, while in response to PWM it was lower for the first 7 days after birth (Table 3) in piglets from sows kept in pens with limited mobility during pregnancy and postpartum. This indicates a reduced proliferative capacity of lymphocytes in piglets from mothers kept in gestation crates. No differences between the two groups were found in lymphocytes and neutrophils response in the third week after the piglets' birth. This observation is in concurrence with the findings of Tuscherer et al. (2002) who studied the impact of chronic stress on proliferation of lymphocytes during one week after birth. Similar effects were also observed in mice and monkeys. However, Braastad (1998) and Krankendonk (2006) suggest that prenatal stress may impair the reactions of both young and mature offspring in stressful situations. They also suggest that this may have an impact on animals' productivity.

Prenatal restraint stress can also cause economic losses arising from increased morbidity and mortality rates during the suckling period (Tuchscherer et al. 2002, Otten et al. 2007) and reduced capacity for wound healing in the offspring (Haussmann et al. 2000). Stress in livestock is definitely an unwanted factor in breeding practice. Its negative impact on the animals has been reported widely, including their performance (Colson et al. 2006, Rutherford et al. 2006), quality (Perez et al. 2002, Plastow et al. 2005) and health (Ekkel et al. 1995, Kanitz et al. 2004). All these factors also had adverse effects on the economic results of farms.

Conclusions

The results of our study show that piglets delivered by sows kept in gestation crates for 100 days exhibited elevated levels of cortisol in plasma and saliva as compared to the piglets from mothers kept in free movement pens. Significant differences in APPs between the two groups show that the elevated levels in the piglets in RM group were due to prenatal stress. The piglets delivered by sows kept in gestation crates for 100 days also exhibited a decreased lymphocyte proliferation index in response to ConA, PHA and PWM. This suggests that the piglets from mothers kept in restriction movement pens will have a weaker immunity barrier compared to the piglets given birth by mothers kept in free movement pens.

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