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Polyherbal formulation mitigates pre-natal stress in sows and boosts immunity in neonatal piglets

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Abstract
Prenatal stress in sows adversely affects the growth and development of newborn piglets. Hence, the current study aimed to assess the effect of a polyherbal formulation (PHF), on prenatal stress in sows and the immune status of the neonatal piglets. PHF contains three Indian traditional Ayurvedic medicinal herbs, *Withania somnifera*, *Ocimum sanctum*, and *Emblica officinalis*, which is known to have adaptogenic properties. Advanced pregnant sows were grouped on 90th day of gestation into control and PHF groups. The PHF group received PHF as a feed supplement at 30g/day/sow during the perinatal period from the 90th day of gestation up to the 7th day post-partum. The cortisol levels were measured in sows on different days during the last week of pregnancy and a week after farrowing. The sows' colostrum and immune status of the neonates were assessed by examining the IgG levels on the day of parturition and on post-partum day 7 respectively. There was a non-significant reduction in the peak of cortisol associated to parturition stress in the PHF treated group. Besides, the use of PHF reduced sow stress improving the IgG status of colostrum and consequently in the serum of neonates. In conclusion, PHF supplementation to advanced pregnant sows can considerably reduce the prenatal stress and augment the immune health in neonatal pigs as well.

Keywords: PHF, cortisol, gestational stress, IgG, immunity

1. Introduction
The modern-day intensive pig husbandry practices have given rise to various stressors acting on sows throughout gestation [1]. Prenatal or gestational stress has a negative impact on the production of sows and harms the performance of the offspring post-natally [2]. The piglets born to such sows suffer from altered physiological and behavioral responses. Maternal stress, especially during the last trimester of gestation, can compromise fetal development and weaken the immune status of the offspring [3]. This affects the growth of piglets during early life and renders them susceptible to neonatal infectious diseases. Also, maternal stress during parturition increases cortisol levels which affect to maternal behaviour and the release of oxytocin and prolactin. Moreover, neonatal diseases can have marked economic consequences in the long run [4]. Therefore, much attention has been given by researchers to develop strategies to combat prenatal stress in pigs.
Recently, plant adaptogens have gained importance in alleviating the stress response and restoring homeostasis in humans and animals. Evidence shows that the adaptogens mainly act on the hypothalamic-pituitary-adrenal axis to exhibit stress-protecting activity [5]. Plant adaptogens have the potential to increase the production performance in sows and piglets; Ivanovsky *et al*. studied effect of plant adaptogen formulation composed of extracts from *Rhaponticum carthamoides*, *Serratula coronate*, and *Filipendula ulmaria* plants on piglets and sows. Their findings depicted that extract supplementation in piglets aged from 40 to 90 days exerted influence on the adaptive-compensatory reactions in the body of piglets and sows, that was proved by an increase in weight gain, livability percentage, and rise in the milk yield of the sows [6]. Moreover, some of the Indian traditional plants like *Withania somnifera*, *Ocimum sanctum*, and *Emblica officinalis* are known to possess adaptogenic properties, as demonstrated in various studies [7]. Besides, they also have potent immunomodulatory actions in the body [8].

Our product containing these three plant parts, had shown potential benefits of ameliorating ammonia stress in zebrafish [9]. In addition, previous studies with this product have demonstrated to protect chickens against heat stress by normalizing the cortisol levels [10].
Therefore, we hypothesized that this polyherbal formulation (PHF) could have a similar protective effect against peripartum stress in sows. Owing to resurgence to search for new strategies to minimize the prenatal stress since prenatal stress in sows adversely affects the growth and development of newborn piglets. the present study was designed to evaluate the possible protective effect of the PHF against prenatal stress in advanced pregnant sows and examine the immune status in the neonatal piglets.

2. Materials and Methods

2.1 Experimental animals

This project was performed in a commercial maternity farm of 3000 danbreed sows. The study population for the present investigation consisted of advanced pregnant sows and the piglets born to these sows. All the procedures, followed in the current study complied with the standard ethical animal production guidelines of the Spanish government.

2.2 Experimental design

The pregnant sows of similar traits on 90th day of gestation were randomly divided into two groups containing ten animals each. The sows in the control and PHF group were given standard feed. In addition, the PHF group sows received a daily adaptogenic complementary feed supplementation.

2.3 Experimental feed

The study was conducted using Phytocee®, a polyherbal formulation in the form of dried powder. This product is registered in Spain as a supplementary feed. This formulation comprised stems of *Withania somnifera*, fruits of *Emblica officinalis*, and the whole plant of *Ocimum sanctum*. It contains active principles like withanolides, polyphenols, and triterpenes that have proven adaptogenic potential. PHF as a supplementary feed was added to the commercial feed at the dose of 30 g per day per sow on top of the feed from the 90th day of gestation up to the 7th day post-partum. The 30 g PHF were transferred into a suitable trough containing the commercial feed (quantity sufficient for ten sows per day) and the preparation was mixed for 2-3 minutes and then thoroughly mixed to homogenization using an electric feed mixing unit for 30 minutes.

2.4 Collection of colostrum

Sows enrolled into the study had a natural milk production with natural variation between sows. As a natural response, most sows allowed the piglets to suckle quickly after reunion. A trained & designated study personnel quietly entered the pen after the sow started her nursing grunt. Then, without disrupting the suckling bout, colostrum was collected in 50 mL sterile scree cap plastic beakers from as many teats as possible at the moment of milk ejection. The colostrum samples were stored at –40 °C until further analysis.

2.5 Collection of blood and serum separation

The whole blood sample (approximately 1.0 mL) was evacuated from the vena jugular vein of sows and collected in tubes containing EDTA as an anticoagulant. The blood samples were subsequently stored at room temperature until they were centrifuged at 3,000 × g for 10 min at 20°C the next working day. Plasma was then stored at –40 °C until further analysis.

2.6 Estimation of cortisol level in sows

The saliva of pregnant sows of both the control and PHF supplement group were collected by using swabs on 7 days and 2 days before farrowing, on the day of farrowing, and post-partum day 7. The cortisol determinations were estimated using an enzyme immunoassay developed and validated at the Endocrinology Laboratory of the Department of Animal Physiology, Faculty of Veterinary Medicine, Complutense University of Madrid. Briefly, microtiter plates (96-well flat-bottom polystyrene) were coated overnight at 4° C with the appropriate antibody dilutions and the plates were washed for 3 times. Then, the conjugate working solutions (CWS) were prepared by diluting conjugate stocks in assay buffer. The standards and saliva samples were diluted in CWS and analysed in duplicate. To achieve a competitive reaction, plates were incubated at room temperature for 2 h. Then the plates were washed 3 times with wash buffer, and Enhance K-Blue TMB substrate (Neogen, Lexington, KY) was added to each well. To stop colorimetric reaction, stop solution was added to each well. Absorbance was read at 450 nm in an automatic microplate reader. Hormone concentrations were calculated by means of software developed for this technique (ELISA AID, Eurogenetics, Belgium). Cortisol concentrations were expressed in ng/ml of saliva.

2.5 Estimation of immunoglobulins (IgG) in colostrum and serum

The colostrum from parturient sows (n=10 per group) and serum from the newborn piglets (n=20 per group) of the control and supplemented groups were analysed on the day of farrowing for the levels of IgG using ELISA. Blood samples were centrifuged at 1,200g, 4ºC for 20 min in order to obtain serum, meanwhile colostrum samples were centrifuged at 4,000g, 4ºC for 60 min. Pig IgG determination was performed using a commercial enzyme immunoassay kit (Pig IgG ELISA kit; Bethyl Laboratories), following the manufacturer instructions. For IgG determinations, samples were diluted as manufacturer recommended: Serum 1: 500000; colostrum: 1:1000000. Briefly, diluted samples and standards were added to a 96 well plate and incubated for 1 hour. Then, plates were washed, and the detection antibody was added and incubated for 1 hour. After incubation time, plated were washed and HRP solution was added and incubated for 30 min. Finally, plates were washed and TMB substrate solution was added to each well. To the reaction, stop solution was added and the plates were read at 450nm in an automatic microplate reader and the resultant absorbances were used to obtain IgG concentrations. These concentrations were determined by constructing a standard dose-response curve. IgG concentrations were expressed in ng/ml for serum and colostrum samples.

2.6 Statistical analysis

The data were compiled and analyzed by independent T – Test using SPSS software (IBM SPSS Statistics Version.21.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Effect of PHF on the cortisol production in sows during gestation and lactation period

Feeding PHF to the advanced pregnant sows resulted in a significant decrease in salivary cortisol levels on days 7 and 2

before farrowing and on the day of farrowing. On post-partum day 7, the cortisol levels in the control and supplement groups did not differ significantly. Due to inter individual variation, salivary cortisol was varied significantly between the groups on all stages. However, the spike of cortisol (fold change) in PHF group is lesser than control group on other days when compared to seven days before farrowing. The obtained data are presented in Table.1.

Table 1: Effect of PHF on cortisol (nmol/l) levels of sows.

Parameters	Control	PHF	P-value
Seven days before farrowing	4.69	1.80	0.002
Two days before farrowing	9.73 (2.1-fold)	2.81 (1.6-fold)	0.002
On the day of farrowing	13.71 (2.9-fold)	4.70 (2.6-fold)	0.073
Seven days after farrowing	8.35 (1.8-fold)	2.02 (1.1-fold)	0.201

3.2 Effect of PHF on immunoglobulins IgG levels in the piglet serum and colostrum

The immunoglobulin IgG levels in the serum of piglets born to sows fed with PHF showed a significant increase compared to the piglets from the control group. Also, the IgG levels in the colostrum of sows increased non-significantly in the PHF supplemented group on parturition to the control group. Table 2 shows the values of IgG on day 7 of farrowing in piglet serum and colostrum.

Table 2: Effect of PHF on IgG (g/L) levels in piglet serum and colostrum

Parameters	Control	PHF	P-value
Piglets' serum	14.33 ± 1.31	21.83 ± 1.24	0.000
Colostrum	217.12 ± 20.53	1456.18 ± 864.81	0.160

4. Discussion

The pregnant sows often experience various kinds of unavoidable stress, even in well-equipped farms. The pre-natal or gestational stress must be managed efficiently to lessen the economic burden of swine farming. Because of this, the use of an adaptogenic plants can be a good alternative to reduce the impact of stress and increase the animal welfare. The salivary cortisol is a valuable marker for stress response during pregnancy [11]. In the present study, we found that the cortisol levels in control pregnant sows were markedly increased in the last week of pregnancy. However, supplementation of PHF from 90th day of gestation up to post-partum day 7 to sows reduced the spike of cortisol levels in saliva during the last week of gestation, indicating a stress-ameliorating property of the supplement. Studies with extracts of *Withania somnifera* have demonstrated glucocorticoid lowering efficiency in a rat model of chronic stress [12]. Similarly, the stress-relieving properties of *Ocimum sanctum* and *Emblica officinalis* in experimental animals are well established [13, 14]. Since the spike of cortisol was very low in PHF supplemented sows in the present study during the last week of gestation, we assume that the components of the PHF, had a synergistic interaction in stress-relieving and adaptogenic properties.

In addition, literature reports evidenced that administration of polyherbal formulation into the diet of piglets aged from 40 to 90 days exerted influence on the adaptive-compensatory reactions in the body of piglets and sows, that was proved by an increase in weight gain, livability of the young, and rise in the milk yield of the sows [6]. Furthermore, supplementation of polyherbal antistressor product in Zebuine cattle breeds significantly reduced the levels of serum cortisol [15]. Moreover, our previous studies demonstrated the antistress and adaptogenic potential of PHF in rats and broilers as well [10, 16]. The increased variation observed within groups after farrowing suggests that unavoidable pain and stress during parturition may affect differently to each animal. However, the statistical tendency observed at the day of parturition suggests that the effect can also be beneficial this critical day.

Prenatal stress affects the immunity and neonatal health in farm animal species; cortisol and other hormones are identified as a modulator for the quantity and quality of colostrum production [17]. Variation on colostrum production by the sow has been studied before with different approaches, where low colostrum producer sows were found. No relationship was found between cortisol and IgG production. However, cortisol was measured from blood samples where the extraction method can affect to its basal levels [18]. In our study, per contrary, hyper colostrum producing sows were detected. Even when these animals' datasets are removed from the supplement group, the IgG in the colostrum showed the positive tendency compared to the supplement group. Further work must be done to investigate the role of cortisol and the IgG colostrum richness in the peripartum period of the sow. A more recent study reported by Merlot *et al.* investigated the relationship between different housing systems, maternal physiology and neonatal survival, and the results revealed that higher neonatal mortality was observed in groups with higher cortisol levels during gestation. Unfortunately, differences on IgG were not detected [19]. None of the studies analyze the IgG levels in the serum of the piglets. Our study suggested that the modulation of cortisol during parturition increase the IgG colostrum richness, and this is transferred to the piglet. Further, other factors such as maternal behaviour and immunomodulatory effects of the polyherbal mixture must be studied.

Summarily, due to the fact of prenatal stress in sows adversely affects the growth and development of newborn piglets and owing to resurgence to search for new strategies to minimize the prenatal stress. This study supplies as scientific evidence based for the adaptogenic and immunity enhancing effects of PHF.

5. Conclusion

PHF supplementation to sows during the perinatal period markedly decreases gestational stress by lowering maternal cortisol levels. It boosts immunity in piglets by elevating the circulating immunoglobulins. We conclude that PHF offers an overall positive effect on pregnant sows and improves piglet immunocompetency during neonatal life.

6. Author Contributions

Conceptualization, GM, JCI; methodology, GM, SC, JCI, RKR; investigation, all authors.; resources, all authors.; writing—original draft preparation, all authors.; writing—review and editing, all authors.

7. Funding

This research received no external funding.

8. Institutional Review Board Statement

Not applicable as no invasive experimental procedure was done in the study according to the definition of the Directive 2010/63/EU.

9. Data Availability Statement

Data are available on request.

10. Acknowledgments
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11. Conflicts of Interest
The authors declare no conflict of interest.

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