Sampling methods for determination of cortisol in pig saliva and their use in the assessment of pig welfare

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Abstract

This study focused on finding new information regarding the assessment of pig saliva cortisol samples in terms of practical effects of the sampling, sample storage conditions, and their laboratory analysis. The study was divided into two experiments. The first experiment was focused on finding the effect of sampling time on cortisol concentrations in pig saliva. The second experiment was focused on determining the effect of storage conditions on the value of salivary cortisol. Before the initiation of the study, we tested which one of the commercially available ELISA kits would be the most suitable for our experiments. Simultaneously, we carried out a pre-study to evaluate the effect of relocation and change in the housing type on the concentration of salivary cortisol in gestating sows. The samples were obtained by oral cavity swabbing, using a standard cotton swab. In the first study, piglets were examined at the age of 4 ± 1 days, and breeding management routine procedures were used as a stress factor. In the second study, the piglets were examined immediately after weaning (at 28 ± 2 days of age). The Cortisol EIA kit was found to be statistically more accurate and thus a more suitable ELISA kit for our experiment. Analysis of the relocation effect and the effect of change in the housing type showed that relocation does not seem to be a stress factor for gestating sows as no significant changes were observed in salivary cortisol concentration ($P > 0.5$); however, the change in the housing type lead to a significant increase in salivary cortisol ($P < 0.001$). In the first study, we determined using the ELISA method that the most significant difference occurred in 40 min ($P < 0.01$), which suggests that the best time for a sampling in order to assess salivary cortisol concentration is 40 min after stress induction by routine procedures. The conclusion of the second study was that in the monitored period of 60 h ($P < 0.05$), cortisol concentration decreased depending on the storage temperature. The decrease started between 48 and 60 h which showed that cortisol is stable in the saliva sample for at least 48 h. These findings will be further applied in our following studies focused on assessment of salivary cortisol concentration after stress induction.

ELISA, storage time, storage temperature, non-invasive

Recently, there has been an increasing interest in reduction of environmental stress for animals. In general, environmental stress in livestock is reduced by increased animal welfare. However, even in a livestock where the level of welfare is high, there is still some residual stress caused by the handling of animals and other necessary procedures. Unfortunately, some essential interventions involved in the breeding management that cause stress are difficult to replace, such as castration, vaccination, handling, tail docking, tooth resection, tattooing etc. In pigs, no study of this type has been published yet. Only one similar study has been carried out so far by Urbanova et al. (2019), focusing on stress in rabbits which showed that animals can get used to repeated manipulation; with each subsequent manipulation there was a lower hormonal stress response.

In addition to welfare protocols, indicators of welfare such as stress induction, can be also monitored by measuring the hypothalamic-pituitary-adrenal axis (HPA) activity. Stress-induced activity of HPA results in an increase of the concentration of stress hormones such as cortisol in blood, other body fluids, and faeces (Cook et al. 1997). As a part of the improvement of welfare, non-invasive sampling methods have been developed.

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Several methods using different types of sampling have been developed to determine concentrations of cortisol in urine, saliva, milk, faeces, or fur (Mormérde et al. 2007). We decided to evaluate the stress level by assessment of cortisol values in saliva samples. The main reason for this choice is the significant correlation between free serum cortisol and salivary cortisol (Cook et al. 1996).

The first aim of the study was to determine the effect of sampling time on cortisol values in pig saliva. The second aim of the study was to determine the effect of sample storage conditions, specifically, the effect of different storage temperatures and timings on the change in cortisol concentrations in saliva samples.

Materials and Methods

The study was divided into two parts. The first study focused on determining the effect of sampling time on cortisol values in pig saliva; more precisely, on finding the most suitable time for sampling after stress induction. In order to assess the cortisol concentration in saliva most accurately, not only the time of sampling is important but also the subsequent storage procedures and sample manipulation. Therefore, the second experiment was focused on determining the effect of storage conditions on the value of salivary cortisol.

Sampling method

In our study, we used crossbreed piglets (Landrace x Czech Improved White Pig). Samples were taken by cotton swabs from the oral cavity. A standard cotton swab was rubbed against the inside of the cheek and under the tongue for a period of 30 s to collect biological material. The use of sponges as described by Strzelec et al. (2011) proved to be unsuitable for sampling in pigs. After sampling, the samples were centrifuged at 800 *g* (Spectrafuge 24D, Labnet International, Inc., Edison, USA) and frozen by dry ice and transported to the freezer (−80 °C).

ELISA kit selection

Before we started with the analysis itself, we focused on finding the most suitable method for determination of salivary cortisol in our laboratory. We chose two commercially available ELISA kits specific for cortisol determination and compared them. One kit (type 1) was specific for cortisol analysis in saliva of all animal species (Cortisol EIA kit) and the other one (type 2) was specific for cortisol analysis in all body fluids of pigs (Pig Cortisol (COR) ELISA kit). In these pre-study experiments, 15 adult pregnant sows were used to compare the results determined by ELISA with two different commercially available kits. A change in the type of housing which usually occurs in breeding before farrowing was used as a stressor. Individual samples were taken by standard cotton swabs from the oral cavity before relocation, after relocation, and 2 h after relocation. After sampling, each sample was divided into two halves and frozen. Subsequently, one half of the samples was processed using Cortisol EIA kit (Boster biological technology Pleasanton, California, USA), and the other half was analysed using Pig Cortisol (COR) ELISA kit (Abbkine, Inc., Wuhan, China). The results were determined after reading from the standard curve which was proposed in the programme free ELISA Software (Elisaanalysis.com). After laboratory analysis of the samples, statistical analysis of the data was performed in the UNISTAT for Excel 6.5 program by determining the Spearman rank-correlation coefficient and F-test. Friedman's two-factor analysis of variance was used for statistical analysis of data evaluating the effect of relocation and housing type change on salivary cortisol concentration in gestating sows.

It was found that there was no significant ($P > 0.05$) difference between data analysed with the type 1 kit (Cortisol EIA kit) and the results obtained by the type 2 kit (Pig Cortisol (COR) ELISA kit). However, it was found that each kit has a different variability of values, and the type 1 kit is statistically more accurate than the type 2 kit.

Sampling time definition

In the first experiment, we used 4 ± 1-day-old male piglets; the age deviation was due to difference in delivery dates between particular sows. Routine procedures, namely castration without anaesthesia, tattooing, and vaccination, were set as stress factors. All routine procedures were performed together during one approximately 15-min long handling at the age of maximum 7 days to be in line with the local legislation and breeding management. All the piglets underwent the above-described procedures at approximately the same age. In this study, the term "routine procedure" includes all the routinely performed procedures such as castration without anaesthesia, tattooing, and vaccination. All male piglets were separated from the sow to the next pen where all of them underwent vaccination against oedema disease caused by shiga toxin-producing *E. coli* (Ecoporc Shiga, Ceva Santé Animale, France) and administration of iron solution for injection (Gleptosil, Ceva Animal Health Slovakia, s.r.o.), both applied intramuscularly. Subsequently, piglets underwent a tattooing of the left ear with ID number of the farm in line with local legislation, followed by castration without anaesthesia during which testicles were removed. After these routine procedures, the piglets were returned

to the pen with the sow. Samples were taken as a group sample; one group sample was taken from all male siblings in one pen. The number of piglets varied from 5 to 7 depending on the frequency of male piglets in one litter. A total of 488 piglets from 80 sows were used in this study. This sampling method was chosen due to the limited maximum volume of produced saliva by one neonate pig, as a volume of 100 µl saliva was necessary for further laboratory testing. All samples taken in this experiment were paired samples, which means they were taken before and after a routine procedure was performed. Samples were taken 10, 20, 30, 40, 50, 60, 90 and 120 min after the performance of routine procedure, i.e. after putting the piglet back to the pen. The experiment was repeated × 10. The laboratory analysis was made with a commercially available ELISA kit (Cortisol EIA kit) for cortisol assessment in saliva of all species. After laboratory analysis, statistical analysis in the UNISTAT for Excel 6.5 with Wilcoxon test was applied.

Storage conditions

The second experiment was focused on the monitoring of changes in cortisol concentrations depending on storage conditions. Samples were taken from piglets aged 28 ± 2 days, just after weaning. In this experiment, 338 piglets in total were used. A mixed sample was formed from the individual samples which was divided into sub-samples after homogenization. Half of them were stored in a thermoblock (Benchmark Scientific BSH 200 My block mini digital dry bath, Balkowitsch Enterprises, Inc., Bismarck, North Dakota, USA) maintaining a temperature of 25 °C, and the other half of the samples were stored in a refrigerator (Cool box C&W 45 l, PENTA CZ s.r.o., Katovice, CZ) at 5 ± 1 °C. Due to an unstable temperature on the pig farm and during transportation, the thermoblock enabling a constant temperature of 25 °C during the whole period of sampling and sample transport to laboratory was used to simulate room temperature. The samples were stored for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min and then later during the study the intervals were extended to 3, 6, 9, 12, 24, 36, 48 and 60 h. All samples were kept frozen after that. This experiment was performed in eight replicates. After laboratory analysis of the samples, statistical analysis of the data was performed in UNISTAT for Excel 6.5 using Wilcoxon test.

Results

In the first experiment, we decided to use the saliva samples for assessment of stress induced by a routine procedure. The mean cortisol values (before and after routine procedures) are presented in the Table 1. The most significant difference between the samples taken at the basal level (before stress induction) and after stress induction was in 40 min.

Table 1. Salivary cortisol concentration in pig saliva.

Sampling time		Cortisol (pg/ml)	*P* value	
			t-test	Wilcoxon test
10 min	Before	9896.30 ± 10301.30	0.0220	0.0020
	After	15166.70 ± 15118.40		
20 min	Before	10528.40 ± 10487.30	0.0256	0.0020
	After	17725.40 ± 16882.10		
30 min	Before	7388.79 ± 7024.30	0.0180	0.0020
	After	18199.20 ± 17311.70		
40 min	Before	10292.73 ± 10194.40	0.0087	0.0002
	After	39057.65 ± 36029.30		
50 min	Before	7047.91 ± 6818.20	0.0089	0.0020
	After	27602.00 ± 25599.30		
60 min	Before	12034.57 ± 12324.90	0.0089	0.0020
	After	36710.84 ± 33800.80		
90 min	Before	7925.25 ± 8672.50	0.0096	0.0020
	After	24359.76 ± 22820.40		
120 min	Before	9620.79 ± 8694.10	0.0249	0.0020
	After	12520.57 ± 11363.20		

All values were expressed as the mean ± standard deviation.

In the second experiment, no significant effect of storage temperature on cortisol concentrations in the sample was found over 60 min. Significant changes in cortisol concentrations due to different storage temperatures did not occur until after 60 h ($P < 0.05$) of sample storage starting with no significant decrease at 36 h ($P > 0.3$). The mean cortisol values at the different times are shown in Table 2.

Table 2. The influence of storage time and temperature.

Storage time	Cortisol (pg/ml)		*P* value
	Room temperature	Fridge	
0 h	1412.47 ± 339.28		< 0.05
3h	1396.86 ± 525.30	1561.96 ± 800.90	< 0.05
6 h	1345.68 ± 454.40	1574.49 ± 748.90	< 0.05
9 h	1391.27 ± 437.40	1462.85 ± 781.90	< 0.05
12 h	1426.51 ± 511.20	1559.43 ± 793.90	< 0.05
24 h	1460.30 ± 644.40	1405.50 ± 620.10	< 0.05
36 h	1443.27 ± 304.70	1601.66 ± 259.70	< 0.05
48 h	1211.58 ± 211.10	1429.25 ± 712.60	< 0.05
60 h	1080.33 ± 240.60	1573.79 ± 469.80	0.033*

All values were expressed as the mean value ± standard deviation. Significant difference ($P < 0.05$) between storage at room temperature and in a fridge for the same time is indicated by asterisk.

Furthermore, data from the pre-study were used to determine the level of stress caused by a change in the type of housing of gestating sows. The sows were moved from group housing to farrowing cages in individual pens. At the same time, it was found that the handling and relocation were not significantly stressful for the gestating sows ($P > 0.5$) as no significant difference was measured between cortisol concentrations before and after relocation. However, a significant difference was found between cortisol concentrations before and 2 h after moving into individual pens ($P < 0.001$). The mean cortisol values are listed in Table 3.

Table 3. The effect of relocation of gestating sows on salivary cortisol concentration.

Sampling	Cortisol (pg/ml)	*P* value	
		Before relocation	After relocation
Before relocation	34100.87 ± 2458.10	/	< 0.05
After relocation	38103.07 ± 4624.50	< 0.05	/
2 h after relocation	47226.80 ± 4654.30	0.0002*	< 0.05

All values were expressed as the mean ± standard deviation. Significant difference ($P < 0.05$) between samples before relocation and 2 h after relocation is indicated by asterisk.

Discussion

With regard to the sampling method, we can say that the samples used for evaluation were not clear saliva but a mixture of all oral fluids. There are many factors as a part of oral fluids besides saliva that impact the laboratory results (e.g., food residues, mouth cavity bacterial microbiota, blood, or the other contaminants) (Lewis 2006; Whembolua et al. 2006). In human studies, these factors can be reduced by starvation or rinsing the mouth with water but unfortunately, in animal studies such reduction is not possible because it

can be an additional stress factor that may affect the results (Whembolua et al. 2006). Magnano et al. (1989) found that in samples taken from breastfed individuals, the level of cortisol might be increased because of sample contamination by breast milk. Since the piglets used in our study were still lactating, we were taking the samples a few minutes after the lactation finished and not during the lactation as the milk might influence the salivary cortisol concentration and it would be impossible to distinguish the origin of cortisol (maternal milk or piglet's saliva). Another factor affecting the cortisol concentration is the circadian rhythm. In pig saliva, the basal cortisol concentration is higher in the morning and lower in the evening (Griffith and Minton 1991).

Based on the results of the first study, the most suitable time interval for sampling was 40 min for analysing saliva cortisol as an indicator of stress level induced by performing a routine procedure. In the published studies, we find different suggestions for the best timing of sampling. Cook et al. (1996) found that the salivary cortisol concentration was at its maximum 5 min after stress induction by fixation with a nose-snare. On the contrary, Coutellier et al. (2007) determined that the highest level of cortisol in saliva was 4–5 h after stress induction by the animal manipulation and regrouping. The important finding is that different salivary biomarkers appear to react differently following various types of stressors (Ott et al. 2014). The salivary cortisol concentration assessment is often used for stress assessment during animal handling, transport, or regrouping (Merlot et al. 2004; Coutellier et al. 2007). For the assessment of stress induced by castration, tooth resection or tail docking, it is more popular to use serum or plasma (Prunier et al. 2005; Sutherland et al. 2012; Backus et al. 2018). At the time when these studies were conducted, there had not been any other published studies focused on saliva cortisol assessment after castration.

The second study showed that saliva cortisol concentration in samples was stable and so it was not necessary to freeze individual samples directly during sampling. There is not much specific information about sample storage conditions. Lewis et al. (2006) reported that cortisol was significantly stable in a centrifuged saliva sample after 5 days at 4 °C and after three months at −20 °C. For longer storage, a temperature of −80 °C is recommended. However, most authors stated that the samples were cooled and transported as soon as possible after collection or immediately frozen.

The secondary result obtained from the pre-study experiments was determination of stress induction by the handling of sows. These sows were not immersed for the first time and therefore, they were probably used to handling to some extent, however, there was an indication of stress from the new environment and the new type of housing. Findings of this study can have an impact on the methodology of pig saliva sampling for saliva cortisol assessment.

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