Exploring the effects of second estrus synchronization and dietary *flushing* on the incidence of ovarian cysts in gilts by using exogenous gonadotropins

Explorando os efeitos da sincronização do segundo estro e flushing *alimentar sobre a incidência de cistos ovarianos em marrãs utilizando gonadotrofinas exógenas*

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Abstract

Estrus stimulation by exogenous gonadotropins (EG) in association with dietary *flushing* is an important tool for the improvement of gilt reproductive performance. However, there is evidence associating both *flushing* and EG with a disturbance in the endocrine balance that could lead to increased ovarian cysts. The aim of this study was to evaluate whether *flushing* or EG might affect the ovulation rate and the incidence of ovarian cysts. Seventy-one gilts were randomly distributed into 2x2 factorial design with four treatments: *flushing* and hormone (wFwH); no *flushing* and hormone (nFwH); *flushing* without hormone (wFnH); and neither *flushing* nor hormone (nFnH). Gilts were slaughtered for macroscopic and histopathological ovary examination approximately five days after artificial insemination. The characterization of these cysts was performed by optical microscopy in the following: follicular cysts (FC), luteinized cysts (LC) or cystic corpora lutea (CCL). The number of ovulations did not differ between treatments. There was no interaction between the factors in any analyzed variable. The frequency of gilts with CCL and LC was not affected by flushing and EG. No difference was found in the incidence of FC, with 12.5% and 5.88% in gilts from wFwH and nFwH treatments, respectively. There were no differences in the proportion of CCL between FC and LC (9.85 vs. 4.22 and 4.22%, respectively). In conclusion, the use of exogenous gonadotropins for second estrus synchronization in gilts, either alone or in association with dietary flushing, does not increase the incidence of ovarian cysts, nor does it decrease the ovulation rate.

Keywords: Cysts. eCG. Follicular atresia. LH. Swine.

Resumo

A estimulação do estro por gonadotrofinas exógenas (GE) associada ao *flushing* alimentar é uma ferramenta importante na melhoria do desempenho reprodutivo de marrãs. Contudo, há evidência da associação do *flushing* com GE levando ao desequilíbrio no sistema endócrino que poderia levar ao aumento de cistos ovarianos. O objetivo deste estudo foi avaliar se o *flushing* ou GE pode afetar a taxa de ovulação e a incidência de cistos ovarianos. Setenta e uma marrãs foram distribuídas aleatoriamente em arranjo fatorial 2x2 com quatro tratamentos: *flushing* e hormônio (cFcH); sem *flushing* e com hormônio (sFcH); com flushing e sem hormônio (cFsH) e sem *flushing* e hormônio (sFsH). Marrãs foram abatidas para exame macroscópico e histopatológico dos ovários, aproximadamente cinco dias após inseminação artificial. A caracterização desses cistos foi realizada por microscopia óptica: cistos foliculares (CF), cistos luteinizados (CL) ou corpos lúteos císticos (CCL). O número de ovulações não diferiu entre os tratamentos. Não houve interação entre os fatores em qualquer variável analisada. A frequência de leitoas com CCL e CL não foi afetada pelo *flushing* e GE. Não houve diferença na incidência de CF, com 12,5% e 5,88 % em leitoas dos tratamentos cFcH e sFcH, respectivamente. Não foram obtidas diferenças na proporção de CCL entre CF e CL (9,85 vs. 4,22 e 4,22%, respectivamente). Em conclusão, a utilização de gonadotrofinas exógenas para sincronização do segundo estro de marrãs, isoladamente ou em associação com o *flushing,* não aumenta a incidência de cistos ovarianos e não diminui a taxa de ovulação.

Palavras-chave: Cistos. eCG. Atresia folicular. LH. Suínos.

Introduction

One of the great advantages of an ideal stimulation and synchronization of estrus in gilts is that it allows for the anticipated culling of non-cycling gilts as market animals, reducing the non-productive days of the herd. Additionally, it enables the management of gilts in order to achieve a desired weight and body condition at breeding, and thereby lowers the number of females incorporated into breeding groups on a weekly basis (GAMA et al., 2005). It was observed that this early stimulation leads to an increased reproductive lifespan in sows from the herd (KOKETSU; TAKAHASHI; AKACHI, 1999; PINESE et al., 2005; PATTERSON; BELTRANENA; FOXCROFT, 2010). The use of exogenous gonadotropins (EG), together with mature boar stimulation, has been an effective tool for attaining better puberty induction and synchronization in comparison with the latter alone (KNOX et al., 2000). Despite the advantages of EG for estrus synchronization, gonadotropins have been associated with an increase in ovarian cyst incidence (KUCHARSKI; JANA; ZEZULA-SZPYRA, 2002; BREEN; RODRIGUEZ-ZAS; KNOX, 2006). For example, EGs are known to have relatively long half-lives (HAFF; THACKER; KIRKWOOD, 2002; JACKSON et al., 2006), and they are most likely associated with an imbalance in the hypothalamus-pituitary-gonadal axis, and can therefore be associated with ovarian cysts. The mechanism is not yet fully understood, but it is suggested that some follicles, which become cystic after exogenous hormone administration under physiological conditions, would undergo atresia (CHUN et al., 1994; LIMA et al., 2006).

Nevertheless, the influence of flushing on ovarian cyst incidence in swine is still unclear, but insulin and IGF-I, which are metabolites for which the plasmatic concentrations increase in response to dietary flushing, play a role in suppressing the onset of apoptosis in follicular cells (CHUN et al., 1994). The flushing procedure consists in giving increased levels of dietary energy to gilts two weeks before ovulation after a short period of food restriction. Flushing could increase ovulation rates (AHERNE; WILLIAMS, 1992; FERGUSON; ASHWORTH; EDWARDS, 2003) and oocyte quality (ZHOU et al., 2010), and therefore may be used as a management tool in swine breeding herds.

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Cox et al. (1987) have established a nutritional effect-mediating role for insulin on the ovulation rate; however, *in vivo* evidence suggesting a role for other hormones or metabolites is limited. The roles of plasma amino acids, free fatty acids, glucose, growth hormone, thyroxine and insulin-like growth factor I (IGF-I) have been proposed primarily by extrapolating the results obtained from in vitro studies of cultured ovarian tissue (BOOTH, 1990.

A characterization of the different classes of cysts is required to achieve greater knowledge about this pathological condition. Accordingly to McEntee (1990), ovarian cysts may be classified as (1) follicular cysts (FC), (2) cystic corpora lutea (CCL) or (3) luteinized cysts (LC). FCs are structures with a diameter larger than 1.5 cm that appear during estrus, when mature follicles fail to rupture. The *antrum* frequently continues to enlarge as a consequence of continuous estrogen-containing fluid production, possibly leading to abnormal sexual behavior in sows (HUNTER, 1980). A cystic corpora lutea forms in the center of the developing corpus luteum; an ovulating papilla is present and the cyst form is generally irregular in outline. LCs develop as a consequence of the partial luteinization of mature follicles, which do not ovulate (MCENTEE, 1990), and they are believed to develop into follicular cysts during later stages

(VANHOLDER; OPSOMER; DE KRUIF, 2006). Unlike CCL, no ovulatory protrusion is visible on the surface of an ovary with LC. The cavity, whether fluid-filled or hemorrhagic, is surrounded by layers of luteal (mostly large and spherical) cells (HUNTER, 1980; MCENTEE, 1990).

Thus, the present study aimed to investigate whether EG administration and/or dietary flushing in gilts might affect the ovulation rate and incidence of ovarian cysts.

Materials and Methods

The experimental use of animals and procedures were approved by the Animal Care and Use Committee of the School of Veterinary Medicine and Animal Sciences at the University of São Paulo (USP), protocol number 784/2005.

Animals and management

The experiment was carried out in the Laboratory of Swine Research, USP. A total of 71 prepubertal 157.48±5.02-day-old gilts (NAIMA, Pen Ar Lan, Espírito Santo do Pinhal, Sao Paulo, Brazil) with body weights of 96.65±7.7 kg were used. Animals were housed in pens (3 gilts/pen) throughout the experiment. Prior to day 1 of the experiment, the animals did not show any symptoms of estrus, and they were therefore considered prepubertal.

Puberty was hormonally induced in gilts to obtain greater homogeneity among the animals (DO LAGO et al., 2005). EG administration consisted of an intramuscular injection of 600 IU eCG (Novormon 5000; Syntex S.A., Luis de Sarro y Avellaneda B1838DQK, Argentina) and a second i.m. injection of 2.5 mg of LH (Lutropin; Bioniche Animal Sciences, Canada) after a 72-h period (GAMA et al., 2005). The day of the eCG administration was considered the first day of the study, which lasted for 32 days. All gilts were fed, on average, at 2.5 kg/animal/day of a replacement ration (6,573.46 kcal ME/day) at the beginning of the trial. The stimulation and detection of the subsequent estrus was performed by using two mature boars (more than 1 year old), which were kept in gilts pens for 15 min twice a day from day 7 until day 25 after the first LH treatment (puberty induction). The onset of estrus was defined as the standing reflex in the presence of a mature boar, and the estrus duration was the standing reflex period.

Two inseminations were performed at 12 and 24 h after the onset of estrus with the heterospermic semen (mixed semen) of four boars (Agroceres PIC, Patos de Minas, Brasil) of known fertility. The inseminating dose contained 4 x 10^9 viable sperm cells diluted in 90 mL of extender (Prolimax; Vetlife Ltd, Nova Odessa, São Paulo, Brasil).

Treatments

The animals were distributed into 4 treatments, which were arranged into a 2×2 factorial according to a completely randomized design. The first factor consisted of the administration of exogenous hormonal synchronization at the second estrus (by intramuscular injection of 600 IU of eCG 16 days after the beginning of the trial, followed by 2.5 mg of LH 72 h later); the other factor consisted of dietary flushing (3 kg/animal/day of replacement breeding, by supplying each animal with 9,860.2 kcal ME/day) from day 7 of the experiment until the day of artificial insemination. Thus, 4 treatments were performed as follows: gilts with flushing and hormones (wFwH, n=16); no flushing with hormones (nFwH, n = 17); flushing without hormones (wFnH, n = 19) and neither flushing nor hormones (nFnH, n = 19).

Ovary rate and cyst incidence evaluations

Gilts were slaughtered approximately 148 h after LH administration and their ovaries were collected from the carcasses in dorsal recumbence to avoid urine reflux to the uterus. The number of ovulations was macroscopically assessed by corpora lutea count. Sagittal sections of the ovaries were fixed in a 10% formalin solution and embedded in paraffin, and 5 μm-thick slices were stained with hematoxylin-

eosin (HE). Ovarian cyst counting and histological cyst characterization were performed with a light microscope (NIKON E-800, 0.5× objective lens, Japan), which was coupled to a Pro-Series High Performance CCD camera and Image Pro Plus Software 4.1 (Media Cybernetics, USA). Images corresponding to each quarter of all the ovaries were acquired and saved for analysis. Follicles with diameters > 1.5 cm were termed follicular cysts; cysts containing luteinic cells, and with fibrous tissue among these cells and cystic cavities were termed cystic corpora lutea; cysts containing one single layer of luteinic tissue and without fibrous tissue were classified as luteinized cysts.

Statistical analysis

The variable number of ovulations was analyzed by ANOVA in SAS (2003). Fisher's exact test was performed to analyze the frequency of ovarian cysts, follicular and luteinized cysts and cystic corpora lutea. Differences were considered significant at $P < 0.05$, and all results are expressed as the mean ± standard deviation (SD).

Results

Ovulation rate

There was no treatment interaction effect on the ovulation number. The mean values and SD for the numbers of corpora lutea were 14.60 ± 5.78, 13.23 ± 4.83, 14.28 ± 5.06 and 13.47 ± 5.57 for treatments with hormones, no hormones, with flushing and no flushing, respectively.

Incidence of ovarian cysts

Eleven of the gilts evaluated (15.49%) developed ovarian cysts. No interaction ($P > 0.05$) between treatments was observed, and therefore, flushing and hormonal treatment were analyzed separately. The incidence of cysts in flushing and non-flushing treatments was 14.29 vs. 16.67%, respectively, and 15.15 vs. 15.79% for animals receiving treatments with hormones and no hormones, respectively, but no significant difference was found (Table 1).

Cystic corpora lutea (CCL)

Interactions between flushing and hormone administration were not observed, as shown in Table 2. There were no significant differences between gilts receiving or not receiving a flushing treatment ($P = 0.71$), and there were also no significant differences between those receiving hormones or and those that did not ($P = 1.00$). It was found a higher incidence of CCL in comparison with LC and FC (9.85, 4.22 and 4.22% of the cysts, respectively).

Luteinized cysts (LC)

The LC incidence was not affected by the treatments, as shown in Table 3. The LC incidence in gilts from the wF and nF groups was 2.86 and 5.56% ($P = 1.00$), respectively, and 3.03 and 5.26% in the wH and nH gilt groups ($P = 1.00$), respectively. There was no interaction between treatments ($P = 1.00$).

Follicular cysts (FC)

The number of gilts presenting FC was not affected by the treatments. The FC incidence was 5.71 and 0% in wF and nF animal groups ($P = 0.23$), respectively, and 9.09% in gilts from the wH group, a higher value in comparison with the nH animals, which did not develop this form of cyst. However, a tendency ($P = 0.09$) was observed between gilts treated with (12.50%) and without (5.88%) hormones; gilts in either wFnH or nFnH groups did not have this form of cyst (Table 4).

Discussion

In this study, no dietary flushing or EG administration effects were observed on the ovulation rate of gilts. Aherne and Williams (1992) concluded that dietary flushing only increases the ovulation rate at second estrus in gilts that had been previously feed-restricted. Conversely, other authors showed that either EG

Table 1 - Frequency of gilts with ovarian cysts per treatment and total

Treatments	N° cysts / n° treated	Frequency (%)	P
wFwH	2/16	12.50	1.00
nFwH	3/17	17.65	
wFnH	3/19	15.79	
nFnH	3/19	15.79	
Flushing			
with	5/35	14.29	0.78
no	6/36	16.67	
Hormones			
with	5/33	15.15	0.94
no	6/38	15.79	
Total	11/71	15.49	

wFwH - with flushing and hormones; nFwH - no flushing and with hormones; wFnH - flushing without hormones; nFnH - neither flushing nor hormones and P – probability.

Table 2 - Frequency of gilts with cystic corpora lutea per treatment and total

Treatments	N° cysts / n° treated	Frequency (%)	P
wFwH	2/16	12.50	0.95
nFwH	1/17	5.88	
wFnH	2/19	10.53	
nFnH	2/19	10.53	
Flushing			
with	4/35	11.43	0.71
no	3/36	8.33	
Hormones			
with	3/33	9.09	1.00
no	4/38	10.53	
Total	7/71	9.85	

wFwH - with flushing and hormones; nFwH - no flushing and with hormones; wFnH - flushing without hormones; nFnH - neither flushing nor hormones and P – probability.

Table 3 - Frequency of gilts with luteinized cysts per treatment and total

Treatments	N° cysts / n° treated	Frequency (%)	P
wFwH	0/16	0	1.00
nFwH	1/17	5.88	
wFnH	1/19	5.26	
nFnH	1/19	5.26	
Flushing			
with	1/35	2.86	1.00
no	2/36	5.56	
Hormones			
with	1/33	3.03	1.00
no	2/38	5.26	
Total	3/71	4.22	

wFwH - with flushing and hormones; nFwH - no flushing and with hormones; wFnH - flushing without hormones; nFnH - neither flushing nor hormones and P – probability.

Table 4 - Frequency of gilts with follicular cysts per treatment and total

Treatments	N° cysts / n° treated	Frequency (%)	P
wFwH	2/16	12.50	0.09
nFwH	1/17	5.88	
wFnH	0/19	0	
nFnH	0/19	0	
Flushing			
with	2/35	5.71	0.23
no	0/36	0	
Hormones			
with	3/33	9.09	0.09
no	0/38	0	
Total	3/71	4.22	

wFwH - with flushing and hormones; nFwH - no flushing and with hormones; wFnH - flushing without hormones; nFnH - neither flushing nor hormones and P – probability.

administration after previous synchronization by Altrenogest (GUTHRIE; PURSEL; WALL, 1997) or dietary flushing (RHODES; DAVIS; STEVENSON, 1991) resulted in increased ovulation rates in gilts.

Ovarian cysts have been associated with decreased reproductive performance in swine breeding herds. When assessing data from 1990 sows and gilts distributed over two farms, Castagna et al. (2004) observed that ovarian cysts were responsible for a higher number of returns to estrus and a decreased adjusted farrowing rate; anestrual sows that were not found pregnant were also influenced by the appearance of ovarian cysts. Karveliene, Zilinskas and Riskevicience (2007) evaluated the ovaries from 150 sows that were culled over a period of 3 months, and they found that 4.7% presented multiple follicular cysts. Thus, it is necessary to evaluate any practice that could be associated with an increase in ovarian cyst incidence to guarantee the feasibility of such practices on commercial pig farms. In the present study, neither flushing nor EG led to an increase in ovarian cysts. However, the analysis of ovarian cysts couldn't be isolated. The cysts must be classified into different categories, because each one of these categories can

be associated with different causes. In the present study, it was found that the proportion of CCL was higher than both LC and FC; nevertheless, the CCL incidence was not influenced by the treatments.

Interestingly, the incidence of FC was 12.50% and 5.88% in gilts in the wFwH and nFwH treatments, respectively; however, neither wFnH nor nFnH gilts developed this form of cyst. Once the frequency of FC in gilts receiving both hormonal treatment and flushing is numerically higher in comparison with gilts that underwent flushing treatment alone, and only the gilts receiving hormones developed FC, an association between EG administration and a higher incidence of FC can be suggested. Some researchers have also reported an increase in the FC incidence following EG administration. Breen, Rodriguez-Zas and Knox (2006) reported the influence of PG600 doses (400 UI of eCG and 200 IU of hCG) on the FC incidence in prepubertal gilts. According to these authors, the number of FCs per gilt increased significantly, and the proportion of gilts with one or more cysts increased numerically as the dose of PG600 was raised from 1 to 1.5 and 2.0 times. Conversely, Knox and Tudor (1999) did not observe any increase in the proportion of gilts with cystic follicles after injecting PG600, whether alone or with norgestomet.

The genesis of FC has been extensively studied in ruminants, and LH is thought to play a pivotal role. Yoshioka, Iwamura and Kamomae (1998) concluded that the absence or mistiming of the preovulatory LH surge at the developmental stages of follicular structures may be associated with the appearance of ovarian cysts. According to Todoroki and Kaneko (2006), another hormonal event involved in cystic degeneration is a relatively high level of pulsatile LH secretion, which promotes continued growth in the dominant follicle. These LH characteristics seem to result from a functional abnormality in the feedback regulation of LH secretion by estradiol, which is caused by hypothalamic insensitivity to estrogen. This regulation is mediated through the absence of stimulation from estrogen receptors (ER) or a reduced number of these receptors, and thus a suboptimal GnRH production (GUMEN; WILTBANK, 2005). Once hCG has a longer biological action than LH in swine (HALL et al., 1993), it is reasonable to suppose that gilts receiving EG have a higher chance of developing cysts than naturally cycling animals. Aside from an imbalanced LH secretion, some authors suggested that alterations in LH receptors may be present in follicles that undergo cystic degeneration. Kawate (2004) observed that the number of LH receptors in granulosa and theca interna cells increases rapidly during the latter stages of normal antral follicular development, but it is reduced in follicular cyst cells; by contrast, luteinized cysts have a comparable number of LH receptors in the theca interna to that of the large antral follicles. In accordance with him, stress is a possible cause of FC in ruminants because (1) higher levels of ACTH might stimulate an increased release of progesterone, which inhibits the release of GnRH, and (2) enhanced cortisol secretion decreases both the number of LH receptors and the estradiol secretion of the antral follicle, inhibiting the positive feedback of estradiol towards the hypothalamus and pituitary gland and suppressing the LH surge. Apparently, follicles that became cystic would undergo apoptosis under normal conditions. Apoptosis is an important physiologic process in the ovary that occurs in atresic follicles (PALUMBO; YEH, 1995), and abnormalities in this process may lead to pathological conditions. In pigs, the atresia of smaller follicles occurs between days 14 and 16 of the estral cycle, following the recruitment of dominant follicles (FOXCROFT; HUNTER, 1985). From day 15 of the estral cycle onwards, the pool of recruited follicles undergoes follicular selection, which will determine those that escape atresia and ovulate, and prolific lines, such as the Chinese Meishan used in the present study, are more prone to having a greater range of follicle sizes escaping atresia (KNOX, 2005).

After injecting 3000 UI of PMSG on day 10 of the estrous cycle into heifers, Van den Hurk et al. (1992) observed that, at 48 h after injection, the mean number of large antral follicles was greater than it was in untreated animals as a consequence of an increase in the number of large and medium-sized non-atretic follicles (follicles whose histological alterations coincided with the occurrence of degenerative changes in the cytoplasm of nearby granulosa cells, which was more frequent in atresic follicles and thus most likely associated with the onset of follicular atresia). The mean number of large atresic follicles also increased after the hormone injection. Isobe and Yoshimura (2000) compared healthy follicles with follicles that underwent atresia or became cystic, and they found that the pattern of cell proliferation decreased in both cystic and atresic follicles, especially in the granulosa layer and theca interna, suggesting a relationship between them. Chun et al. (1994) showed that the preovulatory follicles of rats cultured in serum-free media containing no hormones had an increased apoptotic DNA fragmentation, whereas treating them with hCG or FSH medium suppressed follicular apoptosis in a dose-dependent manner, with 0.1 μg/ml causing a maximum suppression of 60-62%. Taken together, these data led us to speculate whether injecting EG might create an endocrine imbalance to induce follicles that would undergo atresia under physiological conditions to develop into FC, possibly because of the higher stimulation; indeed, Jackson et al. (2006) reported that the half-life of eCG is longer in comparison with the endogenously produced FSH. In addition, Knox (2005) observed that the ovulation rate in prepubertal gilts increases with the dose of PMSG, pFSH and PG600, suggesting that EG may alter the pool of follicles that will undergo selection.

In addition, Chun et al. (1994) found that media containing IGF-I and insulin, intraovarian factors played an autocrine/paracrine role in the control of folliculogenesis, and also suppressed the onset of apoptosis. In the same study, IGF-I induced an increase in the hCG receptor formation in granulose cells, making it a mediator of gonadotropin action towards follicle apoptosis. IGF-binding protein (IGFBP) blocks the action of IGF-I, and it plays a role both in the development of polycystic ovaries in humans and in follicle atresia (VAN DEN HURK et al., 1992). Accordingly to Chun et al. (1994), FSH may enhance granulosa cell maturation by decreasing IGFBP levels, allowing the facilitating action of endogenously produced IGF-I. Lima et al. (2006) demonstrated that the chronic administration of hCG to rats (3 IU of hCG daily, during 22 days), either alone or plus insulin, led to the development of polycystic ovarian syndrome. This syndrome could occur because insulin binds to the insulin receptor substrate proteins, activating a cascade of biochemical reactions that will ultimately result in protein kinase B (Atk) activation, which is involved in cell growth, apoptosis inhibition and granulosa cell survival. hCG and LH enhance the activation of Atk, acting in a synergistic manner with insulin. These data on the influence of insulin and IGF-I on the granulosa cell apoptosis support the hypothesis that dietary flushing may be related to higher ovarian cyst incidence once this practice leads to increased plasmatic levels for both factors (BOOTH; COSGROVE; FOXCROFT, 1996), although no evidence associating dietary flushing and cyst incidence were found in the present study.

Conclusions

The use of exogenous gonadotropins on second estrus synchronization in gilts, either alone or in association with dietary flushing, does not increase the incidence of ovarian cysts, nor does it lead to decreased ovulation rates. Further studies must be carried out to evaluate the physiological alterations promoted by exogenous gonadotropins and the lifespan of similar substances in the blood circulation of gilts.

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