ABSTRACT

The aim of this study was to compare plasma progesterone (P4) concentrations in nonlactating, multiparous Holstein cows (n = 24) treated with 2 types of intravaginal implants containing either 1.0 or 1.9 g of P4 either at the first use or during reuse of the implants after sanitizing the implant by autoclave or chemical disinfection. In a completely randomized design with a 2 × 3 factorial arrangement and 2 replicates, every cow underwent 2 of 6 treatments. Two sources of P4 [controlled internal drug release (1.9 g of P4) from Zoetis (São Paulo, Brazil), and Sincrogest (1.0 g of P4) from Ourofino (Cravinhos, Brazil)] and 3 types of processing, new (N), reused after autoclave (RA), and reused after chemical disinfection (RC), were used. After inducing luteolysis to avoid endogenous circulating P4, the cows were randomized in 1 of 6 treatments (1.9 g of N, 1.9 g of RA, 1.9 g of RC, 1.0 g of N, 1.0 g of RA, and 1.0 g RC). Cows were treated with the implants for 8 d and during this period blood samples were collected at 0, 2, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h. Statistical analyses were performed using Proc-Mixed and the mean ± standard error of the mean P4 concentrations were calculated using the Proc-Means procedures of SAS 9.4 (SAS Institute Inc., Cary, NC). No interaction between treatments was observed. Comparing types of implant, average P4 concentrations during treatments were greater for 1.9 g than 1.0 g (1.46 vs. 1.14 ± 0.04 ng/mL). When types of processing were compared, average P4 concentrations did not differ between autoclaved and new inserts (1.46 vs. 1.37 ± 0.05 ng/mL; respectively), but both were greater than chemically disinfected implants (1.09 ± 0.04 ng/mL). Within 1.9-g P4 inserts, P4 concentrations from autoclaved implants were greater than new, which were greater than chemically disinfected (1.67 ± 0.06 vs. 1.49 ± 0.07 vs. 1.21 ± 0.05 ng/mL; respectively). For 1.0-g P4 implants, P4 concentrations from autoclaved did not differ from new, but both were greater than chemically disinfected (1.20 ± 0.08 vs. 1.24 ± 0.06 vs. 0.97 ± 0.05 ng/mL; respectively). In conclusion, the mean plasma P4 concentration in nonlactating Holstein cows was greater for 1.9 than 1.0 g of P4 and regardless of the type of implant, the autoclaving process provided greater circulating P4 in relation to chemical disinfection, and similar or greater P4 concentrations compared with a new implant.

Key words: hormone, disinfection, device, Bos taurus

INTRODUCTION

Intravaginal progesterone (P4) inserts were initially developed to treat anovular heifers and cows in seasonally calving New Zealand herds with smaller cows with much lower milk production, and luteal phase circulating P4 concentrations could be achieved (Macmillan et al., 1991; Macmillan and Peterson, 1993). However, more recent studies have used these intravaginal P4 implants in high-producing dairy cattle and in whole-herd synchronization programs, with much lower circulating P4 concentrations being achieved (Rabiee et al., 2002a; Gümen and Wiltbank, 2005; Zuluaga and Williams, 2008; Bisinotto et al., 2013). In anovular cows, 2 intravaginal implants, rather than only 1, are required to achieve sufficient circulating P4 and normal fertility (Padula and Macmillan, 2006; Bisinotto et al., 2013; Pereira et al., 2017a,b). Several types of intravaginal P4 inserts are commercially available worldwide, with designs that allow retention within the vagina, usually with a T-shape, and prolonged delivery of P4, usually from P4-impregnated silicone molded over a nylon spine. In nonlactating ovariectomized cows, P4 inserts that have a similar surface area but contain 1.34 versus
1.9 g of P4 release a similar amount of P4, on average, 620 and 610 mg of P4, respectively, over a period of 7 d (Rathbone et al., 2002). These treatments produced circulating P4 of ~4 ng/mL on the day after insertion, with concentrations at ~2.5 ng/mL by 7 d after insertion and few differences due to P4 load (10 to 30% wt/wt; P4:silicone) or presence of additives (liquid paraffin, arachis oil, or polyethylene glycol), as long as surface area was kept constant (Rathbone et al., 2002). However, increasing surface area of silicone available for release of P4 produced a linear increase in circulating P4, indicating the fundamental nature of this aspect of insert design. In anovular high-producing dairy cows, use of a single, new intravaginal P4 insert containing 1.34 g of P4 increased circulating P4 to only 0.8 to 1.0 ng/mL (Cerri et al., 2009; Lima et al., 2009), probably due to the greater P4 metabolism in lactating dairy cows related to elevated liver blood flow (Wiltbank et al., 2006). Thus, surface area for release of P4 and physiology of treated cows seem to be major determinants of the circulating P4 concentrations produced by treatment with P4 inserts.

In many countries, the reuse of intravaginal inserts is a common method to reduce costs of synchronization programs, although not recommended by manufacturers. For example, treatment of cows with a 1.9-g P4 insert for 7 d only removes ~600 mg of P4, leaving ~1.3 g of residual P4 load (Macmillan et al., 1991; Macmillan and Peterson, 1993; Rathbone et al., 2002). However, disinfection of the inserts before reuse is a major consideration, with producers primarily using either chemical disinfection of inserts or high-pressure steam sterilization using an autoclave (Zuluaga and Williams, 2008; Cerri et al., 2009; Long et al., 2009). Oral communication of results with reused P4 implants have been discussed in the scientific community, but publication of these results has generally not occurred due to concern from the manufacturer that off-label use could adversely affect product efficacy, product registrations with governmental agencies, or product sales. Thus, the P4 profiles have not been extensively evaluated in the scientific literature or directly compared with governmental agencies, or product sales. However, disinfection of the inserts before reuse is a major consideration, with producers primarily using either chemical disinfection of inserts or high-pressure steam sterilization using an autoclave (Zuluaga and Williams, 2008; Cerri et al., 2009; Long et al., 2009). Oral communication of results with reused P4 implants have been discussed in the scientific community, but publication of these results has generally not occurred due to concern from the manufacturer that off-label use could adversely affect product efficacy, product registrations with governmental agencies, or product sales. Thus, the P4 profiles have not been extensively evaluated in the scientific literature or directly compared with governmental agencies, or product sales.

Therefore, the objective of this experiment was to compare plasma P4 concentrations in cyclic nonlactating Holstein cows during use and reuse of intravaginal P4 inserts that originally contained 1.9 or 1.0 g of P4. Thus, along with evaluating the circulating P4 concentrations during use of implants with different P4 loads, we also evaluated whether circulating P4 would differ during reuse of the implants that were sanitized by 2 very different methods, using a high-pressure and -temperature autoclave or by chemical disinfection. The hypotheses for this experiment were that (1) plasma P4 concentrations during use of a new 1.9-g intravaginal P4 implant would be similar to the profile for a new 1.0-g intravaginal P4 implant; 2) independent of method of disinfection, plasma P4 concentrations during treatment with a reused implant would be greater for a 1.9-g implant compared with a 1.0-g implant; and (3) independent of type of implant, plasma P4 concentrations would be greater for an autoclaved reused implant than for a chemically disinfected reused implant, based on data from other studies (Cerri et al., 2009; Long et al., 2009).

**MATERIALS AND METHODS**

This experiment was conducted at the Department of Animal Science facilities at Escola Superior de Agricultura “Luiz de Queiroz”/University of São Paulo, located in Piracicaba city, São Paulo, Brazil. The Animal Research Ethics Committee of Escola Superior de Agricultura “Luiz de Queiroz”/University of São Paulo approved all procedures involving cows in this study.

For this study, 24 nonlactating multiparous cycling Holstein cows were used. At the beginning of the experiment, cows averaged 600 kg of BW and a BCS of 3 (Ferguson et al., 1994). Cows were kept in confinement with free access to water and mineral salt, and were fed a TMR maintenance diet (NRC, 2001) based on sugar cane bagasse as forage and concentrate based on corn and soybean meal, minerals, and vitamins.

Cows were randomly assigned to 1 of 6 treatment groups using a completely randomized design with a 2 × 3 factorial arrangement of treatments and 2 replicates, and every cow underwent 2 treatments. We used 2 sources of intravaginal P4 implants [controlled internal drug release (1.9 g) from Zoetis (São Paulo, Brazil), and Sincrogest (1.0 g) from Ourofino (Cravinhos, Brazil)] and 3 types of processing [new (N), reused autoclaved (RA), and reused chemically disinfected (RC)], resulting in the treatments 1.9 g N, 1.9 g RA, 1.9 g RC, 1.0 g N, 1.0 g RA, and 1.0 g RC.

At the beginning of the experiment (d 0), each cow had its estrous cycle synchronized with a new 1.9-g P4 implant that remained for 8 d. At 7 and 8 d after implant insertion, 25 mg of dinoprost tromethamine (PGF₂α; Lutalyse, Zoetis) was administered, and on d 8, after the withdrawal of the P4 implant, a Norgestomet (Crestar; MSD, São Paulo, Brazil) ear implant was inserted, which was maintained for 48 h to avoid ovulation and allow for a complete drop in circulating P4. On d 10, cows were randomized to 1 of 6 treatments. The implants were left within the vagina for 8 d and during this period blood samples were collected.
for circulating P4 measurements at 0, 2, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h. On the last day, after the last blood sampling, P4 implants were removed and Norgestomet was inserted again and maintained for 48 h, together with other PGF<sub>2α</sub> treatments at insertion and withdrawal times. Then, another replicate began on d 20, similar to the first replicate (Figure 1) but with cows randomly assigned to another treatment.

The autoclaved and chemically disinfected implants were previously used in lactating dairy cows for 8 d. After removal, the inserts were washed in clean running water, and air-dried at room temperature. Prior to use in the experiment, the inserts were autoclaved or chemically disinfected. The protocol used to autoclave the P4 implants was similar to the one described by Cerri et al. (2009). Briefly, the inserts were placed in autoclave bags and autoclaved for 15 min at 121°C and 725 mmHg. For disinfection, the implants were dipped for 15 min in 1:2,000 diluted quaternary ammonia (CB-30 TA; Ourofino) and air-dried at room temperature.

Blood samples were collected by puncture of the jugular vein into 10-mL heparinized evacuated tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) for plasma P4 measurements at 0, 2, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h. Blood samples at 0 h were collected immediately before administration of treatments in both replicates on d 10 and 20, respectively, and at 192 h immediately before implant withdrawal. After collection, samples were placed in ice and transported to the laboratory within 2 h. Blood tubes were centrifuged at 1,900 × g for 15 min at 4°C and plasma was frozen at −20°C. Plasma was analyzed for P4 by a solid-phase RIA using a commercial kit (Coat-A-Count; Siemens Healthcare Diagnostic, Los Angeles, CA). A single assay was performed with all samples. The assay sensitivity was 0.01 ng/mL and intra-assay coefficient of variation was 4.6%.

Data were tested for homogeneity of variances and normality of residuals using the GLM procedure of SAS version 9.4 (SAS/STAT, SAS Institute Inc., Cary, NC). Homogeneity of variances followed Hovtest and Welsh methods, and normality of residuals were analyzed using the UNIVARIATE procedure of SAS following the Shapiro-Wilk method.

Concentrations of P4 were analyzed as repeated measures using the MIXED Procedure of SAS. The replicate was considered a random effect and cow within time was the subject effect. The fixed effects of type of implant (1.9 vs. 1.0), type of processing (new, RA, or RC), time, and specific interactions of time with treatments were included in the model, fitting a Kenward-Roger method to calculate the denominator degrees of freedom to approximate the F-tests in the mixed models.

The estimates were calculated to generate the P-values from the adjusted Tukey comparisons of means, although the results are expressed as mean ± standard error of means. Differences were considered significant.
when \( P \leq 0.05 \), whereas a tendency was defined as \( 0.10 \geq P > 0.05 \).

### RESULTS AND DISCUSSION

Although no interaction was detected between type of implant and type of preparation method \( (P = 0.19) \), clear differences were found between the types of implant \( (1.0 \text{ vs. } 1.9 \text{ g P4}; P = 0.0002) \), implant preparation \( (\text{RA vs. RC}; P < 0.0001) \), and time \( (P < 0.0001) \) on circulating P4 concentrations. In addition, interactions on P4 concentrations were detected between types of implant and time \( (P = 0.05) \) and implant preparation method and time \( (P = 0.0002) \). Mean P4 concentration was greater for the 1.9-g P4 than 1.0-g P4 implant and lower for the chemically disinfected than the autoclaved implant during the 8 d of treatments (Table 1).

Our first hypothesis, that plasma P4 concentrations during the use of 2 new intravaginal implants containing 1.9 and 1.0 g of P4 would be similar, was based on the concept that when new inserts with similar surface area are used, even with different P4 loads, they are bioequivalent, having the same overall daily release of P4 during the first week of treatment \( (~0.61 \text{ g; Rathbone et al., 2002}) \). This hypothesis was rejected because the new 1.9-g P4 implant had 20.2% greater \( (P = 0.04) \) circulating P4 concentrations compared with the new 1.0-g P4 implant. Nevertheless, the repeated measures analysis (Figure 2A) did not detect differences at specific times during the 8 d of treatment with new 1.9- versus 1.0-g P4 implants \( (P > 0.10) \). In addition, we found no difference between 1.9- versus 1.0-g P4 implants during the first 4 d (combined analysis; \( P > 0.10 \)) or the last 4 d of treatment \( (P > 0.10) \), although a decrease in P4 occurred during the last 4 d compared with first 4 d of treatment irrespective of P4 load in implant \( (P < 0.0001; \text{Figure 2A}) \).

Our results contrast, somewhat, with other results that show that P4 implants with different P4 load but similar surface area produced similar circulating P4 concentrations during the first week of implant treatment \( (\text{Rathbone et al., 2002}) \). In contrast, residual P4 left in the implant after the first 7 d of treatment has been shown to be distinctly related to the initial amount of P4 in the new implant. For example, Macmillan and Peterson \( (1993) \) showed a quadratic relationship between the initial and residual amount of P4 over an insertion period of 15 d \( (R^2 = 0.953) \). An implant containing 0.69 g of P4 was almost completely depleted of P4 \( (0.07 \text{ g of residual P4}) \), whereas implants with 1.25, 1.86, and 2.67 g of P4 lost \( ~1.0 \text{ g of P4 during the insertion period, leaving dramatically different residual P4 in the used implants (0.31, 0.80, and 1.39 g of P4, respectively). Nevertheless, these previous release rate experiments were done with new P4 implants; the situation may be very different with a reused P4 implant, particularly after the changes in P4 distribution that could occur after the high pressure and heat involved in the autoclave process. One additional factor is that cows with widely varying feed intake and physiology had similar P4 release from a 1.9-g P4 implant during 11 d and similar residual P4 left in the implant after use for 11 d \( (\text{Rabee et al., 2001a,b})\).

In our experiment, we did not evaluate the residual P4 but would expect \( ~0.4 \text{ g of P4 to be released during the first 4-d period from either 1.0- or 1.9-g P4 implants and 0.25 to 0.3 \text{ g of P4 released during the next 4 d, based on previous results (Rabee et al., 2001a,b, 2002b; Rathbone et al., 2002). Thus, our first hypothesis was rejected due to small, but significant, differences in P4 profiles during use of new P4 implants with differing initial P4 loads. In addition, the residual P4 amounts would be expected to be substantially different for 1.9-g \( (~1.2 \text{ g P4}) \) compared with 1.0-g \( (~0.4 \text{ g P4}) \) P4 implants when the implants were going to be reused for the second time in this experiment. Still, the successful reuse of the P4 implants in this experiment, as reflected in the P4 profiles during treatment of cows with reused P4 implants, should not be interpreted to mean that all types of P4 implants, regardless of initial P4 load or release rate, can be successfully used in all types of physiological situations.

Our second hypothesis was that, irrespective of disinfection method, the reused implant from the initial

### Table 1. Plasma progesterone (P4) concentrations (mean ± SEM) between 24 and 192 h during the 8 d of treatments in 24 nonlactating dairy cows after insertion of intravaginal P4 implants (1.9 g or 1.0 g) that were submitted to 3 types of processing [new (N), reused autoclaved (RA), or reused chemically disinfected (RC)]; every cow underwent 2 treatments

<table>
<thead>
<tr>
<th>P4 implant</th>
<th>1.9 g</th>
<th>1.0 g</th>
<th>P-value</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1.49 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.24 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04</td>
<td>1.37 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RA</td>
<td>1.67 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.20 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01</td>
<td>1.46 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RC</td>
<td>1.21 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.97 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02</td>
<td>1.09 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
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<td></td>
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<tr>
<td>Average</td>
<td>1.46 ± 0.04</td>
<td>1.14 ± 0.04</td>
<td>&lt;0.01</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>b</sup>Values in the same column with different superscripts differ \( (P < 0.05) \).
1.9-g P4 implant would produce greater circulating P4 than the reused 1.0-g implant. This was based on the assumption that much greater residual P4 would be available for release during reuse of the 1.9-g compared with the 1.0-g P4 implant. This hypothesis was fully supported by the data for the reused implants. Mean P4 concentrations during the full 8-d period were 39.2% greater \((P < 0.01)\) for autoclaved 1.9- versus 1.0-g P4 implants and 24.7% greater for chemically disinfected 1.9- versus 1.0-g P4 implants (Table 1). This difference between 1.9- and 1.0-g P4 implants was most readily detected in cows treated with the autoclaved implants (Figure 2B), but was not detected using the repeated measures analysis of daily evaluations in cows treated with chemically disinfected implants (Figure 2C). It appears that autoclaving the implant caused more of the residual P4 to be releasable during the reuse period for both 1.9- and 1.0-g P4 implants (RA vs. RC). However, the differences between the 1.9-g RA compared with 1.0-g RA implants at all times after 96 h (Figure 2B) indicates an earlier depletion of residual P4 from the 1.0- versus 1.9-g RA implant. In the reused implants, the initial large increase in circulating P4 concentrations that was observed with autoclaved implants did not occur with chemically disinfected implants, but the profile remained relatively flat throughout the 8-d period, possibly indicating a slower exhaustion of the residual P4, particularly with the 1.0-g P4 implant (Figure 2C).

Previous studies have described the P4 profiles using implants with differing P4 loads but similar surface area in bilaterally ovariectomized nonlactating cows (Rathbone et al., 2002), or when new or used intravaginal P4 implants were used in bilaterally ovariectomized nonlactating cows (Zuluaga and Williams, 2008) or in high-producing dairy cows (Cerri et al., 2009). However, ours is the first comparison of new and reused implants with differing P4 loads that were sanitized by 2 different methods, autoclaving versus chemical disinfection. The autoclaving process may modify the structure of the implant or the location or disposition of P4 within the insert (Zuluaga and Williams, 2008). The increased elution of P4 caused by the autoclaving process may produce more rapid subsequent depletion of the remaining P4, leading to exhaustion of P4 in the 1.0-g but not the 1.9-g P4 implants. Nevertheless, in both previously used autoclaved implants, average P4 concentrations were generally greater than 1 ng/mL during the 8 d of treatment, which should be sufficient to prevent a GnRH-LH surge and may be sufficient to synchronize the emergence of a new follicular wave in some P4-based fixed time AI protocols (Baruselli et al., 2012; Wiltbank et al., 2014), although this will need to be rigorously examined in future experiments.
Our third hypothesis was that circulating P4 would be greater for reused implants that were sanitized using an autoclave rather than chemical disinfection, based on previous reports with autoclaved P4 implants (Zuluaga and Williams, 2008). This hypothesis was accepted. The complete profiles for 1.9-g (Figure 3A) or 1.0-g (Figure 3B) P4 implants demonstrate the effectiveness of autoclaving in causing residual P4 release. The autoclaved 1.9-g P4 implant produced 38.0% greater circulating P4 than a chemically disinfected 1.9-g P4 implant and even produced greater P4 concentrations (12.1%) than a new implant (Table 1). Similarly, the autoclaved 1.0-g P4 implant produced 23.7% greater circulating P4 than a chemically disinfected 1.0-g P4 implant, although we found no difference between new and autoclaved 1.0-g P4 implants (Table 1). Figure 4 demonstrates the differences in circulating P4 during the first 4 d (24 to 96 h) compared with the last 4 d (120 to 192 h) of treatment with reused 1.9- versus 1.0-g P4 implants that were previously autoclaved or chemically disinfected. The autoclaved reused 1.9-g P4 implant produced the greatest P4 concentrations during the first 4 d of treatment (1.83 ng/mL, on average), and this decreased 21.3% during the last 4 d of treatment (1.44 ng/mL). In contrast, cows treated with the chemically disinfected 1.9-g P4 implant had no significant decrease in circulating P4 from the first 4 versus the last 4 d of treatment, but values were significantly lower in both periods for cows treated with a chemically disinfected versus autoclaved 1.9-g P4 implant (Figure 4). For the 1.0-g P4 implant, we noted a dramatic decrease in circulating P4 during the first 4 d versus the last 4 d of treatment with an autoclaved implant (40.4%), but also a smaller but significant decrease in circulating P4 between the first 4 and the last 4 d in cows treated with the chemically disinfected 1.0-g P4 implant (20.2%). In summary, autoclaving compared with chemically disinfecting increased circulating P4 during both the first 4 (32.2%) and the last 4 d (22.2%) of treatment with a 1.9-g P4 implant, but only during the first 4 d (26.2%) and not during the last 4 d of treatment with the 1.0-g P4 implant (Figure 4). Thus, solely based on P4 profile, reuse of P4 implants seems suitable when sufficient residual P4 remains in the implant and the releasable P4 is optimized by autoclaving the implant before reuse.

One other important consideration is that for new and autoclaved P4 implants there is a consistent decrease in circulating P4 concentrations over time after implant insertion ($P < 0.0001$), as previously reported (Macmillan and Peterson, 1993; Cerri et al., 2009). It seems likely that most of the differences in P4 profiles observed in our study were related to alterations in P4 release from the implant, as the vaginal mucosa has high permeability to steroid hormones and P4 subsequently enters the capillaries and blood stream by passive diffu-
sion (Rothen-Weinhold et al., 2000). Indeed, P4 release from a silicone implant seems to follow a zero-order (R² = 0.989) release mechanism with particulate P4 in a saturating concentration at the interface of surfaces (Rathbone et al., 2002). In this regard, treatment with 1, 2, or 3 P4 implants produced corresponding increases in circulating P4 (2× or 3×), with similar depletion of P4 from each of the implants, regardless of the number of implants present in the vagina or the circulating P4 concentration (Macmillan and Peterson, 1993). Nevertheless, differences in animal size (Cerri et al., 2009) and metabolic clearance rate for P4 (Sangsritavong et al., 2002) can potentially alter the circulating P4 achieved in different cows or different experimental situations.

CONCLUSIONS

Mean plasma P4 concentration in nonlactating Holstein cows was greater for 1.9-g than 1.0-g P4 implants. For previously used P4 implants, sanitizing the reused implant using an autoclave produced greater circulating P4 concentrations, compared with chemically disinfected implants, and similar or greater circulating P4 compared with new implants.

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