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Effect of oral administration of pioglitazone on follicular dynamics in Holstein dairy cows

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Abstract

This study investigated the effects of oral administration of pioglitazone (PGT), a specific and synthetic ligand of peroxisome proliferator-activated receptors gamma (PPARγ), on follicular dynamics and corpus luteum (CL) functionality in dairy cows. Cows exhibiting strong signs of estrus after 2 injections of $PGF_{2\alpha}$ (given14 d apart) at d 30 postpartum (n=28) were allotted to four groups (n=7 cows/treatment) and orally received 6 mg PGT/kg body weight/day according to the following protocol: no PGT (control); PGT for 14 d from 7 d before expected estrus (10 d after 1st injection of $PGF_{2\alpha}$) to 7 d after observed estrus (PGT14); PGT for 21 d after observed estrus (PGT21); and PGT for 28 d, 7 d before expected estrus to 21 d after observed estrus (PGT28). During the first follicular wave, number of follicles (total and small) increased in PGT14 and PGT28 cows compared to the control group ($P<0.05$). During the ovulatory wave, number of total and small follicles increased in PGT28 ($P<0.05$) and PGT21 ($P<0.10$) compared with PGT14 and control cows. Size of the largest follicle at first wave was greater in PGT28 ($P<0.05$), PGT14 ($P<0.05$) and PGT21 ($P<0.10$) compared to the control cows. Maximal size of the ovulatory follicle was greater in PGT28 ($P<0.05$) and PGT21 ($P<0.10$) groups compared to the control group. Growth rate of the largest follicle at first wave was higher ($P<0.05$) in PGT-treated cows, while growth rate of the ovulatory wave was higher in PGT28 and PGT21 groups, leading to shorter days from luteolysis to ovulation. Pioglitazone administration did not affect CL size, but increased progesterone (P_4) concentration. The PGT14 and PGT28 cows had higher maximal plasma P_4 concentration and shorter intervals to reach maximal plasma P_4 compared to the control group. In conclusion, oral administration of PGT had some positive effects on follicular development and circulating P_4 levels which may be conducive to better reproductive performance.

***Keywords*:** dairy cow; ovarian folliculogenesis; pioglitazone; progesterone; ultrasonography

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors activated by binding to natural ligands, such as polyunsaturated fatty acids, or synthetic ligands. Thiazolidinediones (TZDs), as specific ligands for PPARs, are used in the treatment of type II diabetes. The binding of TZDs to their receptors, primarily to PPARγ in adipose cells, increases insulin sensitivity (Staels and Fruchart, 2005). A condition of insulin resistance also develops during late gestation in high-producing dairy cows and continues into early lactation (Bell, 1995), which facilitates mobilization of non-esterified fatty acids (NEFA) to mitigate the period of negative energy balance, and at the same time decreases the dry matter intake (Drackley, 1999) with resultant metabolic disorders and low reproductive performance (Bell, 1995; Kaneene et al., 1997).

The PPARs are widely expressed in tissues such as the ovarian follicles, and CL, and exogenous ligands of PPARs may be useful for treating insulin resistance in dairy cows (Smith et al., 2007; Schoenberg and Overton, 2011). In vitro studies suggested that PPARs are involved in many reproductive processes, including steroidogenesis, folliculogenesis, ovulation, fertilization, implantation, and embryo development (Komar et al., 2001; Komar and Curry, 2002; Froment et al., 2003; Komar, 2005; Froment et al., 2006).

Progesterone (P_4) secretion from granulosa cells obtained from cows fed with fat-enriched diets was increased (Wehrman et al., 1991); an effect that is thought to be related to PPAR activation (Singh et al., 2013). Scott et al. (1996) reported that troglitazone (a synthetic PPAR ligand) ameliorated the ovulatory function in polycystic ovarian syndrome (POS). The effectiveness of PPAR agonists on modulation of in vitro steroidogenesis in rodent granulosa cells was also reported (Komar et al., 2001; Froment et al., 2003), mainly through increasing steroidogenic enzyme activity and/or speeding up cholesterol transport (Gasic et al., 1998; Froment et al.,

2003). These findings suggested that natural or synthetic ligands of PPARγ may directly affect the ovary.

Pioglitazone was approved by the USA Food and Drug Administration (FDA) in 1999 and the European Medicines Agency (EMA) in 2000 for the treatment of type-II diabetes (Ferwana et al., 2013). Administration of PGT for patients with type-II diabetes and in POS women resulted in satisfactory outcome (Staels and Fruchart, 2005). People with type-II diabetes are exposed to a higher risk of several types of cancer, including a 40% increased risk of bladder cancer, and it has been postulated that PGT may increase the risk (Giovannucci et al., 2010). However, evidence linking the use of PGT to the increased risk of bladder cancer is controversial. The initial findings for a possible association of PGT and bladder cancer originated from a preclinical study included in the licensing applications which reported a higher number of urothelial hyperplasia in a rat population and malignant tumors in the urinary bladder in the animals treated with PGT (Agency, 2011). Nevertheless, subsequent studies revealed that this is a rat-specific phenomenon and does not pose a urinary bladder cancer risk to humans (El-Hage, 2005; Suzuki et al., 2009; Sato et al., 2011). Similarly, in a cohort study of PGT and cancer incidence in patients with diabetes, Ferrara et al., (2011) found no clear evidence of an association between use of PGT and risk of the incident cancers examined prostate, pancreas, lung/bronchus, colon, non-Hodgkin lymphoma, female breast, endometrial, kidney/renal pelvis, rectal, and melanoma. Moreover, recent comprehensive and meta-analysis studies reported no connection between long-term use of PGT and the risk of bladder cancer and malignancy (Lewis et al., 2015; Filipova et al., 2017). Despite these findings, as described in FDA-approved labels, PGT is still contraindicated in patients with active bladder cancer, should be used with caution in patients with prior history of bladder cancer, and should not be given in patients who develop liver injury (Gourgari et al., 2017).

The TZDs reversed the TNFα-induced insulin resistance in steers (Kushibiki et al., 2001), and improved dry matter intake, plasma glucose and negative energy balance in transition dairy cows (Smith et al., 2007; Smith et al., 2009; Yousefi et al., 2016). A positive effect of TZDs on resumption of postpartum ovarian cyclicity was also reported (Smith et al., 2009; Yousefi et al., 2016). Dietary supplementation of pioglitazone (PGT), a specific and synthetic ligand of PPARγ, improved postpartal ovarian resumption and reproductive performance in transition dairy cows (Yousefi et al., 2016). In addition, short-term supplementation of poly-unsaturated fatty acids (natural ligands of PPARγ) and TZDs (synthetic ligands of PPARs) effectively improved postpartum metabolic condition of dairy cows leading to earlier resumption of ovarian cyclicity and improved reproductive performance (Downing and Scaramuzzi, 1991; Mattos et al., 2000; Singh et al., 2013; Yousefi et al., 2016). These findings confirmed the in vitro studies that reported an increase in PPARγ expression concomitant to the final stage of follicular growth and early CL formation (Viergutz et al., 2000; Froment et al., 2003; Komar, 2005).

Previous investigations suggested that the improved ovarian activity and reproductive performance in TZD-treated transition dairy cows were most likely related to the changes in the animal's metabolism and hormonal profile (Smith et al., 2007; Smith et al., 2009; Schoenberg and Overton, 2011; Yousefi et al., 2016). However, it is not clear whether TZDs directly impact on ovarian follicular dynamics and CL function by activating the PPARs in the ovarian tissues, or their action on reproduction improvement is limited to metabolism and endocrine changes. Therefore, the current study was conducted for better understanding of the effect of a TZD ligand on follicular dynamics and CL function in high-producing dairy cows. In addition, the optimal time during which the TZD treatment should be applied for the greatest impact on follicular dynamics during the pre-estrus, follicular, or luteal phase of the ovarian cycle is clarified.

2. Materials and methods

Under the approval given by Tehran University Ethics Committee, this experiment was conducted at the Dairy Farm Research of the Agriculture and Natural Resources College, University of Tehran, Iran.

2.1. Synchronization of the estrous cycle

Forty Holstein dairy cows (parity 1-3, and 30 days in milk) with no overt signs of disease were blocked by BCS and parity, and allocated to four treatment groups (10 cows per group). However, only 28 cows (7 cows per group) with strong signs of estrus and ovulation confirmed by ultrasonography were used for further observations. Estrous cycles were synchronized by 2 injections of $PGF_{2\alpha}$ (500 μg Cloprostenol sodium, i.m.; Estroplan, Parnell Technologies PTY. LTD., Alexandria, Australia), 14 d apart, started on d 30 postpartum. An average of 3 d was considered as withdrawal required for expression of estrual signs following the second $PGF_{2\alpha}$ injection. Visual observation of standing estrus was conducted 3 times daily (30-min per observation). Ovulation and initiation of the new estrous cycle were confirmed by the absence of a largest follicle (diameter ≥ 10 mm) that had been detected at the previous examination (ultrasonography test), and subsequent CL formation (Dirandeh, 2014).

2.2. Experimental design and treatments

To affect metabolism, at least a 10-14 d administration of TZD ligands is needed (Ghoreishi, 2012; Yousefi et al., 2016). Cow assignment to treatments was balanced for calculated previous 305-d mature-equivalent milk yield and BCS. Treatments included: no PGT (control); PGT for 14 d from 7 d before expected estrus (10 d after 1st injection of $PGF_{2\alpha}$) to 7 d after observed estrus (PGT14); PGT for 21 d after observed estrus (PGT21); and PGT for 28 d; from 7 d before expected estrus to 21 d after observed estrus (PGT28), as shown in Fig. 1. A daily dose

of 6 mg PGT/kg body weight (BW), in the form of a water suspension, was administered orally (at 0800 hour) to PGT cows, and PGT-free water was drenched to the control group. More water was then drenched to insure that PGT was completely ingested.

To adjust PGT level for BW changes during the experiment, the cows were weighed weekly. Pioglitazone hydrochloride (Hetero Drugs, India, Batch No. PHD 0510001) was kindly provided by Darou Pakhsh Co., Tehran, Iran. Based on ~60% bioavailability of orally administered PGT in ruminants (Ghoreishi, 2012; Yousefi et al., 2015; Yousefi et al., 2016) and the efficacy of intravenous administration of 2 or 4 mg TZD/kg BW in influencing the metabolism and performance of transition cows (Smith et al., 2007; Smith et al., 2009; Schoenberg and Overton, 2011), an oral dose of 6 mg PGT/kg BW was chosen. A total mixed ration (TMR; Table 1) was fed ad libitum (NRC, 2001). The cows were milked 3 times per day at 0700, 1400, and 2300 hours, and milk yield was recorded automatically.

2.3. Ultrasonography examination

Ovarian follicular activity was examined daily by transrectal ultrasonography using a real-time linear scanning ultrasound diagnostic system (B mode; Piemedical, Falco 100; 8 MHz transducer). From one day before expected estrus (d 43 postpartum) to 21 d after observed estrus, the ovaries were scanned sequentially. Follicles larger than 3 mm were counted and classified as small (< 5 mm), medium (5 to 10 mm), or large (> 10 mm). Horizontal and vertical diameters of the follicles and CL were recorded and their averages used in the analysis (Dirandeh et al., 2009; Mohammadi et al., 2010). The new follicular wave emergence was defined as the day on which the largest follicle was retrospectively first identified as having a diameter of 4–5 mm (Dirandeh et al., 2009). The day of luteolysis was defined as the day before which serum P_4 decreased to less than 50% of the mean for the four maximum P_4 concentrations in the cycle. Growth rate of the largest follicle was defined as the difference between the

maximal size of the follicle and its size at the first day of the corresponding wave to the period needed to reach maximal size. The period of CL activity was calculated from the first day when P_4 concentration was greater than 1 ng/mL to the day when plasma P_4 started to decrease.

2.4. Blood sampling and P_4 assay

Blood samples were collected from the coccygeal vein in evacuated glass tubes containing EDTA (10.5 mg, Monoject; Sherwood Medical, St. Louis, MO, USA) for 21 d, starting on the first day of the estrous cycle, at 1-d intervals. Within 1 h of sampling, blood plasma was harvested after centrifugation (3000 g, 15 min) and stored at −20°C until analyzed for P_4. Plasma P_4 concentration was measured using a specific ELISA kit (Diaplus Inc., USA), following the manufacturer's instructions. The inter- and intra-assay coefficients of variation were 5.3 and 3.4%, respectively.

2.5. Statistical analysis

Data on continuous variables such as interovulatory interval, diameter of the ovulatory follicle, interval between emergence of last wave and ovulation, growth rate of follicles, days from luteolysis to ovulation, maximal size of CL, maximal concentration of plasma P_4, days to maximal plasma concentration of P_4, period of CL activity, and period of CL activity to maximal plasma P_4 concentration were analyzed by the GLM procedure of statistical analysis system software (SAS, 2004). The data measured over time (mean plasma P_4 concentrations during the estrous cycle, and the number of small, medium, and large follicles) were analyzed by the MIXED procedure. Time of sampling (d) was used in the REPEATED statement. The model included the fixed effects of treatment, time, interaction between treatment and time, and the random effect of cows nested within treatments. The mathematical model was:

$$Y_{ijk}=\mu + T_i + t_j + (T \times t)_{ij} + \delta(T)_{ik} + e_{ijk}$$

in which, y_{ijk} is the observation on cow k at the sampling time j receiving treatment i; μ is the overall mean; T_i is the fixed effect of treatment; t_j is the fixed effect of sampling time j (d); $(T \times t)_{ij}$ is the two-way interaction of treatment i by sampling time j; $\delta(T)_{ik}$ is the random effect of cow k nested within treatment i, and e_{ijk} is the residual random error. Because estrous cycle may be affected by the number of the follicular waves, the effect of treatments on the first and last waves were analyzed separately. The effects of BW, BCS, level of milk yield, and parity measured or assessed at the beginning of the experiment, were used as covariates and/or fixed effects; however, they were removed from the model if their effects were not significant, and the data reanalyzed subsequently. Means were compared by the Tukey's test, and results expressed as the least squares means and SEM. Statistical significance and tendencies were declared at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

3. Results

The effects of oral administration of PGT on follicular dynamics are presented in Table 2. Pioglitazone administration did not affect the interovulatory interval (P>0.05). During the first follicular wave, number of follicles (total and small) increased in PGT14 and PGT28 cows compared to the control group (P<0.05). During the ovulatory wave, number of total and small follicles increased in PGT28 (P<0.05) and PGT21 (P<0.10) compared with PGT14 and control cows. However, treatments did not affect the number of medium and large follicles (P>0.05). Size of the largest follicle at first wave was greater in PGT28 (P>0.05) and PGT14 (P>0.05), and PGT21 (P<0.10) cows compared to the control group. Maximal size of the ovulatory follicle was greater in PGT28 (P<0.05) and PGT21 (P<0.10) groups compared to the control group. Growth rate of the largest follicle at first wave was higher (P<0.05) in PGT-treated cows compared to control cows, and tended (P<0.01) to be higher in PGT28 than PGT21 group. However, growth rate of the ovulatory wave was higher (P<0.05) in PGT28 and PGT21 groups

as compared to the control group. Interval between emergence of last wave and ovulation, and days from luteolysis to ovulation were decreased ($P<0.05$) in PGT28 and PGT21 compared to the control group (Table 2).

Table 3 shows the effect of PGT on CL diameter, functionality, and progesterone concentration. Maximal CL diameter and period of CL activity were not affected by the treatment. Oral administration of PGT increased ($P<0.05$) mean and maximal concentration of P_4 in the cows that received PGT from 7 d before the initiation of the cycle (PGT14 and PGT28) compared to the control animals. However, mean concentration of plasma P_4 tended to increase in PGT21 compared to the control group, and in PGT28 compared to PGT21. Administration of PGT decreased the number of days to maximal plasma P_4 concentration and duration of CL activity to maximal plasma P_4 concentration in PGT28 and PGT14 compared to the control group. However, both PGT14 and PGT28 tended to reach maximal plasma P_4 concentration sooner than PGT21 group (Table 3).

At least a 14 d period of PGT administration (7d pre and 7d post estrus) was required to affect plasma concentration of P_4 (Fig. 2). Following a 14 d period of PGT administration (d 7 of cycle), plasma concentration of P_4 was significantly increased in PGT14 and PGT28 as compared to the control group. The significant difference between PGT28 and the control group was maintained up to d 11 of the estrous cycle, but the slope of P_4 concentration decreased in PGT14 after d 7, resulting in a non-significant difference in P_4 concentration compared to the control group (Fig. 2).

4. Discussion

Previous investigations (Smith et al., 2007; Smith et al., 2009; Schoenberg and Overton, 2011; Yousefi et al., 2016) on the effect of TZDs administration during the transition period in dairy cows reported improvements in the ovarian activity and reproduction, often attributed to the systemic effect of TZDs on hormonal or metabolic status (Smith et al., 2007; Smith et al., 2009).

In the experiment reported here, greater number of small follicles during first follicular wave and increased progesterone concentration were observed in PGT14 and PGT28, but not in PGT21 cows. This suggests the importance of pre-estrual supplementation of PGT on stimulating the ovarian activity which might have resulted in the recruitment of more first wave follicles. This assumption is supported by the higher number of small follicles in PGT28 and PGT21, but not in PGT14 cows, during ovulatory wave; where PGT28 and PGT21 cows were supplemented with PGT before and during the last follicular wave. Meanwhile, the higher number of total follicles observed during the first and last follicular wave may be ascribed to the effect of PGT on the number of small follicles. These data are supported by the findings that prepartal administration of TZDs enhanced the resumption of ovarian cyclicity in postpartum dairy cows (Smith et al., 2009; Yousefi et al., 2016). It has been shown that a period of 10-14 d of PGT supplementation is required to modulate metabolism in dairy cows (Arévalo-Turrubiarte et al., 2012; Ghoreishi, 2012; Yousefi et al., 2016), and PPAR expression is greater in small antral follicles (Froment et al., 2003; Singh et al., 2013). Short-term supplementation of domestic animals with high energy diets or diets supplemented with polyunsaturated fatty acids enhanced the ovarian activity and improved reproductive performance, mainly through activation of PPARγ (Downing and Scaramuzzi, 1991; Mattos et al., 2000; Froment et al., 2006; Singh et al., 2013). In addition, TZDs has been shown to improve insulin sensitively and IGF-I production (Smith et al., 2007; Schoenberg and Overton, 2011; Yousefi et al., 2016) which are crucial for growth of the ovarian follicles (Kawashima et al., 2007).

Greater diameters of the ovulatory follicle and also the largest follicle at first wave in the PGT-treated groups could be explained by the effect of PPAR ligands on follicular development, especially during the final stages of follicular growth (Komar et al., 2001; Komar and Curry, 2002; Froment et al., 2003; Komar, 2005; Froment et al., 2006). It is worth noting that during the first follicular wave, both growth rate and size of large follicles were increased in PGT-treated cows. However, during the ovulatory wave, growth rate and maximum size of ovulatory follicles increased only in PGT28 and PGT21 groups compared with the control and PGT14 groups. This may be an indication of a close relationship between late-stage follicular growth and activation of PPARγ in dairy cows. In agreement with these results, it has been reported that administration of natural ligands of PPARs increased the number of follicle, and stimulated the growth of preovulatory follicle (Singh et al., 2013). Moreover, PPARγ ligands seem to support follicular maturation and CL functionality (Gasic et al., 1998; Viergutz et al., 2000; 2002; Froment et al., 2003).

Positive effects of PGT on follicular development, especially the dominant and ovulatory follicles, has probably decreased duration of last wave emergence and ovulation, and also in the number of days from luteolysis to ovulation, the condition that may lead to maturation of an ovulating oocyte with greater fertility potential (Dirandeh et al., 2015). It has been shown that a prolonged lifespan of the ovulatory follicle under low circulating P_4 results in reduced fertility (Mihm et al., 1994). However, it is not clear why PGT supplementation did not affect the number of medium and large follicles. Probably, by improving the metabolic and endocrine conditions (Smith et al., 2007; Schoenberg and Overton, 2011; Yousefi et al., 2016), PGT stimulated follicular recruitment and increased the number of follicles undergoing growth from resting pool, and subsequently PGT might affected size of large and preovuratory follicles though a direct effect on the receptor, where PPARγ expression in preovulatory follicles progressively increases (Komar et al., 2001; Cui et al., 2002; Komar and Curry, 2002; Froment

et al., 2003; Komar, 2005; Froment et al., 2006). In addition, the greater growth rate of the large follicle at first wave and also ovulatory follicles which caused a greater follicle size during a shorter period may increase estradiol production and enhance domination effect of the largest follicle on subordinate follicles (Lucy, 2007). This may explain the non-significant effect of PGT on the number of medium and large follicle, and also shows that treating cows with PGT may not impair the dominance process in dairy cows as an animal with true dominant follicle (Mihm and Bleach, 2003; Fortune et al., 2004).

Higher level of maximum P_4 concentration observed at shorter time interval after ovulation was probably due to higher efficiency of P_4 production under PGT stimulation. Oral administration of TZD did not affect the CL size, but plasma P_4 levels were 20 to 30% higher than in control animals. In this regard, Smith et al. (2009) showed that prepartal administration of 2-4 mg of TZD/kg BW did not affect postpartal CL size in dairy cow. Moreover, in agreement with this finding, *in vitro* studies suggested that synthetic PPARγ ligands affected the luteal differentiation and functionality rather than cellular proliferation (Komar et al., 2001; Froment et al., 2003). It has been widely accepted that there are no direct correlation between the size of the CL and circulating P_4 (Sartori et al., 2004; De Tarso et al., 2016). However, positive effects of TZDs on *in vitro* hormone synthesis by granulosa (Gasic et al., 1998; Froment et al., 2003) or luteal (Lohrke et al., 1998; Schoppee et al., 2002) cells were reported. *In vitro* studies suggested that PPAR enhanced steroidogenesis in rodent granulosa cells (Komar et al., 2001; Froment et al., 2003), mainly through increasing the expression and activity of the steroidogenic enzymes, and/or speeding up cholesterol transport (Gasic et al., 1998; Froment et al., 2003), such as cyclooxygenase-2 (Meade et al., 1999), plasminogen activator (Xin et al., 1999), or vascular endothelial growth factor (Yamakawa et al., 2000). In agreement with the results of the present study, a 1.6-fold increase in P_4 production in ovine granulosa cells (Froment et al., 2003), and 14-fold increase in P_4 and 30% increase in estradiol

production by rat granulosa cells (Komar et al., 2001) were reported. However, troglitazone inhibited *in vitro* P_4 secretion from the porcine and human granulosa cells (Gasic et al., 1998; Froment et al., 2003). These contradictory findings are likely due to species differences, the state of differentiation of the granulosa cells, differences between *in vitro* models, and/or type of the PPAR ligands used.

It is noteworthy that cows receiving PGT from the first day of cycle (PGT21) recorded lower plasma concentration of P_4 as compared to PGT28 cows that received PGT from 7 d before the initiation of the estrous cycle (Table 1). Similar finding was observed in PGT14 during the first half of the cycle (Fig. 2). The PPARs are expressed at different stage of the follicular development (Cui et al., 2002; Froment et al., 2003; Froment et al., 2006), and their increased expression in preovulatory follicles and CL may impact on the functional capacity of the newly formed CL (Cui et al., 2002). This hypothesis is supported by the positive effect of PGT supplementation on the size of the largest follicles at first wave and ovulatory follicle at last waves in the PGT-treated cows.

In addition to the effects of PGT on ovary which was discussed above, it is worth noting that insulin resistance, starting at the prepartum period is continued into early and mid-lactation of dairy cows (Chalmeh et al., 2015). The degree of insulin resistance at postpartum period results in more net lipolysis from the adipocytes (Chalmeh et al., 2015), and its levels depends on several factors such as lactation number, BCS, level of milk yield, and DM intake (Jamali Emam Gheise et al., 2017). Although these factors were considered in assigning the animals to the treatment groups, and also in the statistical model, direct measuring of insulin resistance and application of higher number of cows per group may be conductive to more precise interoperation of the treatments' effect on dependent variables such as P_4 level and folliculogenesis. Finally, there are several reports published on the effects of TZDs on metabolism and reproduction of dairy cows (Kushibiki et al., 2001; Smith et al., 2007; Smith

et al., 2009; Schoenberg and Overton, 2011; Ghoreishi, 2012; Singh et al., 2013; Yousefi et al., 2015; Yousefi et al., 2016; Jamali Emam Gheise et al., 2017); however, no study was conducted to figure out the level of TZDs released into the cow's milk.

5. Conclusion

In conclusion, oral administration of PGT increased the number of small follicles and diameter of the largest follicle at the first and ovulatory waves. PGT increased postovulatory plasma P_4, but decreased duration of the last wave, and days to maximal concentration of plasma P_4. Pioglitazone treatment at least for 7 d before the initiation of the ovarian cycle had greater effect on the ovarian activity and CL functionality. It seems that by activating the PPARγ, PGT exerted positive effects on follicular development and CL steroidogenesis. It is implied that at least a part of the beneficial effects of TZD on reproductive performance which was reported in the previous studies may be due to the direct effects of PPARγ ligands on follicular dynamics and CL activity. However, further experiments are required to determine the molecular effects of PPARγ ligands on ovarian function and reproduction in dairy cows.

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Figure captions

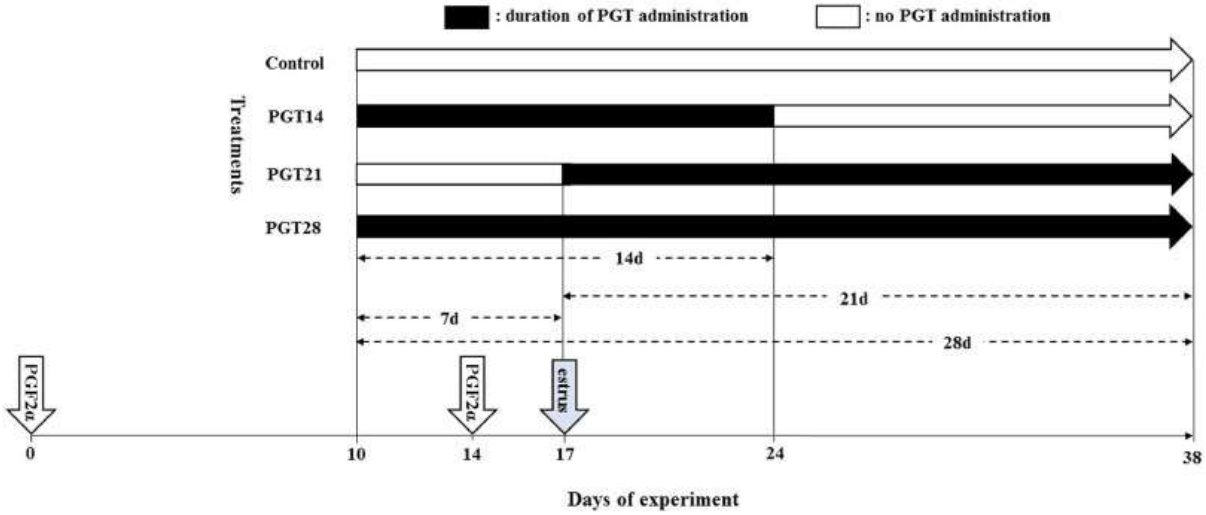

Fig. 1. An outline of the experimental design indicating initiation of treatment administration and duration of pioglitazone (PGT) supplementation. Dairy cows (n=7/group) were not treated (control), or orally treated with pioglitazone (6 mg/kg BW per day) for 14 d started from 7 d before to 7 d after synchronized estrus (PGT14), or for 21 d after observed estrus (PGT21), or for 28 d started from 7 d before expected estrus and continued to 21 d after observed estrus (PGT28). Note: Blood samples were collected daily for 21 d, starting on the first day of the estrous cycle. Ovaries were scanned daily by transrectal ultrasonography from one day before expected estrus (d 16 of the experiment) to 21 d after observed estrus.

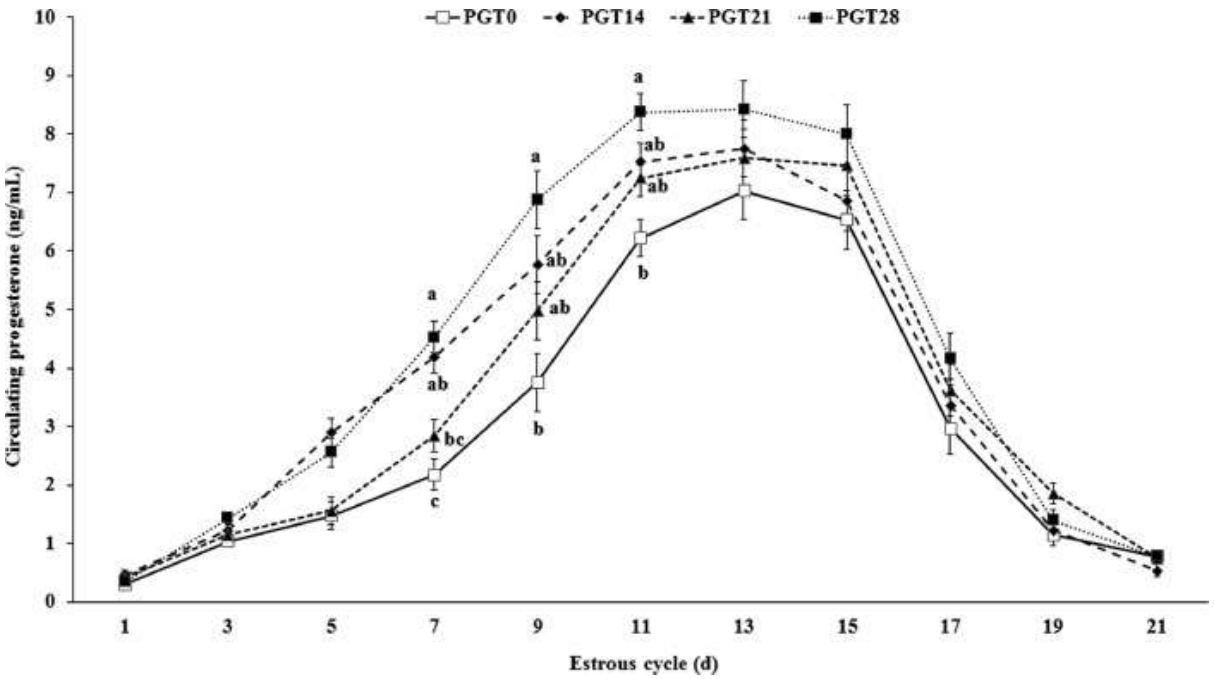

Fig. 2. Effect of daily oral administration of pioglitazone (6 mg/kg BW per day) on plasma concentration of progesterone during the estrous cycle (n=7 cows/group). Details of the treatments are given in Fig. 1. Different letters (a,b) within the each week indicate significant differences $P < 0.05$. Note: P values for the effects of treatment, time, and interaction between treatment and time were < 0.01.

Table 1. Ingredients and chemical composition of the experimental diet

Dietary ingredients	**% DM**
Alfalfa hay (mid-bloom)	20.25
Corn silage (10% grain)	18.28
Ground barley grain	19.73
Ground corn grain	3.46
Beet pulp	5.75
Soybean meal	7.96
Canola meal	7.27
Corn gluten meal	1.73
Meat meal	1.73
Cottonseed, whole	2.94
Wheat grain	2.94
Fish meal	1.11
Megalac (rumen protected fat)	1.73
Wheat bran	0.24
Vitamin and mineral premix[a]	0.76
Sodium bicarbonate	1.00
Salt	0.24
Limestone	0.48
Dicalcium phosphate	0.35
Zeolite	1.73
Biotin supplement	0.09
MycoSorb[b]	0.07
Magnesium oxide	0.17
Diet composition	
DM, %	59
NEL (Mcal/kg DM)	1.63
CP, %	18.5
ADF, %	18
NDF, %	30
NFC, %	35
Calcium, %	1.31
Phosphorus, %	0.63

[a] Contained (per kg): 16,000,000 IU vitamin A; 3,200,000 IU vitamin D; 48,000 IU vitamin E; 24.0 g Mn; 24.0 g Zn; 24.0 g Fe; 12.8 g Cu; 1.44 g I; 0.32 g Se; and 0.32 g Co.

[b] Mycotoxin binder, contained: 95% silicates (calcium silicate, sodium and aluminum silicate, silicate acid); 5% yeast extract.

Table 2. Least squares means for the effect of oral administration of pioglitazone (6 mg PGT/kg BW per day) on follicular dynamics in Holstein dairy cows (n=7 per treatment[a])

Item	Control	PGT14	PGT21	PGT28	SEM
Interovulatory interval (d)	22.28	22.14	21.57	21.45	0.50
First wave					
Number of small follicles (< 5 mm)	$8.62^{b,B}$	$10.90^{a,A}$	$8.90^{ab,B}$	$11.13^{a,A}$	0.86
Number of medium follicles (5-10 mm)	3.46	4.59	4.61	3.41	0.58
Number of large follicles (>10 mm)	0.67	0.97	0.96	1.14	0.25
Total number of follicles	12.78^{b}	16.64^{a}	14.42^{ab}	15.60^{a}	1.11
Size of the largest follicle (mm)	$11.43^{b,B}$	$14.03^{a,A}$	$13.28^{ab,A}$	$14.71^{a,A}$	0.56
Growth rate of the largest follicle (mm/d)	$0.92^{b,C}$	$1.45^{a,AB}$	$1.27^{a,BC}$	$1.56^{a,A}$	0.09
Ovulatory wave					
Number of small follicles (< 5 mm)	$8.21^{b,C}$	$8.87^{b,BC}$	$10.53^{ab,A}$	$11.79^{a,A}$	1.04
Number of medium follicles (5-10 mm)	3.19	3.70	4.90	3.65	0.51
Number of large follicles (>10 mm)	0.68	0.80	0.63	0.91	0.14
Total number of follicles	13.01^{b}	13.20^{b}	16.33^{a}	16.18^{a}	1.08
Maximal size of ovulatory follicle (mm)	$14.58^{b,B}$	$15.02^{b,B}$	$16.85^{ab,A}$	$17.71^{a,A}$	0.52
Growth rate of ovulatory follicle (mm/d)	1.21^{b}	1.28^{b}	1.67^{a}	1.78^{a}	0.10
Interval from last wave to ovulation (d)	11.42^{a}	10.68^{ab}	9.91^{b}	9.80^{b}	0.40
Days from luteolysis to ovulation	5.71^{a}	5.01^{ab}	4.14^{b}	4.50^{b}	0.29

Different letters (a, b) within the same row indicate significant differences $P < 0.05$.

Different letters (A–C) within the same row indicate significant differences $P < 0.10$.

BW = body weight; SEM = standard error of mean.

[a] Details of the treatments are given in Fig. 1.

Table 3. Least squares means for the effect of oral administration of pioglitazone (6 mg PGT/kg BW per day) on corpus luteum (CL) diameter and circulating progesterone levels in Holstein dairy cows (n=7 per treatment[a])

Item	PGT0	PGT14	PGT21	PGT28	SEM
Maximum diameter of CL (mm)	17.05	19.50	17.42	20.21	2.02
Period of CL activity (d)	14.42	14.28	14.91	14.75	0.46
Mean plasma P_4 concentration (ng/mL)	$3.01^{c,C}$	$3.80^{ab,AB}$	$3.59^{bc,B}$	$4.29^{a,A}$	0.17
Maximum concentration of plasma P_4 (ng/mL)	7.07^{b}	7.99^{a}	7.35^{ab}	9.04^{a}	0.60
Days to maximal concentration of plasma P_4 (d)	$13.57^{a,A}$	$11.28^{b,B}$	$13.28^{ab,A}$	$11.04^{b,B}$	0. 58
Period of CL activity to maximal plasma P_4 concentration (d)	$10.57^{a,A}$	$8.10^{b,B}$	$10.28^{ab,A}$	$8.03^{b,B}$	0.86

Different letters (a,b) within the same row indicate significant differences $P < 0.05$.

Different letters (A–C) within the same row indicate significant differences $P < 0.10$.

BW = body weight; SEM = standard error of mean.

[a] Details of the treatments are given in Fig. 1.