



# Evaluation of the relationship between serum paraoxonase-1 activity and superovulation response/embryo yield in Holstein cows

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**ABSTRACT.** In this study, the effect of serum paraoxonase-1 (PON-1) activity on superovulation response and embryo yield was evaluated. The study material comprised 50 Holstein cows aged 3–4 years on postpartum day 90–120 with a body condition score of 3–3.25. A progesterone-based estrus synchronization protocol was initially administered to the selected donors. For this purpose, progesterone source was inserted intravaginally (day 0) and gonadotropin-releasing hormone injection was performed (day 6). Seven days after the insertion of progesterone device, follicle-stimulating hormone injections (total dose of 500 µg in decreasing doses for 4 days) were administered for superovulation. On the morning of the ninth day, prostaglandin (PG) F<sub>2α</sub> was administered, and the progesterone device was removed from the vagina in the evening on the same day. Two days after PGF<sub>2α</sub> administration, fixed-time artificial insemination was performed in the morning and in the evening. On the day of artificial insemination, blood samples were taken from the donors to determine the serum PON-1 activity. Uterine flushing was performed seven days after insemination. The results revealed that the serum PON-1 activity (mean ± SD, 562.71 ± 140.23 U/l) of the cows that responded to superovulation (donors with total corpus luteum count of ≥3 in both ovaries) was higher than those (389.91 ± 80.51 U/l) that did not ( $P < 0.05$ ). On the day of insemination, a positive correlation was determined between serum PON-1 activity and the counts of total corpus luteum ( $r = 0.398$ ), total oocyte/embryo ( $r = 0.468$ ), transferable embryo ( $r = 0.453$ ), and Code I embryos ( $r = 0.315$ ,  $P < 0.05$ ). Unlike the Code I embryos, there was no significant correlation between serum PON-1 activity and the number of Code III embryos. Moreover, no significant difference in the number of Code III embryos between the two PON-1 groups was observed. However, embryo yield and quality were found to have increased with increased PON-1 activity. Therefore, it was concluded that serum PON-1 activity may be associated with superovulation response, embryo yield and quality in donor cows.

**KEY WORDS:** cow, embryo, Holstein, paraoxonase-1, superovulation

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Embryo technology is an assisted reproductive technique involving the transfer of embryos obtained from high genetic merit to recipient animals [9, 19]. The most important factors leading to the widespread use of embryo transfer in animal husbandry include variations in superovulation response and the number of transferable embryos, in addition to the conception rates achieved after transfer among individual animals. These differences considerably affect the implementation cost and feasibility under field conditions [5, 7–9, 22]. Many factors play a role in the occurrence of differences in the superovulation response. Certain factors include hormone type and dosage, duration and route of hormone administration, age, lactation period, breed, days in milk, number of antral follicles in the ovary, presence of dominant follicle, presence of corpus luteum (CL), ovary status at the time of treatment, and nutrition and administration season [7, 8, 22, 35, 38].

It is known that reactive oxygen species (ROS) are involved in several reproductive physiological systems in the body. However, oxidative stress manifests when an imbalance occurs between the amount of ROS and antioxidant capacity in the body [1, 27]. This may negatively influence the reproductive processes including oocyte maturation, folliculogenesis, ovulation, steroidogenesis, embryo development, and luteolysis [2, 18, 33].

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Paraoxonase-1 (PON-1) is a 354-amino acid glycoprotein, primarily synthesized from the liver, with a molecular weight of 43 kDa [14, 16, 21]. PON-1 is a high-density lipoprotein-related antioxidant enzyme. This enzyme protects lipoproteins against fragmented phospholipids generated during the oxidation of ROS and oxidative stress occurring during the hydrolysis of lipid hydroperoxides [25, 40]. Its protective effect is exerted by blocking or delaying oxidation of low-density lipoproteins and high-density lipoproteins. Thus, it inhibits the oxidative stress process and its occurrence [15, 26]. PON-1 has been determined to influence follicular development, ovulation, fertility, uterine health, embryo development, and pregnancy process in cows [12, 20, 23, 34, 41]. Nevertheless, although it has been established in studies that PON-1 activity affects fertility parameters, no study has evaluated its effect on superovulation response in cows. Therefore, the aim of this study is to evaluate the effect of serum PON-1 activity at the time of artificial insemination on the superovulation response and to determine its effect on embryo yield and quality.

## MATERIALS AND METHODS

This study was performed with the permission and approval of Experimental Animals Production and Research Center Ethics Committee of Selcuk University Faculty of Veterinary Medicine.

### Animals

The study was conducted between January and March 2019 in Aksaray (Turkey). The donors used in the study were maintained in paddocks and in groups of five separately from other animals and fed on uniform ration. The rations of the animals were prepared with total mix ration and feeding was done twice a day. Feed amount in the feeder was verified at regular intervals after feeding. Moreover, it was verified whether there were animals that could not reach the feed. The rations included corn silage (30–35% dry matter [DM], 7% crude protein [CP]), alfalfa silage (40% DM, 18% CP), dry clover (90% DM, 20% CP), wheat straw (90% DM, 4.5% CP), vetch dry grass (65% DM, 15% CP), and concentrated feed (19% CP and 2700 kcal / kg DM metabolizable energy [ME]). In total mixed ration, CP was 15% and ME was 2.3 Mcal / kg DM. The nutrient requirements of the animals were calculated as per the national research council [29]. Moreover, the nutritional content of the feeds was checked at regular intervals.

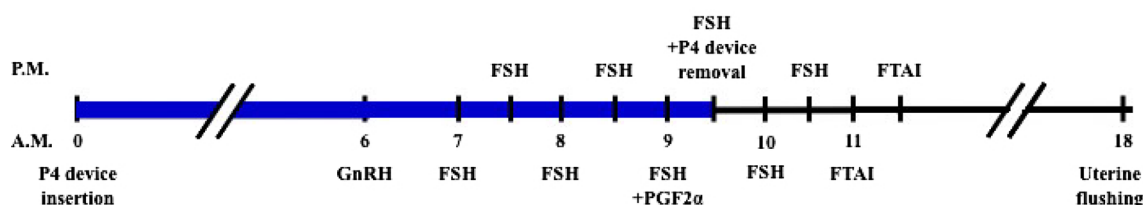
Fifty Holstein cows aged 3–4 years, mean milk yields  $28.55 \pm 4.23$  kg (mean  $\pm$  SD), on postpartum 90–120 days with a body condition score of 3–3.25 were included in the study. Before beginning synchronization, genital organs of the candidate donor cows were evaluated by rectal palpation and ultrasonographic examination (6.0 MHz linear probe, Falcovet, Pie Medical, Maastricht, The Netherlands). During the examinations, cows without any problems in the ovaries, uterus and cervix and with palpable CL in the ovary were selected as donors.

### Synchronization and superovulation protocol

Synchronization involved the administration of progesterone-based (9-day) protocol to the donors (Fig. 1) [3, 6]. First, the progesterone device (1.38 g, Eazi-Breed CIDR, Zoetis, Parsippany, NJ, USA) was placed intravaginally (day 0), and later gonadotropin-releasing hormone (10  $\mu$ g, Buserelin Acetate, Receptal, MSD, Kenilworth, NJ, USA) was intramuscularly injected (day 6). Seven days after the insertion of progesterone device, follicle-stimulating hormone (FSH, Stimufol, Reprobiol SPRL, Ouffet, Belgium) injection was started for superovulation. Totally 500  $\mu$ g FSH was intramuscularly administered in decreasing doses (100–100, 75–75, 50–50, 25–25  $\mu$ g) at 12-hr intervals for four days. Nine days after progesterone administration, prostaglandin (PG) F2 $\alpha$  (Cloprostenol, Interhas, Ankara, Turkey) was intramuscularly given in the morning, and progesterone device was removed from the vagina in the evening of the same day. Fixed-time artificial insemination (FTAI) was performed twice with selected bull sperm at 12-hr intervals 48 hr after PGF2 $\alpha$  administration. All artificial inseminations were conducted by an experienced technician with frozen semen from the same Holstein bull.

### Blood sample collection

During artificial insemination, blood samples (10 ml) were collected from vena coccygea into tubes. These samples were centrifuged at  $3,000 \times g$  for 15 min for serum separation. The separated sera were maintained at  $-80^{\circ}\text{C}$  until analysis.



**Fig. 1.** Synchronization and superovulation protocol applied to donors. (P4: progesterone, GnRH: gonadotropin-releasing hormone, FSH: follicle-stimulating hormone, PGF2 $\alpha$ : prostaglandin F2 $\alpha$ , FTAI: fixed-time artificial insemination).

### *Uterine flushing*

Uterine flushing procedure was performed on the seventh day after artificial insemination. First, the CL counts in the ovaries were determined by ultrasonographic examination and rectal palpation. Donors with total CL count of  $\geq 3$  in both ovaries were considered as superovulation positive. Upper epidural anesthesia was applied to donors who responded to superovulation (5–8 ml lidocaine HCl, Adocaine, Sanovel, Istanbul, Turkey). A balloon catheter (2-Way Foley Catheter, Silicone, 16–20 inc, Minitube, Tiefenbach, Germany) was then inserted into the uterine horn. The uterus was flushed several times with lactated ringer (Polifarma, Tekirdag, Turkey) (1% calf serum + 200 mg kanamycin), and embryos were collected into the filter (EmCon Filter, 75  $\mu\text{m}$ , Agtech, Manhattan, KS, USA).

### *Evaluation and classification of bovine embryos*

The flushed material was taken onto Petri dishes and scanned under a stereomicroscope (Leica S Apo, Wetzlar, Germany). The embryos were evaluated according to the International Embryo Technology Society criteria [10]. The assessment of embryo quality was made according to morphological integrity. Code I (excellent or good) corresponded to the very low levels of irregularity between the cells, a ratio of  $>85\%$  viable embryonic cells, and a round and unfolded zona pellucida. Code II (fair) is characterized by a medium level of irregularity between the cells and a viable cell ratio of 50%. Code III (poor) is characterized by irregularities in the form of the embryo and a viable cell ratio of 25%. Code IV (dead or degenerated) is characterized by extremely dark cytoplasm, nonintact cell membranes, and other significant defects. Unfertilized oocytes (UFO) were designated when there were no signs of cleavage. According to these criteria, Code I, II and Code III embryos were considered to be of transferrable quality.

### *Determination of serum PON-1 activity*

PON-1 activity was measured using commercially available kits (Relassay, Gaziantep, Turkey). Fully automated paraoxonase activity measurement method comprises two different sequential reagents. The first reagent is an appropriate Tris buffer and it contains calcium ion, which is a cofactor of PON-1 enzyme. The second reagent is a newly developed stable substrate solution. The sample is mixed with Reagent 1 and the substrate solution is added to it. The linear increase of the absorbance of p-nitrophenol, produced from paraoxon, is followed at the kinetic measurement mode. Non-enzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. The rate of paraoxon hydrolysis (diethyl p-nitrophenyl phosphate) was measured by monitoring the increase in absorption at 412 nm at 37°C. The amount of generated p-nitrophenol was calculated from the molar absorption coefficient at pH 8.5, which was 18.290  $\text{M}^{-1} \text{cm}^{-1}$ . PON-1 activity was expressed as U/l serum.

### *Statistical analyses*

SPSS 25 (IBM Corp. (Armonk, NY, USA) Released 2017. IBM SPSS Statistics for Windows, Version 25.0) statistical package program was used to evaluate the data. Mean  $\pm$  standard deviation and median (maximum–minimum) percentage values of the variables (Total oocyte/embryo, transferable embryo, Code I, II, III, and IV embryos and UFO counts) were calculated. Variables were evaluated after preconditions of normal distribution and homogeneity of variance were verified (Shapiro-Wilk and Levene Tests). During data analysis, independent two-group *t*-test (Student's *t*-test) was used for comparison of two groups, and Mann-Whitney *U* test was used when preconditions were not met. The relationship between the two continuous variables was evaluated with the Pearson's correlation coefficient and if the parametric test preconditions were not met, the Spearman correlation coefficient was used.  $P < 0.05$  was considered as the significance levels of tests.

## RESULTS

In this study, 12 (24%) of 50 cows used as donors did not respond to superovulation treatments ( $< 3$  CL). Serum PON-1 activities of the responder and non-responder cows were determined to be  $562.71 \pm 140.23$  and  $389.91 \pm 80.51$  U/l, respectively ( $P < 0.05$ ).

Table 1 shows superovulation response, embryo yield, and quality results obtained on the uterine flushing day. Table 2 shows the correlation between serum PON-1 activity and the counts of total CL, total oocyte/embryo, transferable embryos, Code I embryos, Code II embryos, Code III embryos, Code IV embryos and UFO. According to these results, a positive correlation was found between PON-1 and total CL, total oocyte/embryo, transferable embryo, and Code I embryo counts (Fig. 2;  $P < 0.05$ ).

In this study found that the mean serum PON-1 activity on the day of insemination of donors exposed to superovulation was  $510.87 \pm 147.9$  U/l. Donors were divided into two subgroups according to the measured mean PON-1 activity (Table 3). In addition to these results, in donors with PON-1 activity  $> 510$  U/l, the counts of total oocyte/embryo, transferable embryo, Code I and II embryos and fertilization rate were found to be higher than those with PON-1 activity  $\leq 510$  U/l ( $P < 0.05$ ).

## DISCUSSION

PON-1 is known to play a role in many physiological and pathological reproductive conditions [34, 36, 41]. In this study, the effect of the PON-1 activity on superovulation response and embryo yield was investigated. In this study, the average PON-1 activity of superovulated donors was found to be lower than the PON-1 activity obtained in previous studies in cattle [34, 41]. However, the reason for this difference is considered to be attributed to the conditions of the cattle included in other studies such as lactation period, pregnancy, estrus cycle and disease. Moreover, PON-1 activity was reported to be higher in cows that responded to superovulation than those that did not. These results demonstrated that the activity of PON-1 had an impact on superovulation

**Table 1.** Descriptive statistics for the day of uterine flushing in donor cows treated with superovulation

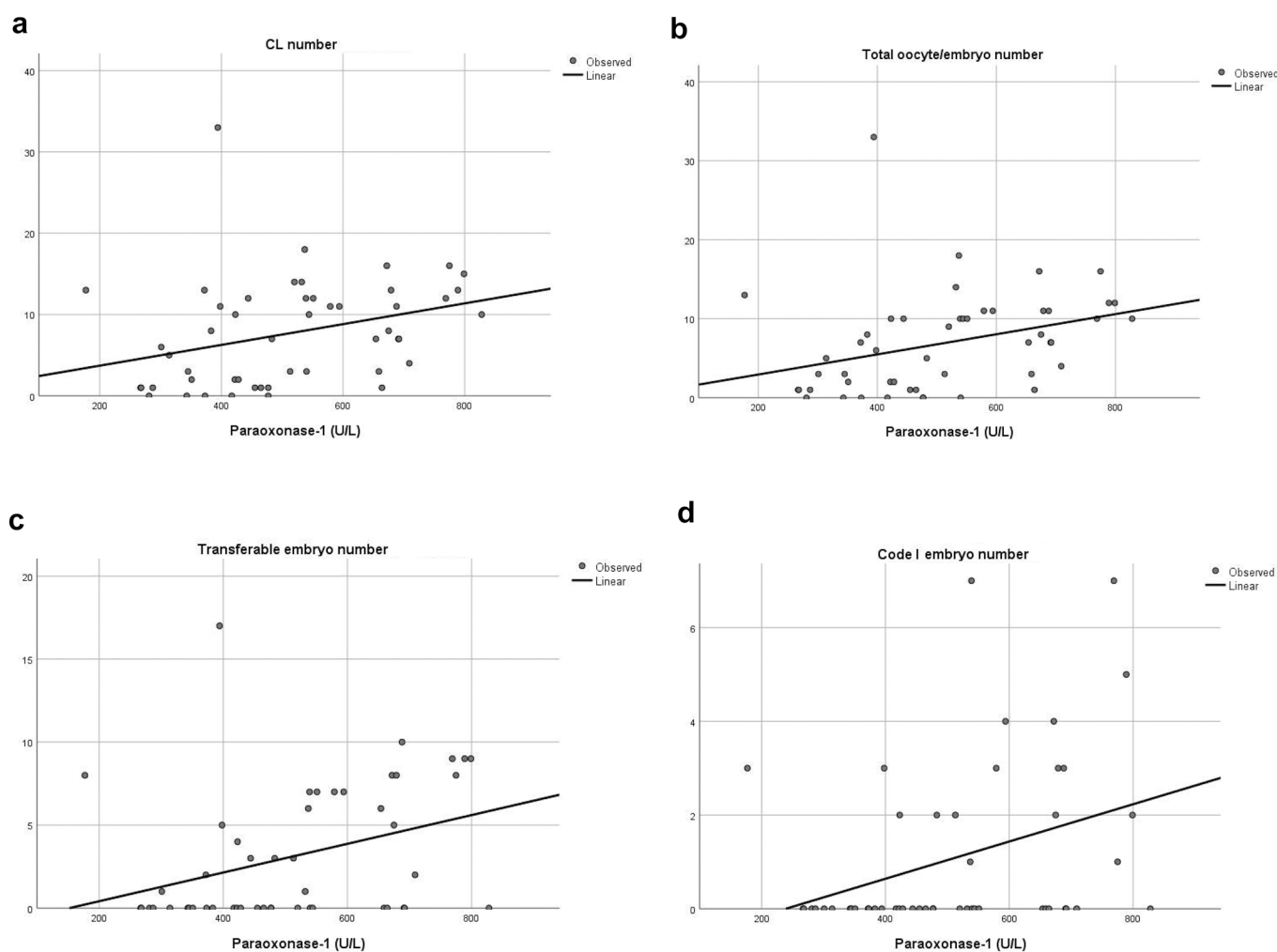
Parameters	N	Mean	Std. deviation	Min	Max
Total CL	50	10.17	3.16	0	33
Total oocyte/embryo	50	8.13	2.96	0	33
Fertilization rate (%)	50	82.56	34.54	0	100
Transferable embryos	50	4.14	2.75	0	17
Code I embryos (Excellent or good)	50	1.71	1.71	0	7
Code II embryos (Fair)	50	1.50	1.06	0	7
Code III embryos (Poor)	50	0.92	0.69	0	10
Code IV embryos (Dead or degenerated)	50	2.47	1.76	0	15
UFO	50	1.53	0.90	0	8

CL, corpus luteum; UFO, unfertilized oocytes.

**Table 2.** Correlation between paraoxonase-1 activity and the counts of total corpus luteum (CL), total oocyte/embryo, transferable embryos, Code I embryos, Code II embryos, degenerating embryos, and unfertilized oocyte

	Total CL	Total oocyte/ embryo	Fertilization rate	Transferable embryos	Code I embryos	Code II embryos	Code III embryos	Code IV embryos	UFO
Paraoxonase-1 r	0.398*	0.468*	0.099	0.453*	0.315*	0.223	-0.008	-0.125	-0.112

\*Correlation is significant at the 0.05 level. UFO, unfertilized oocytes.



**Fig. 2.** The correlation between the paraoxonase-1 activity and the number of corpus luteum (CL) (a), total oocyte/embryo (b), transferable embryo (c) and Code I embryo (d).

**Table 3.** Superovulation findings obtained on the day of uterine flushing based on mean paraoxonase-1 activity

Parameters	Paraoxonase-1 (U/l, mean $\pm$ SD)		P-value
	$\leq 510$ (n=24)	$> 510$ (n=26)	
Total CL	8.62 $\pm$ 3.17	10.8 $\pm$ 5.20	NS
Total oocyte/embryo	6.75 $\pm$ 2.70	9.43 $\pm$ 3.47	<0.05
Fertilization rate (%)	73.49 $\pm$ 25.76	88.32 $\pm$ 15.43	<0.05
Transferable embryos	3.32 $\pm$ 1.14	4.95 $\pm$ 2.68	<0.05
Code I embryos (Excellent or good)	1.30 $\pm$ 0.42	2.14 $\pm$ 0.59	<0.05
Code II embryos (Fair)	1.18 $\pm$ 0.39	1.79 $\pm$ 0.84	<0.05
Code III embryos (Poor)	0.85 $\pm$ 0.25	1.01 $\pm$ 0.76	NS
Code IV embryos (Dead or degenerated)	2.21 $\pm$ 1.16	2.70 $\pm$ 1.23	NS
UFO	1.22 $\pm$ 0.50	1.78 $\pm$ 0.65	NS

CL, corpus luteum; UFO, unfertilized oocytes; NS, not significant.

response. Some studies reported that PON-1 activity may be low because of changes in lipid metabolism [25, 40, 41]. This is because triglyceride, total cholesterol, and high-density lipoprotein-cholesterol concentrations affect PON-1 activity and its effectiveness [41]. Moreover, Turk *et al.* [41] reported that the lower high-density lipoprotein concentration could be one of the causes of reduced paraoxonase activity considering the role of high-density lipoprotein as a carrier of most paraoxonase molecules in the blood. A decreased serum paraoxonase activity could diminish the effectiveness and total capacity of the whole antioxidative system. In this study, it is considered that the low serum paraoxonase activity may have affected the superovulation response by causing a decrease in the capacity and effectiveness of the total antioxidant system. This is because the decrease in the antioxidant capacity and change in the lipid metabolism may lead to an interruption in several reproductive processes. Because decreased antioxidant capacity negatively affects physiological events such as oocyte maturation, follicular atresia, fertilization, embryo development, maintenance and regression of luteal tissue [2, 13, 33].

Studies in humans [28] and cows [34] have reported that paraoxonase activity had an impact on follicle development. Therefore, it is possible that the large number of high-quality follicles during artificial insemination in cattle affect the number of CL and embryos after ovulation. In this study, it was also determined that there was a positive correlation between serum PON-1 activity at the time of artificial insemination and the counts of total CL ( $r=0.398$ ) and oocyte/embryo ( $r=0.468$ ) retrieved on the day of uterine flushing. Moreover, the number of oocyte/embryos retrieved from cows with a PON-1 activity  $>510$  U/l were found to be higher than those with a PON-1 activity  $\leq 510$  U/l. Aydin *et al.* [4] and Okuducu [30] have reported that the number of oocytes retrieved from women with high antioxidant capacity levels was higher. Meijide *et al.* [28] have reported that greater number of oocytes was collected from women with high PON-1 activities on the day of oocyte acquisition. They have proposed that this may be attributed the improved follicle development and quality in women with high PON-1 activities. Moreover, Schneider *et al.* [34] indicated that PON-1 activity in the follicular fluid of estrogen active follicles is high in dairy cattle. Therefore, it was considered that the counts of total CL and oocyte/embryos retrieved on the day of uterine flushing may be higher in cattle with high PON-1 activities during artificial insemination in this study as it supports follicle development and protects the oocytes against oxidative stress.

In this study, it was reported that there was a positive correlation ( $r=0.453$ ) between PON-1 activity and the number of transferable embryos and that the number of transferable embryos increased as the PON-1 activity increased. These results indicate that oxidative stress has harmful effects on oocytes and embryos, and thus a greater number of transferable embryos are obtained in cows with high antioxidant levels because high oxidative stress is known to negatively affect oocyte and embryo development [13, 33, 37]. Moreover, Yoon *et al.* [42] reported that oxidative stress in *in vitro* embryo production reduces blastocyst formation and cell survival rates in bovine. Furthermore, Rincón *et al.* [32] have reported that the absence of paraoxonase during oocyte maturation results in decreased embryo development in *in vitro* embryo production. Moreover, they have reported that depending on the dose administered, the addition of paraoxonase during *in vitro* maturation increases embryo development and the rate of embryos reaching blastocyst [31]. Because Browne *et al.* [11] reported that PON-1 may exert protective effects on overall oocyte competence, especially at the oxidative stress level.

In this study, it was determined that there was a positive relationship between PON-1 activity on the day of artificial insemination and embryo quality. In particular, a positive correlation was found between PON-1 activity and Code I quality embryo count. Unlike the Code I embryos, there was no significant correlation between serum PON-1 activity and the number of Code III embryos. Besides, the number of Code I and II embryos obtained from donors with PON-1 activity  $>510$  U/l was higher than those with PON-1 activity  $\leq 510$  U/l. However, no significant difference in the number of Code III embryos between the two PON-1 groups was observed. These data show that PON-1 activity on the day of artificial insemination has an impact on embryo quality. It has been reported in many studies that oxidative stress has a negative effect on embryo quality [17, 18, 33, 39]. Apoptosis was stimulated and the number of living cells of the embryo decreased, particularly when the oxidative stress increased directly or indirectly in the oviduct and embryo [24, 43]. The increase in the number of dead cells of the embryo led to a decrease in the embryo quality [10]. Moreover, Browne *et al.* [11] reported that serum and intrafollicular PON-1 enzyme activity was positively correlated with embryo quality and blastomere count in women undergoing *in vitro* fertilization procedure. In another study, it has been identified that the addition of PON-1 during *in vitro* maturation in cattle in *in vitro* embryo production improves embryo

development by protecting the oocyte membrane from oxidative stress and peroxidative damage [31].

In conclusion, the serum PON-1 activity measured on the day of artificial insemination is associated to the superovulation response, as well as embryo yield and quality. In other words, high PON-1 activity in cattle was reported to be associated with increased counts of total CL, oocyte/embryo, transferable embryo, and Code I embryo.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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