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—Original Article—

Positive correlations of age and parity with plasma concentration of macrophage migration inhibitory factor in Japanese black cows

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Abstract. Plasma Macrophage migration inhibitory factor (MIF) concentration correlates positively with age, and negatively with self-rated health in women, and optimal MIF concentration may promote proper reproductive function. This study was conducted to evaluate the hypotheses that plasma MIF concentration changes with parturition or postpartum first ovulation, and that age in months and parity correlate with plasma MIF concentration in Japanese black cows. Western blotting utilizing an anti-MIF mouse monoclonal antibody of various tissues and plasma from females indicated that MIF expression was stronger in the anterior pituitary than in other tissues. We developed a competitive EIA utilizing the same anti-MIF mouse monoclonal antibody with sufficient sensitivity and reliable performance for measuring bovine plasma samples. We then measured MIF concentrations in bovine plasma collected from 4 weeks before parturition to 4 weeks after postpartum first ovulation. There was no significant difference in plasma MIF concentration pre- and post-parturition, or before and after the postpartum first ovulation. Plasma MIF concentrations were positively correlated ($P < 0.01$) with parity ($r = 0.703$), age in months on the day of parturition ($r = 0.647$), and age in months on the day of the postpartum first ovulation ($r = 0.553$) when we used almost all data, except for that from a third-parity cow with an abnormally high plasma MIF concentration. We therefore concluded that plasma MIF concentrations may increase with age in months and parity, but do not change either before and after parturition or before and after postpartum first ovulation in Japanese black cows.

Key words: Aging, Anterior pituitary, Biomarker, Pancreas, Ruminant

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Improvement of reproductive performance is required in beef cows as well as dairy cows. Age-related decline in fertility is one important factor in beef cows [1], but little is known about the mechanism underlying this phenomenon in domestic animals. Factors reported to promote age-related fertility decline in women include poor oocyte quality, ovulation failure, increasing incidence of meiotic errors and aneuploidy of gametes and embryos, reduced pregnancy rates, and increased pregnancy wastage [2, 3]. However, thus far no useful blood biomarkers for aging in animals and women have been discovered.

Macrophage migration inhibitory factor (MIF) is a 115–amino-acid peptide with a sequence 93% homologous between humans (National Center for Biotechnology Information reference sequence of human MIF, NP_002406.1) and bovines (NP_001028780.1). MIF is expressed in the spleen, adrenal gland, pancreas, kidney, liver, anterior pituitary, and thymus of rodents and humans [4–6]. Contrary to its historical name, MIF has various roles, including growth-promoting activity in various reproductive cells. Indeed, MIF is also expressed in the ovary, uterus, placenta, oocytes, zygotes, early embryos, and fetus [7–11]. Optimal MIF concentration promotes sperm capacitation, whereas low or excess MIF is inhibitory [12]. Furthermore, follicles synthesize and secrete MIF into the blood, and blood MIF concentration in women is higher in the preovulatory period in than in other phases of the menstrual cycle [11]. Important roles for MIF in establishing pregnancy have been reported in early-stage embryos, ovary, and uterus [13–15].

Recently, blood MIF concentration has begun to be used as a biomarker in various fields of human medicine [16, 17]. In particular, serum MIF level is a useful biomarker for prediction of preterm delivery in women [18]. Furthermore, a recent study revealed that blood MIF concentration correlates positively with age, and negatively with self-rated health in women [19].

Optimal blood concentration of MIF may therefore be necessary for proper reproductive function. However, earlier investigations have evaluated the importance of blood MIF only in humans, and no previous study has measured blood MIF concentration in domestic animals. This study was thus conducted to evaluate MIF protein expression in various bovine tissues, to establish an enzyme immunoassay (EIA) for MIF, and to evaluate the hypotheses that MIF concentration changes with parturition or postpartum first ovulation, and that age in months and parity correlate with plasma MIF concentration in Japanese black cows.

Materials and Methods

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved

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by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University.

Tissue sample collection for western blotting

Postpubertal Japanese Black heifers (n = 3; 26 months old, at the preovulatory phase) were sacrificed for collection of spleen, adrenal gland, pancreas, kidney, liver, anterior pituitary, thymus, a mixture of granulossa and theca cells from a large follicle (18 mm in diameter), regressed corpus luteum, and uterine endometrium. This study utilized heifers at the preovulatory phase because it was very difficult to obtain tissue samples from early postpartum Japanese Black cows, and because we supposed that heifers at this phase would be better suited than those at the corpus luteum phase for determining the main organ that secretes MIF into the blood. Excess tissues surrounding the target tissue were carefully removed, and the target tissue was washed with phosphate-buffered saline (PBS). Samples were frozen in liquid nitrogen and preserved at –80°C until use for protein extraction.

Animals and plasma sample collection for EIA

Japanese Black cows were housed in a free barn, and all had normal parturition. The day of parturition was considered to be day 0. First-parity cows (n = 5, 22.0 ± 0.2 months old on the day of parturition), second-parity cows (n = 7, 41.8 ± 1.6 months old on the day of parturition), and third- or higher-parity cows (n = 9, 82.2 ± 7.0 months old on the day of parturition; the mean and maximum parity were 5 and 8, respectively) were used. The feed volume per cow per day was determined to meet the nutrient requirement of the Japanese feeding standard [20]. Cows were fed daily 5.5 kg rice silage [41.0% dry matter (DM), 1.94 Mcal metabolizable energy (ME) kg^{-1} DM, 5.5% crude protein (CP)], 3.3 kg dried rice straw (42.4% DM, 1.45 Mcal ME kg^{-1} DM, 5.4% CP), and 2.1 kg concentrate (88.0% DM, 3.82 Mcal ME kg^{-1} DM, 22.0% CP) on average. Calves were separated from cows within a few days after parturition. Water and mineral blocks were provided *ad libitum*. Absence of disease, including reproductive disease, was confirmed by daily observation. To determine postpartum ovulation, the ovaries of all cows were examined by ultrasonography and rectal palpation at least 2 times per week from day 7 to 4 weeks after postpartum first ovulation.

Blood samples were collected from the jugular vein of all cows into a tube containing heparin at about 1030 h at least 4 times per week, from one month prior to expected parturition to 4 weeks after postpartum first ovulation. Tubes were centrifuged immediately after blood collection at 800 × *g* for 15 min at 4°C, and the obtained plasma samples were stored at –20°C until analyzed for insulin and MIF.

Protein extraction and western blotting for MIF

The collected tissues and bovine plasma, along with human plasma (Sigma-Aldrich, St. Louis, MO, USA), were ground in liquid nitrogen and homogenized using Tissue Protein Extraction Reagent (T-PER; Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitors (Halt protease inhibitor cocktail; Thermo Fisher Scientific). The total protein content of each sample was estimated using a bicinchoninic acid kit (Thermo Fisher Scientific). The extracted samples (20 µg of total protein from all tissues, and 10 µg of total protein from bovine pituitary glands) were analyzed alongside 250 ng and 500 ng of recombinant human MIF (CYT-596; ProSpec-Tany TechnoGene, Rehovot, Israel) for size comparison and were boiled in a sample buffer solution with reducing reagent (09499-14, Nacalai Tesque, Kyoto, Japan) at 100°C for 3 min, and then loaded onto polyacrylamide gels. Molecular-weight markers ranging from 10 to 170 kDa (Page Ruler prestained protein ladder; Thermo Fisher Scientific) were used to help identify MIF bands. Proteins were electrophoresed through precast sodium dodecyl sulfate polyacrylamide gels (Criterion TGX; Bio-Rad, Hercules, CA, USA) at 200 V for 30 min. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes using a Trans-Blot Turbo transfer system (Bio-Rad). Immunoblotting was performed with anti-MIF mouse monoclonal antibody (Clone 2Ar3, 1: 50,000 dilution; ATGen, Gyeonggi-do, Korea) after treatment with blocking buffer containing 0.1% Tween 20 and 5% nonfat dried milk. The antibody cross-reactivity as reported by the manufacturer was 100% for bovine MIF and < 0.01% for other cytokines and plasma proteins. Antibody incubation was carried out overnight at 4°C. After 3 washes with 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:100,000 dilution; KPL, Gaithersburg, MD, USA) was added and incubated at 25°C for 1 h. Protein bands were visualized using an ECL Prime chemiluminescence kit (GE Healthcare, Amersham, UK) and a charge-coupled–device imaging system (LAS-3000 Mini; Fujifilm, Tokyo, Japan). The MIF band strength of the 500 ng of recombinant human MIF lane was set as 100%, the MIF band strength of the 250 ng of recombinant human MIF lane was set as 50%, and then, the MIF band strength of the other sample lanes were calculated as percentages according to the MIF band strengths of the recombinant human MIF lanes using MultiGauge software (Version 3.0; Fujifilm). After antibodies were removed from the PVDF membrane with stripping solution (Nacalai Tesque), the membrane was blocked and incubated with anti-β-actin mouse monoclonal antibody (A2228, 1:50,000 dilution; Sigma-Aldrich) overnight at 4°C. The membrane was washed, then incubated with the same HRP-conjugated anti-mouse IgG (1:100,000 dilution) at 25°C for 1 h, and the bands visualized using an ECL Prime chemiluminescence kit. The β-actin band strength of the 20 µg of anterior pituitary lane was set as 100%, and the β-actin band strength of the 10 µg of anterior pituitary lane was set as 50%, and then, the β-actin band strengths of the other sample lanes were calculated as percentages according to the β-actin band strengths of the anterior pituitary lanes using MultiGauge software (Version 3.0; Fujifilm). The expression of MIF was normalized to the expression of β-actin in each sample.

Enzyme immunoassay to measure plasma MIF concentration

We followed the EIA method reported for luteinizing hormone [21] to develop a new EIA for measuring plasma concentrations of MIF utilizing the same recombinant human MIF (CYT-596) and the same anti-MIF mouse monoclonal antibody (Clone 2Ar3). The assay buffer was 0.02 M Tris-HCl buffer (pH 7.4) containing 300 mM NaCl, 0.1% (w/v) bovine serum albumin (BSA; EIA grade, Nacalai Tesque), and 0.01% (w/v) triton X-100. Using an EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol, 2 µg of MIF was labeled. Briefly, a reaction mixture was prepared at a 1:20 of

molecular ratio of MIF to biotin, and incubated on ice for 120 min. The reaction mixture was then loaded onto a NAP-5 gel filtration column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) that had been washed twice with PBS (pH 7.4) and once with 1% BSA dissolved in PBS to minimize the loss of labeled MIF inside the column. Labeled MIF was separated from free biotin on the column by elution with PBS, and the 6th to 25th elution drops were collected in 1.5-ml microtubes containing 0.5 ml of assay buffer.

Triplicate 50-µl volumes of 13 MIF standards (0 ng/ml, and 0.06 ng/ml to 125 ng/ml) and 8 plasma samples diluted with assay buffer (0.313 µl to 40 µl each of 20 randomly selected plasma samples brought to a final volume of 50 µl) were added to wells of 96-well microplates (ELISA plate H; Sumitomo Bakelite, Tokyo, Japan) that had previously been coated with 6.25 µg per well of anti-mouse IgG rabbit antibody (Rockland Immunochemical, Pottstown, PA, USA) in a final volume of 150 µl, and blocked with 300 µl/well of 1% BSA. Then, 100 µl per well of the same anti-MIF mouse monoclonal antibody diluted to 1:160,000 in assay buffer was added, and the microplates were incubated overnight at 4°C. After the overnight incubation, 100 µl of the prepared biotin-MIF (0.65 ng/well) diluted in assay buffer was added to the wells and incubated for 4 h at 25°C. After 3 washes with washing buffer (0.9% NaCl containing 0.5 g/l Tween 20), 150 µl HRP-conjugated streptavidin (0.01 unit/well, diluted in assay buffer; Roche Diagnostics, Indianapolis, IN, USA) was added to the wells and incubated for 1 h at 25°C. After 3 more washes, 150 µl tetramethylbenzedine (1.33 µg/well; Wako Pure Chemicals, Osaka, Japan) diluted in 0.1 M sodium acetate buffer (pH 5.0) containing 27 ppm hydrogen peroxide was added to the wells and incubated for 1 h at 25°C for color development. After addition of 50 µl/well of 0.2 M sulfuric acid to stop the reaction, the microplates were read at a wavelength of 450 nm using an iMark microplate reader (Bio-Rad). Sample concentrations were determined by analyzing the obtained optical density (OD) data with appropriate software (RIApro Version 3.34, Hitachi Aloka Medical Ltd., Tokyo, Japan). This assay was repeated 5 times to calculate the intra- and interassay coefficients of variation (CV). Octuplicates of 0 ng/mL standard were also assayed with EIA, and the lower limits of the 95% confidence interval of the OD values (as calculated by the average minus 1.96 times the standard deviation) were adapted to the standard curve in order to calculate the lower detection limit of the assay.

After development of the MIF EIA, duplicate 50-µl volumes of 8 standards (0 ng/ml, and 0.98 ng/ml to 62.5 ng/ ml) or 10 µl of plasma samples plus 40 µl assay buffer were measured in a routine assay.

Insulin assay

EIA kits (Mercodia Bovine Insulin ELISA, Mercodia, Uppsala, Sweden) were utilized to measure plasma insulin concentrations at the following five different time points: the day of parturition, 1 week after parturition, the day of the postpartum first ovulation, 10 days after the postpartum first ovulation, and 20 days after the postpartum first ovulation. The limit of detection was 0.025 ng/ml and the intra- and inter-assay CVs were 3.3% and 8.7%, respectively, at 0.50 ng/ml. This assay has been used to measure changes in blood insulin concentrations in Japanese Black steer before and after glucose treatment [22].

Statistical analysis

Data were analyzed using StatView for Windows (Version 5.0, SAS Institute, Cary, NC, USA). Repeated-measure analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test was performed to evaluate the effect of time, the effect of parity (first, second, third, or higher parity), and the interaction between time and parity on plasma MIF concentrations from 3 weeks before and after parturition. Repeated-measure ANOVA followed by Fisher's PLSD test was performed to evaluate the effect of time, parity, and the interaction between time and parity on plasma MIF concentrations from 3 weeks before and after postpartum first ovulation. Repeated-measure ANOVA followed by Fisher's PLSD test was performed to evaluate the effect of time, parity, and the interaction between time and parity on plasma insulin concentrations at the five different time points. A simple regression analysis and Pearson's correlation analysis were utilized to evaluate the relationship (1) between the plasma MIF concentration on the day of parturition and the cow's age in months on the day of parturition, (2) between the plasma MIF concentration on the day of the postpartum first ovulation and the cow's age in months on the day of the postpartum first ovulation, and (3) between the plasma MIF concentration on the day of parturition and the parity of the cow on the day of parturition. A simple regression analysis and Pearson's correlation analysis were utilized to evaluate relationships between plasma MIF concentration and plasma insulin concentration at each of the five time points. The level of significance was set at $P < 0.05$. Data expressed as mean ± standard error of the mean (SEM).

Results

Western blotting for MIF

Figure 1 depicts representative photographs of the western blotting of MIF (Fig. 1A) and β-actin (Fig. 1B). Western blotting revealed an immunoreactive protein band that migrated with an apparent molecular weight of 12.5 kDa in human recombinant MIF. However, the estimated molecular weights of the immunoreactive bands in bovine pancreas and anterior pituitary were about 14 kDa and 20 kDa, respectively. In the follicle and uterus of preovulatory heifer, the bovine plasma samples, and the human plasma, the immunoreactive protein band appeared at about 25.0 kDa. The anterior pituitary showed the strongest-intensity band among all tissues tested in this study (Fig. 1C).

EIA developed to measure plasma MIF concentration

Figure 2 depicts the good parallelism between the MIF standard curve and the serially diluted plasma mixtures. The detection limit of MIF EIA was 0.69 ng/ml. At 3.15 ng/ml, the intra- and interassay CVs were 7.4% and 10.1%, respectively.

Parturition and the postpartum first ovulation in cows

The deliveries were normal in all cows, and there was neither preterm delivery nor delayed delivery. The differences between the estimated date of delivery and the date of actual delivery were less than 8 days in all cows.

The number of days from parturition to postpartum first ovulation was lower in first-parity cows (15.6 ± 3.7 days) than in second-parity

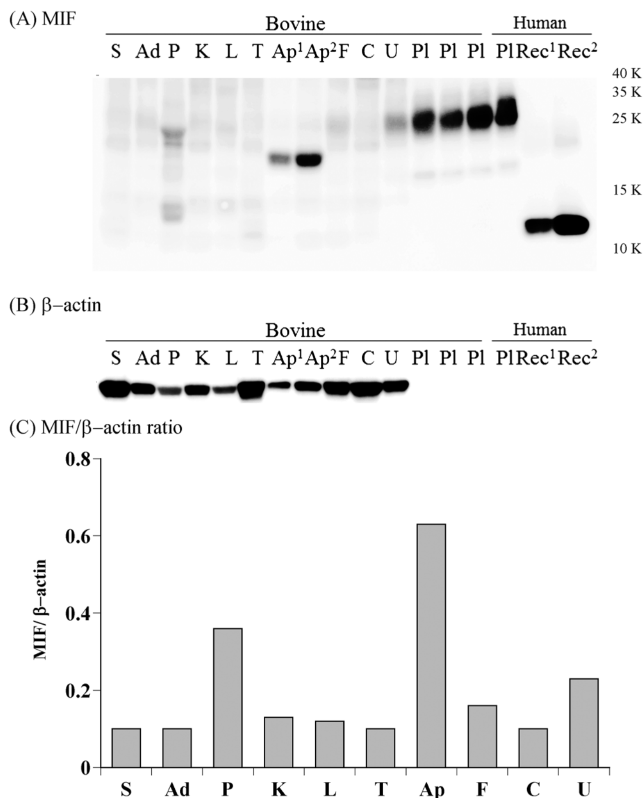

Fig. 1. Representative MIF protein bands detected by western blotting with anti-MIF antibody (A) or with anti-β-actin antibody (B) in various bovine organs (20 µg of total protein from all organs, except for 10 µg of total protein from Ap[1]). S, spleen; Ad, adrenal gland; P, pancreas; K, kidney; L, liver; T, thymus; Ap, anterior pituitary (Ap[1] is 10 µg total protein, and Ap[2] is 20 µg total protein); F, granulosa and theca cells of preovulatory follicle; C, regressed corpus luteum; U, uterine endometrium; Pl, plasma. Human plasma (Pl) and recombinant human MIF protein (Rec[1] is 250 ng and Ap[2] is 500 ng) were utilized for band size and amount comparisons with bovine samples. (C) A graph comparing MIF protein expression in various bovine organs normalized to β-actin.

cows (27.7 ± 4.3 days, P < 0.05) and third- or higher-parity cows (25.3 ± 2.5 days, P = 0.076).

Changes in plasma MIF concentration in cows

Figure 3A shows the changes in plasma MIF concentrations in a cow that had an abnormally high plasma MIF concentration before and after parturition, and before and after the postpartum first ovulation. The cow (third parity) was 56 months old on the day of parturition, and showed the postpartum first ovulation 34 days after parturition. Figure 3B shows the changes in plasma MIF concentrations in a representative cow that had low plasma MIF concentrations before and after parturition, and before and after the postpartum first ovulation. The cow (third parity) was 67 months old on the day of parturition, and showed the postpartum first ovulation 32 days after parturition.

Figure 4 depicts the changes in plasma MIF concentration in cows before and after parturition (Fig. 4A) and before and after postpartum

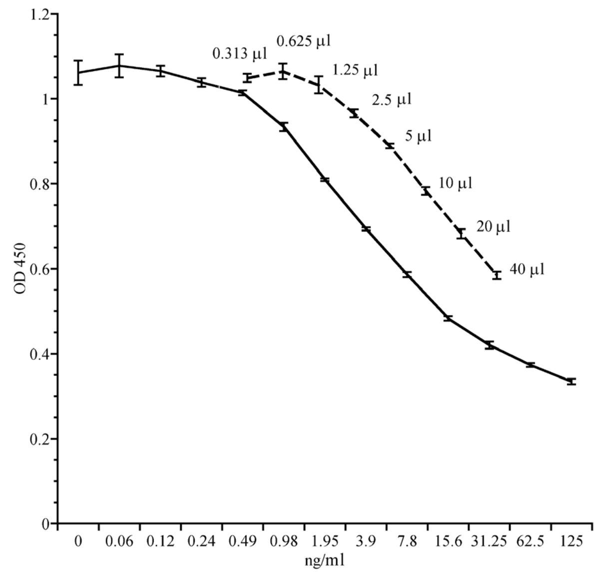

Fig. 2. Comparison between the standard curve (bold line) of MIF EIA and serially diluted plasma samples (dashed line), to evaluate parallelism.

first ovulation (Fig. 4B). The repeated-measure ANOVA did not detect a significant effect of time, parity, or the interaction between time and parity on plasma MIF concentrations before and after parturition. The repeated-measure ANOVA did not detect a significant effect of time, parity, or the interaction between time and parity on plasma MIF concentrations before and after the postpartum first ovulation. The MIF concentration in third- or higher-parity cows showed larger individual differences than that in first- or second-parity cows, as illustrated by the lengths of the vertical lines representing the SEMs.

Relationships between plasma MIF concentration, age, and parity in cows

Plasma MIF concentrations had no significant correlation with parity, age on the day of parturition, or age on the day of the postpartum first ovulation when we used all data, including data from the cow shown in Fig. 3A. However, when we excluded the data from the cow shown in Fig. 3A, plasma MIF concentrations correlated (P < 0.01) with age on the day of parturition (Fig. 5A; r = 0.647) and on the day of the postpartum first ovulation (Fig. 5B; r = 0.553). The plasma MIF concentration on the day of parturition was also correlated (P < 0.01) with the parity of the cow (Fig. 5C; r = 0.703).

Changes in plasma insulin concentration in cows

Figure 6 depicts the plasma insulin concentrations in cows on the day of parturition, 1 week after parturition, on the day of the postpartum first ovulation, 10 days after the postpartum first ovulation, and 20 days after the postpartum first ovulation. The repeated-measure ANOVA detected a significant effect of time (P < 0.01), but no significant effect of parity (P > 0.1), and no significant interaction between time and parity (P > 0.1) on plasma insulin concentrations.

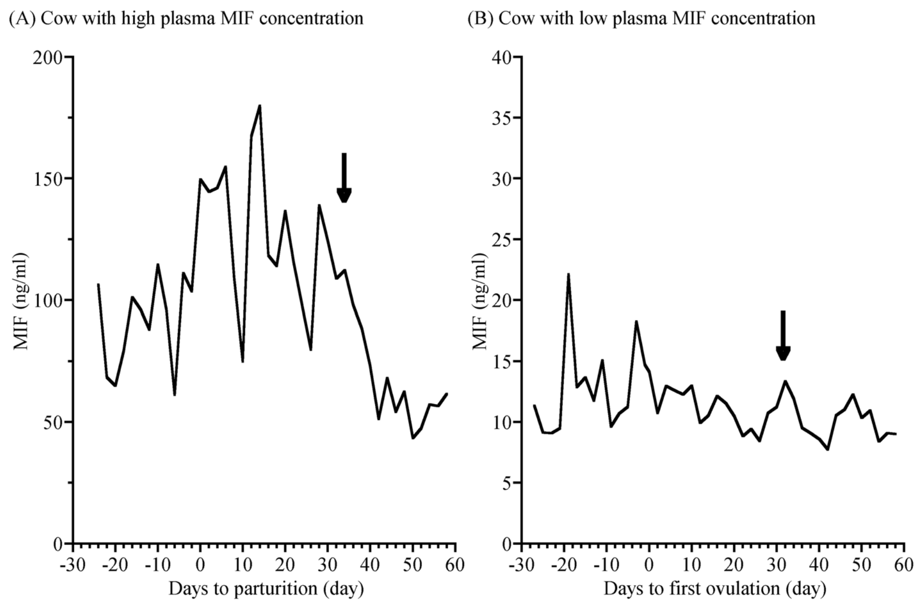

Fig. 3. Changes in plasma MIF concentration in representative cows that had high (A: third parity; 56 months old on the day of parturition) and low (B: 67 months old on the day of parturition) plasma MIF concentrations before and after parturition, and before and after the postpartum first ovulation. Arrows indicate the day of the postpartum first ovulation.

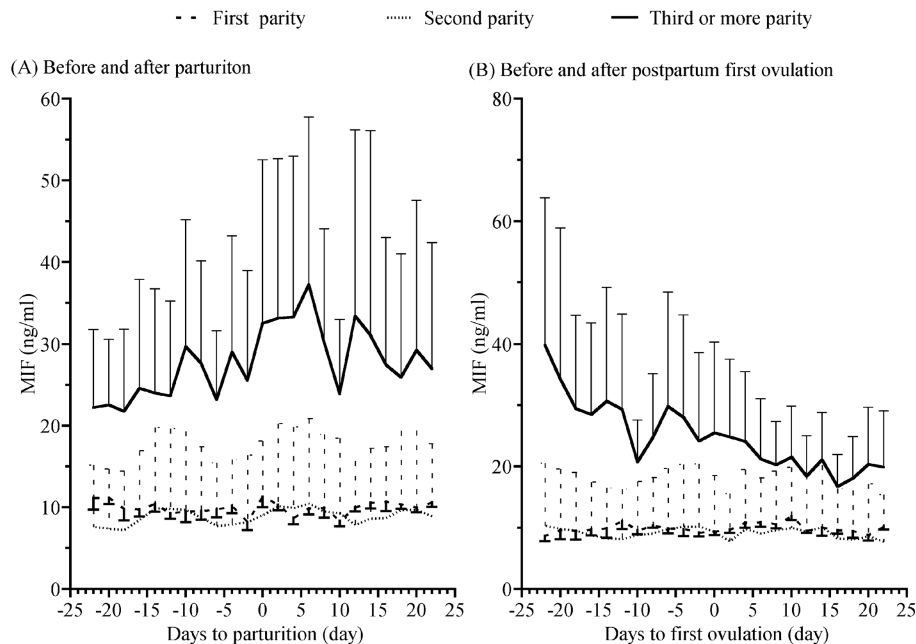

Fig. 4. Changes in plasma MIF concentration before and after parturition (A) and before and after postpartum first ovulation (B) in first-parity cows (n = 5; dashed line, average; bold vertical lines, SEM); second-parity cows (n = 7; dotted line, average; dashed vertical lines, SEM); and third- or higher-parity cows (n = 9; bold line, average; thin vertical lines, SEM). SEM, standard error of the mean.

Relationship between plasma MIF concentration and plasma insulin concentration in cows

Plasma insulin concentrations had no significant correlation with plasma MIF concentrations at any of the five time points when we used all data, including the data from the cow shown in Fig. 3A. Moreover, plasma insulin concentrations had no significant correlation with plasma MIF concentrations at any of the five time points when we used a dataset that excluded the data from the cow shown in Fig. 3A.

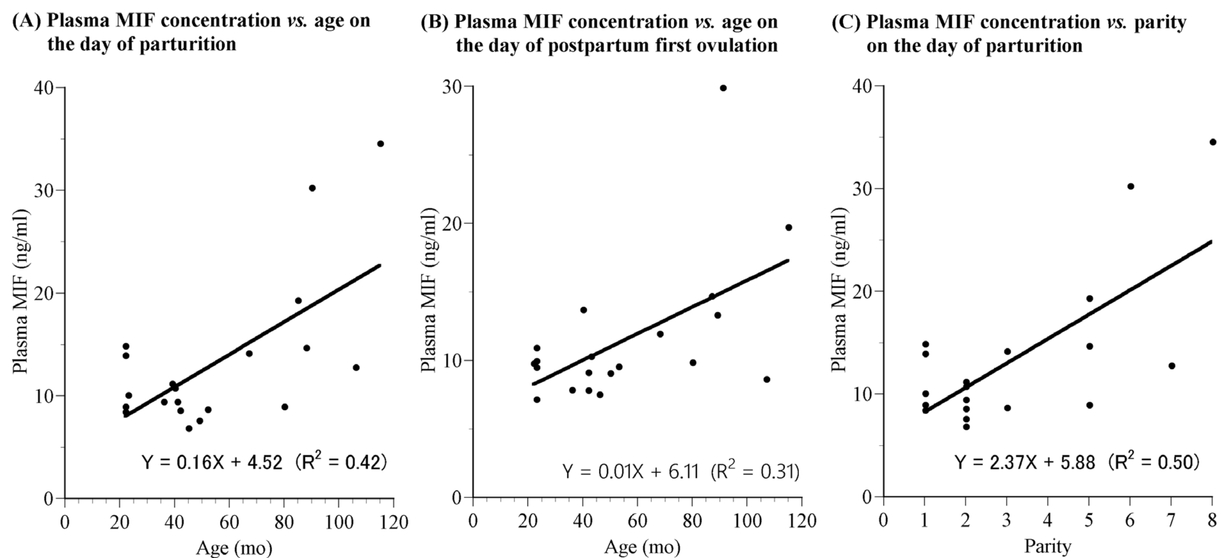

Fig. 5. Significant relationships are shown (A) between the plasma MIF concentration on the day of parturition and the age in months of the cow on the day of parturition, (B) between the plasma MIF concentration on the day of the postpartum first ovulation and the age in months of the cow on the day of the postpartum first ovulation, and (C) between the plasma MIF concentration on the day of parturition and the parity of the cow on the day of parturition.

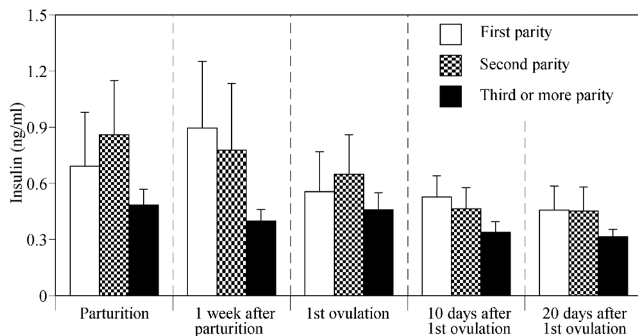

Fig. 6. The mean ± SEM of the plasma insulin concentration on the day of parturition, 1 week after parturition, on the day of the postpartum first ovulation, 10 days after the postpartum first ovulation, and 20 days after the postpartum first ovulation in first-parity cows (n = 5), second-parity cows (n = 7), and third- and higher-parity cows (n = 9).

Discussion

In the present study, we developed an EIA with sufficient sensitivity and reliable performance for measuring MIF concentrations in bovine plasma samples. The concentration of MIF in normal human plasma is about 10 ng/ml [23], similar to that found in first- and second-parity cows in this study. The EIA revealed no significant differences in plasma MIF concentration pre- and post-parturition, or before and after the postpartum first ovulation. No previous studies, even in other species, were available for comparison with these data. Christian *et al*. [19] recently reported that blood MIF concentration correlates positively with age in women. Therefore, plasma MIF concentrations may also correlate with parity and age in months in cows, although we must be cautious of the presence of a multiparous cow with an abnormally high plasma MIF concentration.

MIF mRNA is expressed abundantly in early corpus luteum, and at low levels in anthesis corpus luteum and regressed corpus luteum [24]. Bovine placenta also synthesizes MIF [9]; however, the insignificant difference found in plasma MIF concentration pre- and post-parturition and before and after the postpartum first ovulation suggests that corpus luteum and placenta are unlikely to contribute substantially to circulating MIF. This study clarified MIF protein expression in follicle and uterine endometrium. Preovulatory follicles secrete MIF into the blood, slightly increasing blood MIF concentration in women in the preovulatory period [11]. Bovine endometrial epithelial cells also synthesize MIF [25]; thus, secretion of MIF from follicle and endometrium may contribute to the plasma MIF concentration in cows. However, pancreas and anterior pituitary were found to have stronger MIF protein expression than follicle and uterine endometrium in the present study. Recent studies have revealed that MIF is an autocrine stimulator of insulin secretion [26, 27], although no significant correlation was found between plasma MIF concentration and plasma insulin concentration in this study. MIF is also released from pituitary folliculo-stellate-like cells [28]. The anterior pituitary showed the strongest MIF-immunoreactive band among the tissues evaluated in this study, suggesting that pancreas and anterior pituitary may be the main organs secreting MIF into the blood. Blood MIF concentration correlates positively with age and negatively with self-rated health in women [19]. In the present study, some higher-parity cows had high plasma MIF concentrations, although they did not show clinical signs. Further studies are thus required to measure plasma MIF concentrations in cows with various health conditions.

The molecular weight of human recombinant MIF was estimated

as 12.5 kDa by western blotting; however, the estimated molecular weight of MIF in human plasma, bovine plasma, bovine follicle, and bovine uterine endometrium was 25.0 kDa. Possible reasons for the difference in the apparent molecular weight of MIF may include multiple factors such as glycosylation, binding factors, and sample preparation. MIF is known to exist as a monomer, dimer, or trimer in various human tissues and plasma [29, 30]. The estimated molecular weight of bovine MIF in pancreas and anterior pituitary was higher than that of recombinant human MIF. MIF has a complicated structure, with two antiparallel alpha helices and an additional two beta strands that form a barrel containing a solvent-accessible channel, which has a positive charge and thus binds negatively charged molecules [31]. Therefore, another possible reason for the differences in the apparent molecular weight of MIF may be variations in this intricate 3-dimensional structure.

This study detected MIF protein in uterine endometrium. Wang and Goff [25] reported that interferon-tau stimulates MIF secretion from bovine endometrial epithelial cells *in vitro*. Women with polycystic ovary syndrome have significantly higher plasma MIF concentrations than those without the condition [32], and blood MIF concentration is a useful biomarker for predicting preterm delivery in women [18]; therefore, further study is warranted to evaluate the importance of blood MIF concentration for predicting conception and preterm delivery in domestic animals.

In conclusion, plasma MIF concentrations may increase with age in months and increased parity in Japanese black cows, but were not significantly different either before and after parturition, or before and after postpartum first ovulation.

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