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Original article

Early warning for inactive ovaries based on insulin resistance index, serum adiponectin and leptin in dairy cows

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Abstract

Postpartum inactive ovaries (IO) in dairy cows reduce the economic returns of the dairy industry. It is related to energy metabolism disorder, hormone levels and cytokines.The aim of this study was to evaluate the correlation between insulin resistance (IR), adiponectin (ADPN), and leptin (LEP) at 14 days postpartum to assess the predictive potential for IO risk in dairy cows. Cows at 14 days postpartum were randomly selected and allocated into an insulin resistance group (IR, with IR index > 2.5, n=30) and a non-insulin resistance (non-IR, with IR index < 2.5, n=30). Serum Samples were collected at 14 and 55 days postpartum. Six cows of estrus and six cows of IO were randomly selected for slaughter at 55 days postpartum. Then, adipose and ovary samples were allocated for further experiments. A significant association between IR and IO, with 53.33% prevalence in the IR group compared to 16.67% in the non-IR group. Cows with IR had higher levels of β-hydroxybutyrate, non-esterified fatty acid, and lower levels of glucose, total cholesterol, triglyceride, ADPN, and LEP. Reproductive performance was adversely affected, with IR cows showing longer durations for first estrus and reduced milk yield. ADPN and LEP levels were significantly lower in IR cows, suggesting their role in modulating insulin sensitivity and reproductive functions. The combined analysis of ADPN, LEP, and IR index showed high sensitivity (91.3%) and specificity (87.2%) in predicting IO, highlighting their potential as reliable biomarkers. These observations indicate that IR and serum LEP and ADPN at 14 days postpartum can predict IO in dairy cows.

Keywords: dairy cow, inactive ovaries, insulin resistance, risk warning

Introduction

Reproductive efficiency is a critical factor in dairy production, significantly impacting the economic viability of dairy operations. The transition period, which spans from three weeks before to three weeks after calving, is particularly challenging as dairy cows often experience negative energy balance (NEB), metabolic stress, and insulin resistance (IR) due to increased energy demands for lactation (Butler 2003, Qiao et al. 2024). IR, characterized by a reduced sensitivity to insulin, has been implicated in various reproductive dysfunctions, including inactive ovaries (IO), a condition marked by the absence of estrous cycles postpartum (Butler 2000, De Koster and Opsomer 2013).

IO is a significant reproductive disorder in dairy cows, leading to prolonged calving intervals and reduced milk production (Opsomer et al. 1998). The pathophysiology of IO is multifactorial, with metabolic and hormonal imbalances playing a central role. Specifically, insulin resistance can disrupt glucose and lipid metabolism, negatively affecting ovarian function and overall reproductive performance (Lucy 2001, Butler et al. 2003). Recent studies have highlighted the importance of metabolic health in reproductive outcomes, emphasizing the need for early detection and management of IR to mitigate its adverse effects on fertility (Leroy et al. 2008, De Koster and Opsomer 2013).

Adiponectin (ADPN) and leptin (LEP) are adipokines that regulate energy homeostasis and insulin sensitivity, making them potential biomarkers for metabolic and reproductive health (Houseknecht et al. 1998, Nigro et al. 2014). Adiponectin is known for its insulin-sensitizing properties and has been associated with improved metabolic profiles and reproductive outcomes (Komatsu et al. 2005). Conversely, leptin, which regulates appetite and energy expenditure, is often elevated in cases of obesity and metabolic syndrome, contributing to insulin resistance and reproductive dysfunctions (Houseknecht et al. 1998, Komatsu et al. 2005). Despite the recognized importance of these adipokines, their potential as predictive biomarkers for IO in dairy cows remains underexplored. Research has begun to elucidate the roles of ADPN and LEP in bovine metabolic and reproductive health, suggesting that these biomarkers could provide valuable insights into the early detection of reproductive disorders (Chagas et al. 2007).

This study aims to investigate the predictive value of the insulin resistance index, serum adiponectin, and leptin levels for inactive ovaries in postpartum dairy cows. We hypothesize that cows with higher IR and lower levels of ADPN and LEP will have a higher risk of developing IO. By conducting a cohort study involving 200 *Holstein* cows and assessing their metabolic and hormonal profiles from 14 to 55 days postpartum, we seek to provide new insights into the early detection and management of reproductive disorders in dairy cows. The findings from this study will contribute to the development of effective early warning systems, ultimately enhancing the reproductive efficiency and productivity of dairy herds.

Materials and Methods

Animals

All animal procedures were conducted in accordance with the ethical guidelines and regulations approved by the Institutional Animal Care and Use Committee of Heilongjiang Bayi Agricultural University in Daqing, China (Protocol NO. DWKJXY2024007; approval date: 4 January 2024). The experiment was conducted during the winter months, from January to March 2024, at a large intensive dairy farm located in central Heilongjiang Province, with a herd of 3,125 *Holstein* cows. To elucidate the relationship between postpartum IR and IO in dairy cows, a cohort study was conducted on a randomly selected group of 200 *Holstein* cows. Cows were divided into insulin resistant (IR>2.5, n=30) and non-insulin resistant (non-IR<2.5, n=30) groups at 14-55 days postpartum to identify selected differences in serum energy metabolism, cytokines, and hormonal markers between the two groups and to explain why IR could predict IO. The IR formula calculated was as follows: Fasting insulin (μU/mL) × fasting blood glucose (mmol/L) /22.5, and IR>2.5 was defined as insulin resistance (Holtenius and Holtenius 2007, Friedrich et al. 2012). The lower the IR, the better the energy metabolism level of dairy cows in the cycle, which is conducive to subsequent reproduction. In order to further determine the effects of IR and serum indexes on IO, 6 cows in estrus (E) and 6 cows in IO were randomly selected and slaughtered for follow-up tests at 55 days postpartum. Based on the fluctuations in the number of steps recorded between 50 and 55 days postpartum and clinical signs of estrus, such as mounting behavior or swollen and moist vulvar mucosa, six healthy cows exhibiting normal estrous behavior, with no signs of disease or clinical abnormalities, were selected as the E group. Transrectal ultrasonography was performed on these cows to assess the demarcation between the myometrium and endometrium, uterine wall thickening, uneven uterine texture, and the presence of intrauterine fluid, as well as follicles with a diameter of 15 to 20 mm. For the IO group, six cows were selected based on the absence of significant fluctuations in step count and the lack of clinical estrus detected by the Afimilk farm management system

Table 1. Polymerase chain reaction primer information.

Genes	Primers
ADPN	F:5'-GGAACTTGTGCAGGTTGGAT-3'
	R:5'-GGAACTTGTGCAGGTTGGAT-3'
LEP	F: 5'-CAGTCCGTCTCCTCCTAAACAG-3'
	R: 5'-CGCCAATGTCTGGTCCATCTT-3'

ADPN – adiponectin, LEP – leptin.

between 50 and 55 days postpartum (Afimilk® 3.076, Afimilk, Israel). These cows exhibited no intrauterine fluid, normal ovarian texture and size, follicles with a diameter less than 8 mm, and the presence of uterine endometrial folds on transrectal ultrasonography (Nelson et al. 2017).

The cows were housed in separate tie-stall barns with kiln-dried sawdust bedding and received individually tailored early-lactation total mixed rations (TMR) following parturition. The TMR formulation for early lactation adhered to NRC (2001) standards (NRC 2001). Cows were fed three times daily and milked three times daily at 06:00, 14:00, and 22:00. The components of the TMR diet included soybean hulls 1.50 kg, oat grass 0.50 kg, cottonseed 1.03 kg, alfalfa 2.50 kg, soybean meal 1.30 kg, pressed corn 2.00 kg, molasses 1.00 kg, silage 25.37 kg, corn 3.00 kg, high-yield concentrate 4.09 kg. Feed analysis showed 48.00% of dry matter, 17.70% of crude protein, 7.322 MJ kg^{-1} net lactation production, 22.70% of starch, 31.50% of neutral detergent fiber, 19.00% of acid detergent fiber, 180 g of calcium, and 116 g of phosphorus.

Sample collection

Blood samples were aseptically collected from the tail vein before the morning feeding at 14 days and 55 days postpartum. Each collection involved obtaining 10 mL of whole blood per cow, followed by centrifugation at 1500 × g for 5 min. The resulting supernatant was carefully separated and sub-packed into 1.5 mL Eppendorf tubes. Subsequently, further centrifugation at 12000 × g for 10 min, utilizing a low-temperature high-speed centrifuge, was conducted. The serum was then collected and sub-packed into 1.5 mL cryopreservation tubes, preserving the samples at -80°C for subsequent blood biochemical index detection.

Adipose Samples: At 55 d postpartum, subcutaneous adipose samples were harvested post-slaughter from both groups, namely cows with IO and E. These tissue samples underwent thorough washing with saline. A portion of the adipose tissue was fixed in 10% neutral buffered formalin, while another portion (1 to 2 g) was precisely weighed and promptly stored in liquid nitrogen for subsequent RNA extraction. These samples were reserved for ADPN and LEP in adipose tissue quantitative real-time polymerase chain reaction (RT-PCR) experiments.

Ovary Samples: At 55 days postpartum, ovaries samples were harvested post-slaughter from both groups, and the tissue samples were meticulously rinsed with saline. A segment of the ovary tissue was fixed in 10% neutral buffered formalin, and another part (1 to 2 g) was expeditiously stored in liquid nitrogen for subsequent RNA extraction. These samples were allocated for ADPN and LEP RT-PCR experiments.

Serum biochemical index detection

Serum biochemical indices encompassed energy metabolism markers, specifically: non-esterified fatty acids (NEFA, mmol/L), β-hydroxybutyric acid (BHBA, mmol/L), glucose (Glu, mmol/L), triglycerides (TG, mmol/L), and total cholesterol (TC, mmol/L). The BHBA levels were quantified using a blood ketone meter and ketosis test strips, procured from Yicheng, Beijing, China, with a sensitivity of 93.8%, specificity of 100%, and a Yoden index of 93.8%. The remaining biochemical indices were determined utilizing a commercial kit (Mindray Biomedical Electronics Co. Ltd., Shenzhen, China) and an accompanying fully automated biochemical analyzer.

Quantification of mRNA expression

Total RNA extraction from ovarian and adipose tissue samples was executed using a Trizol RNA extractor (Invitrogen, Carlsbad, CA). Subsequent to RNA isolation, mRNA transcription and cDNA synthesis were accomplished utilizing Oligo(dT) primers and reverse transcriptase AMV (New England Biolabs, Ipswich, MA). RT-PCR was conducted employing Fast Start Universal SYBR Green Master under the following conditions: an initial denaturation at 94°C for 3 min, followed by 40 cycles on the Bio-Rad iCycler ixtm RT-PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA): denaturation at 94°C for 15 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 25 seconds. The primers (Sangon B Company, Shanghai, China) utilized in the experiments are detailed in Table 1.

Table 2. Relationship between 60 IR and ovarian resting cows.

Groups	E	IO	Total	IO rate (%)
IR	14	16	30	53.33
Non-IR	25	5	30	16.67
Total	39	21	60	35

IR – insulin resistance, Non-IR – non-insulin resistance, E – oestrus, IO – inactive ovary.

Table 3. χM-H2test for cohort studies.

$\chi M\text{-}H^2$	*P*-value	RR-value	95% C.I.L	95% C.I.u
7.2039	0.0073	3.2	* 1.3686	7.4823

Enzyme-linked immunosorbent assay

Serum and tissue adipokines as well as hormone indicators were measured using bovine-specific enzyme--linked immunosorbent assays (ELISA). The evaluated adipocytokines comprised leptin (LEP, μg/L) and adiponectin (ADPN, μg/mL) (Song et al. 2021), while the hormone indicators encompassed insulin (INS, mU/mL), insulin-like growth factor-1 (IGF-1, ng/mL), growth hormone (GH, ng/mL), estradiol (E2, pg/mL), and progesterone (P4, ng/mL). ELISA kits for these analyses were procured from Xinfan Company (Shanghai, China) and measurements were conducted using a grating multifunctional microplate reader. Intra-assay coefficients of variation was <10%,inter-assay coefficients of variation was <15%.

Data collection

Age, parity, weight, and milk yield were recorded using specific software (Afifarm, Afimilk, Kibbutz Afikim, 1514800, Israel). Body condition score (BCS) was determined by two qualified field veterinarians using a 5-point scale ranging from 1 to 5 with 0.25-unit intervals, body condition loss (BCL): ΔBCS=BCS2-BCS1) (Edmonson et al. 1989), the postpartum BCL in this experiment was compared to the day of delivery.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics 26.0 software. The χM-H2 test of the cohort study was used to analyse the proportion of IO cows in the IR and non-IR groups and to clarify the correlation between the increase in IR index of the cows and the occurrence of IO. All serum indicators assessed on day 14 and 55 were analysed using a mixed model procedure to account for correlated repeated measures. The model consisted of the fixed IR threshold (IR vs non-IR) and breeding state (IO vs E). To predict the occurrence of IO in dairy cows, Spearson correlation coefficient and receiver operating characteristic (ROC) analysis were employed. The ROC analysis, with a threshold of p-value < 0.05, was utilized to determine the early warning risk index for IO. All data are presented as mean ± standard deviation. A p-value <0.05 indicates a significant difference, while a p-value <0.01 indicates a highly significant difference.

Results

IR assessment of IO risk in dairy cows

As outlined in Table 2, the prevalence of IO in the IR group was notably higher at 53.33% compared to the non-IR group, where it stood at 16.67% ($p<0.01$). The calculated relative risk (RR) value of 3.2 was significantly more pronounced than 1.5, with a 95% confidence interval of (1.3686-7.4823), excluding 1. This outcome demonstrated statistical significance (M-Hχ2=7.2039, p=0.0073). A robust positive correlation between IO and IR was evident in Table 3.

Clinical and reproductive information for IR and non-IR cows

As presented in Table 4, a comprehensive comparison between the non-IR group and the IR group revealed notable distinctions. In the IR group, dairy cows exhibited significantly heightened body condition loss at 14 days and 55 days postpartum ($p<0.05$), along with increased durations for the first oestrus, insemination, days of pregnancy, and calving intervals ($p<0.01$). Conversely, key reproductive metrics, such as average milk yield, number of oestrus occurrences, oestrus rate, and conception rate, were markedly reduced in the IR group ($p<0.01$). No significant differences were observed in age, parity, BCS, and days of first mating between the two groups ($p>0.05$).

Serum biochemical indices of non-IR and IR cows at 14 postpartum days

As delineated in Table 5, cows in the IR group exhibited significantly higher levels of serum BHBA,

Table 4. Clinical data of 60 dairy cows with IR and non-IR.

Project	Non-IR(n = 30)	IR(n = 30)	p-value
Age	4.08 ± 0.13	4.26 ± 0.18	0.422
Parity	2.77 ± 0.12	3.00 ± 0.79	0.224
14 d BCS	3.18 ± 0.07	3.38 ± 0.07	0.267
55 d BCS	2.90 ± 0.08	2.78 ± 0.07	0.098
14 d BCL	0.28 ± 0.07	0.59 ± 0.07	0.004
55 d BCL	0.26 ± 0.06	0.48 ± 0.07	0.032
Daily milk yield, kg/d	41.47 ± 3.67	36.86 ± 3.78	0.001
First oestrus days, days	44.65 ± 1.74	56.62 ± 2.52	0.001
Estrus, number[1]	1.22 ± 0.10	0.79 ± 0.12	0.001
Conception, number[1]	1.73 ± 0.11	2.37 ± 0.13	0.001
First mating days, days[1]	65.33 ± 1.42	66.39 ± 1.26	0.551
Days open, days[1]	93.12 ± 3.15	121.31 ± 3.85	0.001
Calving interval, days[1]	373.13 ± 3.13	401.79 ± 4.55	0.001
Estrus rate[1], %	83.33	46.67	0.001
Conception rate[1], %	59.13	35.11	0.012

BCS – body condition score, BCL – body condition score loss, IR – insulin resistance, Non-IR – non-insulin resistance.
[1] Data were collected from non-slaughtered cows, with 24 cows in each group (n=24).

Table 5. Serum biochemical index of 60 non-IR and IR dairy cows at 14 and 55 days postpartum.

Project	Non-IR(n = 30)	IR(n = 30)	p-value
14 d BHBA (mmol/L)	0.74 ± 0.03	2.07 ± 0.06	<0.001
14 d NEFA (mmol/L)	0.37 ± 0.02	0.79 ± 0.01	<0.001
14 d GLU (mmol/L)	4.79 ± 0.09	2.46 ± 0.04	<0.001
14 d TC (mmol/L)	4.76 ± 0.15	3.41 ± 0.07	<0.001
14 d TG (mmol/L)	0.08 ± 0.01	0.12 ± 0.01	<0.001
14 d INS (μU/mL)	10.88 ± 0.51	24.49 ± 1.06	<0.001
14 d IGF-1 (ng/mL)	94.98 ± 6.61	65.30 ± 6.99	0.003
14 d ADPN (μg/mL)	3.09 ± 0.07	2.16 ± 0.05	<0.001
14 d LEP (μg/L)	5.67 ± 0.07	4.99 ± 0.11	<0.001
55 d E2 (pg/mL)	17.76 ± 0.79	13.93 ± 0.64	<0.001
55 d P4 (ng/mL)	8.32 ± 1.40	4.45 ± 0.86	0.006
55 d GH (ng/mL)	49.18 ± 1.26	44.50 ± 1.25	0.011

IR – insulin resistance, Non-IR – non-insulin resistance, BHBA – β-hydroxybutyrate, NEFA – non-esterified fatty acid, GLU – glucose, TC – total cholesterol, TG – triglyceride, INS – insulin, IGF-1 – insulin-like growth factor 1, ADPN – adiponectin, LEP – leptin, E2 – estradiol; P4 – progesterone; GH – growth hormon.

NEFA, and INS ($p<0.01$) compared to those in the non-IR group. Conversely, serum levels of Glu, TC, TG, ADPN, and LEP ($p<0.01$) were markedly lower in the IR group. Additionally, IGF-1 was significantly lower in the IR group compared to the non-IR group ($p<0.05$).

Reproductive hormones indexes of non-IR and IR cows at 55 postpartum days

As shown in Table 5, E2 and P4 ($p<0.01$), GH ($p<0.05$) were significantly lower in the IR group than in the non-IR group.

Serum biochemicalindexes of E and IO cows at 14 postpartum days

As indicated in Table 6, dairy cows in the IO group exhibited significantly lower average daily milk production and serum levels of INS, IGF-1, ADPN, and LEP ($p<0.01$) compared to the E group. Conversely, IR index was markedly higher in the IO group compared to the E group ($p<0.01$). No significant differences were observed in age and parity between the two groups ($p>0.05$) .

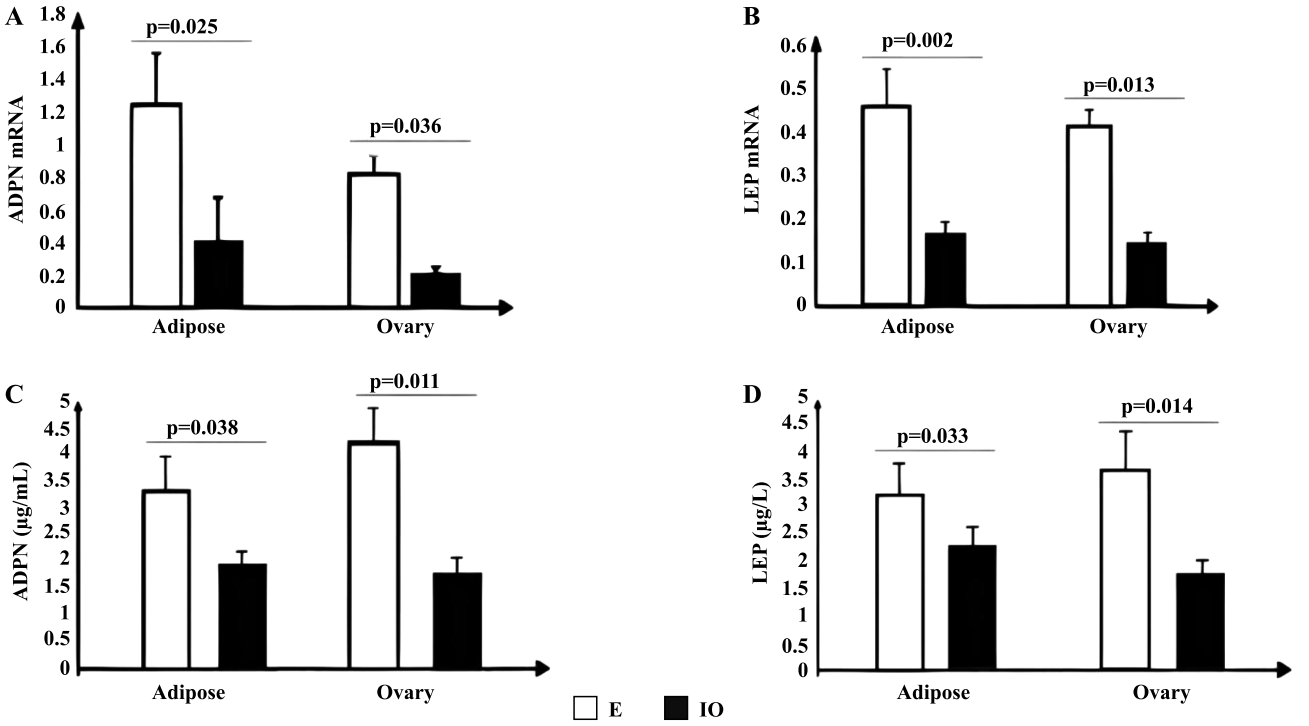

Fig. 1. ADPN and LEP mRNA (A and B) and protein expression (C and D) in adipose and ovary tissue of E and IO groups of cows at 55 days postpartum. E: estrus; IO: inactive ovary; ADPN: adiponectin; LEP: leptin

Table 6. Clinical data of 12 dairy cows with E and IO.

Project	E (n = 6)	IO (n = 6)	p-value
Age	4.18 ± 0.11	4.26 ± 0.13	0.939
Parity	2.74 ± 0.06	2.73 ± 0.22	0.906
Daily milk yield, kg/d	41.53 ± 2.74	36.78 ± 1.59	<0.001
IR index	2.21 ± 0.04	3.02 ± 0.03	0.004
INS (μU/mL)	10.83 ± 0.30	25.06 ± 0.47	<0.001
IGF-1 (ng/mL)	94.34 ± 3.39	65.27 ± 5.28	<0.001
ADPN (μg/mL)	3.10 ± 0.03	2.16 ± 0.04	<0.001
LEP (μg/L)	5.68 ± 0.03	4.89 ± 0.07	<0.001

E – estrus, IO – inactive ovary, IR index – insulin resistance index, INS – insulin, IGF-1 – insulin-like growth factor 1, LEP – leptin, ADPN – adiponectin.

Expression of cytokine genes and proteins in tissues of E and IO cows

As illustrated in Fig. 1, the mRNA expression of ADPN and LEP, in both adipose tissue and ovarian tissue, were significantly lower ($p<0.05$) in the IO group compared to the E group. Similarly, the protein expression of ADPN and LEP in adipose tissue and ovarian tissue of the IO group was significantly lower than that of the E group ($p<0.05$). The outcomes presented demonstrate a consistent low level of the adipokines ADPN and LEP in adipose tissue and ovarian tissue of the IO group, aligning with the observed trend in blood. Fig. 1C and 1D further elucidate the changing pattern of adipocytokines in IOs of dairy cows.

Association between serum biochemical indexesand IO in postpartum cows at 14 days

As delineated in Table 7, IR and serum INS exhibited a positive correlation with IO ($p<0.01$), indicating a link between these factors and the occurrence of IO. Conversely, serum levels of IGF-1, ADPN, and LEP ($p<0.01$) demonstrated a negative correlation with IO.

Risk assessment of postpartum IO in dairy cows

As illustrated in Table 8 and in Fig. 2B, several factors demonstrated substantial early warning value for ovarian inactivity in dairy cows. Table 8 and Fig.2A presented the cut-off value, sensitivity, specificity, and area under the curve (AUC) of relevant indicators for

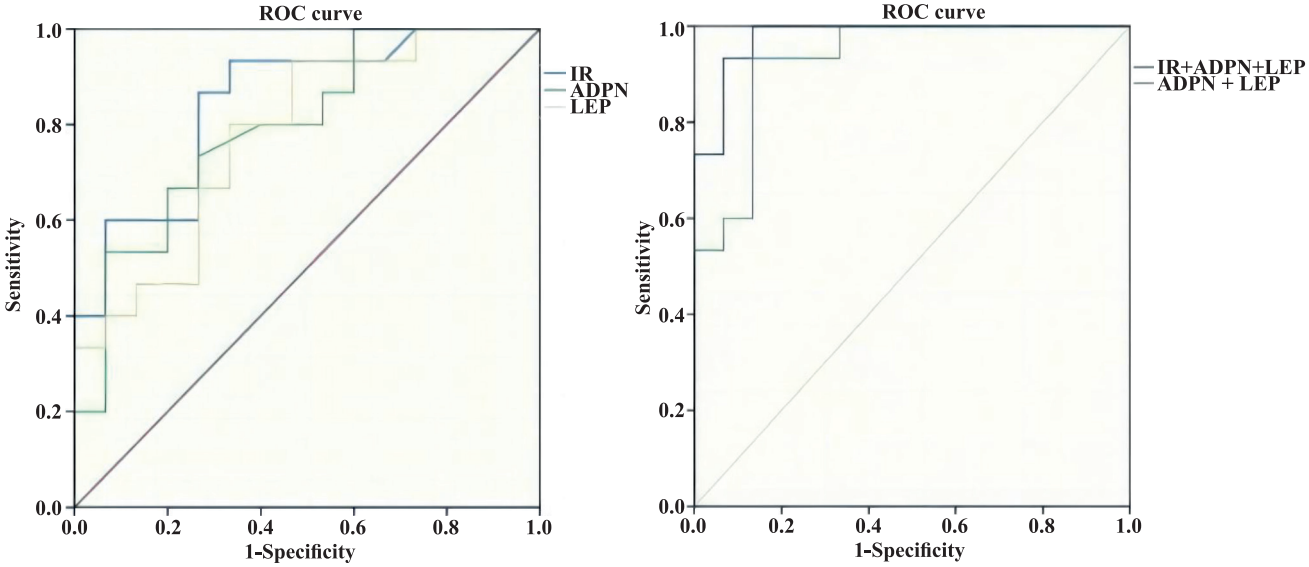

Fig. 2. Area under the curve of serum ADPN and LEP of cows with ovarian disease. IR – insulin resistance, ADPN – adiponectin, LEP – leptin, ROC – area under the ROC curve.

Table 7. Correlation between serum biochemical indexes and IO in dairy cows (n = 12).

Project	Mean	*R*-value	*P*-Value
IR index	2.83 ± 0.53	0.986	<0.001
INS (μU/mL)	17.95 ± 7.44	0.989	<0.001
IGF-1(ng/mL)	79.80 ± 15.76	-0.963	<0.001
ADPN (μg/mL)	2.63 ± 0.49	-0.998	<0.001
LEP (μg/L)	5.28 ± 0.41	-0.991	<0.001

IO – inactive ovarie, INS – insulin, IGF-1 – insulin-like growth factor 1, ADPN – adiponectin, LEP – leptin.

Table 8. The cutoff point, sensitivity, specificity, standard error and area under the ROC curve of ADPN, LEP and IR for diagnosis of inactive ovarian in dairy cows.

Project	Cut-Off	Sensitivity %	Specificity %	SEM	+LR	AUC
ADPN	2.365	0.823	0.857	0.060	6.613	0.778
LEP	5.565	0.733	0.886	0.102	7.563	0.796
IR index	3.796	0.713	0.832	0.071	7.663	0.817
ADPN+LEP	-	0.863	0.867	0.057	5.32	0.885
ADPN+ LEP+IR	-	0.913	0.872	0.046	6.16	0.928

ADPN – adiponectin, LEP – leptin, IR index – insulin resistance index, +LR – positive likelihood ratio, AUC – area under the ROC curve.

predicting inactive ovaries in cows. The optimal early warning values for ovarian static cows were determined using the Youden index.

For serum ADPN levels exceeding 2.365 μg/mL, the sensitivity was 82.3%, specificity was 85.7%, and the AUC was 0.778, indicating significant diagnostic relevance. Serum LEP levels surpassing 5.565 μg/L exhibited a sensitivity of 73.3%, specificity of 88.6%, and an AUC of 0.796, indicating a certain diagnostic significance. An IR index exceeding 3.796 demonstrated a sensitivity of 71.3%, specificity of 83.2%, and an AUC of 0.817, signifying a significant diagnostic capacity.

As illustrated in Fig. 2B, the combined analysis of two cytokines in blood exhibited a sensitivity of 86.3%, specificity of 86.7%, and an AUC of 0.885, signifying a certain diagnostic significance. Moreover, when the two cytokines and the IR index were analyzed in combination for prediction, the sensitivity increased to 91.3%, specificity to 87.2%, and the AUC significantly rose to 0.928, indicating a high diagnostic significance.

Discussion

The study aimed to investigate the predictive potential of IR, ADPN, and LEP for postpartum IO in dairy cows. Our findings indicate that IR significantly impacts reproductive performance and metabolic health, emphasizing the need for early detection and intervention strategies to mitigate the adverse effects on dairy production.

The data demonstrated a clear association between IR and impaired reproductive performance. Cows with IR exhibited prolonged durations for first estrus, insemination, days open, and calving intervals, along with a notable reduction in milk yield and conception rates compared to non-IR cows. It was also found in this study that the incidence of postpartum IO in cows with IR was 53.33% and that in cows with non-IR was 16.67%. High IR increased the risk of postpartum IO by 3.2 times, indicating that IR is a significant risk factor for postpartum IO in cows. These findings align with previous research indicating that IR exacerbates NEB and adversely affects reproductive hormones, ultimately leading to ovarian dysfunction (Baruselli et al. 2016, Mirzaie et al. 2023). Elevated levels of BHBA and NEFA and decreased levels of E2 and P4 in IR cows further corroborate the link between metabolic stress and reproductive inefficiency (Rico et al. 2015). Insulin resistance has been extensively studied in relation to its effects on dairy cow fertility. Previous studies have shown that IR is closely linked to decreased insulin sensitivity, which in turn affects the secretion of IGF-1, a critical hormone for follicular development and oocyte quality (Veerkamp et al. 2003). The lower insulin sensitivity in IR cows may impair IGF-1 signaling pathways, leading to suboptimal follicular growth and increased incidence of ovarian disorders (Gong et al. 2002, Baruselli et al. 2016).

Adiponectin and leptin, critical adipokines involved in energy homeostasis, exhibited significant changes in serum, mRNA, and protein levels, correlating with IO risk. The study found lower ADPN and LEP levels in IR cows, consistent with the role of these adipokines in modulating insulin sensitivity and reproductive functions (Oliveira et al. 2016). The threshold values identified for ADPN (>2.365 μg/mL) and LEP (>5.565 μg/mL) demonstrated high sensitivity and specificity in predicting IO, suggesting their potential as reliable early warning biomarkers (Leiva et al. 2015). Adiponectin is known for its insulin-sensitizing properties, and its decreased levels are associated with increased insulin resistance and metabolic disturbances. In dairy cows, low ADPN levels may contribute to the exacerbation of NEB, further impairing reproductive functions (Abuelo et al. 2016). Similarly, leptin, which regulates appetite and energy balance, has been shown to play a role in reproductive function by modulating the hypothalamic-pituitary-gonadal axis (Yu et al. 1997). Reduced leptin levels in IR cows may reflect an impaired energy status, adversely affecting reproductive hormone secretion and ovarian activity (Trakovická et al. 2013).

The combined analysis of ADPN, LEP, and IR index significantly improved the predictive accuracy for IO. The sensitivity and specificity reached 91.3% and 87.2%, respectively, with an area under the curve (AUC) of 0.928, indicating high diagnostic significance. This combined approach enhances the robustness of early detection strategies, allowing for timely interventions to improve reproductive outcomes in dairy herds (Jorritsma et al. 2003, Leiva et al. 2017). Combining multiple biomarkers can provide a more comprehensive assessment of metabolic and reproductive health. The integration of ADPN, LEP, and IR index allows for the identification of cows at higher risk for IO, facilitating targeted nutritional and management interventions. This approach has been supported by previous research, which highlights the importance of using multiple indicators to predict reproductive disorders in dairy cows (Nebel and Mcgilliard 1993).

The findings underscore the importance of monitoring metabolic and hormonal profiles in postpartum dairy cows. Implementing routine assessments of IR, ADPN, and LEP can facilitate early identification of cows at risk of IO, enabling targeted nutritional and management interventions to alleviate NEB and support reproductive health. This proactive approach can ultimately enhance overall herd productivity and economic returns (De Koster et al. 2013). Early identification of cows at risk for IO allows for timely intervention strategies, such as nutritional supplementation and management adjustments, to improve metabolic status and reproductive performance. Providing balanced diets with adequate energy and nutrient supply can help mitigate NEB and support overall health and fertility. Additionally, monitoring ADPN and LEP levels can help identify cows that may benefit from specific dietary interventions aimed at improving insulin sensitivity and energy balance (Gareis et al. 2020).

Further studies are warranted to explore the mechanistic pathways linking IR, adipokines, and ovarian function. Investigating the efficacy of specific dietary and pharmacological interventions in modulating these biomarkers and improving reproductive performance will provide valuable insights for dairy management practices. Additionally, expanding the sample size and including diverse dairy populations will strengthen the generalizability of the findings (LeBlanc 2014, Kawashima et al. 2016). Future research should focus

on elucidating the molecular mechanisms through which IR, ADPN, and LEP influence ovarian function and overall reproductive health. Understanding these pathways can lead to the development of targeted therapeutic interventions to improve fertility outcomes in dairy cows. Moreover, large-scale studies involving diverse dairy populations will help validate the findings and enhance their applicability across different management systems and environmental conditions (Moyes et al. 2003, Bossaert et al. 2008).

In conclusion, this study substantiates the correlation between IR, adipocytokines (ADPN, LEP), energy metabolism indices (BHB, NEFA, GLU), hormonal markers including INS, E2, P4, IGF-1, GH, and the occurrence of postpartum IO in dairy cows. The identification of risk warning indicators, either singularly or in combination (LEP, ADPN, IR), along with their respective determination thresholds, serves as a crucial scientific foundation for more effective prevention of postpartum IO in dairy cows in the future. Future research should focus on refining these biomarkers and developing targeted interventions to support metabolic and reproductive health in dairy cows.

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