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Early Warning System of Risk in Dairy Cows with Inactive Ovaries

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Abstract

The incidence of Inactive ovaries of dairy cows in China is relatively high. There is no complete early warning system for the occurrence of ovarian quiescence in clinical cows. This test provides early warning indicators for clinical prediction of ovary cessation in dairy cows. This experiment selected blood samples of dairy cows from 60 to 90 days postpartum in the inactive ovaries group and control group. Differential proteins were selected on the basis of proteomics, three energy indexes: AST, Glu, NEFA. Four reproductive hormones: E2, P4, FSH, LH, and four differentially expressed proteins: IGFBP-2, AHSG, APO-A4, and RBP-4. Key enzyme activities: ALDOB, LDHB, ITIH3, GPX3, SPAM1, PKM2. The ELISA test kit was used to detect the content and activity of the above markers in the test bovine serum. Through correlation analysis, binary logistic regression modeling and ROC analysis, a single indicator early warning technique for APOA4 and ITIH3 was established. The early warning values were APOA4 > 28.825 μg/L and ITIH3 > 195.07 ng/L. A multi-index early warning system based on potential biomarkers of APOA4 + ITIH3 and APOA4 + ITIH3 + E2 was established. The former had an early warning value of: APOA4 > 19.55 μg/I; ITIH3 > 191.14 ng/L; the latter has an early warning value: APOA4 > 47.56 μg/L, ITIH4 > 187.80 ng/L, E2 < 69.63 ng/L.

Keywords

Inactive Ovaries of Dairy Cows, APOA4, ITIH3, E2, ROC

1. Introduction

Ovarian quiescence in dairy cows occurs when the follicles appear on the surface of the ovary, and even earlier, stagnant. This hinders the first step in the post-partum pregnancy of the cow during the postpartum period, which results

in an extension of the cow's calving interval [1]. Severe dairy cows will be eliminated in advance because of their reduced breeding performance and lactation performance, causing significant economic losses to dairy farms. In the previous lab, iTRAQ/LC-MS/MS technology was used to study the expression of GPX3, SCGB1D, and PKM2 in dairy cattle's quiescent serum proteomics. ADIPOQ, AHSG, APOA4, FETUB, ALDOB, SPAM1, LDHB, RBP4, IGFBP2 The expression of ITIH3 and GLYCAM1 was up-regulated, and AST, Glu, NEFA, E2, P4, FSH, LH, IGFBP-2, AHSG, APO-A4, RBP-4, ALDOB, LDLHB, ITIH3, GPX3, SPAM1, and PKM2 related factors were determined. Ovarian quiescence has a significant correlation.

At present, there is a bit research on early warning systems for common diseases in dairy cattle in China. The use of biomarkers screened by proteomics technology at home and abroad to establish a disease risk early warning system has become an important method and tool for monitoring the risk of disease. Lin *et al.* [2]. Used proteomics, mass spectrometry, and ELISA methods combined with statistical receiver operating characteristic curve (ROC) analysis to screen and identify differentially expressed proteins in human nasopharyngeal carcinoma. Determination of biomarker markers. Xinhuan Xiao [3] also used a combination of proteomics and ROC analysis to determine that a protein can diagnose early postmortem ovarian quiescence in dairy cows. However, because the differential proteins screened by multiple platforms of proteomics complement each other, and ROC analysis can be used for multi-monitoring and early warning of diseases, this study has carried out multi-index joint warning of disease risk.

At present, there is no complete warning system for the occurrence of ovarian static in dairy cows. This experiment provides an early warning indicator for the prediction of ovarian static in cows.

2. Materials and Methods

2.1. Test Animals

This experiment selected an intensive cattle farm in Heilongjiang, China. Dairy cows were fed a full-mixed diet (TMR) consisting of 8 - 9 *kg* of concentrate, 17 - 20 *kg* of silage, 3.5 - 4.0 *kg* of hay, and 300 - 400 *g* of fat. Its nutritional level is: DM55.60%, crude protein 16%, net milk production 7.322 *MJ/kg*, fat 5.60%, NDF39.10%, ADF20.30%, calcium 180 *g*, phosphorus 116 *g*. The basic information of dairy cows is shown in **Table 1**, on the basis of which the two groups of cows are followed to 60 - 90 days postpartum. The external performances are observed 60 d after delivery and a rectal examination or B-ultrasound examination is performed to monitor the development of follicles. Based on this examination, the abnormal cows were excluded from the study. Finally, 35 cows were selected from the ovaries of the ovaries-free group (IO group) and the healthy control group (CON group).

Table 1. The basic information of test animals from two groups.

Mean index (± standard deviation)	IO (N = 50)	CON (N = 50)	p-value
Postnatal days	72.90 (8.322)	73.82 (9.064)	0.735
Follicular diameter (mm)	2.25 (1.37)	10.82 (2.30)	0.000**
Age	3.06 (1.21)	3.19 (1.35)	0.755
Fetal times	1.75 (0.91)	1.19 (1.06)	0.607
BSC	2.66 (0.25)	2.80 (0.18)	0.053
Daily milk production (kg/day)	34.16 (12.53)	33.04 (7.11)	0.721
BHBA (mmol/L)	0.61 (0.14)	0.48 (0.16)	0.073

2.2. Detection Indicators

All experimental cow blood samples were tested for concentrations of the following indicators in the blood 60 - 90 days postpartum. The kits used in the experiments were purchased from Nanjing Jinyibai Company and tested by ELISA.

Energy Index: Aspartate aminotransferase (AST), Glucosamine (Glu), Non-free fatty acids (NEFA);

Reproductive hormone: E2, P4, FSH, LH;

Differential protein content: IGFBP-2, AHSG, APO-A4, RBP-4;

Key enzyme activities: ALDOB, LDHB, ITIH3, GPX3, SPAM1, PKM2.

2.3. Data Processing

According to the results of the energy index test, a difference analysis was conducted between the two groups. Then, the energy index, reproductive hormones, differentially expressed proteins, key enzymes and ovarian quiescence were established to establish Pearson correlation analysis, and regression analysis was used to determine the energy index's early warning effect on the occurrence of ovarian static.

2.4. Establishment of an Early Warning System for the Risk of Disease

According to the results, ROC analysis was applied to indicators that were significantly related to ovarian static. Through the results of ROC analysis, a single early warning indicator (biomarker) that can be used for risk of inactive ovaries, as well as cutoff values, sensitivity, and specificity, are determined to determine the best early warning system and establish a single indicator disease risk early warning system.

According to the confirmation of a single indicator, based on the optimal forecast indicator, other indicators are added in sequence to perform multiple forecasting. According to the combination of multiple indicators, use probability calculations to determine the coefficients of multiple indicators for disease prediction, apply coefficients for ROC analysis, determine multiple early warning indicators (biomarkers) and cutoff values that can be used for risk of ovarian

static, determine the best early warning system established a multi-index joint disease risk early warning system.

3. Results

3.1. The Level of Blood Energy Indicators

The results of the blood energy index tests of cows (CON) in the inactive ovaries group (IO) and the healthy control group are shown in **Table 2**.

According to the energy index test results, there was no significant difference between the two groups in AST, Glu and NEFA ($p > 0.05$). This shows that the energy metabolism of ovarian stationary cows is normal.

3.2. Blood Reproductive Hormone Levels

The results of the test on blood reproductive hormones of dairy cows in the ovarian static group and the healthy control group are shown in **Table 3**.

According to the analysis of the test results of reproductive hormones, we can see that among the four indicators of reproductive hormones detected between the two groups, there was only a significant difference between the two groups ($p < 0.05$).

3.3. The Level of Major Differential Proteins and Key Enzymes in Blood

Table 4 shows the levels of 10 major differential proteins and key enzyme assays in the blood of dairy cows in the quiescent group and the healthy control group.

Table 2. Serum levels of energy and liver function parameters from two groups.

Detection Indicator	IO (N = 35)	CON (N = 35)	p
AST (U/L)	92.06 ± 17.95	98.94 ± 23.26	0.189
Glu (mmol/L)	3.75 ± 1.40	3.65 ± 0.55	0.686
NEFA (mmol/L)	0.21 ± 0.17	0.26 ± 0.15	0.150

Note: *indicates that there is a significant difference between the two groups ($p < 0.05$); **indicates that there is a very significant difference between the two groups ($p < 0.01$); if there is no shoulder note, the index is in both groups. There was no significant difference between the two groups ($p > 0.05$).

Table 3. Serum levels of reproductive hormones from two groups.

Detection Indicator	IO (N = 35)	CON (N = 35)	p
E2 (ng/L)	60.24 ± 26.66	75.11 ± 24.03	0.017*
P4 (ng/L)	138.39 ± 83.54	137.56 ± 79.10	0.966
E_2/P_4	0.59 ± 0.34	0.75 ± 0.53	0.148
FSH (IU/L)	4.23 ± 1.68	5.15 ± 2.80	0.101
LH (ng/L)	15.22 ± 10.33	19.76 ± 11.20	0.144

Note: *indicates that there is a significant difference between the two groups ($p < 0.05$); **indicates that there is a very significant difference between the two groups ($p < 0.01$); if there is no shoulder note, the index is in both groups. There was no significant difference between the two groups ($p > 0.05$).

Table 4. Serum levels of different proteins and key enzymes from two groups.

Detection Indicator	IO (N = 35)	CON (N = 35)	p
IGFBP2 (μg/L)	42.36 ± 20.43	36.52 ± 11.32	0.144
AHSG (ng/L)	1520.28 ± 506.54	1280.98 ± 466.86	0.044*
APOA4 (μg/L)	42.10 ± 15.99	30.25 ± 9.89	0.000**
RBP4 (μg/L)	15.15 ± 5.84	12.16 ± 3.65	0.013*
ALDOB (ng/L)	711.73 ± 159.41	613.87 ± 132.87	0.007**
LDHB (U/L)	1117.60 ± 237.22	1024.37 ± 148.00	0.053
ITIH3 (ng/L)	181.55 ± 32.19	157.21 ± 24.06	0.001**
GPX3 (ng/L)	307.35 ± 180.69	378.95 ± 246.29	0.170
SPAM1 (ng/mL)	409.99 ± 168.58	351.52 ± 145.39	0.125
PKM2 (ng/L)	478.36 ± 121.43	501.80 ± 215.43	0.577

Note: *indicates that there is a significant difference between the two groups ($p < 0.05$); **indicates that there is a very significant difference between the two groups ($p < 0.01$); if there is no shoulder note, the index is in both groups. There was no significant difference between the two groups ($p > 0.05$).

According to the results of key differential proteins and key enzyme assays, APOA4, ALDOB, and ITIH3 were significantly different between the two groups ($p < 0.01$). The ovarian quiescence group was higher than the healthy control group; AHSG and RBP4 existed between the two groups. Significant difference ($p < 0.05$).

3.4. Correlation Analysis of Energy Index, Reproductive Hormones and Key Different Proteins, Key Enzymes, and Occurrence of Ovarian Metastasis

The results of all testing indicators were analyzed using the Pearson correlation coefficient in the statistical analysis software. The results of the analysis were shown in **Table 5**.

3.5. The Establishment of Binary Logistic Regression and the Determination of Early Warning Indicators

According to the classification of relevant detection indicators, two modules were established to perform binary logistic regression on the data to determine the degree of regression fit and the determination of early warning indicators.

1) Model I

Model I is mainly based on the results of serum reproductive hormone indicators for the determination of binary logistic regression and early warning indicators. According to **Table 6**, the chi-square of the model is 4.841. According to the significance (0.05) and the degree of freedom (df), the chi-squared value can be calculated using the CHIINV (significance, degree of freedom) in EXCEL. The settlement is CHIINV (0.05, 8) = 15.507 and the chi-square statistic is less than the critical value of the chi-square.

According to the analysis results in **Table 7**, the model has a good prediction

Table 5. Correlations analysis among all parameters from groups of dairy cows.

		IO	AST	GLU	NEFA	E2	P4	E2/P4	FSH	LH	IGFBP2	SPAM1	PKM2
IO	R	1	0.166	−0.049	0.176	0.285*	−0.005	0.175	0.197	0.209	−0.177	−0.185	0.068
	p		0.17	0.686	0.146	0.017	0.966	0.148	0.101	0.082	0.144	0.125	0.577
AST	R	0.166	1	−0.077	−0.102	0.018	0.026	−0.192	0.158	0.104	0.062	0.041	−0.002
	p	0.17		0.529	0.403	0.879	0.828	0.112	0.191	0.393	0.608	0.734	0.99
GLU	R	−0.049	−0.077	1	−0.021	0.027	−0.079	−0.013	−0.121	−0.089	−0.075	−0.098	−0.148
	p	0.686	0.529		0.865	0.822	0.515	0.913	0.318	0.464	0.536	0.418	0.222
NEFA	R	0.176	−0.102	−0.021	1	0.244*	0.288*	0.022	0.019	0.338**	−0.109	0.13	−0.063
	p	0.146	0.403	0.865		0.042	0.016	0.854	0.877	0.004	0.37	0.284	0.602
E2	R	0.285*	0.018	0.027	0.244*	1	0.129	0.357**	0.525**	0.448**	−0.088	0.323**	0.222
	p	0.017	0.879	0.822	0.042		0.286	0.002	0	0	0.47	0.006	0.065
P4	R	−0.005	0.026	−0.079	0.288*	0.129	1	−0.691**	0.385**	0.426**	0.209	0.238*	0.225
	p	0.966	0.828	0.515	0.016	0.286		0	0.001	0	0.083	0.047	0.061
E2/P4	R	0.175	−0.192	−0.013	0.022	0.357**	−0.691**	1	−0.099	−0.189	−0.221	−0.094	−0.153
	p	0.148	0.112	0.913	0.854	0.002	0		0.417	0.118	0.066	0.438	0.205
FSH	R	0.197	0.158	−0.121	0.019	0.525**	0.385**	−0.099	1	0.547**	0.104	0.353**	0.475**
	p	0.101	0.191	0.318	0.877	0	0.001	0.417		0	0.391	0.003	0
LH	R	0.209	0.104	−0.089	0.338**	0.448**	0.426**	−0.189	0.547**	1	0.061	0.442**	0.437**
	p	0.082	0.393	0.464	0.004	0	0	0.118	0		0.615	0	0
IGFBP2	R	−0.177	0.062	−0.075	−0.109	−0.088	0.209	−0.221	0.104	0.061	1	0.347**	0.204
	p	0.144	0.608	0.536	0.37	0.47	0.083	0.066	0.391	0.615		0.003	0.091
AHSG	R	−0.242*	0.018	0.031	0.229	0.395**	0.295*	−0.074	0.433**	0.490**	0.238*	0.744**	0.370**
	p	0.044	0.884	0.797	0.057	0.001	0.013	0.543	0	0	0.047	0	0.002
APOA4	R	−0.412**	0.17	0.069	0.148	0.092	0.225	−0.152	0.146	0.151	0.107	0.449**	0.108
	p	0	0.159	0.573	0.22	0.447	0.061	0.21	0.229	0.212	0.38	0	0.371
RBP4	R	−0.297*	−0.038	−0.106	0.165	0.065	0.217	−0.117	−0.114	0.079	0.194	0.311**	0.125
	p	0.013	0.754	0.383	0.172	0.593	0.072	0.335	0.348	0.513	0.107	0.009	0.304
ALDOB	R	−0.320**	−0.066	−0.237*	0.087	0.117	0.268*	−0.15	0.19	0.352**	0.359**	0.365**	0.124
	p	0.007	0.588	0.048	0.472	0.335	0.025	0.215	0.116	0.003	0.002	0.002	0.306
LDHB	R	−0.233	0.369**	0.058	−0.128	−0.001	0.046	−0.117	0.025	−0.095	0.157	0.205	−0.028
	p	0.053	0.002	0.635	0.29	0.993	0.703	0.333	0.839	0.432	0.196	0.089	0.821
ITIH3	R	−0.398**	−0.001	−0.142	0.068	0.234	0.11	−0.123	0.165	0.222	0.124	0.299*	0.17
	p	0.001	0.995	0.239	0.574	0.051	0.364	0.312	0.171	0.065	0.305	0.012	0.16
GPX3	R	0.166	0.116	−0.104	0.255*	0.419**	0.202	−0.055	0.453**	0.534**	0.172	0.580**	0.623**
	p	0.17	0.34	0.39	0.033	0	0.094	0.649	0	0	0.154	0	0
SPAM1	R	−0.185	0.041	−0.098	0.13	0.323**	0.238*	−0.094	0.353**	0.442**	0.347**	1	0.387**
	p	0.125	0.734	0.418	0.284	0.006	0.047	0.438	0.003	0	0.003		0.001
PKM2	R	0.068	−0.002	−0.148	−0.063	0.222	0.225	−0.153	0.475**	0.437**	0.204	0.387**	1
	p	0.577	0.99	0.222	0.602	0.065	0.061	0.205	0	0	0.091	0.001	

Note: R represents the Pearson correlation coefficient, positive is a positive correlation, negative is a negative correlation, shoulder *indicates significant correlation ($p < 0.05$), shoulder **indicates extremely significant correlation ($p < 0.01$), no shoulder Note that there is no significant correlation ($p > 0.05$).

effect on ovary quiescence, which is 62.9%, and the prediction of healthy cows is 571%. This shows that the model can predict ovarian static to some extent.

According to the analysis results in **Tables 8-9**, if the variable E2 is removed from the model, the significance of the change is 0015 < 0.05. This shows that E2 is significantly associated with prevention of ovarian quiescence and cannot be removed.

2) Model II

Model II determines the binary logistic regression and early warning indicators based on the results of serum key differentiating proteins and key enzymes (**Table 10**).

According to the analysis results in **Table 11**, it can be seen that APOA4 is better for warning to healthy cows than ovary stationary cows; after adding GPX3, both groups of warnings are improved, the total percentage is 80%; and further progress in adding RBP4 will reduce the early warning effect. 78.6%; further progress in joining ITIH3 resulted in a noticeable increase in early warning effectiveness, both at 80%; and finally at SPAM1, the total percentage of

Table 6. Hosmer and Leme show test.

Step	Bangla	df	Sig.
1	4.841	8	0.774

Table 7. Classification table.

		Predicted		
		Group		Percentage correction
Observed		IO	CON	
Step 1	IO	22	13	62.9
	CON	15	20	57.1
	Total percentage			60

Table 8. Model if term removed.

	Variable	Log likelihood of the model	Changes in the log-likelihood of −2	df	Change of significance
Step 1	E_2	−48.52	5.889	1	0.015

Table 9. Variables not in the equation.

		Score	df	Sig.
Step 1	P_4	0.124	1	0.725
Variable	E_2/P_4	0.465	1	0.495
	FSH	0.26	1	0.610
	LH	0.659	1	0.417
	Presidential measurement	2.071	4	0.723

Table 10. Hosmer and Leme show test.

Step	Bangla	df	Sig.
1	4.841	8	0.774
2	3.604	8	0.891
3	2.263	8	0.972
4	5.452	8	0.708
5	4.55	8	0.804

Table 11. Classification table.

		Predicted		
		Group		Percentage correction
Observed		IO	CON	
Step 1	IO	21	14	60
	CON	10	25	71.4
	Total percentage			65.7
Step 2	IO	25	10	71.4
	CON	9	26	74.3
	Total percentage			74.3
Step 3	IO	28	7	80
	CON	7	27	77.1
	Total percentage			78.6
Step 4	IO	28	7	80
	CON	7	28	80
	Total percentage			80
Step 5	IO	28	7	80
	CON	8	27	77.1
	Total percentage			78.6

warnings was 78.6%. This shows that the model can predict ovarian static to some extent.

According to the results of **Table 12**, it can be seen that if the variables APOA4, GPX3, RBP4, ITIH3, and SPAM1 were removed in the model, the significance of the changes, although they were successively increased, was still less than 0.05. Note that POA4, GPX3, RBP4, ITIH3, and SPAM1 are significantly related to the occurrence of ovarian quiescence and cannot be removed.

According to **Table 13**, the significance of removing IGFBP2, AHSG, ALDOB, LDHB, and PKM2 was greater than 0.05, indicating that IGFBP2, AHSG, ALDOB, LDHB, and PKM2 could not be used as predictors of ovarian static. According to the establishment of Model II, APOA4, GPX3, RBP4, ITIH3, and SPAM1 can predict ovarian quiescence in the energy index, while IGFBP2,

Table 12. Model if term removed.

	Variable	Log likelihood of the model	Changes in the log-likelihood of −2	df	Change of significance
Step 1	APOA4	−48.52	13.891	1	0
Step 2	APOA4	−47.515	26.597	1	0
	GPX3	−41.575	14.716	1	0
Step 3	APOA4	−42.277	23.367	1	0
	RBP4	−34.217	7.246	1	0.007
	GPX3	−40.322	19.456	1	0
Step 4	APOA4	−36.953	17.351	1	0
	RBP4	−30.903	5.25	1	0.022
	ITIH3	−30.594	4.633	1	0.031
	GPX3	−37.631	18.708	1	0
Step 5	APOA4	−31.484	10.754	1	0.001
	RBP4	−27.579	2.944	1	0.086
	ITIH3	−28.67	5.125	1	0.024
	GPX3	−37.442	22.669	1	0
	SPAM1	−28.277	4.34	1	0.037

AHSG, ALDOB, LDHB, and PKM2 cannot.

According to all the analysis results, among the detected reproductive hormones, major differentially expressed proteins and key enzymes, E2, APOA4, GPX3, RBP4, ITIH3, and SPAM1 can be used as indices for early warning of ovarian rest in dairy cows

3.6. Risk Warning System of Single Indicator

In this experiment, ROC analysis was performed on the above-mentioned early warning indicators, and the Youden value was calculated based on the experimental results. Youden = sensitivity + specificity − 1. Select the critical value of the indicator based on the Youden value. The results are shown in **Table 14**, and the ROC analysis curve is shown in **Figure 1**.

According to the ROC analysis results, the better indicators of early warning effectiveness are APOA4, ITIH3, E2, SPAM1, RBP4, and GPX3. The area under the curve is 0.747, 0.711, 0.673, 0.646, 0.637 and 0.586, respectively. This shows that all eight early warning indicators can provide early warning of ovarian static. The early warning thresholds for each indicator were: APOA4 > 28.825 μg/L, ITIH3 > 195.07 ng/L, E2 < 48.19 ng/L, SPAM1 > 294.255 ng/ml, RBP4 > 15.215 μg/L, and GPX3 < 441.43 ng/L.

3.7. Multiple Indicators Joint Risk Early Warning System

According to the single-indicator warning results, although 8 indicators can provide early warning of ovarian inactivity, in order to explore better warning

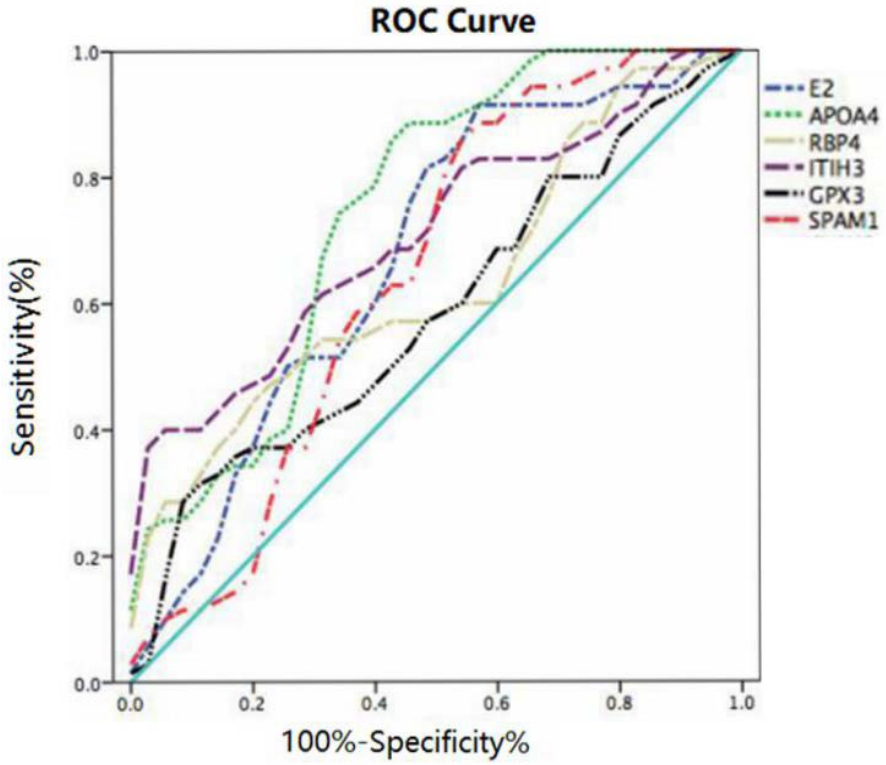
Table 13. Variables not in the equation.

	variable	Score	df	Sig.
Step 1	IGFBP2	0.942	1	0.332
	AHSG	0.002	1	0.968
	RBP4	2.482	1	0.115
	ALDOB	1.905	1	0.167
	LDHB	1.521	1	0.217
	ITIH3	6.587	1	0.01
	GPX3	7.371	1	0.007
	SPAM1	0.04	1	0.842
	PKM2	1.65	1	0.199
	Presidential measurement	21.016	9	0.013
Step 2	IGFBP2	1.903	1	0.168
	AHSG	3.082	1	0.079
	RBP4	6.734	1	0.009
	ALDOB	1.328	1	0.249
	LDHB	3.329	1	0.068
	ITIH3	6.616	1	0.01
	SPAM1	6.705	1	0.01
	PKM2	0.03	1	0.863
	Presidential measurement	18.573	8	0.017
Step 3	IGFBP2	0.849	1	0.357
	AHSG	1.538	1	0.215
	ALDOB	0.292	1	0.589
	LDHB	3.174	1	0.075
	ITIH3	4.69	1	0.03
	SPAM1	3.759	1	0.053
	PKM2	0.002	1	0.967
	Presidential measurement	13.049	7	0.071
Step 4	IGFBP2	1.187	1	0.276
	AHSG	1.674	1	0.196
	ALDOB	0.065	1	0.798
	LDHB	3.784	1	0.052
	SPAM1	4.068	1	0.044
	PKM2	0.305	1	0.581
	Presidential measurement	8.678	6	0.193
Step 5	IGFBP2	0.885	1	0.347
	AHSG	0.785	1	0.376
	ALDOB	0.092	1	0.761
	LDHB	2.862	1	0.091
	PKM2	0.684	1	0.408
	Presidential measurement	5.457	5	0.363

Table 14. The results of single warning indicators from ROC analysis.

Variable	Sensitivity (%)	Specificity (%)	Area under the curve	Demarcation
E_2	91.4	42.9	0.673	<48.19 ng/L
APOA4	88.6	57.1	0.747	>28.825 μg/L
RBP4	42.9	82.9	0.637	>15.215 μg/L
ITIH3	40	97.1	0.711	>195.07 ng/L
GPX3	28.6	94.3	0.586	<441.43 ng/L
SPAM1	88.6	42.9	0.646	>294.255 ng/ml

Note: E_2: Estradiol; APOA4: Apolipoprotein A-IV; RBP4: Retinol-binding protein 4; ITIH3: Inter-alpha-trypsin inhibitor heavy chain H3; GPX3: Glutathione peroxidase; SPAM1: Hyaluronidase.



Note: E2 is estradiol; APOA4 is apolipoprotein 4; RBP4 is retinol binding protein 4; ITIH3 is Inter-*α*-trypsin inhibitor heavy chain H3; GPX3 is glutathione peroxidase; SPAM1 Hyaluronidase.

Figure 1. ROC curve.

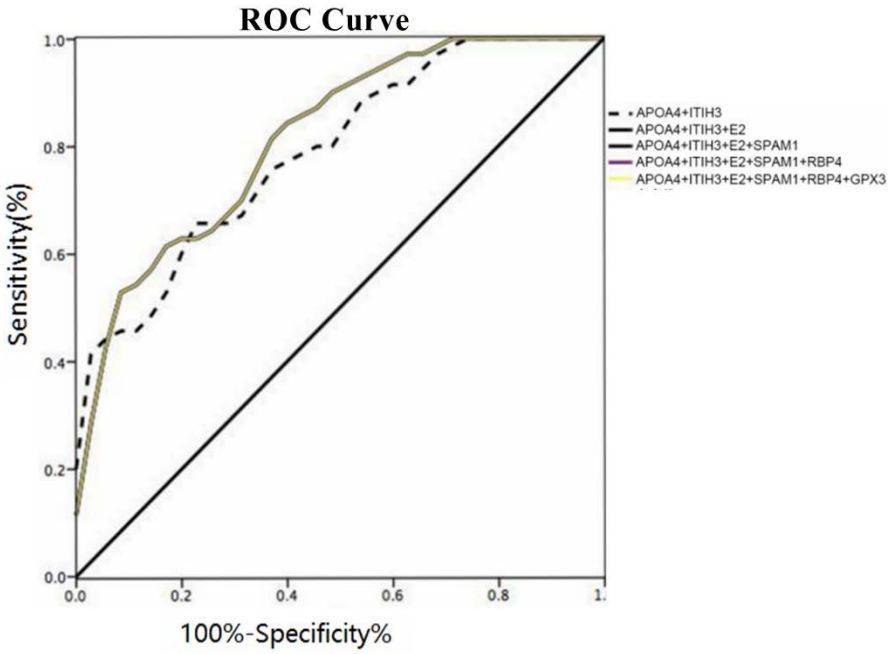
effects, the experiment is based on APOA4 with the best warning effect, followed by the addition of the best indicators for other warnings to fit, and then Perform ROC analysis and establish multiple indicators for early warning of ovarian static. The results are shown in **Table 15**, and the ROC analysis chart is shown in **Figure 2**.

According to the multi-index ROC analysis results, proper combination of various indicators can effectively improve the effect of disease early warning. The sensitivity and specificity of APOA4 + ITIH3 combined with early warning were 65.7% and 80%, respectively. After increasing E2, the sensitivity increased

Table 15. The results of multiple warning indicators from ROC analysis.

Variable	Sensitivity (%)	Specificity (%)	Area under the curve
APOA4 + ITIH3	65.7	80	0.792
APOA4 + ITIH3 + E_2	82.9	62.9	0.816
APOA4 + ITIH3 + E_2 + SPAM1	82.9	62.9	0.816
APOA4 + ITIH3 + E_2 + SPAM1 + RBP4	82.9	62.9	0.816
APOA4 + ITIH3 + E_2 + SPAM1 + RBP4 + GPX3	82.9	62.9	0.816

Note: E2 is estradiol; APOA4 is apolipoprotein 4; RBP4 is retinol binding protein 4; ITIH3 is Inter-*α*-trypsin inhibitor heavy chain H3; GPX3 is glutathione peroxidase; SPAM1 Hyaluronidase.



Note: Due to the fact that the area under the curve of some prediction results is the same, multiple analytical curves overlap. APOA4 is apolipoprotein 4; ITIH3 is Inter-*α*-trypsin inhibitor heavy chain H3; E2 is estradiol; SPAM1 is hyaluronidase; RBP4 is retinol binding protein 4; GPX3 is glutathione Oxide enzyme.

Figure 2. ROC curve from composite warning indicators.

to 82.9% (17.2% above float), but the specificity decreased to 62.8% (downward 17.2%). After continuing to add indicators to the model, it did not affect the effectiveness, specificity, and sensitivity of the warning. Therefore, APOA4 + ITIH3 and APOA4 + ITIH3 + E2 are the two most effective early warning modes.

After the ROC analysis based on the probability values generated by the multi-index model, the Youden value calculation is consistent with the previous experiment. The index data corresponding to the largest Youden value is the boundary value of the diagnostic model. The results are as follows:

1) APOA4 + ITIH3: APOA4 > 19.55 μg/L, ITIH3 > 191.14 ng/L;

2) APOA4 + ITIH3 + E2: APOA4 > 47.56 μg/L, ITIH3 > 1827.80 ng/L, E2 <

69.63 ng/L;

3) APOA4 + ITIH3 + E2 + SPAM1: APOA4 > 47.56 μg/L, ITIH3 > 1827.80 ng/L, E2 < 69.63 ng/L, SPAM1 > 472.07 ng/ml;

4) APOA4 + ITIH3 + E2 + SPAM1 + RBP4: APOA4 > 47.56 μg/L, ITIH3 > 1827.80 ng/L, E2 < 69.63 ng/L, SPAM1 > 472.07 ng/ml, RBP4 > 16.84 μg/L;

5) APOA4 + ITIH3 + E2 + SPAM1 + RBP4 + GPX3: APOA4 > 47.56 μg/L, ITIH3 > 1827.80 ng/L, E2 < 69.63 ng/L, SPAM1 > 472.07 ng/ml, RBP4 > 16.84 μg/L, GPX3 < 456.44 ng/L.

4. Discussion

Ovarian quiescence in cows is usually diagnosed by estrus identification 50 - 60 days after childbirth. The estrus identification method is usually to observe the estrus detection, rectal examination and B-ultrasound, and then use hormones to treat ovarian static cows. The existing problems: 1) The diagnosis of ovarian static is mostly after the occurrence; 2) The rectal examination is more stressful to dairy cows and does not meet the animal welfare requirements; 3) Hormone treatment after ovarian static, the effect is not the same, dairy hormones Residues. In view of the above-mentioned problems of quiescence in the ovaries, this study carried out early warning and analysis of the risk of ovarian static in cows from the aspects of mineral elements, energy indexes, reproductive hormones, and major differential proteins and key enzymes.

In a single indicator early warning, APOA4 and ITIH3 have a better warning effect on the risk of ovarian static. APOA4 + ITIH3 and APOA4 + ITIH3 + E2 have a better warning effect on the risk of ovarian static in multi-index early warning. It is well-known that E2 is a reproductive hormone and is directly related to ovarian disease. However, APOA4 and ITIH3, as representative substances of lipids and enzymes, have a prewarning effect on a single indicator or multi-indicator combination of ovarian static, suggesting their potential role in ovarian function and its application value.

APOA4 is a member of the apolipoprotein A1/C3/A4/A5 gene cluster [4]. APOA4 is a 46 kDa glycoprotein that is almost exclusively produced in intestinal epithelial cells and secreted into the lymph. APOA4 was first identified as a component of chylomicrons and high-density lipoproteins. The members of the APOA4 gene cluster are involved in the metabolism of lipids and lipoproteins, and therefore participate in a variety of physiological and pathological processes in the body.

Inter-α-trypsin inhibitor (ITI) is a blood-derived protein necessary for reproduction in females. It consists of two heavy chains (HC2 and HC3) and core protein bikunin [5]. ITIH3 can be used as a carrier of hyaluronic acid in serum or as a binding protein between hyaluronic acid and other matrix proteins to regulate the localization, synthesis, and degradation of hyaluronan necessary for cells. The only function of Bikunin in binding to ITIH3 is covalent attachment to hyaluronic acid [6], which is the main component of the extracellular matrix

(ECM) but is also secreted into body fluids such as blood and lymph [7]. This is the reason for the detection of ITIH3 in the blood [8]. Hyaluronic acid is a high molecular weight glycosaminoglycan that exists in the ECM with high molecular weight and high hydrophilicity. The complex of serum-derived hyaluronan-associated protein (SHAP) and hyaluronic acid is bound to hyaluronic acid via the ester bond in bikunin [9]. However, no bikunin was found in the purified complex, indicating that it was released during complex formation. Studies have shown that hyaluronic acid and ITI were detected during granulocyte expansion, suggesting that ITI is important during follicular growth [10]. It is speculated that in the development of follicles, the body produces bikunin to bind ITIH3, which in turn binds hyaluronic acid, which in turn promotes the growth of follicles.

5. Conclusion

Based on the correlation analysis, binary logistic regression, and ROC analysis, this experiment established a single early warning index and early warning value of post-natal ovarian rest risk based on APOA4 and ITIH3; established the risk of ovarian static based on APOA4 + ITIH4 + E2 A number of early warning indicators and their early warning values; established a single index and multiple indicator early warning system for the occurrence of post-natal ovarian static in dairy cows, providing a methodological basis for future prediction of post-natal ovarian static in dairy cows.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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