Investigation of some hormones and some biochemical parameters that may be effective in the etiology of Repeat Breeder cows

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SUMMARY

The aim of this study was to determine the role of anti-müllerian hormone (AMH), progesterone, insulin, thyroid stimulating hormone (TSH), triiyodotironin(T3), tiroksin(T4), non-esterified fatty acids (NEFA), beta hydroxy butyrate (BHBA) and glucose concentrations in the etiology of Repeat Breeder (RB) cows. In the study, 60 RB Holstein cows, all housed on the same farm and receiving identical care, feeding, and reproductive management, were inseminated at least three times but did not achieve pregnancy and were confirmed to have no gynecological problems through ultrasonographic examination. Were categorized into three groups based on parity: Group I (1-2 lactation), Group II (3-4 lactation), and Group III (5-6 lactation). During estrus, 10 ml blood samples were collected from all the animals in the study. Blood samples collected on the day of estrus were used to assess serum levels of AMH, insulin, BHBA, NEFA, glucose, T3, T4, and TSH. Additional blood samples were taken on days 9 and 17 post-mating for serum progesterone (P4) analysis. The serum samples were separated and stored at -80°C until analysis. T3, T4, TSH, AMH and insulin levels were measured using an ELISA device, and serum progesterone, NEFA, BHBA and glucose determination were measured using an autoanalyzer. When comparing the results of hormone measurements across lactation groups, it was observed that T3 levels were highest in Group II and lowest in Group III. As for AMH, T4, TSH, insulin, and progesterone hormones, no statistically significant differences were found among the groups. Group I exhibited significantly lower NEFA levels compared to the other two groups, while Group I also had significantly higher glucose levels than the other two groups. In Group II, a statistically significant negative correlation of 69.7% was observed between T4 and AMH. In conclusion, this study determined that T3 hormone, NEFA and glucose levels may play a role in the etiology of RB cows, while AMH and other parameters do not seem to be effective.

KEY WORDS

Anti-Mullerian Hormone; Biochemical Parameters; Repeat Breeder.

INTRODUCTION

Cows that have been inseminated at least three times or more but do not become pregnant and have no detectable abnormalities in the estrus cycle and reproductive organs are defined as Repeat Breeders (RB) (1). Repeat Breeder is an important reproductive problem and a source of economic loss, causing infertility in dairy cows (1,2). The causes of Repeat Breeder Syndrome are complicated and multifaceted; RB cows exhibit a variety of endocrine abnormalities, including abnormal levels of progesterone, estradiol, epithelial growth factor, and luteinizing hormone (3).

Anti-Müllerian hormone (AMH) is a glycoprotein specifically produced in the granulosa cells of developing follicles in the ovary. AMH concentrations and ovarian follicle reserves have a positive relationship (4). It has been reported that AMH concentrations and fertility in lactating dairy cows have a positive relationship, and that pregnancy rates decrease in cows with low AMH concentrations (5,6). Plasma AMH concentration in cows has been reported to be positively correlated with the number of all follicles, the number of small (<5 mm) follicles, and the ovum/embryo count. (7).

Since IGF-I and/or insulin play a key role in the final stage of follicle development, it can be speculated that abnormal levels of these metabolic hormones may lead to follicle dysfunc-

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tion, resulting in follicular regression or cyst formation. (8). Low insulin levels in dairy cows have been linked to reproductive issues including RB, which may delay the return of postpartum cycle activity (9). Therefore, monitoring insulin levels in RB cows may help identify potential factors affecting these animals' infertility (10).

The negative energy balance that occurs after calving in dairy cows is characterized by low blood glucose, high concentrations of non-esterified fatty acids (NEFA) and beta hydroxybutyrate (BHBA). (11). It has been reported that there is a significant negative correlation between NEFA concentrations and AMH concentrations, which increase in negative energy balance and mobilization of body fat reserves in cows, and that AMH concentrations decrease in cows with high NEFA concentrations (12).

The thyroid gland, the most important endocrine gland for regulating metabolism, secretes metabolically active hormones, thyroxine (T4) and triiodothyronine (T3) (13). These thyroid hormones increase the metabolic activity of nearly all bodily tissues. The thyroid gland's activity during the peripartum period is critical for lactation and depends on optimal carbohydrate and lipid metabolism (14). Thyroid hormones also play a role in initiating ovarian activity during the postpartum period (15). It has been reported that T3 and T4 levels are lower in animals with inactive ovaries and low thyroid hormone levels may delay postnatal reproductive functions (15).

The effects of T3, T4, and TSH levels, which significantly impact fertility, and of AMH, accepted as a fertility criterion in humans and animals, on RB cows are still unknown. This study aims to investigate whether AMH, TSH, T3, T4, progesterone hormones, insulin, NEFA, glucose, and BHBA concentrations are effective in the etiology of repeat breeding cows and aims to contribute to the literature and the field of breeding.

MATERIALS AND METHODS

Ethical Clearance

The Local Ethics Council of Animal Experimentation at Tekirdağ Namık Kemal University approved this work (T2020-438).

Animals, Feeding, Housing

This study was conducted on a farm with seven hundred Holstein breed milking cows, each with an average daily milk yield of 35 kg, located in the Tekirdağ region. Among these animals, sixty cows that had been inseminated at least three times but did not achieve pregnancy, exhibited regular estrus, and showed no detectable gynecologic problems in ultrasonographic examinations were selected as study subjects. All animals were raised under uniform care, feeding, and reproductive management protocols. Total Mixed Rations (TMR) were provided to all animals twice daily, ad libitum, and a continuous supply of clean drinking water was made available to them. These animals were categorized into three groups, each containing twenty animals, based on their number of lactations: Group I (1-2 lactations), Group II (3-4 lactations), and Group III (5-6 lactations). All animals within these groups were in the range of 130 to 170 days of lactation. The mean Days in Milk (DIMs) for the groups were 34.85 ± 0.38, 35.00 ± 0.36, and 34.29 ± 0.40, respectively.

Study design

Estrus detection in the cows included in the study was accomplished by assessing the increase in their activity using a pedometer (Nedap Livestock Management, Groenlo, Holland), evaluating the increase in uterine tone through rectal palpation, and identifying the presence of Graff follicles in the ovaries. Following estrus detection, a 10 ml blood sample was collected from the coccygeal vein of each animal, using a holder and vacutainer, and placed in sterile serum tubes. Blood samples were drawn from the coccygeal vein on days 9 and 17 after estrus for progesterone analysis. These blood samples were then centrifuged in a centrifuge device (Rotofix 32 A) at 3000 rpm for 15 minutes, after which serums was separated and stored at -80°C.

Hormonal and Biochemical analysis

T3, T4, TSH, AMH, and insulin serum levels were determined, using an ELISA instrument (Agilent BioTek Epoch, California-USA) following the manufacturer's instructions, from serum samples obtained using bovine-specific ELISA kits (AFG Scientific, Northbrook-ABD). Cobas E701(Roche Diagnostik-Swiss) autoanalyzer was applied to determine the serum progesterone level using the chemiluminescence method. An autoanalyzer (Gesan Chem 200, Campobello di Mazara-Italy) was used to perform biochemical analyses (NEFA, BHBA, and Glucose) on serum samples.

Statistical Analysis

Statistical data were prepared using the SPSS 26 software package (IBM, New York, USA). The results were presented as mean ± standard deviation. The normality of the measurements was assessed using the Shapiro-Wilk test. Given that the measurements did not follow a normal distribution, nonparametric tests were employed. The Kruskal-Wallis H test was used to compare measurements between groups. Following the Kruskal-Wallis test, the Bonferroni Corrected Post-Hoc test was utilized to differentiate between groups. To determine the relationship between AMH and other parameters, Spearman correlation coefficients were calculated. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS

The hormonal state of RB cows in different lactation groups is shown in Table 1. There was a significant difference in serum T3 levels among all groups. It was found that cows in group II had the highest and Group III had the lowest serum T3 levels. When AMH, T4, TSH, insulin and progesterone levels were evaluated, there were no statistically significant differences among the groups.

The biochemical profile of RB cows in separate groups is also shown in Table 1. Serum NEFA concentration was lower in Group I compared to Group II and Group III. Cows in Group I exhibited higher serum glucose levels than the other groups. There was no statistically significant difference in BHBA levels among the groups ($P > 0.05$).

Correlations between AMH and other parameters are displayed in Table 2. In Group II, a statistically significant negative correlation of 69.7% was observed between T4 and AMH ($p < 0.05$). It was noted that as T4 levels increased, AMH concentration decreased in this context. No statistically significant re-

Table 1 - Hormonal and biochemical parameters of Repeat Breeder cows in different groups.

Parameters	Groups	Mean	Std. Dev.	Median	Min.	Max.	*p
AMH(ng/mL)	Group I (n. 20)	141,56	29,19	138,38	102,30	204,10	*,114*
	Group II (n. 20)	163,24	37,33	167,25	85,85	263,90	
	Group III (n. 20)	158,49	47,29	145,80	103,05	254,60	
T3(ng/mL)	Group I (n. 20)	17,93 [b]	4,98	17,48	10,55	28,30	***,020***
	Group II (n. 20)	20,31 [a]	4,38	20,10	13,25	28,55	
	Group III (n. 20)	14,78 [c]	7,09	13,00	,16	28,95	
T4(ng/mL)	Group I (n. 20)	140,69	95,12	131,88	20,40	319,65	*,392*
	Group II (n. 20)	166,76	111,77	155,18	1,00	389,90	
	Group III (n. 20)	122,28	98,80	83,33	1,00	393,70	
TSH(U/L)	Group I (n. 20)	21,70	4,31	19,95	15,00	31,65	*,164*
	Group II (n. 20)	22,53	4,72	22,23	12,80	32,75	
	Group III (n. 20)	19,71	5,01	19,27	11,20	28,75	
INSULIN(IU/mL)	Group I (n. 20)	76,96	18,79	73,47	51,75	124,60	*,109*
	Group II (n. 20)	84,68	17,16	77,93	57,30	124,00	
	Group III (n. 20)	79,30	37,74	65,68	42,70	184,75	
BHBA(mmol/L)	Group I (n. 20)	,41	,27	,39	,16	1,38	*,072*
	Group II (n. 20)	,42	,15	,38	,19	,74	
	Group III (n. 20)	,32	,06	,34	,19	,44	
NEFA(mmol/L)	Group I (n. 20)	,07 [b]	,05	,05	,01	,19	***,003***
	Group II (n. 20)	,11 [a]	,07	,10	,03	,28	
	Group III (n. 20)	,13 [a]	,05	,11	,06	,22	
GLUCOSE(mg/dL)	Group I (n. 20)	57,33 [a]	13,67	59,91	30,31	81,36	***,001***
	Group II (n. 20)	37,62 [b]	15,42	34,56	13,03	76,95	
	Group III (n. 20)	32,97 [b]	8,71	33,60	13,63	49,03	
PRO-1(ng/mL)	Group I (n. 20)	4,50	2,92	3,20	1,10	9,20	*,302*
	Group II (n. 20)	3,01	2,19	2,30	,30	7,50	
	Group III (n. 20)	3,44	2,33	3,10	,30	6,90	
PRO-2(ng/mL)	Group I (n. 20)	6,28	4,88	5,70	,20	16,00	*,591*
	Group II (n. 20)	4,63	3,38	5,00	,10	11,50	
	Group III (n. 20)	4,71	3,51	5,30	,20	12,10	

Kruskal-Wallis H Test significance level between lactation groups
a-c: Displays the difference in groups according to Post-Hoc pairwise comparison test with Bonferroni correction

lationships were found between other parameters and AMH ($p > 0.05$) (Table 2).

DISCUSSION

Repeat Breeder animals are a major problem in dairy cattle breeding, with prevalence rates ranging from 9% to 24% (2). To determine the cause of reproductive failure in cows with undiagnosed clinical signs, the complex etiology of RB necessitates careful evaluation (16). This study aimed to investigate whether some hormones (AMH, TSH, T3, T4, Progesterone) and some biochemical (NEFA, BHBA, GLUCOSE) parameters play a role in the etiology of RB cows. Ribeiro et al. (6) reported a relationship between race and lactation number and plasma AMH concentrations. In our study, we found that AMH concentrations were 163.24 ng/mL for cows in the third and fourth lactations, 141.56 ng/mL for cows in the first and second lactations, and 159.49 ng/mL for cows in the fifth and sixth lactations. There was no statistically significant difference among these groups, which aligns with the findings of Ribeiro et al. (6).

In cows, progesterone plays a crucial role in sustaining pregnancy. Early pregnancy progesterone levels are linked to increased embryonic development, interferon- production, and pregnancy rates (17). The most significant endocrine deviation reported in RB cows is a delay in progesterone secretion and insufficient progesterone secretion (18). According to Ghanem and Nishibori (19), the season did not affect progesterone concentrations in RB cows. In our current study, neither the number of lactations nor age had an impact on progesterone concentrations in RB cows. Additionally, we did not find a significant correlation between AMH and progesterone concentrations, and no other literature on this subject was identified.

It has been reported that thyroid dysfunction affects various organs in the body and can impact male and female gonads, disrupting reproductive physiology and reducing the chances of pregnancy (20). Thyroid hormones are necessary for ovarian follicle activities, as studies in cows have revealed the presence of free fractions of thyroid hormones in the follicular fluid of the ovary (21). Reports indicate that T3 and T4 are crucial for postpartum ovarian activity, and a decline in T3, T4, and IGF-1 levels is associated with decreased reproductive success after giving birth (22). However, there is a limited body of research on thyroid hormone levels in RB cows. In the current study, T4 and TSH did not show statistically significant differences between the groups. However, T3 was found to be significantly higher in the second group compared to the other groups. Additionally, a significant negative correlation of

Table 2 - Spearman correlation coefficients (r) and p-values between the AMH and other parameters in different groups of RB cows.

		Group I	Group II	Group III
		AMH	*AMH*	*AMH*
T3	r	-0.118	0.005	-0.424
	p	0.620	0.985	0.062
T4	r	0.150	-0.697	0.145
	p	0.529	**0.001**	0.541
TSH	r	0.273	0.125	-0.043
	p	0.244	0.600	0.858
Insulin	r	0.068	-0.289	-0.160
	p	0.777	0.217	0.500
BHBA	r	0.056	-0.154	0.438
	p	0.826	0.543	0.061
NEFA	r	-0.038	-0.159	0.232
	p	0.875	0.529	0.339
Glucose	r	0.233	-0.189	0.073
	p	0.323	0.453	0.767
Progesterone I	r	-0.185	0.081	-0.137
	p	0.478	0.775	0.600
Progesterone II	r	-0.146	-0.294	0.262
	p	0.575	0.288	0.310

r: Spearman correlation coefficient

69.7% was observed between T4 and AMH in the second group. These results suggest that further research is needed to better understand the role of T3 and T4 in the etiology of RB cows and their relationship with AMH.

Research indicates that insulin administration in cows leads to an increase in plasma IGF-1 concentrations, which, in turn, promotes follicular growth, corpus luteum formation, and progesterone synthesis (23). Higher pregnancy rates have been reported in RB cows with elevated insulin concentrations, and pregnancy rates can increase with exogenous insulin administration in RB cows (24). In this study, insulin concentrations in RB cows were found to be similar among all groups, and no significant relationship between insulin and AMH was observed. These results suggest that the AMH and insulin concentrations in RB cows are not significantly influenced by the number of lactations and the age of the animals.

Barson et al. (25) reported that serum glucose levels in RB cows were 43.00 mg/dL, which was lower than the levels in normal healthy cows, measuring 53.98 mg/dL. Hypoglycemia in RB cows can have multiple causes, including increased peripheral glucose uptake, gluconeogenesis or glycogenolysis failure, with endogenous hyperinsulinemia being identified as an important contributing factor (26). Serum glucose plays a critical role in regulating fertility, and low glucose levels are associated with decreased fertility rates (27). In the current study, glucose concentrations in Group II and Group III were significantly lower than those in Group I, suggesting that glucose concentrations in RB cows may be age-related.

Various studies in cows have reported a relationship between fertility and NEFA levels, with high NEFA levels observed in cows experiencing fertility problems (28). However, according to Jung et al., NEFA levels in RB cows were not correlated with fertility. In this study, NEFA concentrations in animals from Group II and Group III were significantly higher than those in Group I, indicating an increase in NEFA levels with advancing age in RB cows. Nonetheless, no association between NEFA levels and fertility in RB cows was observed, which aligns with the findings of Jung et al. (29). The higher NEFA levels in middle-aged and older RB cows may suggest underlying feeding issues. It has been reported that high BHBA concentrations in cows increase the number of days in the open and prolong the interval between birth and re-conception, thus negatively affecting fertility (30). In the current study, BHBA values were found to be similar in groups categorized by lactation numbers in RB cows, with no significant differences. Given the lack of existing research on BHBA levels in RB cows in the literature, it is recommended to conduct further studies to explore the role of BHBA levels in RB cows.

CONCLUSIONS

In conclusion, the presented study revealed that T3 hormone, NEFA, and glucose levels may play a role in the etiology of RB cows. Additionally, a statistically significant negative correlation was observed between T4 and AMH in middle-aged RB cows. However, it is evident that further research is necessary to explore the relationships between AMH, thyroid hormones, insulin, glucose, BHBA, and NEFA levels in RB cows.

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Conflict of interest

The authors state that there is no conflict of interest that could damage the impartiality of this experiment.

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