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GDF-9 Gene Expression, Oocyte Qulaity, Hematological and Biochemical Profiles Affected by Feed Restriction as a Biostimulant Method on Rabbits' Fertility

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Abstract: Feed restriction is a good management practice in rabbit breeding not only for the economic importance, but to obtain uniformity in their body weight, avoid fattening and high mortality around parturition and allow a long reproductive life. This study examined the effect of moderate and severe dietary feed restriction followed by refeeding on GDF-9 gene expression in mature and immature oocytes using semi-quantitative real time-PCR. In addition oocyte classification, hematological and some biochemical and hormonal parameters were studied. Results revealed that short feed restriction followed by refeeding enhance the percentage of mature oocytes accompanied by significant increasing in the concentration of GDF-9-mRNA. On the other hand, feed restriction induced normocytic normocromic anemia. In addition, decreased levels of total protein, albumin, blood urea nitrogen and increasing creatinine concentration were observed. All hematological and biochemical parameters returned to its normal values after refeeding. Refeeding can realiminated most of the previous parameters to its normal values. It was concluded that feed restriction and refeeding as a biostimulant method should be applied on rabbits' farms due to its economic importance, increasing animal fertility and has no great effect on clinicopathological parameters.

Key words: GDF-9 Gene Expression · Oocyte Quality · Hematology · Biochemical Profile · Rabbits

INTRODUCTION

Over last few years, important work has been done particularly by the International Rabbit Reproduction Group (I.R.R.G) to set up alternative methods which do not require the use of hormones, in order to increase sexual receptivity at the moment of insemination consequently the productivity of rabbit does. These are called "biostimulation methods" and comprehend a large spectrum of techniques. Up to date, several approaches have been tried such as animal manipulation, feed restriction [1,2] and feeding program [3]. The effect of acute and chronic feed restriction on the regulatory of oocyte quality and reproductive performance has been previously investigated in rabbits [2,4].

Folliculogenesis is controlled by gonadotropins from pituitary and autocrine/paracrine factors that are produced in the ovary. Some of these molecules are synthesized and secreted by the oocytes and act as morphogens that control follicle growth as well as differentiation. The transforming growth factor- (TGF-) superfamily which contains over 35 members, has been shown to be important for regulating fertility [5,6]. Two of the more recent members of this family were identified as having a role in regulation of fertility are the related oocyte-derived family members namely, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15, also known as GDF9B). GDF9 was first shown to be essential for follicular development [7] when lacking GDF9 gene mice were found to be infertile with follicular development blocked at a very early stage [8]. GDF-9 protein was found in oocytes throughout the entire period of follicle development even after ovulation. The exclusive expression of GDF-9 gene in the oocyte is unique among known growth-factor-like molecules and suggested that the oocyte could, at least partially, control follicle development by secreting this putative paracrine factor [9]. Over the last years, with the development of the field of molecular biology a number of investigators have

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examined the effects of nutrient status on gene expression patterns in various animal tissues [10,11] and recently on ovarian and testicular genes [7,12].

In meantime, it has long been known that on protein and/or energy malnutrition, the morbidity and mortality of both humans and animals are increased. This association has consequently given rise to the question, in which extent the composition of the diet affects the hematopoiesis as well as to numerous clinical and experimental investigations about the dependence of biochemical and hematological reactions on the nutrition [1,2,13]. Therefore it was of interest to determine effect of feed restriction as biostimulant method on the fertility of the rabbits through, examination of oocyte quality, alterations in the expression of GDF-9 gene in different types of rabbit's oocytes and some hematological, biochemical and hormonal changes.

MATERIALS AND METHODS

Experimental Design: This study was conducted using 40 mature premiparous non pregnant non lactating female New Zealand White (NZW) rabbits with average body weight of 2.5±30 kg. Rabbits were individually kept in galvanized wire batteries equipped with an automatic drinker and a manual feeder in identical deep-pit compartments, in well naturally ventilated building, in Lab. of Animal Unit, National Research Centre, Giza, Egypt. Rabbits were fed on a formulated pellet ration according to NRC [14]. The chemical analysis of this experimental ration was carried out according to A.O.A.C [15]. The ingredient composition and chemical analysis of the experimental ration is shown in table A.

Rabbits were divided into 3 groups, the 1st group (8 animals) was fed 150 g / day as control. The 2nd and 3rd groups have 16 animals each, the second group received 70 % of the control daily feed intake which equal to 105 g /day and referred as moderate feed restriction group. While 3rd one receive 50% of the control daily feed intake which equal to 75g (referred as sever food restriction). This restriction was extended for 21 days. After that, half of the animals in feed restricted groups were slaughtered for collection of their ovaries. Another half of animals in both restricted groups were subjected to referred for 8 days whereas they fed 150 g. as control. After that all remaining animals in two refed groups and control were slaughtered for collection of their ovaries also. Blood samples were collected from the ear vein 4 times from the tested animals once every 7 days, in addition body weight was determined for the same time intervals.

Examination of Oocyte Quality and Quantity: The ovaries were collected from slaughtered rabbits (control, feed restricted and refeeding groups) and rinsed several times in warm (30-38°C) phosphate buffer saline (PBS). The oocytes were harvested in aspiration media consisting of modified phosphate buffer saline Sigma Chemicals CO. (St Louis, MO, USA). (pH 7.2, M 0.01) supplemented with 3% heat inactivated fetal calf serum(Grand Island, New York, USA). Oocytes were collected by slicing method [16] and classified into three categories Class A is mature oocytes which surrounded by expanded cumulus cell layers; Class B is immature one which completely and partially invested with cumulus cell layers and Class C is that naked oocytes.

Gene Expression Analysis

Synthesis of the cDNA from the Extracted mRNA: mRNA was isolated from both mature and immature oocytes only while denuded ones is excluded due to its poor quality. This isolation was carried using a Dynabead mRNA Direct Kit according to manufacturer's instructions with minor modifications [17]. The method relies on base pairing between the poly A tail of mRNA and oligo deoxy triphosphate (Oligo $dT)_{25}$ sequence bound to the Dynabead's surface. mRNAs were then eluted from the beads by incubation with 15µl sterile water at 95°C for 2 min. Aliquots were used immediately for reverse transcription (RT). RT was performed using 0.5il poly $(dT)_{18}$ primer and 13µl RNA of total volume of 25µl. The reaction was carried out at 37°C for 90 min and finished with a denaturation step at 70°C for 15 min. Afterwards the reaction tubes containing RT preparations were preserved in - 20°C.

Real Time PCR Reaction: n iQ5-BIO-RAD Cycler (Cepheid. USA) was used to determine oocyte cDNA copy number using HPLC GDF-9 primer (F:5'-AAAGACCAGCTGCAGCATCC-3'; R:5'-TGGTGTGAACTGGAGAGCCA-3') according to that previously published [18].

PCR reactions were set up in 25 µL reaction mixtures. The PCR reaction was started by denaturation cycle at 95°C for 3 min, followed by 35 cycles of denaturation step at 95°C for 15 sec, annealing temp at 55°C for 30 sec, extension at 72°C for 30 sec and followed by 71 cycles (dissociation cycle) started at 60°C and increased 0.5 °C every 10 sec till reach 95°C. All raw data were multiplied 50-fold to determine total cDNA copy number in each oocyte. The mRNA levels were normalized on the basis of B- actin mRNA content of albino rats.

Calculation of Gene Expression: First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae (Bio-Rad 2006)

$$Ef = 10^{-1/slope}$$
$$Efficiency\ (\%) = (Ef - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the ΔC_T method if E for the target (GDF-9) and the reference primers (β-Actin) are the same (Bio-Rad 2006): $Ratio_{(reference/\ target\ gene)} = Ef^{C_T\ (reference)} - {}^{C_T\ (target)}$

Hematological and Biochemical Parameters: Blood samples were drawn from the ear vein every week into heparin treated tubes (hematology) or serum separator tubes (serum biochemistry). Hematological parameters include red blood cell (RBC) count, haemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and total white blood cell count were estimated according to the techniques description [19]. Serum biochemical parameters included total protein, albumin, urea nitrogen (BUN), glucose, total cholesterol, high density lipoprotein (HDL) and triglyceride were estimate by enzymatic colorimetric methods [20]. While creatinine was measured by quantitative kinetic method without deprotenization of samples [21]. Concentrations of IGF-1 [22] and leptin [23] hormones were assayed by radioimmune assay.

Statistical Analysis: Data were compared across groups using one way analysis of variance (ANOVA).

Data were expressed as mean and S.D. Levels of significance of $p < 0.05$ and $p < 0.01$ were chosen to identify the significant differences [24] using Statistical Package for Social Sciences (SPSS) version 15.0. *(Overall mean is statistical analysis of all samples in 3 weeks of feed restriction but in GDF-9 table mean both mature and immature oocytes)*.

RESULTS

Examination of Oocyte Quality and Quantity: Result of examination of rabbits' oocyte quality and quantity are presented in table 1. Recovery rate (Total number of oocytes/ovary) is significantly ($p<0.05$) decreased in both restricted group during restriction and even after 8 days of refeeding when compared with control. While classification of the oocytes revealed significant ($p<0.05$) increasing in the percentage of mature oocytes in moderate and severe restricted groups by 3.4 and 6.8% and after refeeding by 6.1 and 10.7%, respectively. On the other hand, significant ($p<0.05$) decrease in immature oocytes percentage by 2.3 and 7.8% after feed restriction and by 4.9 and 8.1% after refeeding was recorded, respectively.

Expression of GDF-9 Gene: Semi quantitative real time-PCR was used for analysis of GDF-9 gene expression in both mature and immature oocytes in all the experimental (Table 2). GDF-9 gene expression analysis in mature oocytes revealed significant ($p<0.05$) decrease in

Table 1: Oocyte recovery rate and classification of New Zealand female rabbits after feed restriction and refeeding.

		Moderate		Severe	
Treatment Categories	Control	Restricted(MR)	Refed(MF)	Restricted(SR)	Refed(SF)
No. of ovaries	12	12	12	12	12
No. of trials	3	3	3	3	3
Total No. of oocytes	221	168	171	194	184
Recovery rate	18.4 ± 0.56	14 .0*±0.28	14.2 *±0.95	16.1*±0.78	15.3 *±0.88
Class A: Mature oocyte					
No.	40	39	41	48	54
Percentage	17.9 % ±0.20	21.3 %*±0.12	24.0 %*±0.60	24.7 %*±0.41	28.6 %*±0.92
Class B: Imature oocyte					
No.	135	96	96	106	97
Percentage	61.4 %±0.35	59.1 %*±0.50	56.5 %*±0.91	53.6 %*±0.99	53.3%*±0.62
Class C: completely denuded oocytes					
No.	46	33	34	40	33
Percentage	20.6 %±1.13	19.4 %*± 0.56	19.4 %*± 0.42	21.6 %± 0.56	18 %*± 0.70

Values represent Means ± standard errors (SE).

* in the same raw in different groups mean significant at < 0.0

Table 2: Semi-quantitative analysis of GDF-9 expression in different types of oocytes of New Zealand female rabbits after feed restriction and refeeding

	Treatment				
		Moderate		Severe	
Types of oocytes	Control	Restricted (MR)	Refed (MF)	Restricted (SR)	Refed (SF)
Mature oocytes	1.27±0.20	1.07*±0.05	1.60*±0.10	1.15*±0.12	1.39*±0.01
Immature oocytes	1.09±0.02	1.48*±0.10	1.51*±0.07	1.19*±0.10	1.66*±0.09
overall mean	1.18±0.10	1.27±0.22	1.56*±0.77	1.17±0.02	1.52*±0.14

Values represent Means ± standard deviations (SD).

· in the same raw in different groups means significant at < 0.05

Table 3: Body weight of New Zealand female rabbits subjected to feed restriction and refeeding

	Body weight (Kg)		
Weeks Of experiment	C	M	S
Initial body weight	2.58±0.24	2.53±0.30	2.50±0.33
1st week of restriction	2.51±0.30	2.41±0.31	2.36±0.23
2nd week of restriction	2.58±0.30	2.37±0.33	2.29*±0.20
3rd week of restriction	2.65±0.32	2.26*±0.33	2.10*±0.33
4th week (Refeeding)	2.71±0.31	2.50±0.20	2.40*±0.21

C ; M and S are control ; Moderate restricted and sever restricted groups respectively

Values represent Means ± standard deviation.

*in the same raw in different groups means significant at < 0.05

the expression level after feed restriction in moderate and severe restricted groups by 15.7 and 9.4% and significantly (p<0,05) increased after refeeding by 25.9 and 9.4%, respectively when compared with control. On the other hand, GDF-9 gene expression in immature oocytes significantly (p<0.05) increased in moderate and severe restricted groups by 35.7 and 9.17% and after refeeding by 38.5 and 52.2%, respectively. Overall mean of GDF-9 gene expression analysis in both restricted groups revealed non-significant change while significant increase after 8 days of refeeding by 32.2 and 28.8% was noticed in moderate and severe restricted groups, respectively.

Body Weight: Weekly determination of body weight is clarified in table 3, significant (p<0.05) decrease in body weight was recorded at the 2nd week in severe restricted group and it reached to 20.75 % in 3rd week of feed restriction. At the same time, 14.7 % decreasing in body weight was recorded in moderate restricted at 3rd week. After refeeding only severe dietary restricted group still recorded 11.43 % significant (p<0.05) decrease than control one.

Hemogram: Results of the erythrogram (Table 4) revealed significant (p< 0.05) decrease in PCV % in severe restricted group starting from first week of feed restriction and in moderate restricted group at 3rd week of feed restriction. While significant (p<0.05) decrease in Hb concentrations appeared at the 3rd week in both restricted groups when compared with control. Overall mean clarified significant (p<0.05) decrease in RBCs' count, PCV and Hb concentrations in both moderate and severe groups when compared with control one, these animals are suffering from normocytic normochromic anemia. After 8 days of refeeding all hematological values return to normal pattern.

Biochemical Parameters: Values of total protein revealed significant (p<0.05) decrease from 1st week of feed restriction in severe restricted group and from 2nd week in moderate restricted one. While significant (p<0.05) hypoalbuminemia appeared only in severe restricted group at the 3rd week of restriction. Glucose levels (Table 5). After 8 days of refeeding serum proteins levels returned to their normal values when compare with control. Serum levels of both BUN and creatinine are clarified in table 5, BUN revealed significant (p<0.05) decrease only in severe restricted group at the 3rd week of restriction and still significantly (p<0.05) decreased after refeeding. At the same time, assay of creatinine

Table 4: Erythrogram of New Zealand female rabbits subjected to feed restriction and refeeding

	Parameters																	
	RBCS (x 106/ µl)			PCV %			Hb (g/dl)			MCV (fl)			MCH (pg)			MCHC %		
weeksof rest.	C	M	S	C	M	S	C	M	S	C	M	S	C	M	S	C	M	S
1st week	4.56± 0.50	4.23± 0.30	4.15± 0.38	37.6± 0.57	36.3± 1.15	36.2*± 0.50	11.3± 0.80	10.7± 1.55	10.4± 0.75	82.1± 0.40	85.9± 1.38	86.1± 1.15	24.6± 0.86	24.4± 0.27	26.1± 0.86	30.2± 1.15	29.8± 0.10	28.8± 1.51
2nd week	4.70± 0.17	4.50± 0.20	4.28± 0.58	37.9± 0.30	36.6± 1.71	36.3*± 1.32	11.4± 0.94	10.9± 0.26	10.6± 0.65	81.9± 3.00	83.5± 3.00	83.4± 3.41	23.9± 0.35	24.2± 0.02	24.8± 0.10	29.8± 0.91	29.4± 1.60	29.3± 0.90
3rd week	4.70± 0.17	4.25± 0.13	4.39± 0.35	37.8± 0.72	35.6*± 1.15	35.5*± 0.57	11.7± 1.00	10.3*± 1.12	10.1*± 0.71	80.0± 1.79	82.4± 4.72	80.8 ± 2.83	24.8± 1.6	23.9± 1.51	23.0± 0.06	30.7± 0.90	28.8± 1.15	28.9± 1.20
Overall mean	4.56± 0.14	4.32*a± 0.22	4.27*a± 0.29	37.8± 0.50	36.3*± 1.0	36.0*± 0.63	11.5± 0.38	10.6*± 1.00	10.4*a± 0.65	81.3± 2.10	83.9a± 3.26	83.4a± 3.42	24.5± 1.03	24.2± 1.20	24.6± 0.85	30.2± 1.33	29.3a± 1.03	29.0a± 1.14
Refeeding	4.83± 0.20	4.73b± 0.55	4.67b± 0.28	37.3± 0.15	36.6± 0.57	36.2± 0.50	12.2± 0.26	11.4± 1.07	11.6b± 1.02	77.8± 3.49	77.2b± 1.32	78.3b± 3.13	24.8± 1.90	24.4± 1.10	24.9± 0.85	31.7± 0.55	31.2b± 2.05	32.2b± 1.72

C = Control ; M = Moderate restricted and S = severe restricted group.
Values represent Means ± standard deviation.
*in the same raw in different groups means significant at < 0.05.
Superscript a, b letters in the same column means significant at < 0.05

Table 5: Levels of some serum biochemicals parameters of New Zealand female rabbits subjected to feed restriction and refeeding.

	Parameters														
	T. protein (g/dl)			Albumin (g/dl)			Glucose (mg/dl)			BUN (mg/dl)			Creatinine (mg/dl)		
Weeks of rest.	C	M	C	C	C	C	C	M	S	C	M	S	C	M	S
1st week	8.93±0.12	8.62±0.16	102.41±3.06	36.02±2.70	1.28±0.29	1.28±0.29	1.28±0.29	3.29±0.23	3.19±0.28	1.350.28	1.60±0.19	1.64±0.29	102.41±3.06	102.31±5.73	98.26±2.60
2nd week	8.82±0.31	8.35*±0.58	101.47±2.87	38.81±2.85	0.98±0.31	0.98±0.31	0.98±0.31	3.20±0.43	3.27±0.41	1.49±0.25	1.62±0.21	1.49±0.28	101.47±2.87	98.93±3.02	97.76±4.02
3rd week	8.82±0.92	8.24*±0.30	99.49±1.43	36.0 ±3.09	1.07 ±0.14	1.07 ±0.14	1.07 ±0.14	2.85±0.44	3.23±0.65	1.58±0.35	1.84±0.27	1.59±0.36	99.49±1.43	99.42±2.82	98.22±3.22
Overall mean	8.87±0.35	8.40*±0.39	101.12±2.70	36.60 ±2.91	1.11±0.21	1.11±0.21	1.11±0.21	3.10*±0.41	3.23*±0.43	1.53±0.25	1.69±0.24	1.58±0.29	101.12±2.70	100.29±4.16	98.07±3.22
Refeeding	8.63±0.48	8.41±0.41	101.46±5.07	37.68 ±3.10	1.22±0.35	1.22±0.35	1.22±0.35	3.13±0.60	3.00±0.42	1.58±0.21	1.73±0.46	1.72±0.26	101.46±5.07	102.22±3.93	100.46±5.31

C = Control ; M = Moderate restricted and S = severe restricted group.
values represent Means ± standard deviations (SD)
* in the same raw in different groups means significant at < 0.05

Table 6: Lipids' profile and Some hormonal assay of New Zealand female rabbits subjected to feed restriction and refeeding.

	Parameters														
	Cholesterol (mg/dl)			HDL (mg/dl)			Triglycerides (mg/dl)			Leptin (ng/ml)			IGF-1 (ng/ml)		
weeks of rest.	C	M	S	C	M	S	C	M	S	C	M	S	C	M	S
1st week	68.11± 9.7	63.86± 18.08	62.15± 13.20	1.11± 0.12	1.20± 0.60	1.05± 0.14	110.84± 27.84	102.04± 16.36	83.40± 6.20	39.39± 5.41	41.84± 1.46	38.93± 5.47	146.75 ± 14.81	129.30± 6.04	116.10*± 17.74
2nd week	64.40± 2.99	61.19± 12.05	65.64± 5.6	1.08± 0.23	0.97± 0.18	1.09± 0.18	102.49± 16.84	105.09± 29.09	88.06± 27.98	38.24± 4.45	38.70± 2.53	40.95± 2.75	138.48 ± 19.94	120.68± 21.58	102.75*± 18.53
3rd week	65.17± 4.00	64.00± 12.92	62.80± 13.51	1.15± 0.21	1.20± 0.19	1.21± 0.13	87.03± 14.28	73.41± 9.26	69.72± 6.23	40.58± 2.01	38.77± 2.16	39.34± 4.79	132.24 ± 11.70	128.24± 11.70	99.50 *± 17.06
Overall mean	65.9± 6.43	62.97± 14.05	63.53a± 10.95	1.11± 0.19	1.12 a± 0.37	1.12 a± 0.16	100.12± 25.46	95.63± 14.72	80.39± 17.93	39.40± 4.20	39.77± 2.46	39.74± 4.25	139.12 ± 16.87	126.00 *± 14.33	104.60*± 17.58
Refeeding	73.93± 16.83	76.34± 12.53	77.66b± 19.03	1.27± 0.11	1.57 b± 0.13	1.47 b± 0.10	104.09± 31.15	115.40± 39.11	112.16± 38.19	42.10± 1.97	37.21± 2.66	39.89± 2.72	130.68 ± 14.50	133.87± 5.79	108.39*± 3.90

C = Control ; M = Moderate restricted and S = severe restricted group.
Values represent Means ± standard deviations (SD).
* in the same raw in different groups means significant at < 0.05
Superscript a &b letters in the same column means significant at < 0.05

concentrations showed parallel significant ($p<0.05$) increase only in severe restricted group at the 3rd week of restriction, while its level was return to normal values after refeeding. Values of lipids' profile showed no significant changes in cholesterol and HDL in both experimental groups while levels of triglyceride are significantly ($p<0.05$) decreased in severe restricted group only all over the period of restriction and even after refeeding when compared with control (Table 6).

Hormonal Levels: Results of hormones (IGF-1 and leptin) are illustrated on table 6. Assay of serum IGF-1 and leptin recorded non significant ($p<0.05$) change between two restricted groups and control one in all experimental periods as well as their overall mean. After refeeding significant ($p<0.05$) increase in leptin concentrations only in both moderate and severe restricted groups was observed when compared with the overall mean of restriction.

DISCUSSION

The current findings revealed significant decrease in recovery rate in both restricted groups and after refeeding in comparison with the control one. This result was in agreement with previous investigation [13] which reported a decrease in the number of oocytes per ovary in rabbits fed on low protein diet. Meanwhile, O'Callaghan [25] added that, low dietary intake resulted in decreasing the number of follicles. Recently, Bernal *et al*. [7] recorded that maternal undernutrition lead to decrease all types of follicles in the ovaries of rat offspring. On the other hand, our result contradicted those results reported by Mahmoud *et al.* [2] who reported a non significant difference between the number of rabbit's oocytes / ovary fed 60% of control for one or two months. The percentage of mature oocyte was significantly increased in feed restricted groups and after refeeding compared to control group, while immature oocytes were significantly decreased. These results were in agreement with McEvoy *et al*. [26] and Mahmoud [2]. The previous results can be explained by the evidence that, altered nutritional regimes prior to mating can influence follicular/oocyte characteristics without altering gonadotrophin secretion per second [27]. Moreover, mechanisms regulating the activation and subsequent growth of primordial follicles still remain poorly understood. However, their growth probably depends on the presence of oocyte/granulosa cell interactions and the secretion of a wide range of local factors (e.g. growth differentiating factor [GDF]-9, bone morphogenetic proteins [BMP], activins, inhibins, basic fibroblast growth factor [bFGF] and epidermal growth factor [28].

Regarding to our knowledge, the current study is the first record of determination of GDF-9 gene expression by semi-qunatitative real time PCR of non-pregnant and non-lactating mature female rabbits during feed restriction and after refeeding. Our result clarified that, GDF-9 expression in mature oocytes was significantly decreased by feed restriction and significantly increased after refeeding. While, in immature oocytes its levels were significantly increased after both feed restriction and refeeding. According to previous record, feed restriction and refeeding has a direct effect on the letulizing hormone (LH) pulsation which declined due to feed deprivation in rabbits for 24 or 48 h and increased by refeeding to match control after only 1-4 h [4]. This fact may help us to explain this finding as LH play as a control key for the GDF-9 expression in which increasing LH pulsation indirectly activate GDF-9 gene which consequently responsible for profilation of cumulus cells and subsequently maturation of oocytes [29]. Non significant decrease of GDF-9 gene expression by feed restriction in overall mean (both mature and immature oocytes) and significant increase by refeeding observed in our study didn't found rescue explanation to this finding but Sharov *et al.* [12] estimated GDF-9 gene concentration in ovaries of rats subjected to 40 % calorie restriction and recorded slight decrease in GDF_9 concentration using DNA microarray. And recently Bernal *et al.* [7] recorded significant decrease in GDF-9 mRNA in ovaries of rats offspring their mothers subjected to under nutrition during pregnancy and /or lactation.

Estimation of body weight in our experimental animals during feed restriction recorded significant decrease when compared with control. This result was in agreement with that recorded in rabbits by Fouad *et al.* [30].

Hematological examination in the present study revealed that significant decrease in RBCs' count, Hb concentration and PCV values, accompanied with normocytic and normochromic anemia. These results were in agreement with that observed in pregnant rabbits subjected to feed restriction [3]. Disturbances in blood parameters may be due to decline in folic acid, vitamin B12 and iron [31]. Moreover Okano *et al.* [32] added more explanations to these findings as long and severe protein deficiency may lead to decrease of serum erythropiotien hormone (EPO) and subsequent reduction of the population size of erythroid precursor cell in spleen. On the other hand, our results differed from others [13, 33] who noticed that, feed or protein restriction has no effect on erythrogram, this differentiation may be due to different applied regime of restriction.

Serum biochemical examination showed that the results of protein profile revealed significant decrease in total protein throughout the period of experiment in two restricted groups accompanied by hypoalbuminemia in severe restricted group at 3rd week of restriction. These findings are in consistent with that reported in rabbits [34]. In the same time Tirapegui *et al*. [35] clarified that the plasma total protein and albumin were closely related to the protein content of the diet as well as feed intake and their levels were associated with their synthesis by hepatic cells in relation to the concentration of serum amino acids. In regards to the previous explanation, low feed intake in our study may be a cause of these changes in protein profile.

In our study, serum glucose concentration showed no significant changes among experimental animals. This result is consistent with that recorded in rabbits [4]

as glucose was maintained at a steady state level in 24h and 48 hr periods of fasting compared to that of ad libitum fed rabbits. Moreover, blood samples taken after 4 days of food deprivation may show no alteration in blood glucose levels in rabbits [36]. Physiologically, in rabbits it is not possible to take a guaranteed fasting sample from a rabbit because they ingest caecotrophs. The digestion of caecotrophs providesa source of glucose as well as they also use volatile fatty acids produced by cecal flora as a primary energy source [34].

In line with Manning *et al.* [37] blood urea nitrogen (BUN) depends on the amount of protein in diet. Moreover, changes in BUN levels are generally opposite in direction to changes in creatinine levels when food is not available. These records confirm our results which showed a significant decrease in BUN concentration at the 3rd week of restriction in severe feed restricted group. Although in our study there is increase in BUN level after refeeding, this increase is still significantly lower in severe restricted group than that of control one, this can be explained by the severe decline of BUN concentration in this restricted group and small period of refeeding as animals can't return to its normal physiologically state. This finding is consistent with that reported by Kouakou *et al.*[38].

Assessment of lipids' profile revealed no significant changes in serum cholesterol and HDL, while significant decrease in triglycerides was detected during all experimental periods in sever restricted group. This finding is in agreement with that reported by Aboelmaaty *et al.* [39]. Decrease in triglycerides more clearly corresponded to the level of dietary restriction and to the percentage of decreases in body weight gain. This suggested that fat metabolism is significantly modified by dietary restriction [40]. Also, across research of different animal species, different rats genotypes [41,42] the data suggested that dietary restriction tends to result in lower total triglyceride more consistently, while the influence on total cholesterol seems in general to be more variable.

Regarding to hormonal assays our study revealed no statistical differences among the three groups in serum concentrations of IGF-1. These results were in agreement with others [2,13]. Circulating IGF-1 levels are relatively stable due to its constitutive pattern of secretion and to its high affinity to binding proteins (mainly IGFBP-3) which prolonged to the half life and titrate the supply of this hormone to its receptors [43]. In addition others [44] recorded that, the changes in serum concentrations of IGF-1 by its relation with IGFBP-3 have been intensively depend on acute changes of plasma glucose, insulin levels.. As well as, serum leptin concentrations in different feed restricted groups showed no significant changes during feed restriction and significantly increased by refeeding. Our finding is consistent with that recently recorded in goats by Aboelmaaty *et al.* [39]. The concentrations of leptin in plasma have been shown to be a direct reflection of the amount of body fat mass and adipose tissue. Although a significant decrease in body weight of restricted groups was recorded in our study but this decrease doesn't necessarily mean loss in adipose tissue mass of animals. Moreover, Marie *et al.* [45] reported that leptinemia is sharply and rapidly decreased by either food deprivation or chronic under nutrition. They also added that, refeeding can increase leptin level above control as reported above in this work.

CONCLUSIONS

Feed restriction followed by refeeding has a significant increasing effect on mature oocyte quality, consequently increasing animal fertility and it accompanied by increasing GDF-9-mRNA concentration after refeeding. On the other hand, moderate and severe (30 and 50 %) feed restriction has significant effects on hemogram and some biochemical parameters but they return to their normal physiological levels after refeeding. Thus, feed restriction and refeeding as a biostimulant method can be applied in rabbit's farms due to its economic importance and increasing animal fertility.

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