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The Diagnostic Value of Acute Phase Proteins in Barki Ewes with Pregnancy Toxemia

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ABSTRACT

Pregnancy toxemia is a fatal metabolic disease resulting in high mortalities in late pregnant ewes. The silent nature of the disease and lack of diagnostic tools decrease the chance of prediction and curing. In this paper, we tried to find out the benefits of APPs, cytokines, and iron profile in diagnosis and prediction of the disease and their relation with other disease metabolic changes. Blood samples were collected from sixty barki ewes housed in Matrouh Research Station and were divided into 3 groups;control, healthy late pregnant, and pregnant toxemic group. Biochemical analysis such as BHB, NEFA, Fb, Cp, Hp, SAA, Tf,ferritin concentrations, cytokines (IL-1α, IL-1β, IL-6, IL-10, TNF-α) were measured and all the data were analyzed statically. The results confirmed that APPs and cytokines are valuable markers for pregnancy toxemia as they have a sensitivity and NPV as 100% and high values of specificity, LR, PPV and accuracy rate. But APPs had higher percentages of increase than cytokines, especially Hp. Moreover, both of APPs and cytokines correlate well with the other metabolic changes of the disease. Regarding the iron status, there was a significant decrease in mean concentrations of serum iron, ferritin, Tf sat. % and a significant elevation in TIBC, UBIC and serum transferrin concentrations in the late pregnant ewes in comparison to the control healthy group. On the other hand, the pregnancy toxemic ewes demonstrated a significant rise in the mean concentrations of serum iron, ferritin, Tf sat. % and a significant drop in TIBC, UBIC and serum transferrin concentrations relative to the late pregnant group.

1. INTRODUCTION:

Pregnancy toxemia (PT) or ovine ketosis is a fatal metabolic disorder affects pregnant ewes and sometimes pregnant does. Shortage of available feeds introduced to ewes at the last third of gestation period is the main cause of pregnancy toxemia. As about 70% of the fetal development occurs at this stage, so the energy demand increases. If the ewe has insufficient feed supply to meet the fetal growth demands and her own demands, hypoglycemia will develop, usage of fat stores will start, hepatic gluconeogenesis will increase, ketone bodies will accumulate in the blood, blood pH will fall and a state of ketoacidosis will arise (Abdalla et al., 2013; Albay et al., 2014; Vasava, et al., 2016).

Toxemic ewes usually stand alone, separate from the other animals, never seeking for feed or water, have poor body condition, show no reflex when approach and by examination acetone odor from breath observed. If the case advanced nervous symptoms will develop like tremors, poor eye reflex, deafness, extrasalivation, grinding teeth and recumbences (Vasava et al., 2016).

In case of death, postmortem examination usually showed the existence of more than one fetus, pale enlarged liver due to severe fatty degeneration, loss of abdominal fat, dehydration and acidic ruminal fluid and urine (Gomez et al., 2015).

The incidence of the disease is higher in ewes carrying multiple fetuses. Some stressors may facilitate the occurrence of the pregnancy toxemia such as sorting, shearing, worming, transportation or sickness (Al-qudah, 2011).

Pregnancy toxemia is a real threat on sheep industry, where it has been recorded all over the world with morbidity rates up to 13% and mortality rates reach approximately 95% among affected ewes. The silent nature of the disease and lack of diagnostic

tools play a leading role in doubling the disease incidence. Hence, it was important to find new diagnostic tools for the disease (Schulz and Riese, 1983).

Acute phase proteins (APPs) are a collection of different proteins synthesized mainly by hepatic cells under the effect of different cytokines and released in the blood circulation as a part of the innate immune response of the host. Different pathological conditions as infection, inflammation and surgery enhance their secretion. Also, metabolic disorders as pregnancy toxemia and diabetic ketoacidosis as well as physiological conditions as pregnancy, parturition and lactation have a positive impact on their production (Aly and Elshahawy, 2016; Czopowicz et al., 2018).

Cytokines are another part of innate immune response composed of polypeptide molecules created mainly by macrophages and lymphocytes but they can be synthesized by other cell types as well. Their role in inflammation is complex, the pro-inflammatory cytokines induce other inflammatory cells to make their functions, coordinate between them to exacerbate the inflammatory reaction, and then the anti-inflammatory cytokines activate to inhibit them to stop the inflammatory process. In pregnancy and parturition, cytokines are concerned with different functions such as arranging gametogenesis, mediating uterine receptivity, orchestrating implantation reactions, taking care of embryogenesis and fetal development and eventually, they evoke inflammatory process to prepare for parturition. Additionally, they were suggested as a target for therapeutic strategies to prevent pregnancy disorders as abortion, pre-eclampsia, preterm labour and fetal brain injury (Orsi and Tribe, 2008; Dutta and Sengupta, 2017).

So, we aimed here frist, to assess the APPs and cytokines accuracy in diagnosis and prediction of pregnancy toxemia in sheep and their relation with other metabolic changes associated with the disease. Second, to monitor the iron status in both late pregnant ewes and toxemic ewes.

2. MATERIAL AND METHODS:

2.1. Animals:

Sixty barki ewes aged from 3-4 years were housed in Matrouh Sustainable Development Center farm, DRC, Egypt and were divided into 3 groups. First group: 20 apparently healthy, non-pregnant ewes and were considered as control group (CG), the second group: 20 healthy late pregnant ewes (LPG) (no symptoms and BHB < 3Umo/L) and had a normal parturition and didn`t suffer from postparturient problems, and the third group: 20 late pregnant ewes were suffering from pregnancy toxemia (PTG) (diagnosed by signs and BHB ≥ 3 Umo/L).

2.2. Samples:

Two blood samples were collected from each ewe before the treatment by jugular vein puncture. The first blood sample was collected in tube contains heparin (20 IU/ml) then centrifugated at 3000 r.p.m. for 20 min at 37 ºC for separation of plasma for estimation of BHB, NEFAs and fibrinogen concentrations. The second blood sample was collected in a clean plain tube without anticoagulant for separation of serum, which used for estimation of the rest biochemical parameters.

2.3. Biochemical analysis:

The serum levels of glucose, liver function test (ALT, AST, ALP), kidney function test (blood urea, creatinine), lipid profile, total protein (TP), albumin, iron (SI) and total iron binding capacity (TIBC) were detected spectrophotometrically using commercial kits supplied by Biodiagnostic company according to manufacturer's instructions.

The plasma levels of beta hydroxybuyrate (BHB), non-esterified fatty acid (NEFAs) were estimated spectrophotometrically using commercial kits supplied by gamma Trade Company according to manufacturer's instructions.

Serum amyliod A (SAA), haptoglobin (Hp) serum concentrations, and plasma fibrinogen (Fb) concentrations, were measured using ELISA kits supplied by IBL International Crop (canda) following the manufacturer's instructions.

Serum caeruloplasmin (Cp) and transferin level (Tf) were measuredin serum byturbidimetric method using Elabscience (USA) kits according to manufactur`s instructions.

Serum ferritin concentrations were evaluated by CLIA method using Abnova (Taipei) kits according to manufacturer's instructions.

Cytokines levels were measured in serum by ELISA kits of RayBiotechcompany following the manufacture's instruction.

Globulin= TP – albumin.

Transferrin saturation percent (Tf sat. %) = serum iron (SI) / total iron binding capacity (TIBC)*100.

-Unsaturated iron binding capacity (UIBC) = TIBC-SI.

2.4. Statistical analysis:

The data obtained from this study was statistically analyzed according to SPSS program version 20 (One-way ANOVA test for comparing of means of the three groups, multiple comparison Tukey`s HSD test for post hoc differences between means and

Pearson's simple correlation method for estimation of correlations between the selected parameters).

-Cut off points, sensitivity, specificity and likelihood ratio (LR) for the measured acute phase proteins and cytokines were estimated between the late pregnant group and the pregnant toxemic group and were calculated using graphed prism version 5 program.

- The positive predictive value (PPV), negative predictive value (NPV) and accuracy rate for them is the result of dividing the number of true positive or true negative or sum of both on the number of total positive or total negative or total population respectively, then multiplied in 100.

-The percentage of increase for each one of them is the result of subtracting the mean value of its concentration in the late pregnant group from the mean value of its concentration in the toxemic group then, dividing the result on the mean value of protein concentration in the late pregnant group multiplied in 100.

-Correlations between the selected parameters were determined with Pearson's simple correlation method. A difference was considerable significant at $P< 0.05$.

3. RESULTS AND DISCUSSION

Pregnancy toxemia is one of the prepartumtroubles, characterized by a sustainable hypoglycemia followed by a serial of metabolic changes include enhanced lipolysis, liver gluconeogensis, ketonemia and ketoacidemia. This is expressed on the animal by the appearance of the clinical signs of the disease (Al-qudah, 2011).

In the current work, pregnant toxemic ewes (four ewes) were found lonely, separated from the rest of the flock, recumbent refused to stand if forced, weak, loss of appetite and body weight. The clinical examination revealed lower ranges of body temperature and pulse rate, rapid respiration, poor eye reflexes and acetone odor breath (Marutsova, 2015; Vasava et al., 2016).

These ewes were induced to labor using 15-20 mg dexamethasone I/M to get rid of the dead fetuses (usually one fetus), after receiving supportive treatment (40 ml glucose 5% and 20 ml Cal- D -Mg I/V) and Na bicarbonate I/V for correction of acidosis and a prophylactic dose of antibiotic (5 ml alamycin L.A. I/M). The supportive treatment was continued for three consecutive days with addition of probiotics to their rations until; they stood up and start to return to normal condition.

The rest sixteen ewes were observed in a more advanced stage, whereas the nervous symptoms appeared (convulsion, spasm, blindness, teeth grinding, salivation, dystocia, stillbirth and some cases had coma (Marutsova, 2015; Vasava et al., 2016). These ewes didn`t respond to the previous treatment, and shortly dead. The post mortem examination of them showed the presence of more than one fetus in its uterus, low abdominal fat and large pale fatty liver (Gomez et al., 2015).

In comparison with the metabolic profile of the three studied groups table (1) cleared that, there were a significant decrease in serum concentrations of glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol between the three groups and in the late pregnant group compared to the control group as well as in the toxemic group compared to the late pregnant group. On contrast, the plasma levels of BHB and NEFAs, serum enzymatic activities of ALT, AST and ALP, serum concentrations of blood urea, creatine, total lipids, triglycerides and phospholipids were significantly increased in both late pregnant and toxemic group.

Table (1): Serum biochemical parameters in non-pregnant, late pregnant and pregnant toxemic ewes. Values are mean±SD.

Parameters	CG	LPG	PTG
Glucose (mg/dl)	85.10±2.73[c]	68.15±2.25[a]	41.70±1.38[a,b]
BHB(Umo/L)	0.31±0.15[c]	1.38±0.01[a]	7.94±1.64[a,b]
NEFA(Umo/L)	0.44±0.76[c]	0.78±0.11[a]	1.84±0.03[a,b]
ALT (U/L)	36.74±1.61[c]	43.45±1.09[a]	48.80±0.81[a,b]
AST (U/L)	26.74±1.61[c]	33.30±1.03[a]	43.33±1.09[a,b]
ALP (U/L)	28.54±0.30[c]	29.69±0.49[a]	33.42±0.21[a,b]
Blood Urea (mg/dl)	24.74±0.73[c]	28.50±0.75[a]	41.43±3.31[a,b]
Creatinine (mg/dl)	0.75±0.11[c]	1.30±0.15[a]	2.35±0.10[a,b]
Total lipids (mg/dl)	353.90±6.10[c]	377.68± 4.36[a]	400.49±4.08[a,b]
Triglycerides (mg/dl)	73.17±2.12[c]	114.77±1.23[a]	137.59±1.47[a,b]
Phospholipids(mg/dl)	159.54±5.64[c]	185.55±3.19[a]	198.42±2.36[a,b]
Total cholesterol (mg/dl)	121.18±1.98[c]	77.36±2.59[a]	64.48±1.77[a,b]
HDL-cholestrol(mg/dl)	86.90±1.39[c]	53.86±1.31[a]	43.96±1.45[a,b]
LDL-cholestrol(mg/dl)	34.29±1.40[c]	23.50±1.29[a]	20.52±0.55[a,b]
TP (g/dl)	6.12±0.09[c]	5.96±0.04[a]	4.92±0.08[a,b]
Albumin (g/dl)	3.99±0.20[c]	2.87±0.06[a]	1.48±0.13[a,b]
Globulin (g/dl)	2.12±0.21[c]	3.08±0.06[a]	3.44±0.14[a,b]
A/G ratio	1.91±0.22[c]	.98±0.240[a]	0.43±0.05[a,b]

[a] (significant with CG), [b] (significant with LPG), [c] (significant between the three groups), considered statistically significant at $P<0.05$.

Table (2): Acute phase protein and cytokines profiles in the three studied groups. Value are mean±SD.

Parameters	CG	LPG	PTG
Fibrinogen (mg/dl)	122.00±8.49[c]	178.00±9.51[a]	255.80±6.50[a,b]
Caeruloplasmin (mg/dl)	2.32±1.18[c]	7.11±0.83[a]	12.01±1.45[a,b]
Haptoglobin (g/dl)	0.15±.02[c]	0.86±0.03[a]	1.69±0.20[a,b]
Serum amyloid-A (mg/L)	2.30±0.15[c]	3.29±0.19[a]	4.30±0.15[a,b]
IL-1α (Pg/ml)	24.05±3.34[c]	59.20±1.45[a]	86.20±3.54[a,b]
IL-1β (Pg/ml)	25.99±2.96[c]	72.20±2.83[a]	108.85±3.04[a,b]
IL-6 (Pg/ml)	24.63±2.92[c]	60.80±2.95[a]	92.00±3.28[a,b]
TNF-α (Pg/ml)	24.91±2.98[c]	77.26±2.06[a]	104.70±5.92[a,b]
IL-10 (Pg/ml)	96.70±4.56[c]	68.31±2.05[a]	46.52±0.56[a,b]

[a] (significant with CG), [b] (significant with LPG), [c] (significant between the three groups), considered statistically significant at P<0.05.

Table (3): Cut off points, sensitivity, specificity, LR, PPV, NPV, accuracy rate and percentage of increase for the estimated APPs and cytokines in the pregnancy toxemic group compared to the late pregnant group (percentage of decrease for IL-10).

Statistical parameters	Fb	Cp	Hp	SAA	IL-1α	IL-1β	IL-6	TNF-α	IL-10
Cut off point	187.5 mg/dl	8.05 mg/dl	0.90 g/dl	3.55 mg/L	60.70 Pg/ml	75.70 Pg/ml	64.80 Pg/ml	79.95 Pg/ml	65.90 Pg/ml
Sensitivity	100%	100%	100%	100%	100%	100%	100%	100%	100%
Specificity	80%	80%	90%	90%	80%	85%	90%	90%	85%
LR	5	5	10	10	5	6.67	10	10	6.67
PPV	83.33%	83.33%	90.91%	90.91%	83.33%	86.96%	90.91%	90.91%	86.96%
NPV	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy rate	90%	90%	95%	95%	90%	92.5%	95%	95%	92.5%
% of increase or decrease	43.71%	68.92%	96.51%	30.70%	31.32%	33.67%	33.91%	26.21%	31.90%

According to many researchers (Gomez et al., 2015; Marutsova, 2015; Vasava et al., 2016), hypoglycemia is expected at the last two months of pregnancy, as rapid fetal growth at this stage consumes most of ewe energy, resulting in this hypoglycemia. By the time, if the pregnant ewes did not receive enough carbohydrate supply, persistent hypoglycemia will induce lipolysis of body fat and hepatic gluconeogensis to find an alternate for glucose to get energy. Thus, keton bodies mainly BHB and NEFAs will accumulated in the blood of affected ewes and a state of ketoacidosis will begin.

These excess amounts of liberated circulating NEFAs and ketone bodies during the course of the disease, primary conjugated in liver then excreted through the kidney. Gradually, they become an overload on the liver and kidney and infiltrated in hepatic cells and the renal tubules resulting in fatty liver formation and irreversible renal damage. This explains the significant increase in serum enzymatic activities of liver enzymes and kidney function tests stated in our research and the positive correlation between BHB and NEFAs and liver enzymatic activities as well as renal function test in the toxemic ewes (table 4) (Gomez et al., 2015; Marutsova, 2015; Vasava et al., 2016).

Concerning the elevated serum liver enzymatic activities in the late pregnant group compared to the control group, this elevation mostly is not hepatic in origin and mainly connected to the increased uterine muscle activity to adapt the rapid increase in the fetal size and getting ready for parturition and the increased ALP placental isoenzyme secretion due to fetal bone formation and development (Aly and Elshahawy, 2016). Similarly, the significant rise in kidney functions tests of the late pregnant ewes in the present data is physiological. As, the hormonal changes associated with pregnancy increase the

blood flow to the kidney and subsequently, increase glomerular filtration rate. This action is important to clear off maternal and fetal wastes (Hussein and Lafayette, 2014; Aly and Elshahawy, 2016**)**.

The obtained hyperlipidemia, hypertriglyceridemia and hyperphospholipidemia in both the late pregnant and toxemic group here, is another outcome of hypoglycemia, increased lipolysis and hepatoneogensis related to late gestation periods and pregnancy toxemia. While, the hypocholesterolemia in the late pregnant group resulted from the unsuitable ration amounts provided to the pregnant ewe and the utilization of LDL- cholesterol in placental steroidogenesis, and was attributed in the toxemic ewes to anorexia and liver insufficiency due to fatty infiltration associated with the disease (Balikci et al., 2009; EL- Hefnawy et al., 2011; Deeb, 2012).

Comparing the proteinogram of the three studied groups, table (1) elucidated a significant hypoproteniema, hypoalbuminemia and reduced A/G ratio between the three studied groups, between the late pregnant group and control group as well as, between the toxemic group and the late pregnant group. These changes in proteinogram in late pregnant ewes were attributed by many researchers to the active placental transportation of amino acids at the last two months of pregnancyfrom mother to her fetus in order to fulfill the fetal developing requirements (Balikci et al.,2009; Hefnawy et al., 2011; Aly and Elshahawy, 2016). Reasonably, These alterations are more noticeable in toxemic ewes because of the catabolic nature of the disease and increased protein degradation, decomposed fetuses and increased protein loss due to terminal renal failure connected with PT (Balikci et al.,2009; Hefnawy et al., 2011; Aly and Elshahawy, 2016).

On the other hand, the significant hyperglobulinemia noticed in the late pregnant ewes compared with the control group, agrees with the common theory that the immune system strongly participates in the parturition mechanism (Dutta and Sengupta, 2017). While, the significant hyperglobulinemia reported in toxemic ewes compared with late pregnant group, clarified that pregnancy toxemia evokes a strong immune response, this is in consistent with the elevated levels of AAPs and cytokines registered in the studied toxemic ewes` blood (Barakat et al., 2007).

The previously mentioned biochemical alterations were translated clinically by weakness, lose of body weight, inability to move, fruity odor breath and nervous symptoms will appear later due to hypoglycemic encephalopathy and the formation of isopropyl alcohol from the acetoacetic acid (Balikci et al.,2009; Vasava et al., 2016).

Table (4,7) illustrated a negative correlation between serum glucose and number of fetuses, plasma BHB, NEFAs, serum globulin, total lipids, triglycerides, phospholipids, ALT, AST, ALP, blood urea, creatinine, SI, Tf sat. %, ferritin, AAPs and pro-inflammatory cytokines as well as a positive correlation between serum glucose and the disease prognosis, TP, albumin, A/G ratio, total cholesterol, HDL-cholesterol, LDL-cholesterol, TIBC, transferrin, UIBC and anti-inflammatory cytokine in the toxemic ewes. This information supports the common belief that the hypoglycemia is the first spark for subsequent biochemical and clinical changes observed in toxemia ewes.

While, the negative correlation between serum glucose and plasma BHB, NEFAs, serum globulin, total lipids, phospholipids, ALT, AST, ALP, blood urea, creatine, TIBC, transferrin, UIBC, AAPs and pro-inflammatory cytokines as well as the positive correlation between serum glucose and TP, albumin, A/G ratio, total cholesterol, HDL-cholesterol, LDL-cholesterol, SI, Tfsat.%, ferritin and anti-inflammatory cytokine in late pregnant ewes (table 5), explained that these metabolic alterations occur normally at the last third of pregnancy but in the presence of certain factors and absence of good management programs, these changes will exaggerated and cause pregnancy toxemia.

Acute phase response is an innate immune response performed by liver against various pathological, physiological and metabolic conditions (Gonzalez et al., 2011). Table (2) elucidated that both late pregnant ewes and toxemic ewes had a prominent acute phase response. This represented by the significant elevation of plasma Fb levels and Cp, Hp and SAA serum levels between the three groups and in the late pregnant ewes and the toxemic ewes when compared to the control group and the late pregnant group respectively. In this aspect, different authors referred to the strong positive effect of pregnancy, parturition and pregnancy toxemia in cow and sheep on AAPs concentrations (Gonzalez et al., 2011; EL-Deeb, 2012; Aly and Elshahawy, 2016). They explained that the high levels of circulating BHB and NEFAs associated to these conditions are a strong

enhancer for the inflammatory immune response. The positive correlation between NEFAs and BHBandAPPsin both late pregnant and toxemic groups gained in this study supports this hypothesis (table 4,5), (Balikci et al., 2009; Albay et al., 2014; Gurdogan et al., 2014; Czopowicz et al., 2018).

The positive correlation between APPs and serum globulin, total lipids, triglycerides, phospholipids, ALT, AST, ALP, blood urea, creatinine, SI, Tf sat. %, ferritin and pro-inflammatory cytokines as well as the negative correlation between APPs and serum glucose, TP, albumin, A/G ratio, total cholesterol, HDL-cholesterol, LDL-cholesterol, TIBC, transferrin, UIBC and anti-inflammatory cytokine (table 4,7) in the toxemic ewes, makes them a good indicator for the metabolic changes of the disease.

Cytokines are cellular polypeptide molecules, closely embedded in the inflammatory immune response. They are the inducer, coordinator and terminator of the inflammatory response(EL-Deeb, 2012). Table (2) revealed that, when comparing the serum concentrations of cytokines between the three groups, there was a significant increase in the serum levels of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α) between the three studied groups and between the late pregnant group related to the control group as well as between the toxemic group related to the late pregnant group. Contrariwise, there was a significant decline in serum levels of anti-inflammatory cytokines (IL-10).

These results are compatible with previous studies, which focused on the physiological importance of pro-inflammatory and anti- inflammatory cytokines in pregnancy establishment, adaptation and parturition, and their pathological role in different pregnancy disorders like sheep pregnancy toxemia (Orsi and Tribe, 2008; EL-Deeb, 2012; Dutta and Sengupta, 2017). They stated that the high concentrations of circulating BHB and NEFAs linked to the late gestation periods and pregnancy toxemia is the direct cause of cytokines enhanced release (EL-Deeb, 2012). This was confirmed in both late pregnant ewes and toxemic ewes by the positive correlation between BHB and NEFAs and the pro-inflammatory cytokines as well as the negative correlation between BHB and NEFAs and anti-inflammatory cytokine (table 4,5).

Table (4,7) described a positive correlation between the pro-inflammatory cytokines and AAPs, globulin, total lipids, triglycerides, phospholipids, ALT, AST, ALP, blood urea, creatinine, SI, Tf sat. % and ferritin as well as a negative correlation between the pro-inflammatory cytokines and serum glucose, TP, albumin, A/G ratio, total cholesterol, HDL-cholesterol, LDL-cholesterol, TIBC, transferrin and UIBC in the toxemic ewes. On the opposite side, there was a negative correlation between the anti-inflammatory cytokine and AAPs, globulin, total lipids, triglycerides, phospholipids, ALT, AST, ALP, blood urea, creatinine, SI, Tfsat. % and ferritin and there was appositive correlation between the anti-inflammatory cytokineand serum glucose, TP, albumin, A/G ratio, total cholesterol, HDL-cholesterol, LDL-cholesterol, TIBC, transferrin and UIBC in the toxemic ewes. This reflects the involvement of cytokines in the pathogenesis of the disease. As the pro-inflammatory cytokines are the main triggers of the acute phase response and other inflammatory immune response. Besides that, the significant drop of IL-10 concentration in serum toxemic ewes in this study (the most potent anti-inflammatory cytokine) takes a part in the extensive inflammatory reaction and predicts a bad prognosis for the cases(Aly and Elshahawy, 2016; EL-Deeb, 2012). This nominates them as a good target for pregnancy toxemia drugs development.

Based on the data of table (3), the selected APPs had 100% sensitivity and NPV. While, Hp and SAA had higher specificity, LR, PPV and accuracy rate than the other two proteins. This likely makes both Hp and SAA better markers for the pregnancy toxemia than Fb and Cp. But, the percentage of increase strongly recommends Hpthan SAA as Hp had the highest percentage of increase as 96.51%.Concerning the cytokines significance in diagnosis and prognosis of the disease table (3) showed that, the measured cytokines had 100% sensitivity and NPV. But IL6 and TNFα had better numbers of specificity, LR, PPV and accuracy rate than the other cytokines. While, percentage of increase was in the sake of IL6 as it had a 33.91% percentage of increase.

The comparison between APPs and cytokines value in the diagnosis and prognosis of pregnancy toxemia revealed that they have the same sensitivity and NPV as100%, and relative values of specificity, LR, PPV and accuracy rate. But the percentage of increase tends to favor of APPs, especially Hp. This result is in agreement with previous suggestions of utilization of APPs and cytokines in detection and evaluation of the disease (EL-Deeb, 2012; Gurdogan et al., 2014). In addition to that, the negative correlation between the measured APPs, pro-inflammatory cytokines and the disease prognosis as well as the positive correlation

between the measured APPs, pro-inflammatory cytokines and the number of fetuses in the toxemic ewes (table 4) confirmed their value in evaluation of the diseased cases.

Regarding the iron status in the late pregnant group, results of this study depicts a significant decrease in mean concentrations of SI, ferritin, Tf sat. % in late pregnant ewes relative to control healthy group. Conversely, they had a significant elevation in TIBC, UBIC and serum transferrin concentrations (table 6).

Many researchers demonstrated similar results, they assign this hypoferremia and hypoferritinemia (depletion of iron stores) to the increased fetal demand and the expanded red mass at the last third of gestation. Logically, this depletion followed by Tf sat. % drop and TIBC, UBIC rise. Whereas, serum transferrin concentrations will be enhanced in order to compensate these alterations (Zafar, 2008; Biswas et al., 2016). On the contrary, the studying of iron status in pregnancy toxemic ewes described a significant rise in mean concentrations of SI, ferritin, Tf sat. % relative to the late pregnant group, and a significant drop in TIBC, UBIC and serum transferrin concentrations (table 6).

Different opinions were recorded around the association between pregnancy toxemia and hyperferremia and hyperferritinemia. The first opinion attributed them to the hepatic damage connected with the pregnancy toxemia and the dependant release of the Kupffer cells stored iron. The positive correlation between hepatic enzymes and SI and ferritin reported in this research coincide with this postulate (table 7), (Zafar, 2008; Biswas, et al., 2016). Another opinion connects between the acute phase response related to the PT and the increased levels of ferritin, as ferritin is a positive acute phase reactant. This assumption was strengthen by the positive correlation between acute phase proteins and ferritin obtained in this work (table 7), (Zafar, 2008; Biswas et al., 2016).

In addition to the above mentioned causes, the oxidative stress which usually detected during the pregnancy toxemia course seems to have a role in this hyperferremia and hyperferritinemia. As the liberated free radicals destruct RBCs, causing excess iron release which followed by extreme ferritin formation (Biswas et al., 2016). Interestingly, the excess free iron is incorporated in the oxidative stress mechanism and the pathogenesis of the disease(Abdalla et al., 2013). This iron overload resulted in a prominent increase inTf sat. % and reduction in TIBC, UBIC and serum transferrin concentrations (Zafar, 2008).

Finally, we can conclude that; first pregnancy toxemia is a mangemental disease in the first place and the stage of the disease and number of fetuses affect strongly on the response to treatment. Second, the estimation of APPs and cytokines is helpful in diagnosis and prognosis of pregnancy toxemia in sheep but APPs are better than cytokines and Hp is the best among the estimated APPs. But the combination between Hp and another APP will be more worthy. Cytokines may be a new target for the therapeutic strategies of the disease. Third, the evaluation of iron status of the late pregnant ewes and toxemic ewes is crucial to take the necessary precautions to prevent the occurrence of the disease.

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Table (4): The correlation between the estimated APPs, cytokines and the metabolic profile in PTG(Pearson's correlation test).

parameters	Glucose	BHB	NEFAs	Fb	Cp	Hp	SAA	IL-1α	IL-1β	IL-6	TNF-α	IL-10
The prognosis	0.669*	-0.662*	-0.649	-0.734*	-0.707*	-0.707*	-0.673*	-0.638*	-0.657*	-0.657*	-0.694*	0.721*
No of fetuses	-0.789*	0.839*	0.866*	0.887*	0.860*	0.845*	0.859*	0.884*	0.895*	0.895*	0.861*	-0.822*
Glucose	1	-0.956*	-0.959*	-0.957*	-0.946*	-0.961*	-0.949*	-0.955*	-0.951*	-0.951*	-0.967*	0.953*
BHB	-0.956	1	0.977*	0.945*	0.949*	0.976*	0.948*	0.978*	0.983*	0.983*	0.984*	-0.968*
NEFAs	-0.959*	0.977*	1	0.951*	0.976*	0.979*	0.974*	0.991*	0.989*	0.989*	0.990*	-0.968*
ALT	-0.975*	0.953*	0.953*	0.915*	0.938*	0.959*	0.930*	0.955*	0.949*	0.949*	0.962*	-0.958*
AST	-0.968*	0.965	0.976*	0.938*	0.973*	0.983*	0.965*	0.978*	0.973*	0.973*	0.990*	-0.981*
ALP	-0.907*	0.960*	0.939*	0.926*	0.931*	0.955*	0.936*	0.947*	0.966*	0.966*	0.960*	-0.945*
Blood Urea	-0.907*	0.922*	0.956*	0.923*	0.923*	0.934*	0.918*	0.968*	0.960*	0.960*	0.942*	-0.896*
Creatinine	-0.925*	0.933*	0.962*	0.936*	0.920*	0.940*	0.934*	0.962*	0.958*	0.958*	0.955*	-0.918*
Total lipids	-0.942*	0.967*	0.983*	0.933*	0.961*	0.970*	0.959*	0.978*	0.986*	0.986*	0.982*	-0.956*
Triglycerides	-0.710*	0.670*	0.731*	0.727*	0.700*	0.682*	0.722*	0.708*	0.961*	0.691*	0.707*	-0.684*
Total cholesterol	0.944*	-0.907*	-0.958*	-0.924*	-0.961*	-0.940*	-0.948*	0.946*	-0.940*	-0.940*	-0.942*	0.915*
HDL-cholestrol	0.946*	-0.948*	-0.976*	-0.948*	-1.00*	-0.971*	-0.990*	-0.974*	-0.974*	-0.974	-0.981*	0.968*
LDL-cholestrol	0.914*	-0.948*	-0.912*	-0.922	-0.951*	-0.920*	-0.956*	-0.899*	-0.908*	-0.908*	-0.933*	0.938*
Phospholipid	-0.764*	0.814*	0.832*	0.728*	0.806*	0.826*	0.790*	0.833*	0.837*	0.837*	0.839*	-0.827*
TP	0.879*	-0.918*	-0.951*	-0.893*	-0.927*	-0.924*	-0.907*	-0.959*	-0.949*	-0.949*	-0.932*	0.898*
Albumin	0.945*	-0.959*	-0.951*	-0.951*	-0.926*	-0.949*	-0.954*	-0.939*	-0.951*	-0.951*	-0.965*	0.953*
Globulin	0.971*	0.975*	0.971*	0.962*	0.959*	0.981*	0.966*	0.971*	0.975*	0.975*	0.988*	-0.978*
A/G ratio	0.953*	-0.961*	-0.965*	-0.952*	-0.948*	-0.961*	-0.972*	-0.949*	-0.958*	-0.958*	-0.977*	0.968*
Fb	-0.957*	0.945*	0.951*	1	0.948*	0.942*	0.962	0.958*	0.955*	0.955*	0.958*	-0.934*
Cp	-0.946*	0.949*	0.976*	0.948*	1	0.971*	0.990*	0.974*	0.974*	0.974*	0.981*	-0.968*
HP	-0.961*	0.976*	0.979*	0.942*	0.971*	1	0.965*	0.982*	0.982*	0.982*	0.990*	-0.979*
SAA	-0.949*	0.948*	0.974*	0.962*	0.990*	0.965*	1	0.966*	0.968*	0.968*	0.990*	-0.965*

Statistical significance of correlations * was recorded at ($P < 0.05$).

Table (5): The correlation between the estimated APPs, cytokines, iron status and the metabolic parameters in LPG(Pearson's correlation test).

parameters	Glucose	BHB	NEFAs	Fb	Cp	Hp	SAA	IL-1α	IL-1β	IL-6	TNF-α	IL-10
Glucose	1	-0.772*	-0.915*	-0.893*	-0.898*	-0.947*	-0.946*	-0.917*	-0.941*	-0.947*	-0.947*	0.933*
BHB	-0.772*	1	0.915*	0.934*	0.892*	0.911*	0.889*	0.925*	0.923*	0.911*	0.896*	-0.926*
NEFAs	-0.915*	0.915*	1	0.944*	0.936*	0.969*	0.962*	0.954*	0.977*	0.969*	0.963*	-0.971*
ALT	-0.919*	0.926*	0.969*	0.966*	0.967*	0.990*	0.977*	0.972*	0.984*	0.990*	0.980*	-0.980*
AST	-0.939*	0.916*	0.958*	0.974*	0.957*	0.989*	0.977*	0.983*	0.989*	0.989*	0.975*	-0.980*
ALP	-0.946*	0.831*	0.927*	0.911*	0.894*	0.945*	0.939*	0.941*	0.943*	0.945*	0.928*	-0.932*
Blood Urea	-0.798*	0.801*	0.847*	0.834*	0.832*	0.869*	0.859*	0.849*	0.856*	0.869*	0.860*	-0.863*
Creatinine	-0.917*	0.925*	0.954*	0.991*	0.940*	0.985*	0.965*	1.00*	0.975*	0.985*	0.970*	-0.979*
Total lipids	-0.954*	0.864*	0.952*	0.940*	0.967*	0.973*	0.967	0.957*	0.965*	0.973*	0.968*	-0.966*
Triglycerides	0.331	0.121	0.198	0.222	0.278*	0.270	0.278	0.227	0.251	0.270	0.276*	-0.273
Total cholesterol	0.909*	-0.929*	-0.952*	-0.993*	-0.966*	-0.983*	-0.966*	-0.998	-0.974*	-0.983*	-0.971*	0.976*
HDL-cholestrol	0.920*	-0.911*	-0.948*	-0.985*	-0.966	-0.979*	-0.966*	-0.994*	-0.970*	-0.979*	-0.965*	0.968*
LDL-cholestrol	0.888*	-0.938*	-0.946*	-0.990*	-0.956*	-0.977*	-0.956*	-0.992*	-0.968*	-0.977*	-0.966*	0.975*
Phospholipid	-0.689*	0.667*	0.756*	0.733	0.782	0.731	0.782*	0.750*	0.762*	0.731*	0.788*	-0.696*
TP	0.919*	-0.747*	-0.867*	-0.830*	-0.892*	-0.890*	-0.892*	-0.886	-0.889	-0.890*	-0.875*	0.881*
Albumin	0.974*	-0.791*	-0.913*	-0.924*	-0.957*	-0.957*	-0.957*	-0.934*	0.949*	-0.957*	-0.954*	0.942*
Globulin	-0.933*	0.841*	0.938*	0.911*	0.954*	0.957*	0.954*	0.935*	0.954*	0.957	0.944*	-0.945*
A/G ratio	0.183	0.301	0.282*	-0.288	0.207	0.253	0.207	0.295	0.225	-0.253	-0.209	0.312
SI	0.963*	-0.762*	-0.887*	-0.901*	-0.913*	-0.928*	-0.930*	0.917*	-0.889*	-0.854*	0.982*	0.972*
TIBC	-0.955*	0.882*	0.962*	0.965*	0.979*	0.982*	0.967*	-0.976*	-0.946*	0.942*	0.982*	0.972*
Transferrin	-0.902*	0.864*	0.917*	0.934*	0.940*	0.939*	0.945*	-0.920*	0.900*	0.898*	0.939*	0.940*
Tf sat. %	0.947*	-0.769*	-0.898*	-0.906*	-0.918*	-0.938*	-0.938*	0.915*	-0.881*	-0.865*	-0.938*	-0.941*
UIBC	-0.876*	0.851*	0.903*	0.896*	0.920*	0.919*	0.900*	-0.912*	0.868*	0.1	0.1	0.1
Ferritin	0.840*	-0.820*	-0.865*	-0.902*	-0.885*	-0.888*	-0.889*	0.866*	-0.872*	-0.867*	-0.888	-0.895

Statistical significance of correlations * was recorded at ($P < 0.05$)

Table (6): Comparison between the iron statuses in the three studied groups. Value are mean±SD.

Parameters	CG	LPG	PTG
SI (µg/dl)	106.39±2.46[c]	55.68±2.29[a]	85.55±1.54[a,b]
TIBC (µg/dl)	327.39±2.16[c]	359.02±6.17[a]	333.63±2.39[a,b]
Transferrin (mg/dl)	124.65±2.74[c]	153.30±8.31[a]	86.01±10.16[a,b]
Tf sat. %	32.64±0.66[c]	15.50±0.49[a]	25.64±0.46[a,b]
UIBC(µg/dl)	220.50±2.24[c]	303.36±4.78[a]	248.08±2.60[a,b]
Ferritin (ng/ml)	13.60±1.04[c]	10.73±0.40[a]	44.45±1.41[a,b]

[a](significant with CG), [b] (significant with LPG), [c] (significant between the three groups), considered statistically significant at P<0.05.

Table (7): The correlation between the iron status and glucose, BHB, NEFAs, liver enzymes, APPs, cytokines in PTG (Pearson's correlation test).

Parameters	SI	TIBC	Transferrin	Tf sat.%	UIBC	Ferritin
Glucose	-0.908*	0.946*	0.924*	-0.947*	0.969*	-0.927*
BHB	0.959*	-0.965*	-0.965*	0.978*	-0.961*	0.945*
NEFAs	0.968*	-0.973*	-0.961*	0.980*	-0.976*	0.966*
ALT	0.925*	-0.941*	-0.936*	0.948*	-0.941*	0.930*
AST	0.955*	-0.966*	-0.968*	0.980*	-0.973*	0.964*
ALP	0.924*	-0.936*	-0.885*	0.958*	-0.954	0.938*
Fb	0.880*	-0.955*	-.901*	0.934*	-0.976*	0.943*
Cp	0.947*	-0.985*	-.943*	0.972*	-0.972*	0.995*
Hp	0.966*	-0.975*	-0.957*	0.986*	-0.975*	0.963*
SAA	0.931*	-0.981*	-0.930*	0.968*	-0.977*	0.986*
IL-1α	0.962*	-0.977*	-0.964*	0.978*	-0.972*	0.970*
IL-1β	0.965*	-0.977*	-0.952*	0.983*	-0.975*	0.971*
IL-6	0.965*	-0.977*	-0.952*	0.983*	-0.975*	0.971*
TNF-α	0.967*	-0.981*	-0.964*	0.992*	-0.985*	0.976*
IL-10	-0.959*	0.965*	0.950*	-0.981*	0.966*	-0.965*

Statistical significance of correlations * was recorded at ($P < 0.05$).