Sahel Journal of Veterinary Sciences

Pathology and Oxidative Stress Changes Associated with Pregnancy Toxaemia in Ewes

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ABSTRACT

Pregnancy toxaemia is often associated with negative energy balance, and ewes carrying multiple lambs are at risk due to high energy requirements. Deficient nutritional intake with a consequent body fat mobilization for energy, results in production of reactive oxygen species which leads to pathologic changes. This study aimed to evaluate the pathology and oxidative stress changes associated with pregnancy toxaemia in ewes. A total of 33 animals aged 2-3 years with mean weight of 40 ± 0.43 to 60 ± 0.50 kg, were selected from different private breeders' farms in Ilorin. Twenty-three animals that showed signs of pregnancy toxaemia (recumbence, weakness, and restlessness) were selected and labeled as group A. Ten healthy non-pregnant ewes were also labeled as group B. There was a significant increase in packed cell volume (PCV%), mean corpuscular volume (MCV), and glucose level in ewes with pregnancy toxaemia, while there was a significant decrease in superoxide dismutase (SOD), and catalase (CAT) levels in pregnancy toxaemia group. Postmortem findings included, congested ocular mucous membrane, congested and enlarged lungs, liver and spleen, congested intestinal mucosa and brain meninges, enlarged heart and dilated ventricles, atrophy of fat along the coronary grove of the heart, pale and enlarged liver, excess abdominal fat, and two dead fetuses. On the other hand, the histopathological examination of the pregnancy toxaemia group showed livers with oedematous areas and regular border vacuoles. The lungs were also congested and oedematous There is tubular necrosis and areas of congestion in the kidneys. In conclusion, this study reveals significant hematological and biochemical changes indicative of oxidative stress, alongside pathological findings in various organs such as the liver, lungs, and kidneys. These results underscore the complexity of pregnancy toxaemia and emphasize the need for further research to develop better management strategies.

Keywords: Congestion; Fetuses; Oxidative stress; Oedema; Pregnancy; Toxaemia;

INTRODUCTION

Pregnancy toxaemia, also known as ketosis or twin-lamb disease, is a metabolic disorder characterized by the development of ketonemia and ketonuria in pregnant ewes, typically occurring during the last trimester of gestation (Ji *et al.,* 2023). This condition poses a significant threat to the health and well-being of both the ewe and the developing fetuses, often resulting in increased morbidity and mortality rates. Understanding the underlying pathological and oxidative stress changes associated with pregnancy toxaemia is crucial for developing effective diagnostic and therapeutic strategies. The occurrence of pregnancy toxaemia is linked to a variety of factors, including nutritional and hormonal imbalances, stress (Abd-Elghany

et al., 2011; Abba *et al.*, 2015; Khan *et al.*, 2021), and metabolic demands associated with carrying multiple fetuses, especially in the last 4-6 weeks of gestation (Rodoifo *et al.*, 2019; Akraiem *et al.*, 2020). The required maintenance energy at the late gestation is between 600 to 700 g per day of metabolized energy for twins (Robinson, 1980). A comprehensive investigation into the pathological alterations during pregnancy toxaemia sheds light on the intricate interplay between metabolic pathways and the physiological changes occurring in affected ewes. Research in this field has highlighted the pivotal role of oxidative stress in the pathogenesis of pregnancy toxaemia (Ravarotto *et al.*, 2021). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen

species (ROS) and the antioxidant defense mechanisms (Pizzino *et al*., 2017). Several studies have been reported on pregnancy toxaemia in sheep and goats (Mongini and Van Saun, 2023). Histopathological examinations have revealed notable changes in various organs, including the liver and kidneys, indicating the systemic impact of pregnancy toxaemia. Hepatic lipidosis, vacuolar degeneration, and necrosis are commonly observed in affected ewes, underlining the severity of metabolic derangements (Rodolfo *et al*., 2019). Furthermore, renal alterations, such as tubular degeneration and interstitial inflammation, contribute to the overall pathological complexity of this condition. This study aims to deepen our understanding of the pathological changes associated with pregnancy toxaemia in ewes. By elucidating the pathological changes and oxidative stress investigation strives to contribute valuable insights that may inform targeted interventions to mitigate the impact of pregnancy toxaemia on ewe health and reproductive outcomes.

MATERIALS AND METHODS

Ethical Approval

Ethical approval for this study was obtained from the University of Ilorin Committee on Animal Use and Care, University of Ilorin, Ilorin, Nigeria (Ethical code-UREC/FVM/2023/035). All applicable international, national, and/or institutional guidelines for the collection of blood samples from ewe were appropriately followed.

History and Sample Collection

The study was conducted on private breeder farms in Ilorin, Kwara State, Nigeria, where previously reported cases of pregnancy toxaemia in ewes had been reported to the University of Ilorin Veterinary Teaching Hospital. A total of 32 ewes aged 2-3 years and mean weighing 40 ± 0.43 to 60 ± 0.50 kg, were selected from these farms. The ewes were managed intensively with access to feed and water *ad libitum.* Twenty-three ewes exhibited signs of pregnancy toxaemia, including recumbence, weakness, and restlessness, were categorized into Group A, while 10 healthy non-pregnant ewes were labeled as Group B. Throughout the study period, three ewes from Group A succumbed to pregnancy toxaemia died and underwent postmortem examination.

Determination of Haematological Parameters

For this study, 5 mLs of blood sample was collected through jugular venepuncture from each animal. Three mLs of serum was transferred to a non-heparinized sample bottle for determination of oxidative stress makers. The collected blood was allowed to clot and centrifuged at 1000 × g for 10 minutes using micro-centrifuge 5418 R (Eppendorf, Ontario, Canada), to obtain serum for analyses. The remaining 2 mL was transferred to a sterile capped tube, containing ethylenediaminetetraacetic acid anticoagulant (Greiner Bio-One, Frickenhausen, Germany) and used for haematological evaluation as described by Adam *et al*. (2019). Packed cell volume (PCV), and total and differential leukocyte counts were determined as described previously (Coles, 1980). Mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated from

the total red blood cell count, PCV, and haemoglobin concentration as described by Adam *et al*. (2019).

Determination of Blood Glucose

Using a glucometer as described by the manufacturer, a small drop of blood collected from the ewes were placed on the test strip. The strip was then inserted into the glucometer and waited for 5 minutes to display the blood glucose reading. All the values obtained were recorded as displayed on the device (Gavrilovic *et al*., 2011).

Determination of Oxidative Stress Makers

Superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (TAC) were all measured by a colorimetric technique using a Bio-diagnostic kit, in the USA. The malondialdehyde (MDA) was assayed using a commercial Bio-diagnostic kit, USA as described by the method of (Marnett, 1999); Nawito *et al*. (2016), and was expressed as SOD (U/ml), CAT (U/L), TAC (mM/L), and MDA (mmol/ml).

The serum was homogenized with phosphate-buffered saline at a ratio of 1:10 (w/v) using homogenizer for 5 minutes. The homogenate was mixed with TBA reagent at a ratio of 1:1 and incubated at a suitable temperature of around 95-100°C for 30 minutes and formed a colored complex. The mixture was heated to a temperature of about 95-100°C for 30 minutes and allowed to cool. The homogenate was then centrifuged at the revolution of about 10,000 x g for 10 minutes. The supernatant was collected and analyzed spectrophotometrically at a suitable wavelength of 532nm for measuring the absorbance of the MDA-TBA adduct. A calibration curve is prepared using known concentrations of MDA standards. The calibration curve was used to convert absorbance readings into MDA concentrations. The MDA concentration was normalized to the protein content which provided results as MDA concentration per unit of protein.

Postmortem Examination

The dead carcasses were sent to the necropsy unit of the Veterinary Pathology Department for postmortem examination. During the postmortem examination, most of the findings included a bodily condition with a rough hair coat, a slightly dehydrated, congested ocular mucous membrane with a soiled hind leg, and diarrhoeic faeces. There was severe serous atrophy of fat on the subcutaneous layer of the abdomen of pregnant toxaemia ewes that died during the study. The trachea was slightly hyperaemic with excessive froth in the airways. The lung was slightly enlarged and severely congested. The heart was slightly enlarged with fat atrophy along the coronary grove. The ventricles of the heart were dilated. The liver was also enlarged and congested. The mucosal surface of the intestine was congested with mild catarrh inflammation in one of the animals. Semi-solid ingesta distended the rumen. Excessive abdominal fat that weighed 2.8 ± 052 kg from one of the animals when removed from the viscera. The spleen was also enlarged and congested. The head of the animal was bilaterally dissected into equal halves in which the nasal cavity showed the presence of a larva of *Oestrus ovis* in the nasal turbinate. The meninges and brain tissue were congested. There was the presence of two dead

foetuses in the uterus. The estimated gestation age of the fetuses is about 120 days using mobile ultrasound machine.

Gross Examination

The liver, lungs, heart, fat, kidneys, and brain samples were examined for gross abnormalities. The collected samples were thoroughly inspected using visualization, palpation, and multiple systemic incisions when and wherever required. Texture, consistency, color, adhesion, pattern, distribution, and number of lesions were recorded. Gross tissue changes were observed, meticulously recorded, and photographed with a Pentax 18-55mm digital camera manufactured in the Philippines by Hoya Corporation. Representative tissue samples containing lesions were stored in 10% neutral buffered formalin for histopathological studies for at least 24 hours Adam *et al*. (2023).

Histopathology Examination

After 24 hours, the fixed tissue sections were cut into pieces (3 mm thick) and dehydrated using ascending grades (70%, 80%, 90%, and 100%) of alcohol for 15 min, followed by clearing in absolute xylene and embedded in paraffin wax. Sections of 4–5 microns in thickness were cut and stained using Harri's haematoxylin and eosin methods. Finally, the stained slides were examined at 4, 10, and X100 magnifications for the presence of characteristic and/or suggestive lesions using an ordinary light microscope. The different forms of lesions were then classified according to the involvement of anatomical sites and the nature of the inflammatory exudate and reaction present Adam *et al*., (2023).

Data Analyses

Values obtained were expressed as mean $\pm$ SEM and subjected to statistical analysis using Students' t-test. Descriptive statistic was also use and expressed values as percentage. GraphPad prism version 5.0 (San Diego, California, USA) was used for the analyses. Values of $P < 0.05$ were considered significant.

RESULTS

The results of the study revealed significant differences in various hematological parameters between the pregnant toxaemia ewes (Group A) and the non-pregnant ewes (Group B). Group A exhibited a notable decrease in packed cell volume (PCV) compared to Group B (25.80 $\pm$ 1.22% vs. 36.40 $\pm$ 1.34%, respectively; $P < 0.05$). Although the mean hemoglobin level in Group A was numerically lower than that in Group B (8.20 $\pm$ 0.36 g/dL vs. 10.10 $\pm$ 0.22 g/dL), the difference was not statistically significant ($P > 0.05$). Similarly, the red blood cell count (RBC) was lower in Group A compared to Group B (10.30 $\pm$ 0.16 x10^{x12}/L vs. 12.20 $\pm$ 0.12 x10^{x12}/L), with no significant difference noted ($P > 0.05$). A significant decrease in the mean corpuscular volume (MCV) was observed in Group A

(25.04 $\pm$ 1.32 fL/cell) compared to Group B (34.06 $\pm$ 1.25 fL/cell; $P < 0.05$). However, no significant difference was found in the mean corpuscular hemoglobin (MCH) between the two groups. The mean corpuscular hemoglobin concentration (MCHC) in Group A was lower than that in Group B (30.40 $\pm$ 1.12 g/dL vs. 32.51 $\pm$ 1.14 g/dL), although the difference was not statistically significant ($P > 0.05$). Furthermore, Group A exhibited a higher mean total white blood cell (WBC) count compared to Group B (9.17 $\pm$ 0.18 x10^{x9}/L vs. 6.34 $\pm$ 0.15 x10^{x9}/L), with no significant difference observed ($P > 0.05$). The lymphocyte count was lower in Group A than in Group B (7.41 $\pm$ 1.10 x10^{x13}/μL vs. 5.30 $\pm$ 1.52 x10^{x13}/μL), showing no significant difference between the groups ($P > 0.05$). Additionally, Group A showed a higher neutrophil count compared to Group B (9.10 $\pm$ 1.64 x10^{x13}/μL vs. 6.01 $\pm$ 1.23 x10^{x13}/μL), with no significant difference observed ($P > 0.05$). Notably, the mean glucose levels were significantly lower in Group A (38.110 $\pm$ 3.265 mmol/L) compared to Group B (44.21 $\pm$ 3.142 mmol/L; $P < 0.05$) as shown in Table 1. Also, the mean superoxide dismutase (SOD) activity in Group A ewes was 501.99 $\pm$ 8.215 U/mL, while in Group B ewes, it was 560.06 $\pm$ 15.36 U/mL. SOD is an enzyme crucial for neutralizing superoxide radicals. Remarkably, the SOD level in Group A was significantly lower than that in Group B ($P < 0.05$). Conversely, catalase (CAT) activity in Group A was 113.40 $\pm$ 18.325 U/mL, notably lower than the value in Group B (356.81 $\pm$ 12.95 U/mL), with a significant difference observed ($P < 0.05$). However, the malondialdehyde (MDA) level in Group A (15.745 $\pm$ 0.87 mmol/L) did not significantly differ from that in Group B (12.42 $\pm$ 0.45 mmol/L; $P > 0.05$). Finally, the total antioxidant capacity (TAC) in Group A (0.56 $\pm$ 0.16 mML) was not significantly different from that in Group B (0.82 $\pm$ 0.13 mML; $P > 0.05$) as depicted in Table 2. Various lesions were observed during post-mortem examination. The percentage score of congested ocular mucous membrane was found to be 4.4%, while lesions of congested and enlarged lungs accounted for 17.4% of observed cases. Additionally, enlargement of the heart and dilated ventricles scored 4.3% among the observed lesions, with atrophy of fat along the coronary groove amounting to 13%. Congestion and enlargement of the liver were estimated at 13%, while congested intestinal mucosa mirrored a percentage score of 4.4%, suggesting potential gastrointestinal issues or infections. Excess abdominal fat was notable, comprising 17.4% of the observed lesions, possibly indicating nutritional imbalances or metabolic disorders. The percentage of congested and enlarged spleen was also 4.4%, indicative of systemic infections or immune system-related conditions. Furthermore, congested brain meninges were observed, representing 4.4% of the lesions. Finally, the percentage of dead fetuses found in the uterus was recorded at 8.7%. These findings offer insights into the pathological conditions observed during the examination (Table 3).

Table 1: Mean haematological parameters of pregnancy toxaemia compared with non-pregnant ewes.

Parameters	Group A	Group B
PCV (%)	25.80 ± 1.22[a]	36.40 ± 1.34[b]
Hb (g/dL)	8.20± 0.36[a]	10.10 ± 0.22[a]
RBC (x10 ^{12}/L)	10.30± 0.16[a]	12.20 ± 0.12[a]
MCV (f/cell)	25.04 ± 1.32[a]	34.06 ± 1.25[b]
MCH (pg/cell)	7.96 ± 0.53[a]	10.23 ± 0.34[a]
MCHC (g/dL)	30.40± 1.12[a]	32.51 ± 1.14[a]
WBC (x10^9/L)	9.17± 0.18[a]	6.34 ± 0.15[a]
Lymphocytes (x10^{13}/μL)	7.41± 1.10[a]	5.30 ± 1.52[a]
Neutrophils (x10^{13}/μL)	9.10 ± 1.64[a]	6.01 ± 1.23[a]
Glucose (mmol/L)	38.110 ± 3.265[a]	44.21 ± 3.142[b]

SEM = standard error of the mean, PCV packed cell volume, Hb= haemoglobin, RBC= red blood cell, MCHC = mean corpuscular haemoglobin concentration, MCV= mean corpuscular volume, WBC= white blood cell, Group A= Pregnancy toxaemia ewes, and Group B= Non-pregnant ewes. a, b, = Values of mean ± SEM in the same row with different superscripts are significantly (P < 0.05) different.

Table 2: Mean oxidative stress makers' values of pregnant and non-pregnant toxaemia ewes

Parameters	Group A	Group B
SOD (U/mL)	501.99 ± 8.215[a]	560.06 ± 15.36[b]
CAT (U/mL)	311.40 ± 18.325[a]	356.81 ± 12.95[b]
MDA (mmol/L)	15.745 ± 0.87[a]	12.42 ± 0.45[a]
TAC (mML)	0.56 ± 0.16[a]	0.825 ± 0.13[a]

SOD=Superoxide dismutase, CAT=Catalase, MDA=Malondialdehyde, TAC=Total antioxidant capacity, Group A=pregnant ewes, Group B=non-pregnant. a, b, = Values of mean ± SEM in the same row with different superscripts are significantly (P < 0.05) different

Table 3: Gross lesions observed during post-mortem examination of pregnancy toxaemia ewes.

Postmortem gross lesions	Number of lesions	Percentage score (%)
Congested ocular mucous membrane	1	4.4
Congested and Enlarged Lungs	4	17.4
Enlarged heart and dilated ventricles	1	4.3
Atrophy of fat along the coronary grove	3	13
Congested and Enlarged Liver	3	13
Pale and enlarged liver	2	8.6
Congested intestinal mucosa	1	4.4
Excess abdominal fat	4	17.4
Congested and enlarged spleen	1	4.4
Congested brain meninges	1	4.4
Dead foetuses in the uterus	2	8.7

DISCUSSION

Pregnancy toxemia in ewes occurs when the animal is unable to satisfy the glucose requirements of both the feotus and placental unit leading to hypoglycemia and increased levels of ketone bodies in the blood. The diagnosis of this metabolic disorder depends on the gestation age, clinical signs, and determination of some hematological and biochemical parameters. The results of this study indicate that ewes diagnosed with pregnancy toxaemia exhibit significant changes in hematological parameters compared to non-pregnant ewes. For instance, the lower PCV, Hb, RBC, MCV, MCH, and MCHC in pregnancy toxaemia ewes suggest anemia, which may be due to the metabolic disorder affecting red blood cell production. This agreed with the result of Radostits *et al.*, 2007 who reported that anemia is associated with pregnancy toxaemia in small ruminants. In addition, a decrease in the level of RBCs and Hb may be a result of the reduced erythropoiesis in pregnancy toxaemia (EL-din and EL-sangery, 2005). Also, the higher neutrophil count

in pregnancy toxaemia ewes suggests a potential inflammatory response, possibly associated with the metabolic disorder. Leukocytosis and lymphocytosis in pregnancy toxemia could also be attributed to the presence of acute and chronic inflammations (Gavan *et al.*, 2010). Lymphocytosis in the present study was like that described by the previous study Tharwat and Al-Sobayil, 2013. The increases in WBCs, neutrophils, and lymphocytes in this study agree with results earlier reported by Abba *et al.*, 2015 and Tharwat and Al-Sobayil, 2013 who hypothesize that this increase was as a result of metabolic acidosis, infection, inflammatory reactions, and tissue necrosis of the liver. Neutrophilia in the present studies was in accord with the explanation of Smith and Sherman, 2009 but was contrary to Tharwat and Al-Sobayil, 2013. The increase in neutrophils may be due to hepatic lipidosis which exposes hepatocytes and triggers inflammation. In addition, it increases the oxidative burden and release of fibrogenic cytokines (Smith and Sherman, 2009). However, the lower glucose levels in pregnancy toxaemia ewes indicate

impaired glucose metabolism, a characteristic feature of this disorder. All these findings highlight the physiological changes associated with pregnancy toxaemia in ewes. However, the lower glucose levels in pregnant sheep were also associated with reduced gluconeogenesis by the liver from glucogenic precursors such as propionate, which emanated from rumen fermentation (Dzadzowski *et al*., 2015). Again, with the gestation progress, the level of blood glucose decreases because foetus requires more glucose as the sole energy source estimating up to 70% of the maternal production (Kaneko *et al*., 2008). Therefore, with advancing pregnancy, blood glucose levels tend to decrease since the foetus demands glucose as the primary energy source, consuming up to 70% of maternal production (Kaneko *et al*., 2008).

Figure 1: Gross pathological findings of ewe with pregnancy toxaemia showing dead twin fetuses (A), excessive accumulation of abdominal fat (**B**), icteric liver (C), and congested brain (D).
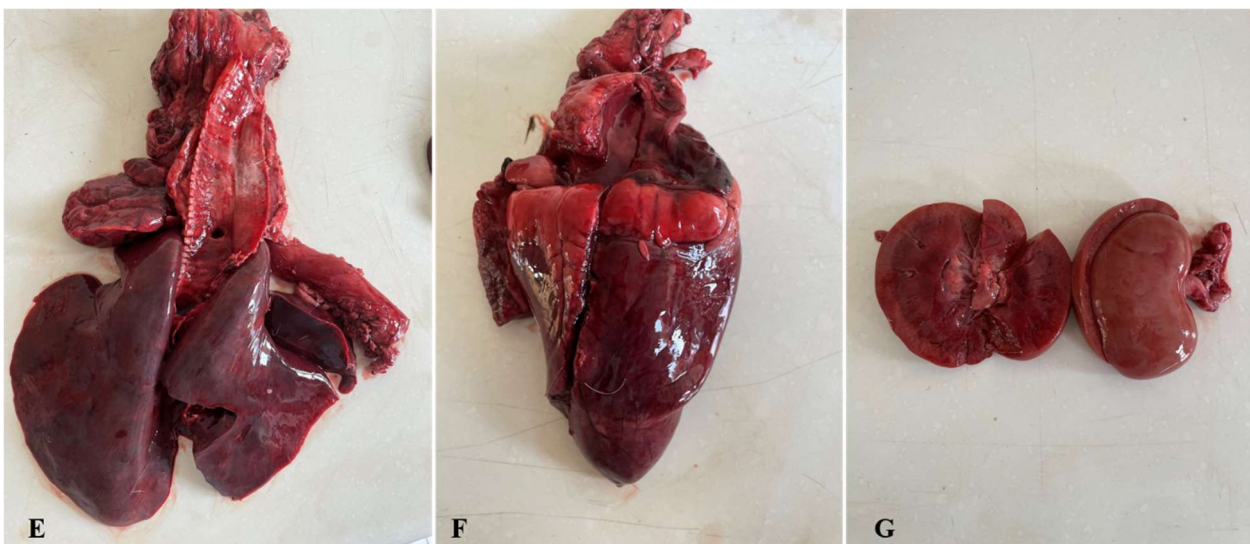

Figure 2: Gross pathological findings of ewe with pregnancy toxaemia showing the lungs that are slightly enlarged and congested (E). Heart is congested with atrophy of fat along the coronary grove (F). The kidneys were congested and slightly enlarged (G).
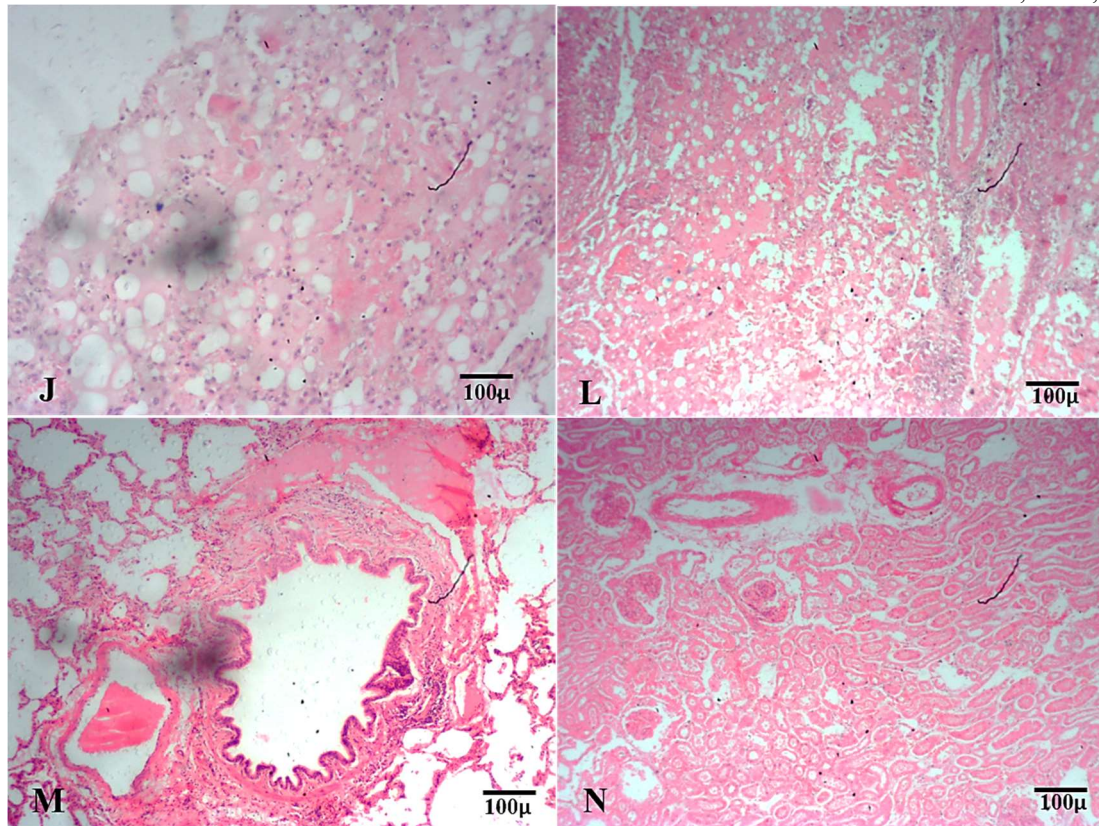
Figure 4: J-N; Photomicrograph of the liver, lung, and kidney of ovine who died of pregnancy toxaemia. The liver shows regular border vacuoles with areas of oedema (**J and I**). The lungs show congestion and areas of oedema (**M**). The kidneys show evidence of congestion with areas of tubular necrosis (N), H&E x100.

Emphasizing the need for careful monitoring and intervention to manage this metabolic disorder during pregnancy. The present study showed lower levels of SOD, CAT, and TAC, along with higher MDA, collectively suggest increased oxidative stress in pregnancy toxaemia ewes. The reduced activity of SOD and CAT indicates compromised antioxidant defence mechanisms in pregnant toxaemia ewes, making them more susceptible to oxidative damage. The elevated MDA levels as shown in the result indicate higher lipid peroxidation in pregnancy toxaemia ewes, potentially contributing to cellular damage. This also reflects lipid peroxidation in sheep with pregnancy toxemia compared with healthy pregnant and healthy non-pregnant ewes. This may be ascribed to a decrease in antioxidant protection of ewe as gestation progresses up to lactation and was accompanied by increased generation of pro-oxidants, thus exposing the animals to oxidative stress (Pilarczyk *et al*., 2012). It was also associated with a decreased level of antioxidants, together with the presence of high concentrations of ketone bodies in the blood.

From our findings the lower TAC implies a diminished overall antioxidant capacity in pregnancy toxaemia ewes, further supporting the notion of increased oxidative stress. The clinical effects of our results suggest that pregnancy toxaemia in ewes is associated with oxidative stress, which can have detrimental effects on various physiological processes. In this study again, the presence of lesions in multiple organs (lungs, heart, liver, spleen, intestines, brain, and reproductive system) suggests a systemic condition rather than localized issues. The lesions in the lungs and heart indicate possible respiratory and cardiovascular distress, which could be associated with various diseases or conditions including pregnancy toxaemia in the ewe. Again, the findings in this study including atrophy of fat, congested liver, and excess abdominal fat suggest potential nutritional imbalances or metabolic disorders affecting the overall health of the animals. The excess fat and pale gross lesions found in the abdominal visceral and livers of the animal with pregnancy toxaemia agree with those reported by Kabakci *et al*. (2003), who in animals with good body condition, when necropsied, observed the liver with pale yellow coloration, enlarged liver and easily friable, suggesting extensive fatty infiltration. In conclusion, this study reveals significant hematological and biochemical changes indicative of oxidative stress, alongside pathological findings in various organs such as the liver, lungs, and kidneys. These results underscore the complexity of pregnancy toxaemia and emphasize the need for further research to develop better management strategies.

Acknowledgment

Conflict of interest

The authors declare that there are no conflict of interest.

Authors' Contribution

AM, AAA, AJA, and OMO were involved in carrying out the investigation, methodology, and writing of the manuscript. LFM, RIA, BRB, and IGM were involved in the editing of the drafted manuscript. OIA and BA analyzed the data. All authors reviewed, read, and approved the manuscript.

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