SUITABILITY OF SALINE-ADENINE-GLUCOSE-MANNITOL ADDITIVE IN CITRATE-PHOSPHATE-DEXTROSE-ADENINE FOR STORING PACKED RED BLOOD CELLS OF GOATS

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Suitability of Saline-Adenine-Glucose-Mannitol Additive in Citrate-Phosphate-Dextrose-Adenine for Storing Packed Red Blood Cells of Goats"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Suitability of Saline-Adenine-Glucose-Mannitol Additive in Citrate-Phosphate-Dextrose-Adenine for Storing Packed Red Blood Cells of Goats"** is a record of research work done independently by Anaz S.R. (18-MVM-31), under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the Advisory Committee of **Anaz S. R. (18-MVM-31)**, a candidate for the degree of Master of Veterinary Science in Veterinary Clinical Medicine, Ethics and Jurisprudence agree that this thesis entitled "**Suitability of Saline-Adenine-Glucose-Mannitol Additive in Citrate-Phosphate-Dextrose-Adenine for Storing Packed Red Blood Cells of Goats**" may be submitted by **Anaz S.R. (18-MVM-31)** in partial fulfillment of the requirement for the degree.

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EXTERNAL EXAMINER

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ANAZ S. R

DEDICATED TO MY BELOVED GUIDE

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Introduction

1. INTRODUCTION

Goat, the poor man's cow, due to its adaptability in harsh environments, good productivity and being non-competitive to humans for their resources, became the first farm animal to be domesticated. As per 20th livestock census, goat population in India was around 148.88 million, an increase of 10.14 per cent from the previous census. It is the second largest livestock animal group in India accounting for 27.8 per cent of total livestock population (20th livestock census, 2019).

Anaemia is a debilitating and often life threatening condition in goats. Anemia results in reduction in red blood cells with inadequate oxygen supply to the vital organs. Anaemia in goats in our setting is commonly associated with endo and ectoparasites and haemoparasites. With considerable reduction in haematological indices, specific treatments with regard to the etiology alone may not suffice in such cases. Blood transfusion becomes an important life-saving procedure under such a circumstance.

With rise in emotional and commercial value of farm animals, blood transfusions are being increasingly recognized and used. This emergency procedure should be timely executed to get better results. As the need overweigh the availability, veterinary transfusion should progress from fresh whole blood transfusion to targeted component therapy with safe and reliable products. Timely unavailability of healthy donors, increased awareness related to proper screening, incompatibility and storage lesions has led to the development of different blood products, specific storage medias and veterinary blood banks. One unit of whole blood collected from individual donor can be separated into different components and stored prior to transfusion. Use of these stored products, called component therapy, benefits multiple recipients and prevents the wastage of valuable biological material.

Among the components, most commonly used one is packed red blood cells. It is preferred mainly in euvolemic anemia cases. Prolonged storage of these cells cause alteration in its biochemical and biomechanical properties called storage

lesions. Introduction of different anticoagulants with additives restrict the changes and prolong the storage viability to a maximum extent with minimum storage lesion and provide a post transfusion viability of 75% as recommended by United States Food and Drug Administration.

Storage studies of packed red blood cells using commercially available citrate phosphate dextrose adenine, saline adenine, glucose mannitol (CPDA-SAGM) blood bags has been established in case of humans and dogs. Such storage studies in farm animals especially goats are scarce. Therefore, the present study was carried out with following objective.

- Study the suitability of saline-adenine-glucose-mannitol (SAGM) additive in citrate- phosphate-dextrose-adenine (CPDA) for storing packed red blood cells (pRBCs) of goats.

Review of Literature

2. REVIEW OF LITERATURE

2.1 ANAEMIA IN GOATS

Burke *et al.* (2007) reported that FAMACHA chart, which compare the colour of mucous membrane with score range from 1 to 5 where 1 and 2 considered normal, is validated in goats.

Goklaney (2012) opined that most common clinical manifestation in goats is anaemia. It was also suggested that anaemia was the major problem affecting productivity.

Berthelsson (2017) stated that for the evaluation of anaemia, packed cell volume is used, which determines the package of erythrocytes in the total blood volume.

Argolo *et al*. (2018) opined that gastro-intestinal nematodes causing severe health problems in goats could be managed better by the integrated parasitic control. However, in severe anemia cases (less than 11% globular volume), blood transfusion ensures the survival.

Anaemia is a common condition in ruminants due to the affection of other organs rather than defects in erythropoiesis (Katsogiannou *et al.,* 2018).

2.1.1 Etiology

Katoch and Mandial (2003) reported that in field condition, one of the most common clinical manifestation in animal is anaemia. It is mainly due to helminthosis, mineral deficiencies, haemoprotozoans and ectoparasites.

Braun *et al.* (2010) reported that common causes of haemolytic anaemia in ruminants included haemoparasites, leptospirosis, and bacterial toxins.

Ermillo and Smith (2011) opined that parasitism was the most common cause of anaemia.

Joseph (2011) opined that anaemia in goats was linked with external and internal parasitism and had major clinical significance in goats. Overall prevalence of parasitic anaemia infection was around 20%, among this 85 % are of gastro intestinal parasites.

Feeding of bovine colostrum to lambs may cause immune mediated haemolytic anaemia (Katsogiannou *et al.,* 2018).

2.1.2 Clinical Findings

According to Katoch and Mandial (2003), anaemia manifested with pale mucous membrane, low haematological indices and decrease in plasma levels of Mg, P, K, Fe and Cu.

Haemoparasite conditions in goats were mostly subclinical. But sometimes goats showed severe anaemia with clinical picture similar to cattle, which is more frequent in goats suffering from any underlying cause (Hornok *et al.,* 2007).

Polizopoulou (2010) opined that inadequate oxygen transport in small ruminants was indicated by tachycardia, tachypnea and exercise intolerance.

Chronicity of anaemia reduced the manifestation of clinical signs, which was positively related with amount of blood loss (Gianniti *et al*., 2014).

2.1.3 Management

In acute haemorrhage with less than 50 per cent blood loss, tissue re-oxygenation could be achieved by administration of packed red blood cells (pRBCS) along with volume expansion using crystalloids (Lanevschi and Wardrop, 2001).

As the donor erythrocytes had a life span of only eight days in goats, transfusion effects were considered temporary. However, normal bone marrow regeneration of erythrocytes took place within five days (Smith, 2009).

In critical conditions due to massive haemorrhage or acute anaemia with the evidence of hypovolemia, to regain the tissue perfusion and organ oxygenation, red blood cell transfusion was extremely important (Lion *et al.*, 2010).

Conditions like acute traumatic haemorrhage, surgical blood loss, parasitism, haemorrhage from drugs and toxins, immune mediated anaemia and neonatal isoerythrolysis lead to life threatening anaemia, which could be managed by whole blood transfusion (Tocci, 2010).

Ermillo and Smith (2011) concluded that in critical conditions, anaemia could be managed with transfusion, decision for which was based on the prognosis of the underlying condition, patient stability, availability of suitable donor and economics of herd.

According to Kisielewicz *et al*. (2014), requirement of transfusion in euvolemic anaemia was to counteract hyperlactemia due to inadequate perfusion.

2.2 STORAGE OF BLOOD

Raat and Ince (2007) observed that extended storage of blood increased the oxygen-haemoglobin affinity with resultant reduction of oxygen unloading capacity after transfusion.

Blood component separation was done using refrigerated centrifuge and the rate of the sedimentation was influenced by some external factors like centrifugation time, rotor size, and revolutions per minute (RPM) (Roback *et al.*, 2011).

According to Kakaiya *et al*. (2011), weight of the blood in blood bag was obtained by subtracting the weight of blood bag with anticoagulant from the weight of blood bag after collection.

Species difference in morphology and metabolism of RBCs resulted in variations of storage time among species (Ekiz *et al*., 2012).

Food and Drug Administration stated the validity of stored blood is determined by the survival of 75 per cent of transfused erythrocytes after 24 hours along with less than one per cent haemolysis recorded at the end of specified storage period (Obrador *et al*., 2015).

Pre-cooling of refrigerated centrifuge should be done before the initiation of the component separation procedure (Yagi and Holowaychuk, 2016).

In case of goats, transfusion using whole blood or stored blood did not affect lipid peroxidation values, renal and liver functions (Fonesca *et al*., 2018)

2.2.1 Anticoagulants

Lanevschi and Wardrop (2001) opined that acid citrate dextrose (ACD), citrate phosphate dextrose (CPD) and citrate phosphate dextrose adenine (CPDA-1) were the commonly used preservatives. Constituents in these preservatives extended the storage time by favoring the viability of RBCs up to 3 to 5 weeks depending on the preservatives used.

Morris *et al*. (2002) reported that the concentration of glucose and lipids of the stored blood can be influenced by the anticoagulants used.

The most commonly used anticoagulant in the initial days of transfusion was ACD. Addition of phosphate and increased pH derived the CPD which stabilize the 2,3-DPG with augmented the shelf life. Addition of adenine to CPD replenished ATP concentrations by restoring adenine nucleotide pool which further enhanced the storage period (Mudge *et al*., 2004).

Obrador *et al*. (2015) remarked that blood storage was done *ex vivo* for the first time in 1915 after the discovery of anticoagulant sodium citrate.

In citrate based anticoagulants with additive solutions, shelf life of packed RBCs can vary between species from 33 to 42 days at a temperature range of 4 ± 2^0C (Ferreira *et al*., 2018).

2.2.1.1 Citrate Phosphate Dextrose Adenine -1

Mudge *et al.* (2004) reflected that assessment of biochemical and haematological parameter of RBCs stored in CPDA-1 indicated the improvement in viability and it was a better medium for storage.

In veterinary medicine, for collection and storage, most common anticoagulant combination is citrate, phosphate, dextrose, adenine (CPDA-1). It inactivates the spontaneous coagulation and provided substrate for metabolism to extend the RBC viability (Sousa *et al*., 2013).

Tavares (2013) found that blood bag used in veterinary medicine and human blood bank is same. Among them CPDA-1 blood bag was preferred for goats.

Tavares *et al*. (2019) noticed that goat blood remained viable in CPDA-1 blood bag upto 42 days under refrigeration of around 3^0C.

2.2.1.2 Additive Solutions

Saline – adenine – glucose (SAG) was developed by Hogman to preserve human RBCs and extend the storage life to 5 weeks. Mannitol addition to SAG produced saline adenine glucose mannitol (SAGM). Storage of RBCs upto six weeks was made possible with SAGM (Hess and Greenwalt, 2002).

Optisol, having dextrose, adenine, mannitol and sodium chloride provided nutrition for RBCs. Storage of canine and human RBCs upto 42 days was possible (Gibson and Abrams, 2012).

Sparrow (2012) stated that mannitol in SAGM improved membrane stability of RBCs and reduced haemolyis.

Obrador *et al*. (2015) opined that ATP depletion progressed from day 10 to 44 during storage of erythrocytes which can be delayed by the addition of SAGM.

Mustafa *et al.* (2016) observed considerable increased fragility of RBCs with increased period of storage in SAGM in humans

Brugue *et al*. (2018) reported that addition of SAGM to RBCs delayed the loss of ATP and improved packed RBCs shelf life up to 44 days.

2.2.2 Storage of Whole Blood

Mudge *et al.* (2004) reported that storage of blood was done in temperature controlled blood bank refrigerator which prevented wide fluctuations and maintained in range of 1 to 6^0 C.

Hughes *et al.* (2007) stated that fresh whole blood can be transfused within 24 hrs of collection at room temperature or refrigerated within 8 hrs. After it is stored, it becomes stored whole blood (SWB)

Whole blood from bovine, canine and equine can be collected and stored in CPDA-1 for upto 35 days in a temperature range of 1 to 6^o C (Sousa *et al*., 2013).

Stored whole blood more than 14 days may affect the haemostatic function. So along with the SWB, components preferably platelets should be administered to promote the haemostatic function (Cap *et al*., 2018).

Tavares *et al*. (2019) observed that goat whole blood showed more resistance to changes with storage conditions compared to other species.

2.2.3 Storage of Packed Red Blood Cells

One unit of whole blood after centrifugation yielded 150-200ml of pRBCs after removing plasma in dogs (Lanevschi and Wardrop, 2001).

Concentration of RBCs in storage was inversely related with the recovery of transfused erythrocytes (Hess and Greenwalt, 2002).

World Health Organization guidelines (2005) recommended that storage time of pRBCs was 30-40 days under 4^oC in humans.

D'Alessandro *et al*. (2010) noticed that RBC processing and its transfusion has progressed to a great extent after the introduction of plastic bags, additive solutions and leucocyte reduction filters.

Herring *et al.* (2013) observed that compared with fresh transfusion, RBCs older than two weeks caused deleterious effects on the recipients in humans.

According to Radwanski *et al*. (2014), for the redistribution of packed red blood cells in stored media, it should be gently mixed at least once in a week.

Warming of stored RBC prior to transfusion was not encouraged as it may cause damage to proteins, clotting factors and resulted in bacterial contamination (Poder *et al*., 2015).

Storage recommendation for feline RBC with citrate containing bags along with additive solutions was in the temperature range of 2-6^{o}C for 30-42 days (Jagodich and Holowaychuk, 2016).

According to Stefanetti *et al*. (2016), packed RBCs in dogs should be processed in refrigerated centrifuge at 5000g for 20 min at 4^{o}C within 4 hours of collection.

2.3 STORAGE LESIONS

Hess and Greenwalt (2002) reported that storage lesions can vary among individuals of the same species under same storage conditions, due to the difference in RBCs metabolism among them.

Blasi *et al*. (2012) observed changes in biochemical and biological properties of RBCs on storage of blood termed as storage lesions.

Changes in the biochemical, biomechanical and immunologic events in the erythrocytes and its storage media which could affect the effectiveness of transfusion, are collectively called as storage lesions (Almizraq *et al*., 2013).

Healthy blood donor and quality of blood components are the main factors which led a successful transfusion. Quality of components could be achieved by implementing standardized protocols which reduce the storage lesions to the maximum extent (Ferreira *et al*., 2014).

Cellular integrity of stored RBCs depend on the quality parameters like, VPRC, haemolysis and bacterial contamination (Brugue *et al*., 2018).

Marchi *et al*. (2019) opined that human parameters were used widely in veterinary medicine as there were no proper guidelines for the quality assessment and storage of blood bags.

Shelf life of stored blood is determined by the alterations in the haematological and biochemical parameters (Spada *et al*., 2019).

2.3.1 Biochemical Studies

According to Beutler (2000), elevation and reduction in the following biochemical parameters like potassium, lactate, pH, ATP and 2,3-DPG indicated leakage and haemolysis of RBC.

Collaborative biochemical and biomechanical changes will detrimentally affect the performance of transfused erythrocytes after storage (Berezina *et al*., 2002).

Bennett-Guerrero *et al*. (2007) opined that changes noticed during the storage of packed RBCs included elevation of potassium, lactate, decrease in deformability, pH drop etc. Among these, pH, lactate and potassium variation were noticed from the beginning of storage.

Barshtein *et al*. (2011) reported that biochemical changes alter the RBC membrane structure, resulting in increased haemolysis during storage.

2.3.1.1 pH

Hess and Greenwalt (2002) stated that pH ≥ 6.2 was required for normal metabolic activities of human erythrocytes. pH ≥ 6.65 was the value limit for stored blood. pH value above 7.2 of stored blood induces the 2,3- DPG synthesis affecting ATP.

Mudge *et al*. (2004) reported that most of the erythrocyte metabolism is anaerobic with the production of lactic acid which finally resulted in acidic pH of stored blood.

Hess *et al.* (2009) opined that glucose degradation for the production of energy during storage with lactate and hydrogen ion as metabolite led to reduction in pH of the blood.

Decrease in pH resulted in glycolysis inhibition through which ATP is produced by the erythrocytes (D'Alessandro *et al*., 2010).

Heinz *et al.* (2016) observed that, the decline in pH of stored blood from day one onwards was due to the acidic pH of anticoagulant (CPDA) and the additive solutions (SAGM) which may reduce the pH even below the physiologic levels.

2.3.1.2 Reduced Glutathione Estimation

The balance between the formation of GSH and glutathione disulphide (oxidised glutathione) act as a buffer to prevent oxidative stress. Alterations in this balance during storage will overcome the antioxidant defence of the cells (Lion *et al.*, 2010).

Nazifi *et al*. (2009) observed that reduced glutathione, which is a tripeptide thiol, is an important antioxidant in living cells with co-factor activity for several antioxidant enzymes.

Kozlova *et al*. (2015) opined that principle component of erythrocyte antioxidant system was the intracellular reduced glutathione (GSH), reductions of this during storage aids the oxidative process in RBC.

2.3.1.3 Malondialdehyde Estimation

Simsek *et al.* (2006) recorded that unsaturated fatty acids in the blood cells were unstable and formed various compounds. One among them was MDA, which was used as an indicator for lipid peroxidation and indirectly oxidative stress in blood cells.

Nazifi *et al*. (2009) observed that MDA is one of the end product of lipid peroxidation and it was used widely to indicate cellular lesions. Reference values of MDA indicated that oxidative stress was less in goats as compared to carnivores and may be due to the difference in diets.

Pandey and Rizvi (2011) reported that measuring MDA in human RBCs was a useful marker in assessing the extent of lipid peroxidation.

Imbalance between antioxidants and pro-oxidants resulted in oxidative damage. This is due to free radicals like hydroxyl ions in case of lipid peroxidation which resulted in the loss of membrane integrity of RBCs (Ayala *et al*., 2014).

Mustafa *et al.* (2016) opined that Thiobarbituric Acid Reactive Substance (TBARS) assay was used for determining the lipid peroxidation as MDA, a major by product was a thiobarbituric acid reactive substance.

According to Fonesca *et al*. (2018), MDA is the parameter used to determine the extent of lipid peroxidation. Oxidative lesion of erythrocytes with haemolysis as the consequence was proportional to the formation of MDA.

2.3.1.4 Potassium Levels

The variation in cellular sodium and potassium levels would be stabilised after transfusion within 24 hours for sodium and few days for potassium (Lion *et al.,* 2010).

Blasi *et al*. (2012) reported that increased potassium values in the supernatant during storage was due to the ATP depletion and low temperature affecting the pump kinetics. Decrease in pH during storage inhibited glycolysis through which ATP was synthesized.

Sousa *et al*. (2013) observed that species-wise difference was noticed regarding intracellular potassium levels of erythrocytes concentration for ovine, bovine and equine compared with canines.

Lacerda *et al.* (2014) observed an increase in potassium levels in canine blood on storage.

Post transfusion hyperkalaemia resulted in cardiac arrest in humans. A low pH resulted in leakage of potassium from intracellular to extracellular space which resulted in hyperkalaemia during storage (Yang *et al*., 2019).

Estimation of the potassium in the supernatant of stored medium of erythrocytes served as a useful marker to access the quality of stored erythrocytes. Storage at lower temperature affected the ATPase enzyme activity which led to reduced activity of sodium potassium pump resulting in intracellular elevation and reduction in sodium and potassium respectively (Fonesca *et al*., 2018)

2.3.1.5 Glucose Levels

According to Tavares (2013), low consumption of glucose by caprine erythrocytes prevented pronounced reduction in glucose during the storage period.

Crestani *et al*. (2018) observed that normally glucose concentration of the stored blood reduced due to glucose consumption by RBC for the production of ATP, but in CPDA-1, dextrose in it maintained the glucose in elevated levels even at the end of storage.

Post transfusion studies revealed that nutrients used in the blood bag for storage is used as energy source especially glucose. Elevation of glucose after transfusion was promptly balanced by liver (Fonesca *et al*., 2018).

2.3.2 Haematological Studies

Haematogical values of stored blood depended on many factors including time gap between collection and analysis, storage solutions and storage temperature (Antwi-Baffour *et al*., 2013).

2.3.2.1 Volume of Packed Red Cells

Ekiz *et al.* (2012) opined that the average VPRC of packed RBCs stored in additive solution was 55-65 per cent and it may show difference based on the donor VPRC, residual plasma in packed RBC stored bag and the proportion of additive solution added to the bag.

It has been reported in human studies that due to degenerative changes during storage, entry of water into the cell leads to increase in RBC volume with time leading to elevated packed cell volume (Antwi-Baffour *et al*., 2013).

2.3.2.2 Haemolysis of RBC

Hess and Greenwalt (2002) reported that haemolysis indicated the failure of storage system of RBCs which can be reduced by stability enhancers like mannitol, citrate and hypotonic solutions.

Hogman *et al.* (2006) stated that spherocyte RBCs had higher osmotic fragility compared to normal discoid RBCs. As the sphere shape had the minimum surface to volume ratio, it resulted in rapid haemolysis with feeble osmotic stress.

High concentration of haemoglobin vitiated the renal, vascular, myocardial and central nervous system. Therefore, haemolysis should be minimized to enhance the quality of blood product (Buehler and D'Agnillo, 2010).

Maximum permitted haemolysis percentage of human blood units at the end of storage is one per cent due to the reported harmful effects of free haemoglobin (Kakaiya *et al*., 2011).

Osmotic fragility test was carried out to measure the degree of resistance of erythrocytes to different concentration of saline in an ascending order (Blasi *et al*., 2012).

Ferreira *et al*. (2014) stated that standardization of velocity and time for centrifugation is important as variation lead to haemolysis.

Corsi *et al*. (2014) opined that incremental effects of pro-inflammatory substances were principally released from leucocytes and platelets and the depleted ATP deleteriously affected the integrity of RBC membrane during storage.

Heinz *et al*. (2016) stated that in the storage of pRBCs, most important parameter used for assessing the viability was the percentage of haemolysis.

Hofbauer *et al*. (2016) opined that RBCs membrane integrity can be assessed by its osmotic resistance, which was influenced by species, breed, age and storage time.

Haemoglobin values did not vary with morphology whereas VPRC variations were recorded. Therefore oxygen carrying capacity of packed RBC was better assessed with haemoglobin estimation (Ferreira *et al*., 2018).

2.3.3 Morphological Studies

Extent of rheological disturbances directly depended on the number of RBCs with altered geometry. Progressive change in morphology of RBCs were noticed after two weeks of storage with significant reduction in deformability index and elevated values of haemolysis and acidosis in humans (Berezina *et al*., 2002).

According to Frank *et al*. (2013), structural changes during storage of erythrocytes were irreversible unlike biochemical changes.

Relevy *et al.* (2008) opined that RBC should pass through capillaries with half of its diameter for delivering oxygen. Therefore, deformability of biconcave disc shape is a critical characteristic feature required to accomplish adequate perfusion.

D'Alessandro *et al*. (2010) stated that morphological changes in packed RBCs were generally classified into reversible and irreversible. The reversibility was inversely proportional to the storage time of pRBCs.

Deformability was the element which aided the survival of RBCs while entering the splenic circulation. Hence, the reduction in deformability due to storage is considered as a major factor for the survival of transfused erythrocytes (Obrador *et al*., 2015)

2.3.3.1 Light Microscopy

Galey *et al*. (1978) opined that faulty preparations may cause the formation of echinocytes and spherocytes, but acanthocytes was only noticed in some diseased conditions.

Increase in viscosity of blood during storage was due to progressive shape changes of cells from discoid to less deformable echinocytes (Sollberger *et al*., 2002).

According to Barger (2010), in small ruminants especially goats, poikilocytosis was a common finding and it is considered to be normal.

Antwi-Baffour *et al*. (2013) reported that during storage progressive changes of cells were noticed from its normal biconcave discoid shape to echinocytes (spiculated) or spherocytes (spherical) shapes.

2.3.3.2 Scanning Electron Microscopy

Polliack (1981) reported that morphological alteration in SEM studies due to ageing, ionic changes especially with calcium, contact with glasses, biochemical variations were collectively termed as spheroechinocytic transformation.

Berezina *et al*. (2002) opined that RBC shape like echinocyte and stomatocyte can be reversed to normal discocyte under certain conditions. But, spherocyte, ovalocyte, spheroechinocyte and degenerated shapes were irreversible.

Scanning electron microscopic studies of pRBCs in SAGM on 0^{th} day showed normal biconcave disc shaped cells. Due to prolonged storage, majority of cells changed to spherocytes and echinocytes and it became irreversible after 28 days of storage (Mustafa *et al*., 2016).

2.3.4 Culture Studies

After following all the aseptic measures to prevent the bacterial contamination in blood components, the prevalence of contamination reported in human was 0.04 to 2 per cent (Engelfriet *et al*., 2000).

Bacterial contamination result in variation of pH and lactate which affected the storage life of packed RBCs (Mudge *et al.*, 2004).

Kessler *et al.* (2010) opined that if discolouration was noticed in the blood bag under storage, it needed to be referred for culture and quality control studies. As most of the recipients were debilitated or immune-compromised, microbial contamination may cause severe complication during transfusion.

Most commonly recorded contamination of blood bags was caused by gram negative aerobic endotoxin producing pathogens (Herring *et al*., 2013).

Brecher and Hay (2005) reported that the contamination of the blood bag start from the skin flora of the donor during collection. Making the skin completely aseptic was difficult.

Storage temperature of blood around 4^0C is suitable for the proliferation of psychrotolerant bacterial species like *Pseudomonas* and *Serratia.* Growth was clinically irrelevant in the beginning of storage period to significant at the end which caused sepsis in transfused patients (Stefanetti *et al*., 2016).

Haematological and microbiological studies are of immense use in assessing the quality of stored blood used for transfusion. Rectification of the errors would improve the shelf life of the blood (Marchi *et al*., 2019).

Materials and Methods

3. MATERIALS AND METHODS

The present study was carried out in the Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Sciences, Mannuthy during the period of October 2019 - September 2020.

3.1 DESIGN OF THE STUDY

Whole blood was collected from the healthy adult goats to study the shelf life of packed RBCs in SAGM.

3.1.1 Selection of Animals

Ten apparently healthy goats weighing 35 to 60 kg within an age range of 4 to 8 years were selected from University Goat and Sheep Farm, Mannuthy and other organized farms in Thrissur district. All the goats were dewormed and the health status of each animal was evaluated by physical examination and laboratory analysis including complete blood count, examination of blood smear and faecal sample.

3.1.2 Blood Collection and Storage Studies

Whole blood units were collected in commercial triple blood bags consisting of a primary bag for the collection of 350ml of blood with 49mL of CPDA-1 anticoagulant (tri-sodium citrate, sodium phosphate, dextrose and adenine), an empty bag for plasma storage, and 1 bag with 100 mL of additive solution containing saline, adenine, glucose, mannitol (SAGM) (Plate 1). The animals were restrained physically and placed in upright position. The puncture area over a jugular vein was clipped of hair and aseptically prepared using chlorhexidine and alcohol. Jugular venepuncture using 16 G needle in the dorso cranial direction, allowed blood to flow by gravity into the collection bag, which was gently inverted to mix the blood with the anticoagulant at every 50 mL of collected volume (Plate 2). Once the total volume was obtained, the tubing was manually stripped to allow for anticoagulation of the tubing and a knot was made at 3 locations in the tubing, 1 cm apart. The collected whole blood units were

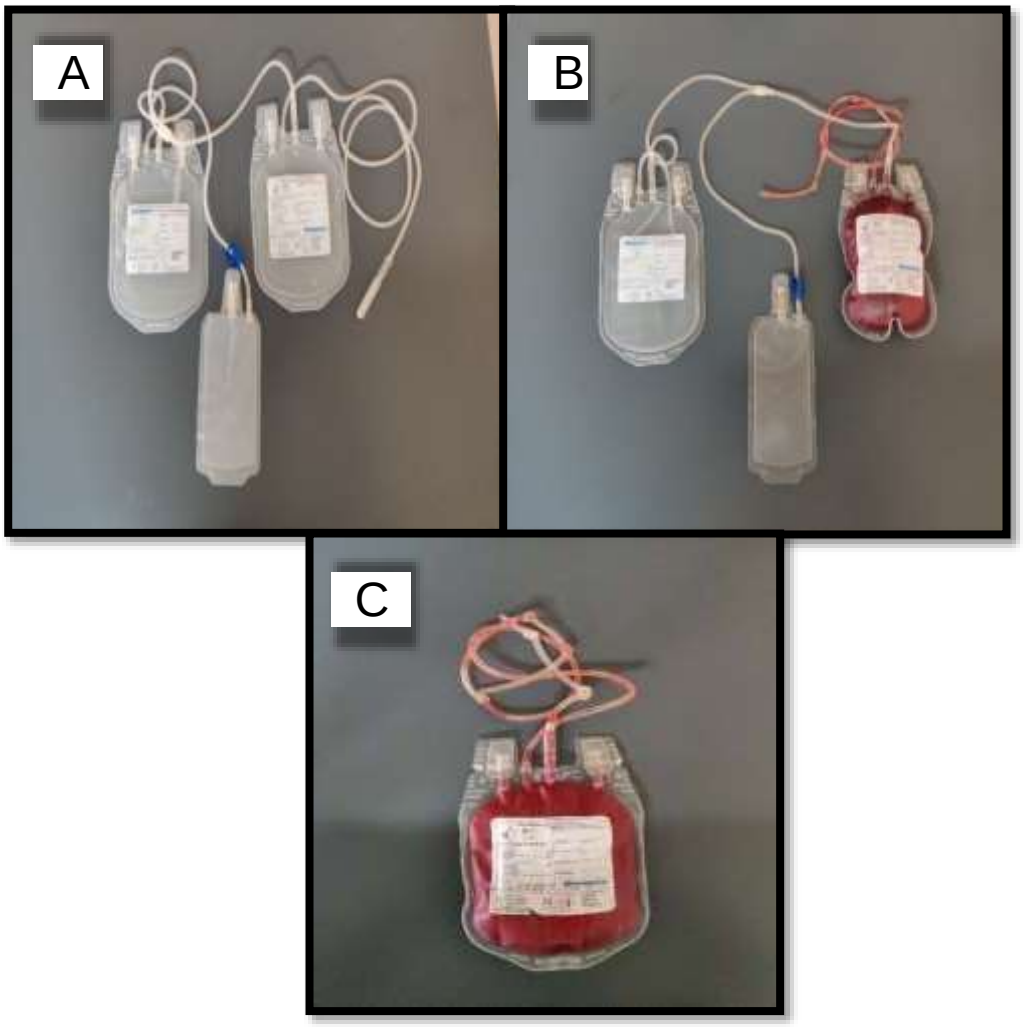


Plate 1. Donato CPDA Double Blood Bag with SAGM (350 ml) (A) Empty bag (B)With whole blood (C) With pRBC

Plate 2. Blood collected from healthy donor

immediately stored using an insulated plastic chiller icebox keeping a constant temperature and processed within six hours.

After gentle mixing, the triple bag system was placed in the centrifuge cups, avoiding plastic folds and ensuring a symmetrical distribution of weight with a tolerated difference of 0.2 g between opposite cups. The units were centrifuged at 5,000 × *g* for 7 minutes, at 4°C using FTBC- 6100R Blood Bank Refrigerated Centrifuge (Plate 3). After centrifugation, it was carefully removed and hung on the hooks of plasma expressor (Plate 4). The valve towards the empty satellite bag was opened and the front panel of the plasma extractor was slowly pressed against the bag to remove the plasma from the primary collection bag. The fluid path was closed by tying the tubes. The additive solution SAGM in the second satellite bag was transferred to the primary bag that contained the packed RBCs by opening the second valve and thus making the fluid pathway patent (Plate 5). All these procedures were done inside the horizontal laminar air flow bench to reduce the contamination. The volumes of blood units were calculated on the basis of their weight, assuming that 1 mL of whole blood (WB) weighs 1.053 g and that 1 mL of packed RBCs weighs 1.085 g (Ferreira *et al*., 2018). Finally the packed RBCs suspended in SAGM additive were stored at 4 ± 2^0c in a dedicated refrigerator for up to 42 days for storage studies (Plate 6). During the storage period, the bags were daily inverted manually, to mix the blood with the preservative solution.

3.2 PARAMETERS STUDIED

The faecal sample and blood smear examinations were carried out before the start of the study. Red blood cell samples were removed aseptically for the analysis using gamma irradiated bottles every two weeks from day 0, 14, 28, 42 of storage for evaluating haematological parameters, biochemical parameters, RBC morphology and for culture studies.

Plate 3. FTBC - 6100R Blood Bank Refrigerated Centrifuge

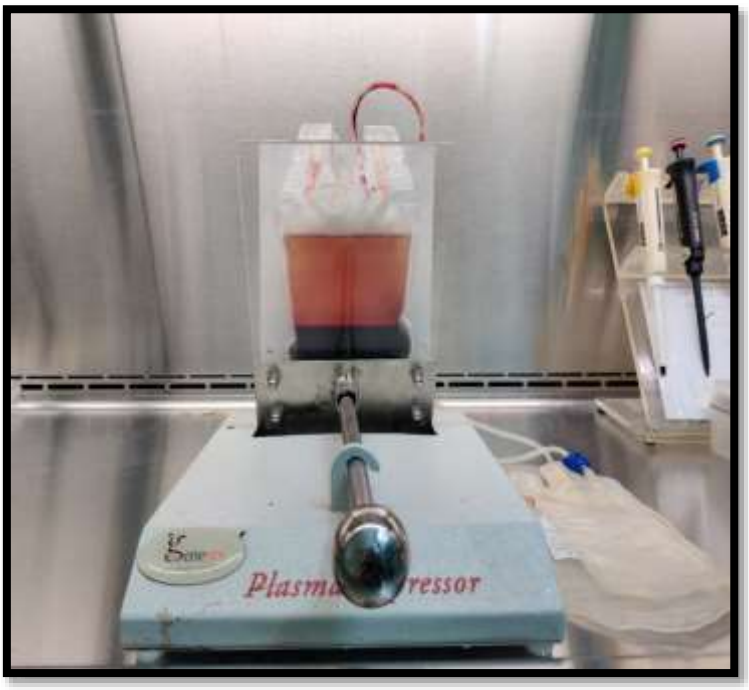


Plate 4. Genesys Manual Plasma Expressor

Plate 5. SAGM addition inside Laminar Air Flow Cabinet

Plate 6.Genesys Blood Bag Refrigerator

3.3 PROCEDURES

3.3.1 Clinical Examination

Detailed history and results of clinical examination including body condition, mental attitude, status of superficial lymph nodes, rectal temperature, pulse, respiration and colour of conjunctiva or oral mucous membranes were recorded for the primary evaluation of the animals. Normally goats have an alert, attentive, and inquisitive mental attitude.

3.3.2 Laboratory Examination

3.3.2.1. Faecal Sample

Faecal samples were collected directly from rectum using moist cotton swabs and transported to laboratory.

3.3.2.1.1 Screening for Gastrointestinal Parasites

The faecal samples were homogenised with distilled water and examined immediately after collection.

3.3.2.1.1.1 Direct microscopic examination

Thin smears of faecal swabs were prepared on glass slides. The slides were then examined under low power objective of light microscope.

3.3.2.1.1.2 Centrifugation technique

The faecal samples collected from goats were diluted in distilled water and centrifuged at 3000 rpm for two minutes. The supernatant was discarded and a drop of the sediment was examined under the low power objective of light microscope.

3.3.2.2 Blood Smear Examination

Thin peripheral blood smears were prepared from selected animals, air-dried and fixed in methanol.

3.3.2.2.1 Staining of Blood Smears

3.3.2.2.1.1. Fields stain

Fields stain solution A and B (Nice chemicals private limited, Kochi) were used to stain blood smear.

3.3.2.2.1.2. Fields staining technique

Two coplin jars were filled with Field's stain A and B solutions separately. Peripheral blood smears were fixed in methanol for one minute and air dried. This was followed by coupling of stain B and allowing it to act for 30 seconds. After washing the slides in running tap water, stain A was added and allowed to act for 30 seconds. The slides were further washed, air dried and observed under 100 X objective of a light microscope (Olympus, CH20i and Leica, DM 500).

3.3.3 Evaluation of Haematological Parameters

A complete blood count, including the parameters such as haemoglobin (g/dL), volume of packed red cells (%), total leukocyte count (10^3/µL), red blood cells (10^6µ/L), differential leukocyte count (%), mean cell corpuscular volume (µm^3), mean corpuscular haemoglobin (pg), mean corpuscular haemoglobin concentration (g/dL) and platelets (10^3/µL) were estimated on the day of collection. ORPHEE Mythic 18 Vet CBC Machine was used for the analysis (Plate 7).

3.3.3.1. Haemoglobin

Estimation of haemoglobin was done using the principle of haemoglobin to cyanmethemoglobin using the AGAPPE kit.

3.3.3.2 Haemolysis of RBC

Haemolysis of RBCs was estimated using osmotic fragility test. The osmotic fragility test is used to measure erythrocyte resistance to haemolysis while being exposed to varying levels of dilutions of a saline solution (Bain *et al*., 2016).

Plate 7. ORPHEE Mythic 18 Vet Haematology Analyser

- Reagent Preparation

i. Preparation of stock solution of buffered sodium chloride

To prepare a stock solution of buffered sodium chloride, osmotically equivalent to 100g/L (1.71 mol/L) NaCl.

1. Weighed the following chemicals and added water to make a final volume of 1000ml
    a) Sodium chloride – 90g
    b) Di-sodiumhydrogen phosphate–13.65g
    c) Sodium dihydrogen phosphate dihydrate – 2.34g
2. The bottle was stored at 4 °C.
3. Salt crystals formed during storage must be thoroughly re-dissolved before use.

ii. Preparation of working solution of buffered sodium chloride

1. Diluted 100 ml of the stock solution with 900 ml of distilled water to made a diluted stock solution with a final concentration of 10g/ litre.
2. Working solution was prepared as follows:-

No.	Buffered NaCl reagent solution	Diluted stock solution	Distilled water
1	0.0% NaCl (Water)	Nil	200 ml
2	0.1% NaCl	20 ml	180 ml
3	0.2% NaCl	40 ml	160 ml
4	0.3% NaCl	60 ml	140 ml
5	0.4% NaCl	80 ml	120 ml
6	0.5% NaCl	100 ml	100 ml
7	0.6% NaCl	120 ml	80 ml
8	0.7% NaCl	140 ml	60 ml

9	0.8% NaCl	160 ml	40 ml
10	0.9% NaCl	180 ml	201

- Procedure
  a) Delivered 5.0 ml of each of the ten saline solutions into 5 ml test tubes
  b) Added 20 µl of well mixed blood and mix immediately by inverting the tubes several times, avoiding foam
  c) Incubated for 30 minutes at room temperature (26-28 °C). Mix again and centrifuge the tube at 800g for 10 minutes
  d) Removed the supernatant and estimated the amount of lysis in each test tube using a spectrophotometer at a wavelength of 540nm
  e) Percentage haemolysis was calculated by assuming 100 per cent haemolysis in 0.0% Nacl solution and 50 percent haemolysis was calculated

3.3.3.3 Volume of Packed Red Cells

Volume of packed red cell is the percentage of volume of blood occupied by the red blood cells, which was determined by using Wintrobes method (Bain *et al*., 2016). Under centrifugal force components of blood will settle and get packed according to specific gravity in a suspension of plasma fluid

- Procedure
  a. Packed RBCs in blood bag was drawn into Pasteur pipette and introduced in the wintrobes from the bottom to 0 or 10 mark above
  b. Place the wintrobes tube in the centrifuge machine and other wintrobes tube filled with water in the opposite side so as to balanced it
  c. Centrifuged the tube at the speed of 3000rpm for 30 minutes
  d. After 30 minutes stop the centrifuge, take out the tube and noted the readings

3.3.4 Biochemical studies

3.3.4.1 pH

Blood pH was estimated with portable Oakton waterproof pH meter (as per user manual). Potentiometric pH meters measure the voltage between two electrodes and display the result converted into the corresponding pH value. They comprise a simple electronic amplifier and a pair of electrodes

- Procedure (as per the user manual)
  a. Pressed the ON/OFF button to switched the Testr on
  b. Dipped the electrode about 2 to 3 cm in to the packed RBCs suspension. Stirred and let the reading stabilize
  c. Noted the pH value or pressed HOLD/ENT button to freeze the reading. To release the reading, pressed HOLD/ENT again
  d. Pressed ON/OF to turn off Testr

3.3.4.2 Estimation of Erythrocyte Reduced Glutathione (GSH)

Level of GSH in erythrocyte suspension was determined as per Bain *et al*., 2016). This method is based on the development of a yellow colour when 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) is added to sulphydryl compounds. The colour is fairly stable for about 10 minutes and the reaction is little affected by variation in temperature. Reduced glutathione is slowly oxidised in solution, so only fresh lysates should be used for the assay.

- Reagent preparation

a. Lysing solution

Dissolve 1g EDTA in 1.0 litre of distilled water.

b. Precipitating solution

Dissolve metaphosphoric acid sticks, 1.67 g; disodium EDTA dihydrate,0.221g and sodium chloride, 30g in distilled water and make up to a final volume of 100 ml.

For rapid dissolution, add the reagents to boiling water and the solution is stable for at least three weeks at 4°C. If any EDTA remain undissolved, the clear supernatant should be used.

c. Disodium hydrogen phosphate solution (300 mmol/l)

Mix 2.129 g of disodium hydrogen phosphate anhydrous in 50 ml of distilled water. This solution is freshly prepared at the time of analysis.

d. 5,5'-dithiobis-2-nitrobenzoic acid

Dissolve 20 mg of DTNB in 100 ml of 34mmol/l trisodium citrate solution. This solution is stable for up to three months at 4°C.To prepare 34 mmol/l trisodium citrate (pH 8.0), dissolve 1g of trisodium citrate in 100 ml distilled water.

- Procedure

1. Added 0.2 ml of red blood cell suspension to 1.8 ml of lysing solution in a test tube to prepared a haemolysate and allowed to stand at room temperature for three minutes for lysis to be completed
2. A reagent blank is made using saline instead of red cell suspension
3. Added 3.0 m**l** of precipitating solution, mix well and allowed to stand for a further five minutes
4. Filtered the solution through a single-thickness Whatman No.42 filter paper
5. Added 1.0 ml of clear filtrate to 4.0 ml of freshly made_disodium hydrogen phosphate solution
6. Recorded the absorbance at 412 nm (A1)
7. To the mixture added 0.5 ml of DTNB reagent and mixed well by inversion

8. Recorded the absorbance at 412 nm (A2) within 10 minutes and calculated the change in absorbance (ΔA412) for the test as well as the blank. Substracted the change in absorbance for the blank from change in absorbance for the test to calculate the final absorbance of the sample tested
9. Calculated the concentration of GSH from the standard curve developed using glutathione standard
10. GSH in nmol/ml (A) = (absorbance of sample / absorbance of standard) × (total standard/ sample volume)
11. GSH in µmol/g Hb = (A/Hb in g/ml) ÷ 1000

3.3.4.3 Estimation of Erythrocyte Malondialdehyde (MDA)

Level of lipid peroxides in erythrocyte suspension was determined by estimating MDA using the method of Okhawa *et al*., (1979); as modified by :- Al-Azzawie and Alhamdani (2006). Thiobarbituric acid (TBA) reacts with lipid peroxides and MDA to form a red coloured pigment with maximum absorbance at 532 nm and can be determined by colorimetry. 1,1,3,3 tetramethoxypropane was used as a standard since it can be converted to malondialdehyde quantitatively by reacting with TBA.

- Reagent preparation
  a. Preparation of erythrocyte suspension: Erythrocytes were washed three times with PBS of pH 7.4. The packed RBCs after the third wash is used for the assay
  b. Preparation of 8.1% sodium dodecyl sulphate (SDS) solution: Mix 2.025 g of SDS in 25 ml distilled water
  c. Preparation of 20% acetic acid with pH 3.5: Add 20 ml of glacial acetic acid to 80 ml of distilled water and adjust the pH to 3.5 with sodium hydroxide crystals

d. Preparation of 0.8% aqueous solution of TBA: Mix 0.2 g of TBA in 25 ml of distilled water. Fresh solution is prepared every day
e. Preparation of 1% butylated hydroxytoluene (BHT) in absolute alcohol: Mix 100 mg butylated hydroxytoluene in 10 ml of absolute alcohol

- Procedure

1. Take 0.1 ml of erythrocyte suspension in a 10 ml screw-capped pyrex tube
2. Added to the erythrocyte suspension, 0.1 ml of 8.1% SDS, 0.75 ml of 20% acetic acid with a pH of 3.5 and 10µl of 1% w/v BHT in absolute alcohol
3. To this added 0.75 ml of 0.8% aqueous solution of TBA
4. Added 0.3 ml distilled water (to make the final volume to 2.0 ml), homogenized and heated in a water bath at 95°C for 60 minutes
5. Cooled the mixture in tap water and added 2.5 ml of n-butanol/pyridine (15:1) and 0.5 ml of distilled water
6. Vortexed vigorously and centrifuged the mixture at 3000 rpm for 15 minutes
7. Measured the absorbance of n-butanol/pyridine phase, the upper layer, at 532nm in a spectrophotometer
8. Calculated the concentration of MDA from the stan dard curve developed using TMP as standard
9. MDA in nmol/ml = (absorbance of sample / absorbance of standard) × (total standard/ sample volume)

3.3.4.4 Glucose

The supernatant glucose estimation of packed RBC stored in SAGM was carried out using glucose estimation kit- GenX GLUCOSE-ML (GOD-PAP Trinders method).

3.3.4.5 Potassium

Potassium estimated from the supernatant of packed red blood cells stored in CPDA SAGM blood bag using Easylyte analyser, which is a completely automated microprocessor controlled electrolyte system using ion selective electrode (ISE) technology.

3.3.5 Morphological Studies

3.3.5.1 Light Microscopy

Red blood cell smears were prepared from stored CPDA-SAGM blood bag in 0, 14, 28 and 42 days from the day of collection, air-dried and fixed in methanol.

3.3.5.1.1 Staining of Blood Smears

3.3.5.1.1.1 Fields stain

Fields stain solution A and B (Nice chemicals private limited, Kochi) were used.

3.3.5.1.1.2 Fields staining technique

Two coplin jars were filled with Field's stain A and B solutions separately. Red blood smears were fixed in methanol for one minute and air dried. This was followed by coupling of stain B and allowing it to act for 30 seconds. After washing the slides in running tap water, stain A was added and allowed to act for 30 seconds. The slides were further washed, air dried and observed under 100 X objective of a light microscope (Olympus, CH20i and Leica, DM 500). The variations in the number of cells was classified as 1+ (1-10 cells), 2+ (11-20 cells) and 3+ (> 20 cells) and minimum 1000 cells were counted (Palmer *et al*., 2015).

3.3.5.2 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy scans a narrow, tapered electron beam back and forth over the specimen. When the beam strikes a particular area, surface atoms

discharge a tiny shower of electrons called secondary electrons, and these are trapped by a detector. Secondary electrons strike a material in the detector that emits light when struck by electrons (the material is called a scintillator).The flashes of light are converted to an electrical current and amplified by a photomultiplier. The signal is digitized and sent to a computer, where it can be viewed

- Procedure
    1. Take 1.5mL packed RBC washed twice with PBS (3000 rpm for 5min)
    2. Take 100µL of washed pRBC
    3. Prepared 10mL 2.5 % gluteraldehyde (1mL 25% gluteraldehyde made upto 10mL using PBS)
    4. Mixed 100µL of washed RBC pack with 5mL of 2.5% gluteraldehyde and incubated at 37ºC for 24hrs
    5. Mixture was washed with PBS (3000rpm for 5min)
    6. To 100µL pellet added 1% osmium tetroxide in PBS (50mg:5mL) and incubated it for 2hrs
    7. Mixture was washed thrice with PBS
    8. To 100µL pellet added 2mL of 30% ethanol and incubated for 20 min
    9. Centrifuged for 20 min and take 2ml out
    10. Vortex 100µl pellet for 20 min with 50% alcohol
    11. Centrifuged for 20 min and take 2ml out
    12. Vortex 100µl pellet for 20 min with 70% alcohol
    13. Centrifuged for 20min and take 2ml out
    14. Vortex 100µl pellet for 20 min with 90% alcohol
    15. Centrifuged for 20min and take 2ml out
    16. Vortex 100µl pellet for 20 min with 100% alcohol
    17. Centrifuged for 20min and take 2ml out
    18. To pellet added 1 or 2 drops of 100% ethanol
    19. 5µl of sample was smeared in carbon sheet and stubbed

20. Dried the stubbed sample for one hour in vacuum
21. Sputter coating was done

3.3.6 Culture Studies

Culture and identification of microorganisms was performed as per Agzie *et al*. (2019)

- Procedure:
  1. One ml of blood from the bag were dispensed into separate, sterile bottles containing 10ml of Brain-Heart Infusion broth (Plate 8)
  2. The suspension was incubated aerobically at 37^{o}C for up to 3 days and observed for the signs of bacterial growth on 48 and 72 hours
  3. Samples showing signs of bacterial growth, a gram stain was made and examined microscopically
  4. At the same time the samples were sub-cultured using standard methods on to Blood agar (BA) and Macconkey agar (MA)
  5. Then BA and MA plates were incubated aerobically, at 37^{o} C for up to 72 hours
  6. Plates were inspected for bacterial growth at 48 and 72 hours

3.4 STATISTICAL ANALYSIS

The statistical analysis of data obtained was done using computer software Statistical Package for Social Sciences (SPSS), version 24.0. Repeated measures ANOVA and pair wise comparison was done by using least significant difference test. Regression analysis was done for fitting linear trend in osmotic fragility test.

Plate 8. Brain heart infusion broth used for blood culture studies

Results

4. RESULTS

Present study was undertaken to determine the suitability of SAGM additive in CPDA for storing pRBCs of goats. Blood samples collected from 10 healthy goats maintained at University Goat and Sheep farm, Mannuthy and other organized farms in Thrissur district were utilized in the present study.

4.1. HEALTH STATUS OF THE SELECTED ANIMALS

Before the start of the study, selected goats were dewormed. Health status of each animal was evaluated with detailed clinical examination, haematological analysis, peripheral blood smear and faecal sample examination. The goats selected were active and alert with normal vital parameters. Haematological indices of the goats in the study were within the normal range and as in table 1.

Blood parameters	Minimum	Maximum	Mean ± SE	Reference range*
WBC	5.3	14.3	9.41 ± 0.83	4-13
LYM	3.5	11.0	5.34 ± 0.68	2-9.1
MON	0.3	0.7	0.51 ± 0.06	0-0.5
GRA	1.1	4.0	2.57 ± 0.25	1.7-5.4
RBC	12.8	15.4	14.27 ± 0.85	8-18
HGB	10.5	12.4	11.17 ± 0.70	8-14
HCT	25.7	32.8	29.5 ± 2.15	19-38

Table 1: Haematological indices of selected goats

*Bain *et al*., (2016).

4.2 BIOCHEMICAL PARAMETERS

The mean value of biochemical parameters of blood samples taken on day 0 and 14, 28 and 42 of blood storage is as in table 2. Repeated measures ANOVA was carried out to find out any changes in the variables at different period of time. If F-value was found to be significant, pair wise comparison was done by using least significant difference test.

4.2.1 pH

The mean pH values of the stored sample did not show a significant variation ($p > 0.05$) during the study. The values declined from 7.18 ± 0.03 (day 0) to 7.13 ± 0.03 (day 42).

4.2.2 Glucose

Mean glucose value of stored blood reduced significantly from 650.4 ± 18.45 mg/dL (day 0) to 535.5 ± 16.92 mg/dL (day 42). The decline in glucose values was significant between the study periods also ($p < 0.01$).

4.2.3. Potassium

Mean value of supernatant potassium increased from 0^{th} day to 42^{nd} day. The values increased from 4.02 ± 0.22 mmol/L on day 0 to 13.15 ± 0.57 mmol/L on 42^{nd} day. A significant increase ($p < 0.01$) was recorded.

4.2.4. Malondialdehyde

Mean MDA value of day 0, 14, 28 and 42 were 10.47 ± 1.13, 15.15 ± 1.36, 20.59 ± 1.60, 28.16 ± 1.76 µmol/L respectively. Comparing the values, significant variation was noticed statistically.

4.2.5. Reduced Glutathione

The reduced glutathione values showed a significant increase throughout the storage period ($p < 0.01$). The increase was from 0.02 ± 0.01 µmol/g of Hb (day 0) to 0.10 ± 0.02 µmol/g of Hb (day 42).

Table 2. Variation of bio-chemical parameters during storage

Variables	Day 0	Day 14	Day 28	Day 42	F-value (P-value)
pH	7.18 ± 0.03	7.18 ± 0.03	7.14± 0.03	7.13 ± 0.03	1.317^{ns} (0.289)

Glucose (mg/dL)	650.4±18.45[a]	607.5±18.92[b]	573.2±22.09[c]	535.5± 16.92[d]	20.707** (<0.001)
Potassium (mmol/L)	4.02 ± 0.22[d]	6.96 ± 0.27[c]	9.61 ± 0.39[b]	13.15± 0.57[a]	160.61** (<0.001)
MDA (µmol/L)	10.47±1.13[d]	15.15±1.36[c]	20.59±1.60[b]	28.16± 1.76[a]	33.342** (<0.001)
GSH (µmol/g of Hb)	0.02 ± 0.01[d]	0.05 ± 0.01[c]	0.08 ± 0.02[b]	0.10 ± 0.02[a]	46.62** (<0.001)

** Significant at 0.01 level (P<0.01); ns - Non-significant (P>0.05)
Means having different letter as superscript differ significantly within a row

4.3 HAEMATOLOGICAL PARAMETERS

The average volume of pRBCs obtained from the samples 220 ± 20 ml. The minimum volume obtained was 200ml. The mean haematological changes of blood samples taken at 0, 14, 28 and 42 day of blood storage is as in table 3.

4.3.1 Haemoglobin

Mean value of haemoglobin on day 0, 14, 28 and 42 were 19.53 ±0.41, 19.53 ±0.41, 19.72 ± 0.41 and 19.67 ± 0.41 g/dL, respectively. The values showed no significant difference statistically from day 0 to 42 (p>0.005).

4.3.2 Volume of Packed Red Cells (VPRC)

Mean VPRC value at 0, 14, 28 and 42 were 55.20 ±1.24, 55.8 ± 1.20, 55.8 ± 1.20 and 55.8 ± 1.20 per cent respectively. No significant variation could be observed durring study period (p>0.005).

Table 3. Variation in haematological parameters during storage

Variables	Day 0	Day 14	Day 28	Day 42	F-value (P-value)
Haemoglobin (g/dL)	19.53 ±0.41	19.53 ±0.41	19.72 ± 0.41	19.67± 0.41	0.573^{ns} (0.637)
Volume of packed red cells (%)	55.20 ±1.24	55.8 ± 1.20	55.90 ± 1.39	55.8 ± 1.20	0.635^{ns} (0.599)

ns - Non-significant (P>0.05)

4.4. HAEMOLYSIS OF RBC

Haemolysis of RBCs was determined by the fragility of RBCs by measuring resistance to haemolysis while being exposed to varying levels of dilutions of a saline solution (Plate 9). The data obtained from the selected samples as presented (Fig 1) was used to obtain the mean cell fragility (MCF) of the selected animals from day 0 to day 42. The concentration of saline for promoting 50 per cent haemolysis is termed as MCF.

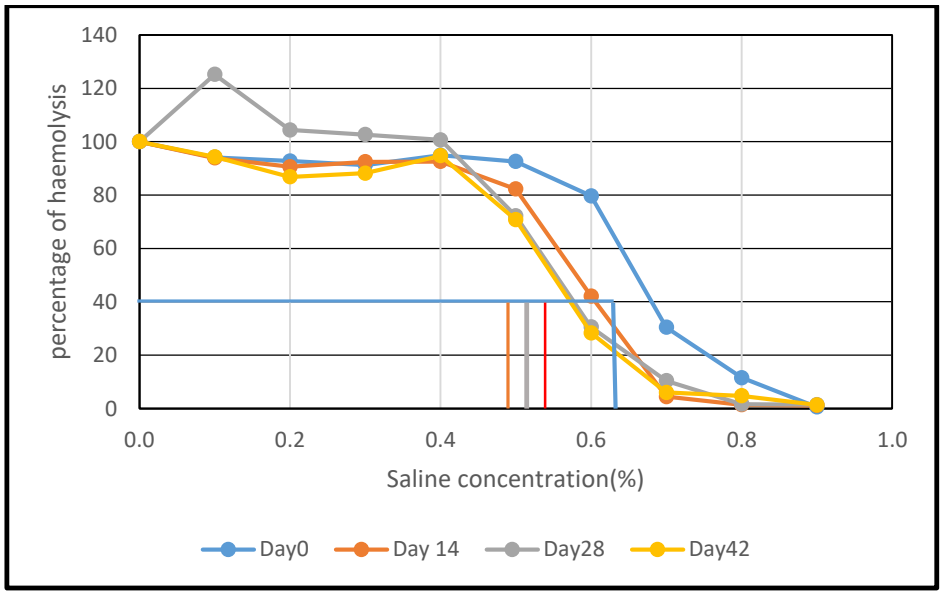


Fig 1. Osmotic fragility curves of sample stored from Day 0 to 42

Plate 9. Haemolysis of RBC exposed to varying levels of saline dilution

Linear equations were fitted for each period and the equations are as in Table 4. Using the fitted equations, concentration capable of promoting 50 per cent of haemolysis was estimated and is presented in table 5.

Table 4. Models for predicting percentage of haemolysis at different stages of storage

Dependent variable	Fitted model	R^2
Percentage of haemolysis at Day 0	Y =118.498 – 110.456 X	0.864
Percentage of haemolysis at Day14	Y =118.157 – 129.044 X	0.910
Percentage of haemolysis at Day 28	Y =132.232 – 149.57 X	0.929
Percentage of haemolysis at Day 42	Y =115.436 – 128.690 X	0.925

Y- dependant variable (percentage of haemolysis), X- independent variable (concentration of saline)

Coefficient of determination (R^2) as given in Table 3 indicated that on day 0, 86 per cent of the variability in the dependent variable percentage of haemolysis was due to the variation in concentration. About 91 per cent variability in percentage of haemolysis on day 14 can be explained by the variability in concentration. On day 28 it was 93 per cent and on day 42 it was 92.5 per cent. Higher R^2 throughout the study is an indication of good fit of the predicted equation.

After fitting the equation, concentration capable of promoting 50 per cent of haemolysis is estimated by putting Y as 0.5 and then X value is estimated by using the fitted equation for all the four period of time and is as given in Table 4.

Table 5. Concentration capable of promoting 50 per cent of haemolysis in each period

Period	Concentration capable of promoting 50 percent of haemolysis
Day 0	0.620
Day 14	0.528
Day 28	0.550
Day 42	0.508

Based on the above results mean cell fragility did not show any major change from 14 to 42 of storage.

4.5 MORPHOLOGICAL STUDY

4.5.1 Light Microscopy

Blood smears were observed under oil immersion objective of microscope on day 0, 28 and 42 of collection. Red blood cells were classified based on the shape and size of the cells, which included macrocytes, microcytes, echinocytes, schistocytes, spherocytes and dacrocytes. Among these, echinocytes, schistocytes, spherocytes and dacrocytes did not show any significant change in its number from day 0 to 42. Anisocytosis and poikilocytosis was the predominant variation.

4.5.1.1 Anisocytosis: Number of anisocytes increased from day 0 to 42 during storage (Plate 10 A). Number of cells per field increased proportionally with the storage time (Table 6).

4.5.1.2 Poikilocytes: Same pattern of increase as of anisocytes was (Table 6) noticed during the storage period as in Table 7 (Plate 10 B).

4.5.1.2.1 Acanthocytes: Irregularly shaped poikilocytosis with spicules (acanthocytes) increased from day 0 to 42 (Plate 10 C). No 3+ variations were recorded during any stage of the study (Table 8).

Table 6. Variation in Anisocytes during storage

Anisocytes/1000 cells	Day 0 (nos)*	Day 0 (per cent)	Day 28 (nos)*	Day 28 (per cent)	Day 42 (nos)*	Day 42 (per cent)
1+ (1-10)	5	50	4	40	3	30
2+ (11-20)	3	30	3	30	4	40
3+ (>20)	2	20	3	30	3	30
Total	10	100	10	100	10	100

* nos- Number of field

Table 7. Variation in Poikilocytes during storage

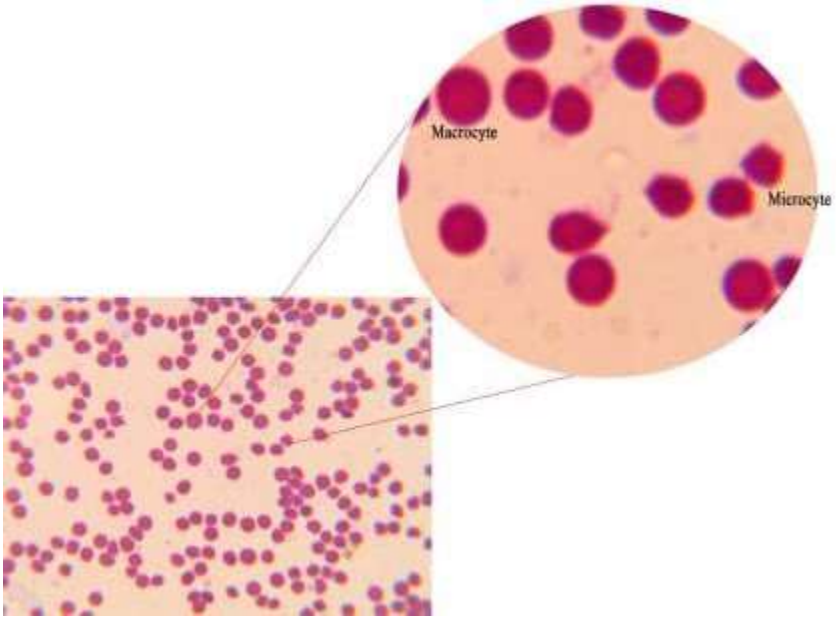
Poikilocytes/1000 cells	Day 0 (nos)*	Day 0 (per cent)	Day 28 (nos)*	Day 28 (per cent)	Day 42 (nos)*	Day 42 (per cent)
1+ (1-10)	5	50	4	40	3	30
2+ (11-20)	3	30	3	30	4	40
3+ (>20)	2	20	3	30	3	30
Total	10	100	10	100	10	100

* nos- Number of field

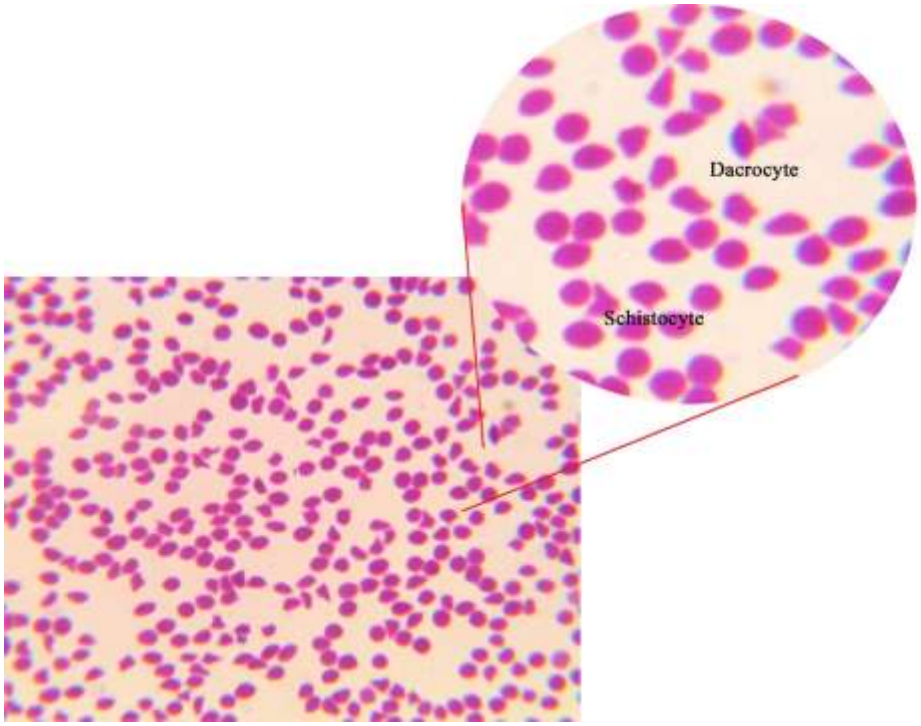
Table 8. Variation in Acanthocytes during storage

Acanthocytes/1000 cells	Day 0 (nos)*	Day 0 (per cent)	Day 28 (nos)*	Day 28 (per cent)	Day 42 (nos)*	Day 42 (per cent)
1+ (1-10)	10	100	8	80	7	70

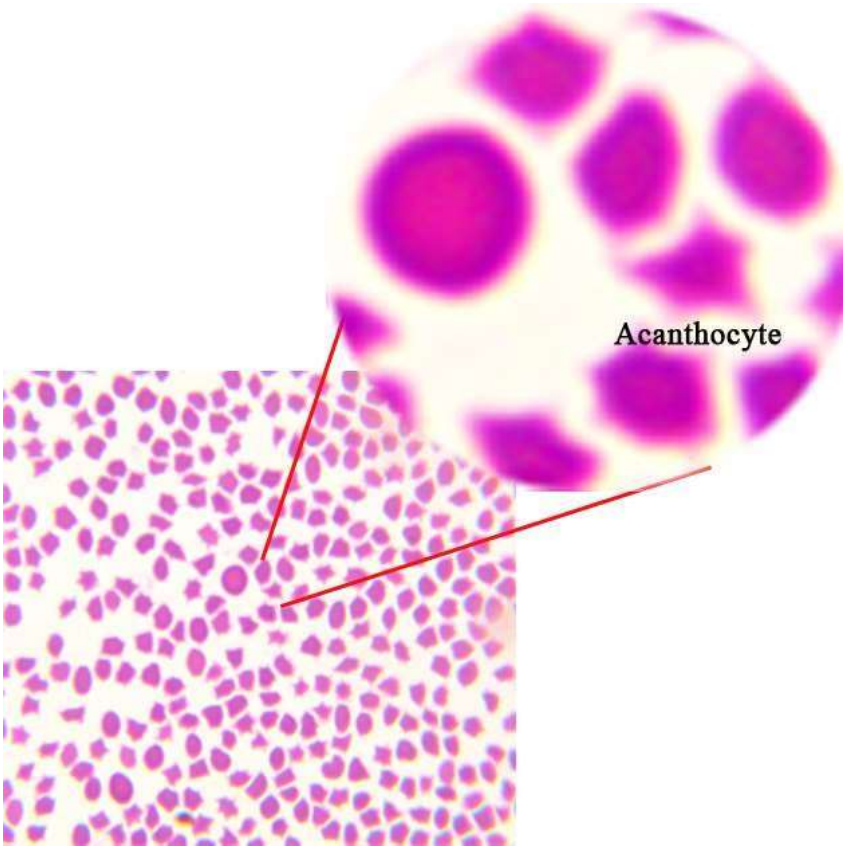
Plate 10. Field stained pRBC smears using oil immersion objective of microscope (100X)



10A. Blood smear with anisocytosis on day 0 of storage



10B. Blood smear with poikilocytosis on day 42 of storage



10C. Blood smear with acanthocytes on day 42 of storage

2+ (11-20)	0	0	2	20	3	30
3+ (>20)	0	0	0	0	0	0
Total	10	100	10	100	10	100

* nos- Number of field

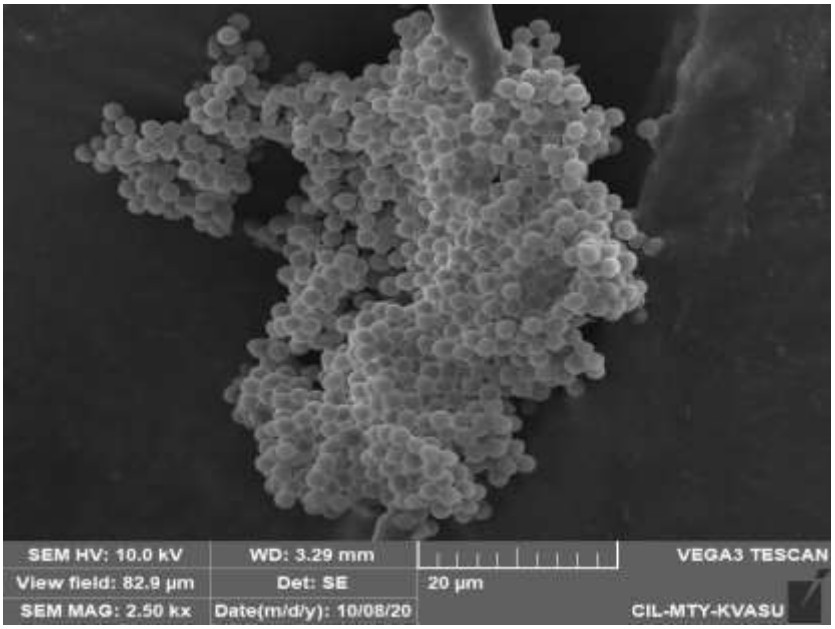
4.5.2 Scanning Electron Microscopy

The selected samples were taken on 0, 28 and 42nd day of storage period for SEM study. The morphology of day 0 cells was considered as the normal morphology (Plate 11 A). In this study, no major changes of cell morphology was noticed on 28th and 42nd days from day 0 (Plate 11 B). Most of the cells were spherical (Plate 11 C). Acanthocytes and dacrocytes were the common abnormal cells that were recorded (Plate 11 D), however these variations were present from day 0 itself.

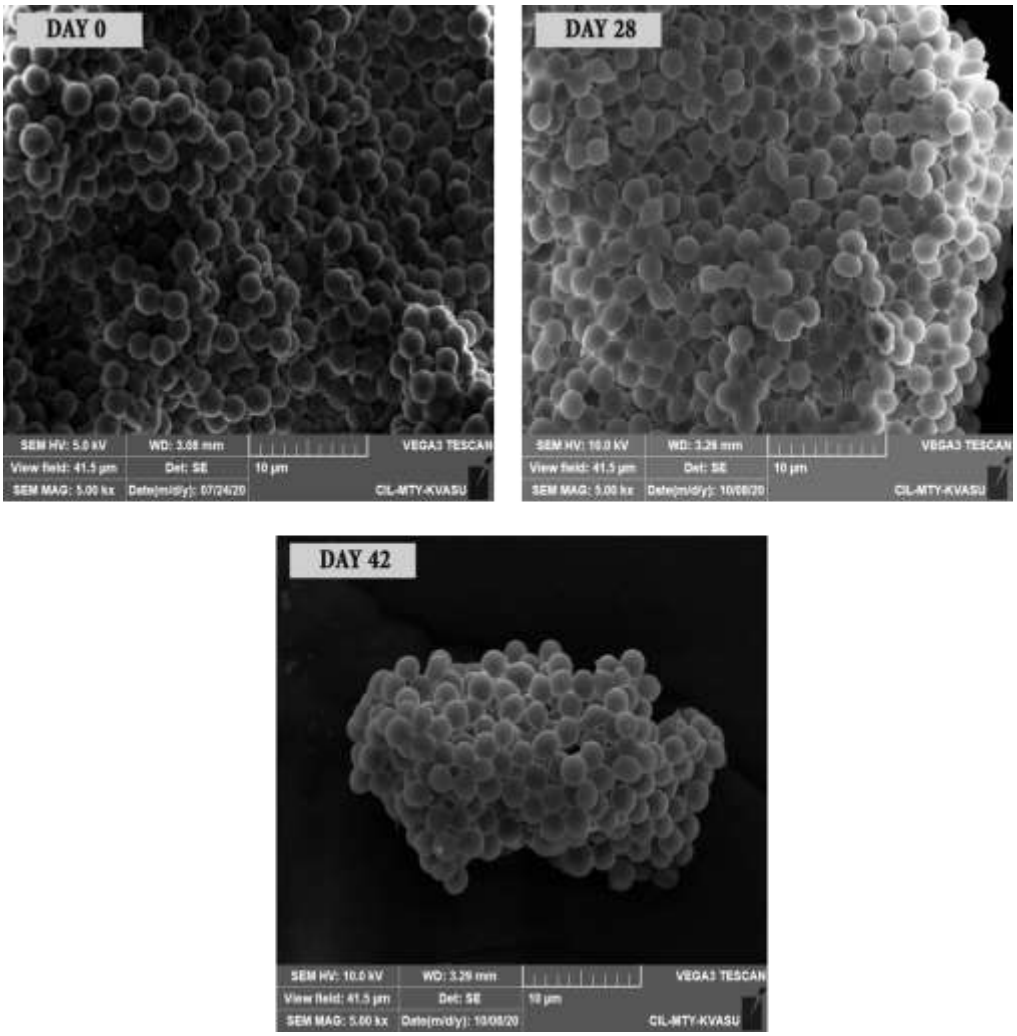
4.6 CULTURE STUDIES

All the selected samples were taken for culture studies on day 0, 14, 28 and 42 days of storage period. The samples were incubated and inspected at 48 hrs and 72 hrs of culture using blood agar and Macconkey agar. No growth was recorded during the study period. One sample that was positive for *Staphylococci* spp on day 0 of storage was discarded.

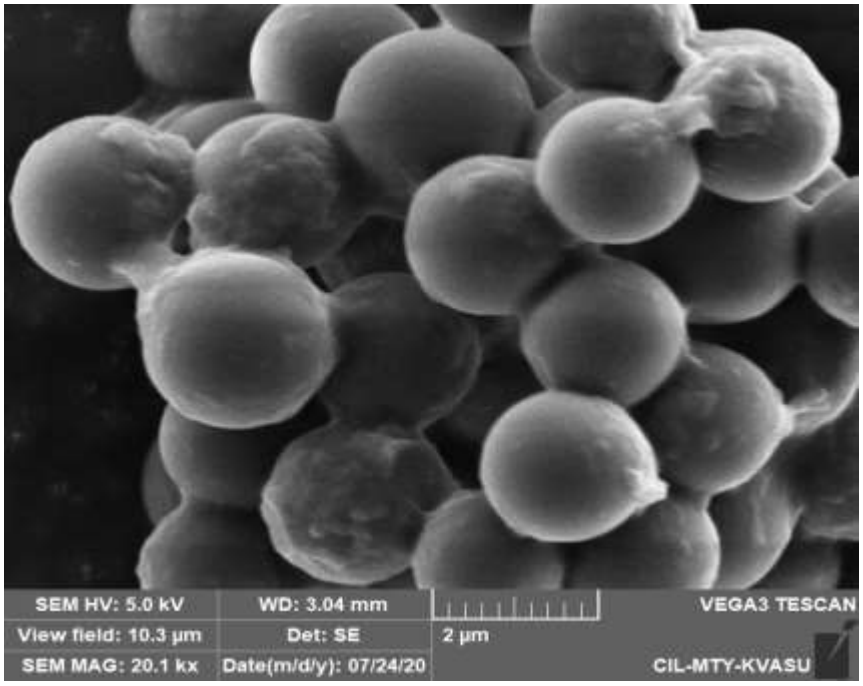
Plate 11. Scanning electron micrograph of erythrocytes stored in blood bags with SAGM



11A. Normal morphology (day 0 of storage)- 2500X

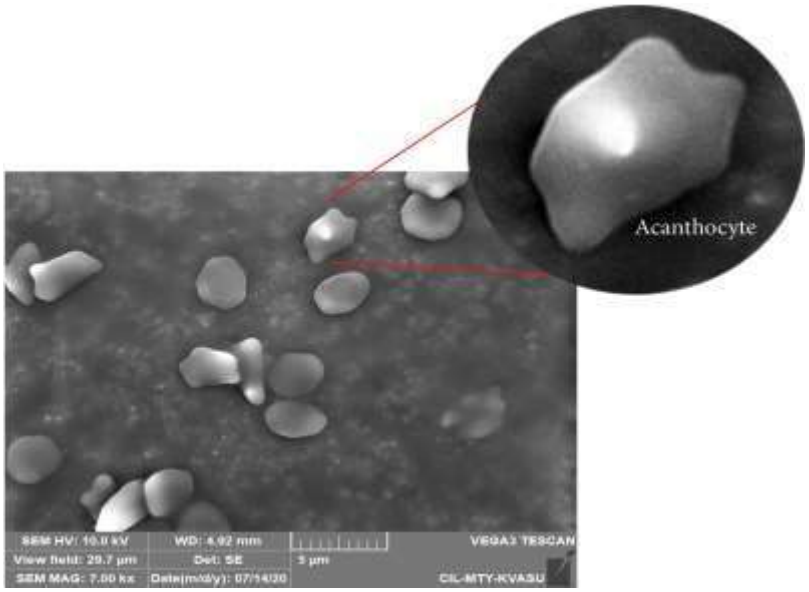


11 B. Representative RBCs morphology on day 0, 28 and 42 of storage (5000X)

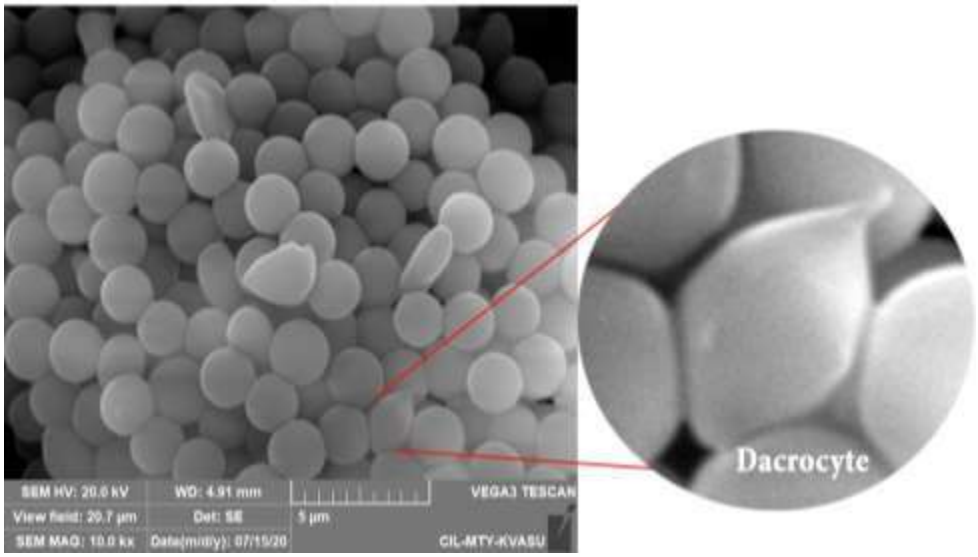


11C. Spherical cells (day 0 of storage)-20000X

Plate 11D. Abnormal cells



Acanthocytes (7000X)



Dacrocyte (10000X)

Discussion

5. DISCUSSION

The following section discusses the results of the research work, “Suitability of saline-adenine-glucose-mannitol additive in citrate-phosphate-dextrose-adenine for storing packed red blood cells of goats”.

5.1 HEALTH STATUS OF SELECTED ANIMALS, BLOOD COLLECTION AND STORAGE

The donor animals for the collection and storage of blood should be selected as per the health status, weight and be screened for blood borne parasites (Mudge, 2010). Selected animals should have VPRC value and other haematogical values within the reference range of selected species (Balcomb and Foster, 2014). In accordance with the above studies the selected animals in the study were clinically healthy, haematogical indices were in normal range and negative for any blood borne parasites. Therefore, the blood collected was used as representative samples of donor animals for storage studies.

The mean volume of blood in relation to body weight in goat was estimated as 0.076 L/kg (about 8 per cent of body weight) (Luethy *et al*., 2017). According to Mudge (2010), twenty percentage of total blood volume can be collected for transfusion without any risk to the donor. In this study, 350ml of blood was collected using CPDA-SAGM blood bags for storage. As per the findings of Sousa *et al*. (2013) and Tavares *et al*. (2019), CPDA-1, is a suitable anticoagulant for the storage of sheep and goat blood respectively, under refrigeration. Moreover, Brugue *et al*. (2018) opined that addition of SAGM to RBCs improved its shelf life (up to 44 days) by reducing its ATP loss.

5.2 BIOCHEMICAL PARAMETERS

The biochemical parameters assessed in this study were pH, glucose, potassium, MDA and GSH.

5.2.1 pH

In this study, mean pH value decreased from day 0 to 42 during storage, though it was not significant. The mean value was in the range 7.18 ± 0.03 to 7.13 ± 0.03. Hess and Greenwalt (2002), opined that pH value above 7.2 will favor 2,3-DPG production and may affect the ATP synthesis and permissible lower pH limit for the stored blood was 6.65. Reduction in pH values occur as RBCs are metabolically active and associated with glucose breakdown and anaerobic metabolism. Lactate and hydrogen ions were produced on storage of blood (Mudge *et al*., 2004; Hess *et al*., 2009). However, as the pH value decline was not significant and within the limits, the stored blood could be used for transfusion.

5.2.2 Glucose

The mean value of supernatant glucose reduced as the storage period increases. This is in agreement with Tavares (2013), who observed decrease in glucose values during storage from day 0 to 42.This reduction might be due to the consumption of glucose by the metabolically active erythrocytes as an energy source. However, it has been recorded that glucose consumption of caprine erythrocytes is low in comparison to other species. Higher ATP reserves of caprine erythrocytes may also result in non-decline of glucose levels (Kaneko *et al*., 2008).

Even at the end of storage period, significant reduction in glucose was noticed in the mean value from 573.2 ± 22.09 mg/dL on 28th day to 535.5 ± 16.92 mg/dL on 42nd day. This suggests that RBCs are metabolically active and fit for transfusion. The excess glucose in the nutrient media at the end of storage period was easily balanced post transfusion by the liver (Fonesca *et al*., 2018).

5.2.3 Potassium

A significant increase was recorded in the supernatant potassium values during the study. In this study, on 42nd day of storage the supernatant mean potassium value was 13.15 ± 0.57 mmol/L, *vis-à-vis* day 0 value of 4.02 ± 0.22

mmol/L. During blood storage, a slow and continuous leakage of intracellular potassium to the plasma has been recorded. This has been considered to be associated with failure of Na^+- K^+ ATPase pump (Opoku-Okrah *et al*., 2015). Species wise difference in intracellular potassium level has been reported (Sousa *et al*., 2013). Opoku-Okrah *et al*. (2015) stated that plasma potassium level of blood stored in CPD solution can increase by 0.5-1.0 mmol/L per day. Extracellular potassium value can be used as a marker for assessing the quality of stored erythrocytes as the ATP depletion and improper refrigeration may result in elevation of potassium in ECF as suggested by Fonesca *et al*. (2018). Perusal of literature did not reveal any similar study for caprine pRBC storage with regard to potassium levels. If the canine and human potassium levels increase are considered for comparison, the potassium increase in the present study is slow and constant. Further, the values of the present study can be used as reference values for caprine pRBC storage studies. The post transfusion hyperkalaemia will become normal within few days of transfusion (Lion *et al*., 2010).

5.2.4 Malondialdehyde

The mean MDA values increased significantly throughout the storage from 10.47 ± 1.13 µmol/L (day 0) to 28.16 ± 1.76 µmol/L (day 42). As it is an end product of lipid peroxidation, quantifying the MDA in stored blood was a useful marker to determine the extent of lipid peroxidation (Simsek *et al*., 2006; Nazifi *et al.*, 2009; Pandey and Rizvi, 2011). Malondialdehyde formation indicated the loss of phospholipid from RBC membrane. Proportionating the MDA values, oxidative damage and resultant haemolysis can be assessed and viability of the cells in the storage media can be predicted (Fonesca *et al*., 2018). Increase in MDA values in the storage of pRBCs in SAGM of human blood has been reported (Chaudhary and Katharia, 2012). However in a similar study of human pRBCs, increase was found to be insignificant (Mustafa *et al*., 2016). Similar studies with regard to stored caprine pRBCs are lacking. Oxidative damage can result in change of osmotic fragility and formation of echinocyte and

spheroechinocyte (Sharifi *et al*., 2000). No such variation was recorded in the present study. Osmotic fragility variation and morphological changes of RBCs has been discussed in the specified section. It has been suggested that, addition of membrane interacting antioxidants like vitamin E analogue has beneficial effects in reducing lipid peroxidation level of RBCs stored in SAGM as suggested by Antosik *et al*. (2018).

5.2.5 Reduced Glutathione

Erythrocytes in blood bank under storage are highly susceptible to oxidative damage as it contains high amount of reactive oxygen and it is regulated by the antioxidant system, which fails as the storage prolongs due to deficient cellular energy (Roback *et al*., 2011). According to Kozlova *et al*. (2015), among the antioxidants, principal one is the intracellular reduced glutathione (GSH) and reduction in this value favours oxidative changes in RBCs. In the present study, a significant increase in GSH was recorded with storage. Huyut *et al*. (2016) reported that MDA value and GSH value were negatively correlated. Reduced glutathione studies with respect to caprine pRBC stored in SAGM is lacking. Increase in GSH during the study period suggests antioxidant activity to manage the oxidative stress. According to Roback *et al*. (2011), the oxidised form of GSH is GSSG, which is not an antioxidant but it can be converted back to GSH by using NADPH reducing equivalent derived from phosphate pentose pathway. It was also reported that supplementation of amino acid precursors can also stimulate GSH synthesis.

Two other antioxidants reported were α- tocoferol and ergothioneine, which are obtained from the diet or some unknown mechanisms and not synthesized by the erythrocytes. These were relatively stable during the storage. Additive used in the study, SAGM contains mannitol which act as an antioxidant by scavenging hydroxyl radical (OH) in various systems (Antosik *et al*., 2018). Therefore, several factors influence the GSH values and no specific conclusion could be drawn with regard to the increase recorded.

5.3 HAEMATOLOGICAL PARAMETERS

Cellular integrity and shelf life of stored erythrocytes can be determined by assessing the haematogiocal parameters (Brugue *et al*., 2018; Spada *et al*., 2019). According to Antwi-Baffour *et al.* (2013), these parameters are highly sensitive to several factors, *viz*. storing temperature, storage solution, time gap between the analysis and collection. In this study, VPRC and total Hb of the stored erythrocytes were assessed.

5.3.1 Volume of Packed Red Cells

The volume of packed red blood cells can vary with the donor, residual plasma and amount of additive solution added. In canines, the VPRC values of pRBCs was in the range of 55-65 per cent (Ekiz *et al*., 2012). In the present study, no significant variation was recorded in VPRC values. Antwi-Baffour *et al.* (2013) opined that during storage, degenerative changes may cause variation in membrane permeability leading to entry of water into cells which increase VPRC *via* elevated RBC volume. No degenerative changes of pRBCs as indicated by variation in VPRC was present in this study.

5.3.2 Haemoglobin

In this study, the mean value of haemoglobin was 19.53 ± 0.41g/dL (day 0), 19.67 ± 0.39 g/dL (day 14), 19.72 ± 0.41 g/dL (day 28) and 19.67 ± 0.41 g/dL (day 42). No significant variation was recorded during the study period. Haemoglobin is considered to be a more reliable indicator of oxygen carrying capacity in comparison to the VPRC values as the latter is influenced by morphological changes (Ferreira *et al*., 2018). According to the guidelines of Council of Europe (2011) and UK blood transfusion services (2005), in human transfusion, each unit of blood should contain Hb value of 40g minimum in 80 per cent of the collected samples. Ferreira *et al*. (2014) in an extensive study of

canine pRBC, extrapolated these guidelines to assess the minimum Hb value required in pRBC. It was opined that minimum Hb value required was 18.2 g/dl. All the samples obtained in the present study had values above 18.2 g/dl, indicating the suitability of the storage media as well as validating the centrifugation procedure.

5.4 HAEMOLYSIS OF RBCS

Resistance to haemolysis of RBCs was measured using osmotic fragility test. In this test, degree of resistance was measured by treating the erythrocytes in varying concentration of saline in ascending order (Blasi *et al.*, 2012; Mustafa *et al*., 2016). Concentration capable of promoting 50 per cent haemolysis was 0.620 per cent (day 0), 0.528 per cent (day 14), 0.550 per cent (day 28) and 0.508 per cent (day 42). Osmotic fragility was found to be higher on day 0. This is contrary to finding of Mustafa *et al*. (2016). It was reported that osmotic fragility increased as the storage progressed and RBCs stored at 42^{nd} day had the least resistance to hypotonicity. Studies with respect of haemolysis of caprine RBCs stored in CPDA-SAGM blood bag is lacking. Reduction in osmotic resistance of the RBCs is due to morphological changes. Transformation of normal discoid cells to spherocytes resulted in decreased surface area to volume ratio (Hogman *et al*., 2006). In the present study, no major morphological alterations were noticed in SEM and light microscopy. The storage media contains citrate and mannitol which are stability enhancers (Hess and Greenwalt, 2002). As research with regard to haemolysis of caprine RBCs stored in SAGM are lacking, it is concluded that extensive research with larger number of caprine samples may be required to reach an unequivocal opinion.

5.5 MORPHOLOGICAL STUDY

Deformability of the cells was one of the crucial factors that helped the RBCs to pass through the splenic circulation. Major morphological changes may affect the post transfusion viability of erythrocytes (Obrador *et al*., 2015). Relevy *et al*. (2008) reported a similar opinion that deformability of the cells is

critical for passing through the minute capillaries with the size half the diameter of erythrocytes to accomplish proper perfusion of the tissues. In the present study, assessment of the structural changes during storage was done with light microscopy and scanning electron microscopy.

5.5.1 Light Microscopy

Under standard blood bank conditions during storage, RBCs undergo various reversible or irreversible morphological alterations which may affect the post transfusion viability and other complications in recipients (Berezina *et al*. (2002). Light microscopy aids the visual assessment of cell shape and is considered as the gold standard technique (Pinto *et al*., 2019). As per the study of Palmer *et al*. (2015), different systems are used to describe the findings of light microscopy like simple descriptions, present / absent and semi quantitative methods like few, moderate and many. In this study, it was described based on the number of cells per field. Different type of cells noticed were macrocytes, microcytes, acanthocytes, echinocytes, schistocytes, spherocytes and dacrocytes. Among these, variations were noticed during the storage period for macrocytes, microcytes and acanthocytes resulting in anisocytosis and poikilocytosis.

5.5.1.1 Anisocytosis

Anisocytosis is a condition of increased variability in size of RBCs. It may be larger than normal (macrocytes), smaller than normal (microcytes) and increased frequency of both the cells (Palmer *et al*., 2015). In the present study, mild to moderate increase was noticed during storage. According to Jones (2007), in ruminants, moderate anisocytosis was normal. Palmer *et al*. (2015) reported that hypochromasia may result in microcytes but, in this study haemoglobin level is stable and was adequate for RBCs survival. In storage studies, macrocytes are considered as artifacts (Whitney and Weidmeyer, 2012). In ruminants macrocytosis has been recorded associated with conditions including non-regenerative anaemia, vitamin B_{12}, folic acid and cobalt

deficiencies (Roland *et al*., 2014). Formation of macrocytes and microcytes was not related to membrane damage or storage (Bain *et al*., 2016).

5.5.1.2 Poikilocytosis

In this work, same pattern of increase in poikilocytes was recorded as for anisocytes. Barger (2010) reported that in goats, poikilocytosis was a common finding. According to Palmer *et al*. (2015), abnormal shaped cells are called poikilocytes and it was not a specific abnormality. Based on the prominent shape observed, it can be correlated with the cause. As per the reports of Valenciano *et al*. (2014), RBC fragmentation and membrane abnormalities result in the formation of echinocyte, stomatocyte and acanthocytes. Among the poikilocytes in this study, acanthocytes were prominent and showed an increase during the storage period.

Acanthocytes are irregularly shaped round hyperchromic cells with 2-20 spicules of different length, shape and thickness. As suggested by Bain *et al*. (2016), it can be formed due to abnormal phospholipid or with inherited abnormalities of membrane proteins in the cells. Oxidative damage as evidenced by increase in MDA in the present study, can be considered as a cause of acanthocytosis. However, no 3+ variations were recorded, suggesting that the changes were not severe. Oxidative damage alone cannot be inferred as the cause of acanthocytosis (Warry *et al*., 2013).

5.5.2 Scanning Electron Microscopy

Among the domestic animals, central pallor and biconcavity was not prominent with an exception in dogs. In goats, erythrocytes shows flat surface with little surface depression (Harvey *et al*., 2012). In the present study, no major morphological changes were noticed from the 0^{th} day to the end of storage period. Most of the cells were normal compared to goat erythrocyte morphology. Few abnormal cells, acanthocytes and dacrocytes were noticed from the beginning itself, indicating that it was not a storage defect. Moreover, in healthy goats

moderate poikilocytosis is commonly recorded (Barger, 2010). In a study in humans by Mustafa *et al*. (2016), pRBCs stored in CPDA-SAGM on 0^{th} day showed normal erythrocyte morphology. It was reported that by 28 days of storage majority of these cells became spherocytes and echinocytes and these changes were irreversible. No such morphological variations were recorded in the present study.

Berezina *et al*. (2002) in a study of human blood stored in adenine saline solution opined that spherocyte, ovalocyte and sphero-echinocytes were irreversible changes and it affected the post transfusion viability. Leonart *et al*. (1997) reported that morphological alterations occur in RBCs with depletion of ATP. Spherocytes thus formed was an irreversible variation. No such morphological variation was recorded in the present study. Specific studies with regard to SAGM related caprine stored RBC SEM studies is lacking. In comparison to the light microscopic study, SEM studies can be considered superior in detecting morphological variations. The morphological changes recorded in RBCs in the present SEM study were few and cannot be considered to influence the storage viability.

5.6 CULTURE STUDY

During this study, one sample was positive of *Staphylococci* spp on the day 0 of the storage and was discarded. Kessler *et al*. (2010) opined that if any discoloration was noticed in the blood bag, it should be checked for contamination. Because, even a mild contamination may cause severe post transfusion complication in recipients, who are mostly debilitated or immune-compromised. According to Dargere *et al*. (2018), a contaminant is a non-pathogenic bacteria which had entered during the collection or processing of blood. Among them, most prevalent were *Staphylococci* spp followed by *Bacillus* spp. Though non- pathogenic and irrelevant in the beginning, it becomes the cause of true bacteraemia as the concentration increases. It also results in variation in pH and lactic acid in blood bags affecting the quality and shelf life (Mudge *et al*., 2004; Stefanetti *et al*., 2016). Clinical and Laboratory Standard

Institute (2007), recommended that the rate of blood contaminant should not exceed 3 per cent of total blood culture and it can be reduced to a great extent by a proper collection protocol. Based on the reports of Brecher and Hay (2005), making the skin completely aseptic was difficult. The ten samples in the study did not show any growth during the storage period. As the discarded sample was positive of *Staphylococci* spp on day '0' of storage it can be inferred to be a skin contaminant. It is suggested that standard aseptic precautions need to be maintained during the phlebotomy procedure for blood collection. Moreover, it is recommended that stored blood is cultured at periodic intervals to ascertain its suitability for transfusion with regard to bacterial contamination.

Summary

6. SUMMARY

The study entitled 'Suitability of saline-adenine-glucose-mannitol in citrate-phosphate-dextrose-adenine for storing packed red blood cells of goats' was conducted in Department of Veterinary Clinical Medicine Ethics and Jurisprudence, College of Veterinary and Animal Sciences , Mannuthy, during the period of 2019-2020.

Ten apparently healthy goats weighing 35 to 60 kg within an age range of 4 to 8 years were selected for the study from University Goat and Sheep Farm, Mannuthy and other organized farms in Thrissur district. All the goats were dewormed and the health status of each animal was evaluated by physical examination and laboratory analysis including complete blood count, examination of blood smear and faecal sample.

Whole blood was collected from the selected animals using commercially available CPDA-SAGM blood bags under aseptic conditions. Each of these units were centrifuged at 5,000 × *g* for 7minutes, at 4°C using FTBC- 6100R Blood Bank Refrigerated Centrifuge. After centrifugation, plasma was extracted in empty bag using plasma extractor from primary blood bag and SAGM was added from the satellite bag using the valves. After separation, pRBCs with SAGM was stored at 4 ± 2^0C in a dedicated refrigerator for 42 days and suitability of the blood in the blood bag was analysed every two weeks from day 0 to 42. The parameters used to assess the suitability in this study was broadly classified into biochemical, haematological, haemolysis of RBC, morphology and cultural studies.

Biochemical parameters assessed in this study are pH, glucose, potassium, MDA and GSH. Among these, pH was stable throughout the study without showing any significant change. A significant reduction was noticed in glucose values during the storage from 650.4 ± 18.45 mg/dL (day 0) to 535.5 ± 16.92 mg/dL (day 42) as glucose was used by the cells. Rest of the parameters increased significantly from 0^{th} day to 42^{nd} day. Malondialdehyde value increased from 10.47 ± 1.13 to 28.16 ± 1.76 µmol/L and GSH from 0.02 ± 0.01 to 0.10 ± 0.02 µmol/g of Hb.

Selected haematological parameters were haemoglobin (Hb) and volume of packed red cells (VPRC). Both of the parameters were steady during storage and did not show any significant change. The mean value of Hb was 19.53 ± 0.41 to 19.67 ± 0.41 g/dL and VPRC was 55.20 ± 1.24 to 55.70 ± 1.44 per cent on day 0 and 42 respectively.

Haemolysis of RBCs was determined by osmotic fragility test. A graph was plotted by percentage of haemolysis in the X axis against concentration of saline in Y axis to find mean cell fragility. Mean cell fragility did not show a major change from day 14 to 42 of storage.

Morphological study of RBCs was done using light and scanning electron microscopy on 0, 28 and 42 days of storage. In light microscopy studies, blood smear samples were observed under oil immersion objective. Red blood cells were classified based on the shape and size of the cells. The cell noticed were macrocytes, microcytes, acanthocytes, echinocytes, schistocytes, spherocytes and dacrocytes. Among these, echinocytes, schistocytes, spherocytes and dacrocytes did not show any significant change in its number during storage period. Anisocytosis and poikilocytosis was the predominant variation.

In scanning electron microscopy, no major abnormal changes were noticed and most of the cells was spherical comparable to normal goat cells. Some abnormal cells like acanthocytes and dacrocytes were observed.

Cultural study was done on day 0, 14, 28 and 42 days of storage period. The samples were incubated and inspected at 48 hrs and 72 hrs of culture. One sample was positive for *Staphylococci* spp, suggestive of skin contamination and was discarded. No growth was recorded in the ten samples used for the research work during the study period.

Based on the findings of the present study, it can be concluded that

- The storage media, CPDA-SAGM can be used for the storage of goat blood upto 42 days was fit for transfusion. Hence the storage media is suitable.

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SUITABILITY OF SALINE-ADENINE-GLUCOSE-MANNITOL ADDITIVE IN CITRATE-PHOSPHATE-DEXTROSE-ADENINE FOR STORING PACKED RED BLOOD CELLS OF GOATS

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ABSTRACT

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(Veterinary Clinical Medicine, Ethics and Jurisprudence)

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ABSTRACT

In veterinary transfusion medicine, studies related to farm animal transfusions are scarce. Due to the rise in emotional and commercial value, need of transfusion in this area is increasing day by day. The present study entitled 'Suitability of saline-adenine-glucose-mannitol additive in citrate-phosphate-dextrose-adenine for storing packed red blood cells of goats' envisages the identification of a suitable storage media for goat blood upto 42 days.

Ten apparently healthy goats weighing 35 to 60 kg within an age range of 4 to 8 years maintained at University Goat and Sheep Farm, Mannuthy and other organized farms in Thrissur district were selected for the study. Whole blood units were collected from the selected animals using commercially available CPDA-SAGM blood bags under aseptic conditions. After centrifugation of each unit (350ml), plasma was separated and SAGM were added to pRBCs and stored at 4 ± 2^0C in dedicated refrigerator upto 42 days. For analysing the parameters, samples were collected from the bag every two weeks from the day of collection to day 42. Light and scanning electron microscopic (SEM) studies were carried out on day 0, 28 and 42 of storage. Biochemical and haematological parameters, RBC haemolysis assessment, morphological variation and cultural studies of stored blood was carried out.

The pH of the stored blood remained stable whereas glucose showed a significant reduction. Potassium, malondialdehyde and reduced glutathione increased throughout the storage. Haemoglobin and volume of packed red cells did not show any significant change during storage. Haemolysis of RBCs was analysed using osmotic fragility test. The mean cell fragility of pRBCs did not show a major change from day 14 to 42 of storage. Anisocytosis and poikilocytosis was recorded in light microscopy. In SEM only a few acanthocytes and dacrocytes were recorded. Storage related variations were recorded in the samples as the storage period progressed. The study suggests that CPDA-SAGM is a suitable storage media for caprine pRBCs.

Acad. Form No.19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Faculty of Veterinary and Animal Sciences

PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis:

Suitability of saline-adenine-glucose-mannitol additive in citrate-phosphate-dextrose-adenine for storing packed red blood cells of goats

2. **(a) Title of the departmental/ KVASU research project of which this forms a part:**

Not Applicable

(b) Code No. if any, and order by which the departmental /KVASU research project is approved:

Not Applicable

3. **a) Name of student:**

Anaz S.R.

b) Admission No:

18-MVM-31

c) Name of the Discipline:

Veterinary Clinical Medicine, Ethics and Jurisprudence

4. a) Name of Major Advisor (Guide):

Dr. N. Madhavan Unny

b) Designation:

Associate Professor,
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Thrissur- 680 651

5. Objective of the study:

Study the suitability of saline-adenine- glucose-mannitol (SAGM) additive in citrate- phosphate-dextrose-adenine (CPDA) for storing packed red blood cells (pRBCs) of goats

6. Practical/Scientific utility:

Anaemia remains an important cause of mortality in farm animals irrespective of the etiology. Treatment of primary cause may often be ineffective as there is a considerable reduction in haematological indices that may not sustain life. Blood transfusion is considered as a life saving procedure in such circumstances. However donor animals are not easily available. Studies on blood transfusion and that of its products in goats is scarce. Recent advances in component transfusion in animals have produced encouraging results with lesser chance of transfusion reactions.

Saline-adenine-glucose-mannitol additive has been used in storing pRBCs in human beings and certain species of animals. Storage lesions and variations can considerably affect the longevity and utility of RBCs. The current study is aimed at assessing the suitability of SAGM for storing pRBCs. The study would establish the time frame for storing caprine pRBCs in SAGM. The research work would provide a guideline with regard to storing goat pRBCs in SAGM for saving the life of anaemic goats.

7. Important publications on which the study is based:

Superior additive solutions are capable of increasing the storage time of blood and blood products (Kurup *et al.*, 2003).

Raat and Ince (2007) observed that haemoglobin oxygen affinity increases with storage of blood thus reducing the oxygen unloading capacity after transfusion.

Boudreaux (2010) documented that canine whole blood could be stored at 4°C for upto 35 days depending on the anticoagulant preservative solution used.

Barshtein *et al*. (2011) reported that biochemical changes alter the RBC membrane structure, resulting in increased haemolysis during storage.

Joseph (2011) opined that anaemia due to loss of blood in goats

was associated with external and internal parasitism and had major clinical significance in goats.

Pandey and Rizvi (2011) reported that measuring malondialdehyde in human RBCs was a useful marker in assessing the extent of lipid peroxidation.

Sparrow (2012) stated that mannitol in SAGM protected the RBC membrane and reduced haemolysis which enabled storage of human RBCs upto six weeks under refrigeration.

Lacerda *et al.* (2014) observed an increase in potassium levels in canine blood on storage.

Mustafa *et al.* (2016) observed considerable increase in fragility of RBCs with period of storage in SAGM in humans.

8. Outline of the technical programme:

Ten apparently healthy adult goats with normal haemogram will be utilised for the study. Blood will be collected in standard CPDA blood bags. The blood collected will be centrifuged at 5000Xg for seven minutes in a temperature controlled blood bank centrifuge for the separation of RBCs.

Plasma expressor will be used to separate the plasma component following standard procedure as for other species (Kurup *et al*., 2003). The desired volume of plasma will be removed from the primary bag. The additive solution SAGM (100 mL for 450 mL blood bag) will be introduced to the primary CPDA containing blood bag having concentrated red cells. The pRBC will be stored at 4°C in a blood bag refrigerator.

Samples will be withdrawn from the pRBC bags on day 0, 14, 28 and 42. Biochemical, haematological and membrane fragility studies will be carried out (Obrador *et al*., 2015; Mustafa *et al*., 2016). Microscopic changes in pRBC will be analysed using light and scanning electron microscopy on day 0, 28, and 42 of storage. Culture studies of stored pRBCs will be carried out. The data

collected will be analysed statistically to determine the variations associated with storage using SPSS version 24.0.

9. Main items of observations to be made:

1. Biochemical parameters
    a. pH
    b. Reduced glutathione in pRBC (µmol/g of Hb)
    c. Malondialdehyde in erythrocytes (µmol/L)
    d. Potassium level (mmol/L) in supernatant
    e. Glucose level (mg/dL) in supernatant
2. Haematological parameters
    a. Volume of packed red cells (%)
    b. Haemoglobin level (g/dL)
3. Light and scanning electron microscopic changes of pRBCs
4. Haemolysis of pRBCs (%)
5. Presence of bacteria in culture

10. Facilities:

a) **Existing**: Facilities of College of Veterinary and Animal Sciences, Mannuthy will be utilized
b) **Additional facilities required**: Chemicals and Biologicals

11. Duration of study:

Four semesters

12. Financial estimate:

Cost of chemicals and biologicals : Rs. 20,000/-

Contingencies: Rs. 5,000/-

Total: Rs. 25,000/-

Signature of student

Project coordination group to which the proposal is to be placed:

Animal Disease II

Signature of Major Advisor

Mannuthy,

16.06.2020

Name and signature of members of the Advisory Committee

1. Dr. N. Madhavan Unny
   Associate Professor

2. Dr. Usha Narayana Pillai
   Professor and Head

3. Dr. Arun George
   Assistant Professor

4. Dr. R. Thirupathy Venkatachalapathy
   Professor

APPENDIX - I

References:

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APPENDIX-II

Time frame of work

Semester -I

1. Collection of literature
2. Preparation of project proposal

Semester-II

1. Collection of literature
2. Standardisation of procedures

Semester -III

Study of storage lesions of pRBC in goats and analysis of samples

Semester -IV

1. Study of storage lesions of pRBC in goats and analysis of samples
2. Compilation of data and interpretation of results
3. Finalisation, writing and submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animal Act (Amendment, 1998).

Place: Mannuthy

Date: 16-06-2020

Dr. N. Madhava Unny

(Major Advisor)

CURRICULUM VITAE

Name of candidate	:	Dr. Anaz S R
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Educational status	:	BVSc & AH, undergoing MVSc

Publications made

- **S.R. Anaz**., E. Niyas., S. Afsal., N. Madhavan Unny., N.P. Usha. 2019. Blood transfusion in the management of bovine theileriosis [abstract]. In: *Compendium, 26th Annual Convention of Indian Society of Animal Production and Management;* 23rd and 25th January, 2019, Mannuthy. Kerala Veterinary and Animal Sciences University.p.350.
- **S.R. Anaz**., M.V. Anjaly., N. Madhavan Unny., Sukanta Datta., N.P. Usha. 2019. Blood transfusion in the management of anaemia associated with theleriosis and strongylosis in a buck. In: *Proceedings of Veterinary Practice to Address Challenges of Climate Change and Antibiotic Resistance in Animal Health Care and Food Safety*; 9th and 10th November, 2019, Pookode. College of Veterinary and Animal Sciences, Indian Veterinary Association. Pp. 137.
- **S.R. Anaz**., T. Juby., M.E. Anugraha., V.H. Shyma., N. Madhavan Unny., N.P. Usha. 2020. Leptospirosis in a calf: A case report [abstract].In: *Compendium, 38th Annual Convention and National Symposium of Indian Society of Veterinary Medicine 2020*; 5th February, 2020, Bengaluru. Veterinary College, Hebbal, Bengaluru. Pp. 96.

Accepted article for publication : Effect of storage on biochemical parameters of packed red blood cells of goats in citrate phosphate dextrose adenine/ saline adenine glucose mannitol

Membership in professional societies : Kerala State Veterinary Council

Indian Veterinary Association, Kerala