



Characterization of yolk sac proteins of *Bos indicus* cattle embryos

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ABSTRACT. The yolk sac is an embryonic membrane that is essential for the embryo's initial survival in many mammals. It also plays an important role in the production of proteins necessary for development. We studied proteins of the yolk sac in bovine embryos at up to 40 days of gestation. We examined the yolk sac of 17 bovine embryos at different gestational periods, measuring α -fetoprotein, α -1-antitrypsin, and transferrin. This experiment was carried out by Western blot technique, associated with electrophoresis on a 6% sodium dodecyl sulfate polyacrylamide gel. Mouse monoclonal antibody anti-human- α -fetoprotein, mouse antibody anti-human-transferrin and rabbit polyclonal anti-human- α -1-antitrypsin were used as primary antibodies, and conjugated peroxidase as a secondary antibody. We detected the three proteins in some of the yolk sac samples; however, the bands in some specimens (samples) were weak, maybe a result of poor antigen-antibody reaction, since the antibodies used in this study were not

specific to bovine proteins. The fact that weak bands appeared might be due to a weak cross-reaction.

Key words: Yolk sac; Western blotting; Proteins; Alpha-fetoprotein; Alpha 1-antitrypsin; Transferring

INTRODUCTION

Bos indicus is considered a different species in comparison to *Bos taurus* due to its morphology, physiological peculiarities and ecological characteristics; it is considered the world's largest breed, with about 200 million in India and showing a continuous expansion in tropical countries. Nowadays, the Zebu breed is considered an icon in tropical cattle investigation due to its considerable economical production (Bezerra, 1976), and *in vitro* embryo production and transfer techniques have been increasing and improving more and more, although the culture medium used in this technique usually affects embryo development after fertilization and interferes with metabolism and important gene expression (Alves et al., 2003). The extra-embryonic membranes (allantois, amnion, chorion, and yolk sac) are responsible for the fetal-maternal communication and placental formation (Wolf et al., 2003). The yolk sac is responsible for early embryo survival in many mammal species, producing important proteins for their development. The proteins are polymers that are involved in numerous biological functions and also determine cell structure and shape. Besides, the proteins catalyze many chemical reactions and control membrane permeability, to regulate metabolite concentration and to control genetic function (Zaha et al., 2003). Specialized proteins can play antibody, toxin, hormone, anticoagulant, and elastic fiber functions as well (Alberts et al., 2006).

The fetal membranes, chorion, allantois, amnion, and yolk sac, represent the membranous structures involved in placental development (Bowen and Burghardt, 2000). These membranes can establish intrinsic connections during placental development, which could be transient or permanent, depending on the animal species (Assis Neto, 2010). The placenta represents a tissue apposition or fusion, responsible for fetal-maternal nutrition and residual excretion. The morphological efficiency of the fetal membrane is related to the large superficial apposition area of layers, and even though the maternal and fetal blood are close, they are morphologically separated by the fetal membrane cell layers (Björkman, 1973).

The yolk sac plays a major role in embryonic development in mammals. The extra-embryonic portion is responsible for initial hematopoiesis. In rodents, it is the main fetal-maternal membrane for exchanges during gestation. This capacity to synthesize and secrete proteins in the embryonic and extra-embryonic spaces is frequently used in experimental embryology and reproduction toxicology (Gulbis et al., 1998). Salisbury and Vandermark (1964) affirm that the young embryo could persist for a short time due to the nutritive absorption of the yolk sac and uterine milk, and as long as development continues, a well-developed nutritive system is required. The three embryonic layers derived from diverse embryonic tissues, as well as the protective layers involved, have a nutritive function. The blood vessels of the yolk sac are responsible for transporting the nutrients from the uterine milk to the embryo. The functional activity of the yolk sac is short-term, whose function is developed by the allantois. Barone (1976) affirms that the yolk sac undergoes an early involution and degeneration in ruminants and no vestige remains after the second week to the end of pregnancy. It has been observed that in domestic ruminants, such as cattle, sheep and goats, proteins produced by

extra-embryonic membranes have a mediator function in some fetal-maternal interactions for the establishment and development of gestation. The bovine yolk sac is well developed by the 20th day of gestation, and afterwards, its regression begins (Godkin et al., 1988). The yolk sac of domestic mammals is initially large but involutes with placental progression. Despite being a transitory structure, the yolk sac shows important functions, such as permanent placental nutrition until its complete formation, especially in equine and carnivores. Some of the yolk blood vessels are incorporated by the embryo forming the adult visceral blood vessels. Its endodermic portion originates the primordial germ cells (primary sex cells), which migrate to the gonads and also show characteristics similar to the steroidal hormone-producing cells (Latshaw, 1987). The yolk sac could have functions of the liver in embryonic development before fetal liver maturation and produce a variety of fetal liver proteins (Gulbis et al., 1998).

MATERIAL AND METHODS

After the evisceration of the animals in the abattoir, the uterus were identified and incised. Afterwards, the embryo and its membranes were collected and placed in plastic containers that were kept in a box with ice. The yolk sac was characterized macroscopically, collected and frozen at -20°C or placed in physiological solution for protein extraction. The liver was also collected, sampled and its fragments (0.5 cm) were placed in 1.5-mL conical tubes for protein extraction.

Estimation of fetal period

The gestational period was estimated following the procedure described by Evans and Sack (1973) and Noden and Lahunta (1972), measuring the sacral-occipital axis, i.e., the distance between the nuchal crest and the last sacral vertebra (crown-rump, CR). Following the measurements, the embryos were grouped according to the measurements and to the gestational period (Table 1).

Table 1. Relationship between the estimated gestational period and size of embryo.

Embryo	Size (cm)	Gestational period (days)
Embryo 1	0.87	15-20
Embryo 2	1.3	20-30
Embryo 3	1.73	30-40
Embryo 4	3.62	50-60
Embryo 5	2.1	30-40
Embryo 6	2.01	30-40
Embryo 7	0.4	0-15
Embryo 8	3.4	50-60
Embryo 9	3.3	50-60
Embryo 10	0.2	0-15
Embryo 11	1.9	30-40
Embryo 12	1.3	20-30
Embryo 13	1.7	30-40
Embryo 14	2.2	30-40
Embryo 15	19.0	30-40
Embryo 16	2.9	40-50
Embryo 17	3.5	50-60
Embryo 18	0.60	10-20
Embryo 19	1.25	20-30
Embryo 20	1.75	30-40
Embryo 21	2.77	40-50

Protein extraction

The fragments of yolk sac and liver were boiled in a solution composed of 1 g SDS, 0.3362 g EDTA and 1.21 g Tris in 100 mL distilled water, after adding 500 μ M DDT, for 20 min. In these cases, a persistence of fragments was observed, and a maceration technique was applied to dissolve the remaining fragments; afterwards, the suspension was centrifuged for 15 min at 13,500 rpm. The resulting supernatant was transferred to other tubes, identified and considered to be the pure total protein extracted from the samplings. For Western blotting, a 1:1 mixture of the protein extract and sample buffer dye was used. This dilution was separated and frozen at -20°C .

Protein quantification

Protein was determined with the Quick Start Bradford Protein Assay kit (BioRad). Concentrations of bovine serum albumin (BSA; 2.0, 1.5, 1.0, 0.75, 0.5, 0.25, and 0.125 mg/mL) were used for the standard curve. The analysis was performed with the aid of a spectrophotometer at 595 nm.

Polyacrylamide gel electrophoresis

The method applied here followed the descriptions of Laemmli (1970). Polyacrylamide gel electrophoresis was performed on 6 and 7.5% gels. The 6% separation gel was prepared with 5.425 mL water, 2.5 mL buffer, 2.0 mL 30% acrylamide, 2.5 μ L TEMED, and 75 μ L 10% APS, and the 7.5% gel (for fetuin protein) was prepared with 4.925 mL water, 2.5 mL buffer, 2.5 mL 30% acrylamide, 2.5 μ L TEMED, and 75 μ L 10% APS. Stacking 4% gel was made with 1.585 mL water, 625 μ L buffer, 287.5 μ L 30% acrylamide, 2.5 μ L TEMED, and 31 μ L 10% APS. Aliquots of 20 μ L sample and 5 μ L molecular weight standard were loaded, according to Bio-Rad (161-0324) instructions, i.e., 5 to 10 μ L molecular weight standard for 4 to 20% gels. During the initial part of electrophoresis (10-20 min), gels were run at 70 V for protein compaction, and the voltage was then increased to 100 V and in the last 10 min to 120 V. The gel was stained with Coomassie blue (250 mL methanol, 100 mL acetic acid, 0.5 g Coomassie blue R-250 in 11 mL distilled water) for 3 x 30 min up to 100 min.

Transfer to nitrocellulose membrane

Before the antibody reaction, the membrane was blocked in 5% milk. The primary antibody was diluted in TTBS and applied to the membrane overnight. The secondary antibody was also diluted in TTBS and applied to the membrane for 1 h. Mouse anti-human α -fetoprotein monoclonal antibody (AFP; Serotec, MCA1863HT), mouse anti-human transferrin antibody (BD, 612124) and rabbit anti-human α -1-antitrypsin polyclonal antibody (A1AT; Abcan, AB922) were applied as primary antibodies, and anti-mouse- and anti-rabbit-peroxidase conjugate as secondary antibody (GE, NIF825).

RESULTS

A total of 21 embryos were collected and grouped in accordance with the size and ges-

tational period. Gross anatomy of yolk sac was performed in relation to the fetal membranes studied; the chorion showed non-vascularization and appeared as an external membrane. The allantois appeared as a transparent membrane, totally separated from the chorion and showing a few blood vessels. The amnion also appeared as a non-vascular transparent membrane surrounding the embryo (Figure 1A and B). The yolk sac was observed as a thin and transparent membrane, well developed on the 40th day of gestation, being proximal to the umbilical vessels and connected to the embryo's intestine. The yolk sac was composed of the vitelline duct, a central part and two projections (Figure 1B).

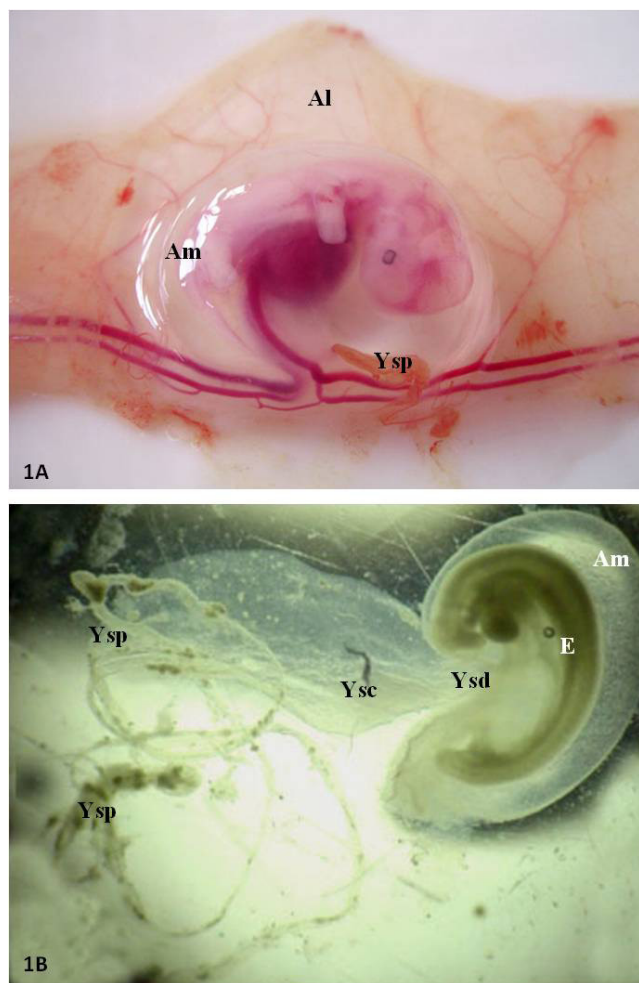


Figure 1. Photograph of the bovine embryo and its fetal membranes. **A.** Embryo with 2.1 cm crown-rump (CR) (35 to 40 days). Scale bar = 2.0 cm. **B.** Embryo with 0.4 cm CR (15 to 20 days); observe the yolk sac composed by the vitelline duct (Ysd), a central part (Ysc) and two projections (Ysp). Scale bar = 0.2 cm. Note in both figures the embryo (E), allantois (Al), amnion (Am) and yolk sac (Ysd, Ysc, Ysp).

After the 40th day of pregnancy, its size drastically diminished, which made it more difficult to visualize starting on the 50th day of gestation, since it became thinner and transparent, characterizing the involution process.

Protein quantification

Protein was assayed by the Bradford method, in which different concentrations of BSA were used as standards, as indicated in Table 2; the standard curve was used to determine the quantity of protein in each sample (Table 3). The largest protein concentrations were measured in the yolk sac of embryos of 5, 8 and 16, with gestational periods of 30-40, 40-50 and 50-60 gestational days, respectively.

Table 2. Measurements of absorbance at 595 different concentrations of bovine serum albumin (BSA).

BSA concentration ($\mu\text{g/mL}$)	Absorbance at 595 nm
0	0.003
125	0.143
250	0.226
500	0.445
750	0.583
1000	0.924
1500	1.012
2000	1.177

Table 3. Determination of proteins from different samples of bovine embryo's yolk sac and its absorbance at 595 nm.

Samples	Absorbance at 595 nm	Concentration ($\mu\text{g/mL}$)
Liver	0.822	1197
Embryo 5	0.666	937
Embryo 6	0.536	720.33
Embryo 7	0.536	720.33
Embryo 8	0.599	825.33
Embryo 9	0.193	148.66
Embryo 10	0.397	488.66
Embryo 11	0.519	692
Embryo 12	0.555	752
Embryo 13	0.528	707
Embryo 14	0.467	605.33
Embryo 15	0.368	440.33
Embryo 16	0.548	740.33
Embryo 17	0.441	562

Protein analysis

The Western blot technique associated with polyacrylamide gel electrophoresis applied to the protein analysis enabled to observe the existence of AFP, A1AT and transferrin proteins in the yolk sac. AFP, 70 kDa, was observed in the samples of yolk sac of cattle embryos 5 and 6 demonstrated in Figure 2. Transferrin, a protein of 85 kDa, showed bands practically in all samples at different intensities; it may be due to a cross-reaction of the primary antibody to B-galactosidase of 111 kDa (Figure 3). A1AT, a protein that has a molecular weight

of approximately 52 kDa, being between the BSA protein of 59 kDa and carbonic anhydrase of 30 kDa. After development with ECL (Figure 4) were reactive in some samples, and no nonspecific reaction was observed due to the position of this protein on the gel, corresponding to the desired molecular weight.

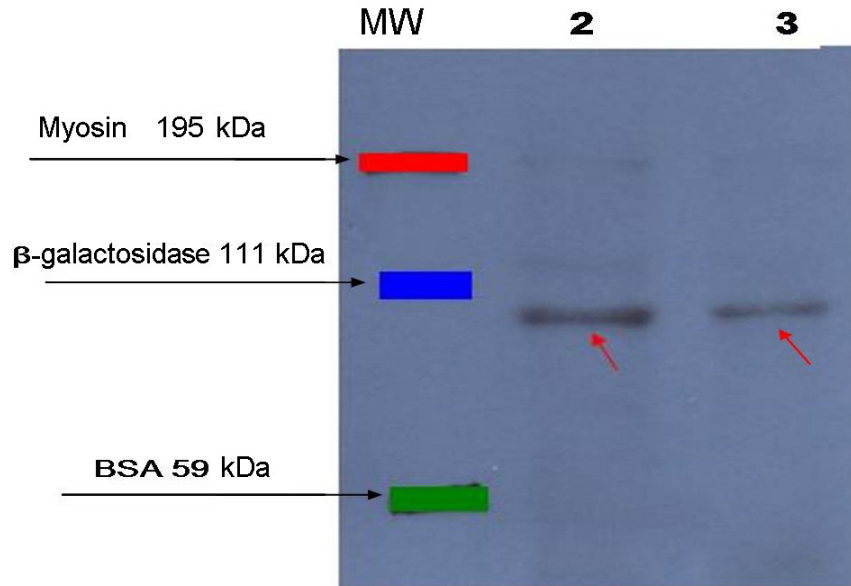


Figure 2. Membranes after ECL showing the molecular weight (MW) and samples of the yolk sac of embryos 2 and 3 at an estimated gestational period from 30 to 40 days. Red arrows indicate the α -fetoprotein in the samples 2:03 with a molecular weight of 70 kDa.

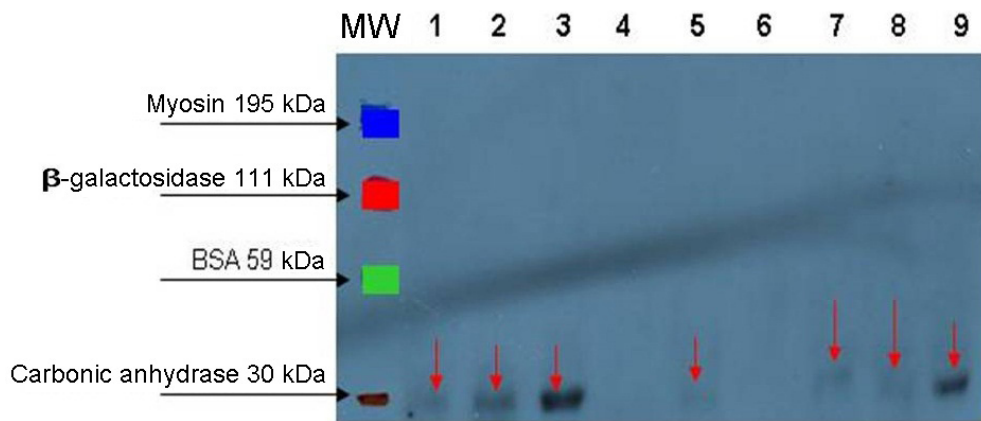


Figure 3. ECL revelation showing the molecular weight Kaleidoskop (MW) and samples of the yolk sac of bovine embryos (2-9). In a sample of the fetal liver, sample 1 has gestational period estimated from 15 to 20 days; samples 2, 3 and 8, from 30 to 40 days; samples 4 and 7, from 5 to 10 days; samples 5 and 6, from 50 to 60 days, and sample 9, from 20 to 30 days. Red arrows indicate specific nonreactions.

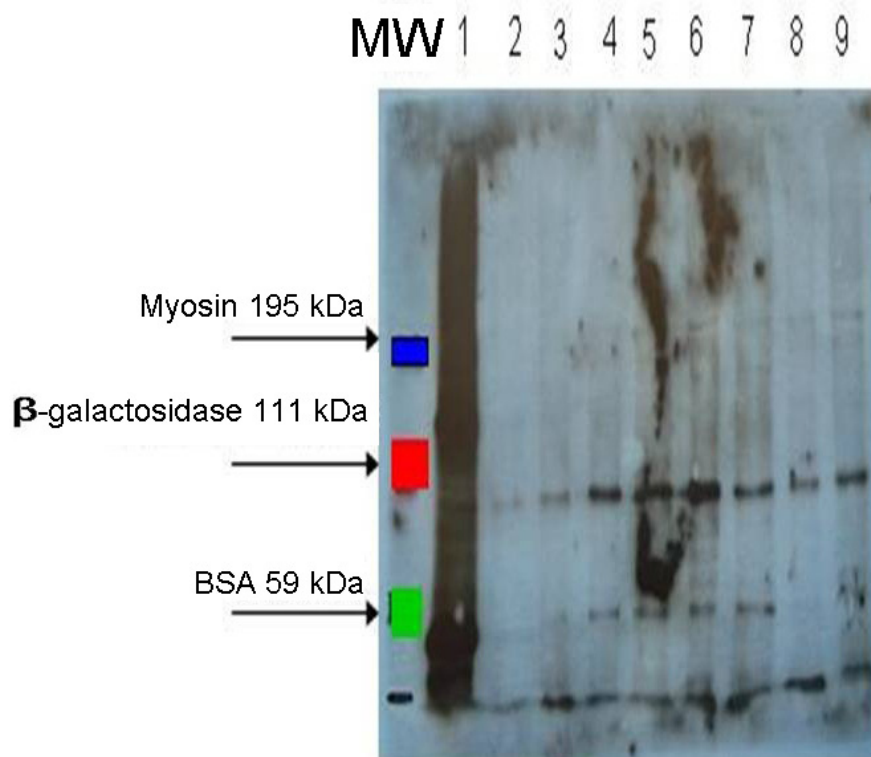


Figure 4. ECL revelation showing the molecular weight Kaleidoskop (MW) and samples of the yolk sac of bovine embryos (1-9). In a sample of the fetal liver, sample 2 has an estimated gestational period from 0 to 15 days; samples 3, 4, 5, 6, from 20 to 30 days; samples 7 and 8, from 30 to 40 days, and sample 9, from 40 to 50 days.

DISCUSSION

Macroscopic description of the yolk sac

The gestational periods were estimated in accordance with the methods proposed by Evans and Sack (1973) and Noden and Lahunta (1972), measuring the distance of the sacral occipital, taking as reference the nuchal crest at one end and the last sacral vertebra at the opposite end (CR). Based on the measurements of embryos, clusters were obtained in 6 different gestational periods. Analyzing a table described by Evans and Sack (1973) and Noden and Lahunta (1972), CR measurements show values close to those described in this study, but for lack of more recent and detailed literature, we cannot be sure of the exact gestational period of each embryo. Based on our findings, the yolk sac, a thin and transparent membrane, is shown to be developed enough to the 40th day of gestation, where it is close to the umbilical vessels of the embryo and connected to the gut of the embryo. Until about the 40th day of gestation, the yolk sac is composed of three parts: a vitelline duct, connection to the intestine of the conceptus, a center and two projections (Ruesse et al., 1992; Mançanares, 2007; Assis

Neto, 2010). Its size decreases dramatically, which makes it difficult to view from 50 days of gestation, where the process of devolution is well characterized, making it into a very thin and transparent membrane.

According to studies by Barone (1976), the yolk sac regresses and degenerates very early in ruminants, and as of the second week their remains are not found until the end of pregnancy. Latshaw (1987) describes that the yolk sac of domestic mammals is initially large, but it subsides when the placenta permanently develops. Although this is a transitional structure, the yolk sac has several important functions, such as to nourish the permanent placenta until it is formed, where it is especially important in horses and carnivores. Initially, the yolk sac is large, vascular, where it is completely surrounded by the coelom, and completely separated from the vascularized chorion around the 20th day of gestation in the cow, with only a transitory existence, since it is displaced by the early rapid growth of the allantoic sac.

The same observations were made by Bryden et al. (1972) and Noden and Lahunta (1972), who noted that in ruminants the yolk sac is large and vascularized and is completely separate from the chorion around the 20th day of pregnancy in the cow. Around the 25th day of pregnancy, it is reduced to a solid structure like a string. In domestic mammals, the yolk sac is transient, but is of great importance to the embryonic and maternal placenta exchange process before the maternal placenta becomes permanent. As the cavity expands, the effective transfer area of the vitelline placenta decreases, and when the formation of the extra embryonic cavity is complete, the separation of mesoderm at the point of attachment between the chorion and the yolk sac is destroyed. At this time, the vitelline placenta no longer exists.

Protein analysis

Little information was found in the literature searched regarding the presence of these proteins in the yolk sac of the bovine embryo. The descriptions are more related to the proteins produced by human and mouse yolk sac. Liu et al. (1991) found that the yolk sac of mice produces specific proteins identical to the bovine fetal serum proteins, such as fetal bovine transferrin AFP, A1AT, α -acid glycoprotein. According to Thomas et al. (1990), the visceral yolk sac and fetal liver of mice synthesize proteins such as transthyretin, transferrin and AFP, but a larger amount of these proteins is produced by the yolk sac in early pregnancy. Studies by Gitlin and Perricelli (1970) demonstrated that the yolk sac of rabbits produces proteins such as prealbumin, albumin, transferrin, AFP, and A1AT.

When grown, the yolk sac of human embryos with 5.5 weeks of gestation possesses large quantities of the proteins: albumin, prealbumin, AFP, A1AT, and transferrin. Slightly smaller amounts are present in the cultured embryonic yolk sac with 8.5 weeks of gestation, and cultures of embryos of 11.5 weeks contain prealbumin and a small amount of albumin, AFP and A1AT. Before 4 weeks of gestation, the human yolk sac appears as a well-defined bladder and liver by the hepatic ducts and buds that synthesize prealbumin, albumin, AFP, A1AT, transferrin, and other proteins. Little is known about the proteins synthesized by the yolk sac of bovine animals. Therefore, we used the above cited researches related to the proteins produced by the yolk sac of mouse and human as a reference to select the proteins under study.

The yolk sac has been recognized for a long time as having the fetal liver for hematopoiesis and production of protein during early embryonic development (Liu et al., 1991). Farrington et al. (1997) describe some vital functions of the visceral yolk sac for embryonic

growth and differentiation, such as digestion and absorption of nutrients, maternal protein synthesis, and formation of the vitelline circulation and intravascular secretion of nutrients. During our studies, we observed the presence of some proteins in some samples of the bovine embryo yolk sac.

In our results, AFP has a molecular weight close to 111 kDa, which is the molecular weight of the protein β -galactosidase, being close to the molecular weight studied by several authors ranging from 69 to 72 kDa, which is close to the molecular weight determined by some authors, ranging from 69 to 72 kDa. Gulbis et al. (1998) describe this as a human protein having a molecular weight of 70 kDa, which is close to that found by Milunsky (1992) of 69 kDa and by Alpert et al. (1972) of 72 kDa. Regarding the test done with the primary monoclonal antibody anti-mouse for human AFP (SERO-MCA1863HT - 1 mL), with no reaction with the other samples, we can assume that in the other samples, there was a lack of this protein or that the primary antibody had spoiled, which was unlikely to happen.

Previous tests with the other primary anti-mouse monoclonal antibodies for human AFP (Vector laboratories, VP-A104), also showed no reaction. Since this antibody had not been tested in Western blotting, we cannot guarantee that it worked in our samples. According to Gulbis et al. (1998), AFP is an embryonic glycoprotein produced in the second month of pregnancy, where it is the main plasma glycoprotein synthesized by hepatocytes during development (Hau et al., 1981). Studies by Milunsky (1992) showed that this protein is synthesized by the gestational sac (yolk sac), gastrointestinal tract and mainly the fetal liver, but small amounts can be produced by the kidneys and placenta. According to Zizkovsky (1975), AFP is a protein synthesized by the yolk sac and fetal liver of most mammals, with still unknown function.

The biological function of AFP in the fetus remains unknown. It was believed to have only immune function in preventing rejection of the fetus by the mother (Daffos and Forestier, 1988). Yachnin et al. (1980) conclude that the AFP is immunosuppressive. Gorin et al. (1981) studied fetuses and concluded that this protein may be comparable to serum albumin in adults, the major protein for the developing fetus, which is synthesized by the liver and embryonic yolk sac. After birth, AFP concentrations decrease drastically to levels that are barely detectable in adult non-pregnant women. The decrease in this protein results in a gradual decrease of its rate synthesized by the liver and, in the case of rodents, the loss of the yolk sac. However, the synthesis of AFP in adult liver is resumed during its degeneration, and in specific tumors, such as hepatomas and teratocarcinomas. It has also been reported that there are structural and functional similarities between AFP and albumin, which led to the suggestion that AFP serves as a fetal albumin.

High concentrations of AFP and albumin in plasma help control plasma osmolality and intravenous fluid. AFP is also related to the mother's immune response and protection of the fetus in rodents, and to the effects of maternal estrogens. Lowseth et al. (1991) comments that this protein acts in the protection of neural cells from high levels of estrogen during fetal development. They further noticed that the level of AFP is significantly increased in hepatocellular carcinoma and embryonic tumoral cells in humans, which help in the diagnosis and monitoring of response and therapies. Samples of the yolk sac of the bovine embryo showed the presence of transferrin, which is close to β -galactosidase with a molecular weight of 111 kDa. Because the antibody is not specific it can be difficult because it probably cross-reacts weakly with bovine protein. In fact, nonspecific bands appeared when the membrane reacted

with the antibody, which may be explained by the blocking time. According to Brock et al. (1976), this protein has a molecular weight of 85 kDa and is mainly synthesized by the liver; it is involved in iron transport and shows a bacteriostatic effect *in vitro*. Also, *in vivo* experiments suggest that this mechanism may contribute to defense against pathogenic bacteria, indicating thus another possible physiological function of this protein. According to Streu et al. (2000), transferrin, a glycoprotein present in high concentrations in amniotic fluid, is involved in iron transport during pregnancy, meeting increased fetal iron demand.

It also suggests that this glycoprotein plays a role in the control of the progesterone production, modulating the endocrine function of trophoblasts. According to Carlson (1994), the placental surface has specific receptors for this protein. Apparently, the iron moves from transferrin on the placental surface, and is then actively transported to the fetal tissues.

We noted that in most samples (samples of yolk sac of bovine embryos 5, 6, 7, 9, 11, 12, 14, 15, and 16) there was human antibody reaction with bovine protein, and that this reaction appeared to be stronger in samples 3 and 9, representing the embryos with gestational periods of 0-15 days and 20-30 days, respectively. This protein has a molecular weight close to that of carbonic anhydrase, 30 kDa. According to Aires (1999), A1AT, a glycoprotein with a molecular weight of 52 kDa, is mainly produced by hepatocytes, which release into the circulation about 2 g protein daily. The main function of A1AT is to inhibit neutrophil elastase, a serine protease that has the ability to hydrolyze elastin fibers in the lung. This enzyme inhibits the action of other enzymes that break down proteins. The deficiency of A1AT allows the action of other enzymes affecting the lung tissue. According to Takashina et al. (2004), A1AT is a protease inhibitor produced by epithelial cells of the amnion during pregnancy and the hepatocytes throughout the life of the individual. Vatne et al. (1996) proved that A1AT acts as an inhibitor of elastase and trypsin, but at different binding sites. Its importance in inflammatory pulmonary diseases has also been noted, where it has the capacity to inhibit the migration of macrophages. A deficiency of this protein, with an unknown etiology, triggers clinical manifestations of different grades, such as liver cirrhosis and chronic obstructive pulmonary emphysema, and even panniculitis. This deficiency can be diagnosed in the gestational period in humans, by assessing the level of this protein (Richmond and Zellner, 2005). We cannot be sure about the size of the proteins under study. Bands were revealed, which may represent some other specificity, with a different molecular weight, since the primary antibodies used were not specific antibodies, and thus, the bands observed could be dimers or chains of the proteins studied.

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