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Comparative Evaluation of Alkaline Phosphatase, Lactate Dehydrogenase and Total Protein Between Fetal and Adult Sheep Liver and Their *In Vitro* Cultures

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ABSTRACT

ALP, LDH and protein production are important hepatic functional markers. Hepatocytes proliferation is positively correlated with ALP activity, and synthesis of liver-specific proteins. Hepatocytes release LDH in distress. Current study investigated ALP and LDH activities and total protein concentration in fetal and adult sheep liver tissues and their cultured hepatocytes, as well as the day-wise variations in the ALP activity and total protein concentration in their culture media during their proliferation. Liver tissues were processed by homogenization, and ultrasonication. Processed liver tissues were then subjected to ultracentrifugation. Supernatant was analyzed for ALP activity, LDH activity, and total protein concentration. Hepatocytes were cultured for one week. Their proliferation was characterized by analyzing these parameters in the culture medium on different days. ALP activity was higher in fetal liver tissues. LDH and total protein concentration was higher in adult liver tissues. These parameters increased after culture and were significantly higher in cultured hepatocytes. Day-wise analysis of the culture medium showed that ALP activity reached the maximum values earlier for fetal hepatocytes than that of adult. LDH activity was absent in culture medium, but total protein concentration reached maximum values on seventh day in both the cultures. Present work concluded that the ALP activity increases in the hepatocytes during the cell cuture. Moreover, it is a good marker to investigate hepatocytes proliferation, and fetal hepatocytes have a higher proliferation potential than adult hepatocytes.

HIGHLIGHTS

- ALP, LDH and protein secretion by hepatocytes are useful hepatic functional markers.
- All these parameters increased on *in vitro* hepatocytes culture of fetal and adult sheep.
- ALP activity and protein secretion was higher in culture of fetal hepatocytes.

Keywords: Alkaline phosphatase, Lactate dehydrogenase, Total Protein, Fetal, Adult, Liver

The ruminant liver can be affected by various diseases like plant toxicoses, liver flukes, mycotoxicosis, etc.. Liver diseases may progress into fatal liver failure from insufficient hepatic regeneration. Hepatic progenitor cells have shown great usefulness for cell therapy. In the liver, regeneration can occur either by replication of mature hepatocytes or by the proliferation of the local stem cell/progenitor cell population. Main hepatic epithelial cell population consists of hepatocytes, which are most abundant, and cholangiocytes (Zhang *et al.,* 2021).

Some important hepatic functional markers include alkaline phosphatase, lactate dehydrogenase, and protein production (Desmond Burke, 2002). ALP is a membrane-bound enzyme, which catalyses the hydrolysis of phosphate monoesters at alkaline pH. It is present in the canalicular membrane of hepatocytes (Lowe *et al.,* 2022). Tissue non-specific ALP is abundant in hepatic tissues (Sharma *et al.,* 2014). LDH is an oxidoreductase enzyme located in small amounts in various active organs (Shimizu, 2008). LDH had been assayed during drug

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toxicological studies in either supernatant media or within cells as hepatocytes released it in distress (Tolman *et al.,* 1978). In addition to producing intracellular proteins, hepatocytes are also responsible for generating and secreting most of the plasma proteins (Trefts *et al.,* 2017). Use of total protein is recommended to test the hepatic functions (Hayden and van Heyningen, 2001). Zhang *et al.* (2012) reported that measuring LDH, albumin, and urea levels in the supernatant of the cell culture can assess hepatocyte function.

As much literature is not available regarding the daywise variation in the amounts of ALP secretion by hepatocytes during culture, thus, present study was planned with the aim to investigate ALP, LDH, and total protein concentration in fetal and adult sheep liver tissues and their cultured hepatocytes, as well as the day-wise variations in the ALP activity and total protein concentration in the culture media of fetal and adult liver progenitor cells during their proliferation.

MATERIALS AND METHODS

Sampling

Present investigation was conducted at Department of Veterinary Physiology, MVC Chennai, Tamil Nadu. Liver samples (n=22) from adult sheep of age group one to three years were collected from Chennai Corporation Abattoir, Perambur and aborted fetuses (n=22) were collected from various sheep farms in Chennai. Afterwards, they were washed with PBS supplemented with 50µg/ml gentamicin sulphate and transported in sufficient ice to laboratory.

Liver tissue ALP activity, LDH activity and total protein concentration

Liver tissue was processed by the method of Keller (1973) for enzyme estimation. Briefly, liver tissue samples were rinsed in ice-cold saline, and homogenized as a 1:10 dilution with a 0.25 M sucrose solution for 3 minutes in an Omni-Mixer and then ultrasonicated for 4 minutes in a Branson-Sonifier, both with cooling jacket (0-2˚C). Homogenates were then centrifuged for one hour at 105000 g in a refrigerated ultracentrifuge (0-2˚C). The supernatant was analyzed immediately for ALP activity (ALP Assay Kit, MAK447, Sigma), LDH activity (LDH Assay kit, MAK066, Sigma) and total protein concentration (Protein Assay Kit TP0300, Sigma) by using autoanalyzer spectrophotometer (Biochemical autoanalyser, BioSystems A15 model). The cell culture media were also analyzed for ALP and LDH activities and total protein concentration by the same method.

Isolation of cells and cell culture

Isolation and culture of liver cells were performed according to the Hristova *et al.* (2009) protocol with some changes. In the current study, the pellet containing hepatocytes was resuspended and seeded in RPMI-1640 medium (RPMI-1640, Sigma-Aldrich, R6504), supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, F2442) and antibiotic-antimycotic solution (Hi-Media, A002). The cells were cultured for one week at 37 $^{\circ}$C, 5% CO_2 in 25-cm^2 culture flasks (Sigma-Aldrich). The proliferation of the cells in the culture was characterized by analyzing the ALP, LDH and total protein concentration in the culture medium on different days of the hepatocytes culture.

STATISTICAL ANALYSIS

Differences in ALP, LDH and total protein concentrations between before and after cell culture, as well as day-wise variations in ALP activities and total protein concentrations in culture media, in case of both fetal and adult sheep livers, was compared by one-way ANOVA and Duncan's multiple comparison test. The differences between ALP activities and total protein concentrations of fetal and adult hepatocytes in vitro cultures on different days of cell culture were analyzed by independent t test. All the tests were performed using SPSS, USA (16.00).

RESULTS AND DISCUSSION

Hepatic tissue ALP activity, LDH activity, and total protein concentration

All these parameters increased significantly ($P<0.05$) by the end of culture (Table 1). Before cell culture, fetal liver tissue samples showed significantly higher ($P<0.05$) mean ALP activity (2.17±0.06 IU/g) when compared to adult liver tissue samples (0.85 ± 0.06 IU/g). Similarly, mean ALP activity of cultured fetal hepatocytes (8.75±0.14 IU/g) was significantly higher ($P<0.05$) as compared

Table 1: Mean ± SE values of ALP activity, LDH activity, and total protein concentration of fetal and adult sheep liver hepatocytes before and after their in vitro culture

Parameters	Source of hepatocytes			
	Fetal Liver (n=22)		Adult Liver (n=22)	
	Before culture	After culture	Before culture	After culture
ALP activity (IU/g)	2.17 ± 0.05^{b}	8.75 ± 0.14^{d}	0.85 ± 0.06^{a}	4.86 ± 0.16^{c}
LDH activity (IU/g)	6.93 ± 0.18^{a}	16.77 ± 0.37^{b}	63.94 ± 3.88^{c}	100.77 ± 0.51^{d}
Total Protein concentration (mg/g)	68.45 ± 0.81^{a}	750.1 ± 9.41^{b}	86.68 ± 2.93^{a}	970.73 ± 9.82^{c}

^{a,b,c,d} within rows vary significantly from each other at least at $P<0.05$.

to that of cultured adult hepatocytes (4.86±0.15 IU/g). Keller (1973) reported a lower tissue ALP activity (0.45 IU/g) in adult sheep. Similarly, Collis and Stark (1977) also reported comparatively lower ALP activity (0.3 IU/g of liver wet weight) in the adult pig. However, Clampitt and Hart (1978) recorded higher tissue ALP activity (5 IU/g of liver wet weight) in adult sheep. ALP activity recorded in the liver, arise in it and did not accumulate from extrahepatic sites (Combes and Schenker, 1969). Sharma *et al.* (2014) observed significantly higher levels of ALP in young kids, which shows higher ALP activity in organs at young age. Moog (1965) reported that ALP activities were more extensively distributed in the embryo and all the embryonic tissues were rich in ALP levels at the early stages of development. These findings corroborate the observations of the present investigation. Moreover, higher ALP activity observed in the fetal liver is related to the fact that the normal rate of metabolism in tissues of the body is accompanied by a low level of ALP activity and higher levels of ALP activity could occur during hyperactivity of a tissue or organ (Collis and Stark, 1977). It can be inferred from these findings that the fetal liver cells have higher ALP activity, which can be due to their higher rate of metabolism and more proliferation potential.

The mean LDH activity of the adult liver tissue (63.94 ± 3.88 IU/g) was significantly higher ($P<0.01$) than fetal liver tissue (6.93 ± 0.18 IU/g). Similarly, after culture, the mean LDH activity of cultured adult hepatocytes (100.77±0.51 IU/g) was significantly higher ($P<0.01$) than that of cultured fetal hepatocytes (16.77±0.37 IU/g). Current findings corroborated with Keller (1973), who reported a tissue LDH activity of 64.26 IU/g of liver weight in adult sheep. In anaerobic conditions, LDH catalyses the conversion of pyruvate to lactate. Lactate is released into the bloodstream and delivered to the liver, where LDH completes the Cori cycle by converting lactate to pyruvate (Puranik *et al.,* 2021). As the animal attains the adult age, there may be an increase in blood lactate. This may contribute to an increase in hepatic LDH activity with the increase in age of the animal. Our result of low hepatic LDH activity during the fetal stage as compared to higher hepatic LDH activity at an adult age corroborates the findings of Singh and Kanungo (1968) who reported that the LDH activity increased in the supernatant fraction of the rat liver from a day old to 96 weeks of their age. The major LDH isoenzyme present in the liver is LDH-5 (Khan *et al.,* 2020). The proportion of LDH5 and LDH4 in fetal rat liver may vary according to the amount of oxygen available to the tissue (Arizmendi *et al.,* 1983). LDH-5/ LDH-4 ratios were nearly equivalent in the normal and regenerated adult rat liver (7.14, 6.41), but substantially lower in fetal rat liver (2.50) (Allalouf *et al.,* 1986), which in turn suggests a lower LDH 5 activity in the fetal liver as compared to adult liver. These reports indicate a lower LDH activity in the fetal liver as compared to the adult liver, which was in agreement with the current findings.

The mean total protein concentration of adult liver tissue (86.68 ± 2.93 mg/g) was non-significantly higher ($P>0.05$) than that of fetal liver tissue (68.45 ± 0.81 mg/g), but it was significantly higher ($P<0.05$) in cultured adult hepatocytes (970.73±9.82 mg/g) than cultured fetal hepatocytes (750.09±9.41 mg/g). Present findings corroborated with Tanaka *et al.* (1978) who reported that on culture, the rates of synthesis and secretion of the protein of both fetal and adult liver cells increased. Different reports with contradictory findings are available regarding the effects of age on liver protein synthesis. Ricca *et al.* (1978) reported a significant decrease in the rate of in vivo protein synthesis between 3 and 15 months of age in female Sprague-Dawley rats. Goldspink and Kelly (1984) reported an increase in the

fractional rate of protein synthesis by liver with age in male CD rats. Mays *et al.* (1991) reported that the mean fractional synthesis rate for the total protein pool within rat liver was unaltered from 1 to 15 months of age, after which it increased significantly up to 24 months of age. However, in the present investigation, the insignificant increase in protein concentration in adult liver samples may be the result of protein accretion in adult liver.

ALP activity, LDH activity, and total protein concentration in culture medium

ALP activity is estimated day-wise in the culture medium of hepatocytes culture for seven days to assess the proliferation rate of fetal and adult liver hepatocytes (Table 2).

Table 2: Day wise Mean ± SE values of ALP activities in culture media of fetal and adult sheep liver hepatocytes

Days of Culture	ALP Activity (IU/L)		t-test value
	Fetal Liver (n=22)	Adult liver (n=22)	
1	72.95 ± 0.47^{fX}	61.51 ± 0.49^{fY}	16.66^{**}
2	82.79 ± 0.67^{eX}	68.62 ± 0.57^{eY}	15.99^{**}
3	89.48 ± 0.67^{dX}	78.33 ± 0.55^{dY}	12.75^{**}
4	98.01 ± 0.65^{bX}	86.60 ± 0.80^{eY}	11.05^{**}
5	109.72 ± 0.94^{aX}	95.90 ± 0.73^{bY}	11.57^{**}
6	94.88 ± 0.57^{cX}	106.03 ± 1.31^{aY}	7.76^{**}
7	93.73 ± 0.58^{cX}	97.05 ± 0.87^{bY}	3.15^{**}

^{a,b,c,d,e,f} within columns, and ^{X,Y} within rows vary significantly from each other at least at P<0.05.

It increased significantly (P<0.05) in the fetal hepatocytes culture medium from first day (72.95±0.47 IU/L), and reached the highest value on fifth day (109.72 ± 0.94 IU/L), but insignificantly declined from sixth day (94.88 ± 0.57 IU/L) to seventh day (93.73 ± 0.58 IU/L) of culture. Similarly, the ALP activity in the adult hepatocytes culture medium increased significantly (P<0.05) from first day (61.51±0.49 IU/L), and reached the highest value on sixth day (106.03 ± 1.31 IU/L). However, it decreased significantly (P<0.05) on seventh day (97.05± 0.87 IU/L) of culture. The ALP activities in the culture medium of fetal hepatocytes were significantly higher (P<0.01) than that of adult hepatocytes on all days. Several studies have indicated the involvement of ALPs in cellular events such as the regulation of cell growth, apoptosis, and cellular migration during embryonic development (Sharma *et al.,* 2014). Horiuti *et al.* (1992) reported that ALP activity is positively correlated with hepatocytes proliferation. Lowe *et al.* (2022), stated that increased hepatic ALP activity occurs due to increased translation of the mRNA of ALP, and increased secretion of ALP occurs via canalicular leakage into the hepatic sinusoids. This ALP activity regulation was due to contact phenomena and crowding of the cells. Terryn *et al.* (2007) reported that ALP activity in seven-day-old primary proximal tubular cells culture is about 20 times more than in 14-day-old primary cultures, indicating the degree of differentiation is dependent on time in culture, and decrease in ALP with time takes place due to differentiation. ALP activity measured in seven-day-old cultures of proximal tubular cells is approximately 4 times higher than freshly isolated proximal tubule cells. These reports corroborate well with the findings of the current study that ALP activity initially increased and later decreased in the culture medium. In this study, LDH activity was assayed in the culture media on different days of cell culture, however, it was found to be absent in it.

Day-wise estimation of total protein concentration was performed in culture media of fetal and adult hepatocytes to assess their proliferation. It increased steadily from day one till the last day of culture (Table 3).

Table 3: Day wise Mean±SE values of total protein concentration in culture media of fetal and adult sheep liver hepatocytes

Days of Culture	Total Protein concentration (g/dl)		t-test value
	Fetal Liver (n=22)	Adult liver (n=22)	
1	0.72 ± 0.01^{fX}	0.65 ± 0.01^{gY}	6.48^{**}
2	0.80 ± 0.01^{eX}	0.72 ± 0.01^{fY}	9.08^{**}
3	0.87 ± 0.01^{dX}	0.80 ± 0.01^{eY}	7.81^{**}
4	0.93 ± 0.01^{cX}	0.87 ± 0.01^{dY}	6.06^{**}
5	0.98 ± 0.01^{cX}	0.95 ± 0.01^{cY}	4.64^{**}
6	1.39 ± 0.03^{bX}	1.01 ± 0.01^{bY}	9.57^{**}
7	1.56 ± 0.02^{aX}	1.32 ± 0.02^{aY}	6.71^{**}

^{a,b,c,d,e,f} within columns, and ^{X,Y} within rows vary significantly from each other at least at P<0.05.

It increased from day 1 (0.72±0.01 g/dl) and reached the highest value on day 7 (1.56±0.02 g/dl) in the culture medium of fetal hepatocytes. Similarly, it increased from day 1 (0.65±0.01 g/dl) and reached the highest value on

day 7 (1.32±0.02 g/dl) in the culture medium of adult hepatocytes. The differences between days within both the groups were highly significant (P<0.05), except between day 4 and day 5 in the fetal group. Moreover, it was significantly higher (P<0.01) in the culture medium of fetal hepatocytes than that of adult hepatocytes on all days of culture. Isolated hepatocytes synthesize liver-specific proteins, such as albumin, fibrinogen, transferrin (Jeejeebhoy *et al.,* 1975), transcobalamin II (Savage and Green, 1976), and lipoproteins (Tarlow *et al.,* 1977). Nishimura *et al.* (2010) reported the secretion of albumin to the culture medium in hepatocytes culture. Goldspink *et al.* (1985) reported a 50% decrease in protein synthesis by rat liver from fetal stage to 26 months of age, which is in agreement with the findings of the present study that a higher level of protein secretion was observed by the fetal hepatocytes.

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