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Original Research

Effect of Thermal stress on Functional Properties of Caprine Hepatocytes Culture *in Vitro*

Phad Ashok Vyankati, Anand Laxmi*

.Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal-132001, Haryana, India

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Abstract

Liver is involved in several vital functions such as synthesis, secretion, storage, metabolism of plasma proteins, detoxification of ammonia to urea. Digestion of liver tissue with collagenase to separate hepatocytes was done by non perfusion technique. The activity of enzyme Lactate Dehydrogenase (LDH) as a marker for assessing the integrity of cell membrane, concentration of albumin, urea and transforming growth factor beta (TGF-β) in the supernatant of hepatocyte culture were used as markers of functionality of hepatocytes. The present study was conducted to evaluate the effect of thermal stress on caprine hepatocytes culture on all the above mentioned parameters at different time intervals. At first, caprine hepatocyte culture was optimized at 37°C. After optimization, the hepatocytes culture was subjected to three different temperatures i.e. 37°C, 40°C and 42°C in different CO_2 incubators under controlled humidified conditions. The culture was performed in 6 well plastic culture dishes. Supernatant was harvested at 24, 48 and 72 hours respectively from the hepatocyte cultured plates for estimation of the different mentioned parameters. It was observed that LDH activity at 37°C was greater at 48 hours and decreased by 72 hours of culture, when hepatocytes culture incubated at 40 and 42°C, activity of LDH temporally increased significantly ($P<0.01$) by 48 hours and was maintained till 72 hours, there was a significant increase in the concentration of albumin ($P<0.01$) and urea ($P<0.05$) at 72 hours of incubation at 37°C, whereas at 40°C and 42°C decrease in the secretion of urea and albumin was observed. Least Square Mean concentration of hepatocytes TGF-β was significantly greater ($P<0.01$) at 42°C when compared with LSM value at 37°C.The net effect observed was that when caprine hepatocytes were subjected to hyperthermic conditions, the function of hepatocytes decreased and TGF β secretion was significantly greater at 40°C and 42°C, indicating that caprine hepatocytes suffered from thermal stress over a period of 48-72 hours of incubation at temperatures higher than 37°C. It was also reflected by the significant decrease in the viability the cells at 42°C post 48 hours of incubation.

Keywords: Albumin; Caprine; Hepatocytes; LDH; TGF-β

Introduction

In mammals, liver is the central organ for energy metabolism, synthesis of plasma proteins, detoxification of ammonia to urea and for many metabolic, synthetic and storage functions. Hepatocytes culture therefore serves as a useful model to study pharmacological and toxicological effects (Aninat *et al*., 2008; Nussler etal., 2001). Spotorno *et al*. (2006) isolated bovine hepatocytes by non perfusion technique, involving disaggregation of cells with collagenase solution, instead of perfusion technique. In the present study also hepatocytes were obtained from goat liver piece by non- perfusion technique. Studies with hepatocytes has revealed that estimation of albumin, urea and lactate dehydrogenase (LDH) in the supernatant of culture can act as indices of optimal hepatocytes function. In hepatocytes, transforming growth factor beta (TGF-β) has been associated with inhibition of cell proliferation in regulating the excessive proliferation of hepatocytes (Oberhammer *et al*., 1991) during hyperthermic conditions. TGF-β is part of a complex cellular program, which establishes thermo-tolerance to protect cells from further insults (Kathleen *et al*.,1993).TGF- β causes increase in reactive oxygen species (Herrera *et al*., 2003) which may cause stress.

The purpose of this study was to evaluate the effect of different temperatures 37°C and > 37°C i.e. 40°C and 42°C on the morphology and physiology of hepatocytes in terms of LDH activity and concentration of albumin , urea and TGF-β in hepatocytes culture. A temperature of 37°C is considered an optimum temperature for carrying out *in vitro* experiments. Temperatures higher than 37°C may

*Corresponding author: Anand Laxmi

E-mail address: dnana44@yahoo.co.in

create thermal stress reflecting change in functional properties.

Materials and methods

Petri-dishes and six well culture dishes were purchased from M/s Genetix Bitotech Asia Pvt. Ltd. New Delhi. All the other plastic wares were purchased from Axiva Sichem, Bitotech., New Delhi. The culture media used for the culture of caprine hepatocytes was Dulbecco's modified eagle's medium with Ham's F12 nutrient mixture.The other solutions used were RPMI-1640, Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (DPBS). The additional supplements added to culture media, included insulin, tri-iodo-thyronine sodium, dexamethasone and antibiotics penicillin and streptomycin which were purchased from Sigma Chemicals Pvt. Ltd., New Delhi. Enzymes Collagen Type IV and Trypsin-EDTA, were also purchased from Sigma chemical Co. Fetal bovine serum was purchased from Hyclone (Logan, Utah, USA). For the assay of LDH enzyme activity, estimation of albumin and urea in hepatocytes culture, kits were purchased from Span Diagnostics Ltd. Gujrat,INDIA. EIA Kit for the estimation of TGF β I was purchased from Biotech Inc. USA.

Liver from healthy goat was collected immediately after slaughter. The caudate lobe of the liver was excised. The excised piece was washed and briefly dipped in 70% alcohol and aseptically transferred to phosphate buffered saline present in a flask maintained at 4°C. Approval from Ethic's committee was obtained for carrying out research.

Isolation of caprine hepatocytes

Hepatocytes isolation was performed by non perfusion technique. Liver piece was finely minced with scissors, and washed with Ca++, Mg++ free PBS until blood clots were removed. The sample was transferred to a conical flask and treated with collagenase Type IV solution (500ug ml^{-1} in HBSS-HEPES buffer - 50 ml collagenase solution per 2-5g of tissue) and softly stirred for 10-15 min. The cell suspension was filtered through cheese cloth and centrifuged at 50g for 8 min. at 4°C. The cells pellet was washed with PBS containing antibiotic solution. Finally the pellet was suspended in DMEM/Ham's F12 medium containing 10% Fetal bovine serum, 24mM $NaHCO_3$, 2mM L-Glutamine, 100nM Dexamethasone, 1µmol/L Insulin Transferrin, Pencillin and Streptomycin were added as antibiotics.

Exposure of hepatocytes culture to different temperatures

Hepatocytes were seeded in to six well plates at a density of 4.5 $x10^5$ viable cells/cm^2 per well in 2 ml of DMEM/Ham's F12 medium. The cells were cultured at 37ºC, 5% CO_2 in a humidified atmosphere under controlled conditions in a CO_2 incubator. After 4-6 hours of seeding, cells firmly attached to the bottom of the plate. It was observed that 85% of the cells were viable.

The monolayer of hepatocytes thus formed was washed with HBSS. After the washing step, DMEM/Ham's F12 containing 10% FBS was added to each well. The mentioned procedure was repeated for three plates. Each hepatocytes plate was subjected to different temperatures i.e. 37°C, 40°C and 42°C in different CO_2 incubators. The experiment was repeated six times and each time, samples were run in duplicates. The supernatant from each well was collected at 24, 48 and 72 hours respectively.

The supernatant was used for the assay of LDH, albumin and urea at different temperatures and also at respective different time intervals. TGF-β was also estimated by Enzyme Immuno Assay. All the parameters were estimated using commercially available kits and according to manufacturer's protocol.

Statistical analysis

Data was statistically analyzed by SAS software, version (9.1) of the SAS system for windows operating system, SAS Institute Inc., Cary, NC, USA. Data is expressed as Mean ± SE and analyzed by ANOVA, considering the different temperatures and time of incubation as variables. Results exhibiting significant effect were compared by the least significant difference pair wise multiple comparison test. Difference was considered statistically significant at $P<0.01$.

Results

The activity of LDH, metabolic parameters like al-

bumin and urea production was estimated in cell culture supernatants of caprine hepatocytes culture at different time periods i.e. 24, 48 and 72 hours maintained at three different temperatures i.e. 37°C, 40°C and 42°C.

Lactate Dehydrogenase:

Least square mean (LSM) of the LDH activity at 42°C was significantly greater (P<0.01) than the activity observed at 40°C and 37°C. The enzyme activity at 40°C being significantly greater than the activity at 37°C .The leakage of LDH enzymes was determined by the activity of LDH in the supernatant of cell culture. The activity of LDH at 48 hours was significantly greater (P<0.01) than the activity observed at 24 and 72 hours. At 40°C and 42°C also, the activity of LDH significantly increased (P<0.01) from 24 to 72 hours; with no significant difference between 48 and 72 hours (Table 1).

Albumin

The Least Square Mean values for albumin secretion at different temperatures when compared, were significantly different. The LSM values at 40°C and 42°C were significantly less (P<0.01) than the LSM value at 37°C, while no significant difference was observed between 40 and 42°C. At 37°C the concentration of albumin in supernatants of hepatocytes culture was significantly greater at 72 hours when compared with concentration of secreted albumin at 24 and 48 hours. At 40°C, no significant difference was observed between different concentrations of albumin from 24 till 72 hours of incubation. At 42°C, the concentration of albumin decreased (P<0.01) at 48 hours when compared with 24 hours, which decreased further at 72 hours, though observed to be not significant (Table 2).

Urea

The LSM concentration of urea at 40 and 42°C was significantly less (P<0.05) than the LSM concentration at 37°C. The concentration of urea in the supernatants of hepatocyte cell culture increased significantly (P<0.05) at 72 hours when compared with concentrations at 24 and 48 hours respectively. At 42°C, the concentration of urea decreased significantly (P<0.05) at 48 hours and then increased at 72 hours (Table 3).

Table 1. Mean± SE of LDH activity (IU/ml) in caprine hepatocytes culture

Duration of incubation (Hours)	Temperature (°C)		
	37	40	42
24	$79^{c} \pm 0.59$	$120.67^{b} \pm 1.45$	$161.33^{a} \pm 1.45$
48	$89.33^{a} \pm 0.88$	$143^{b} \pm 2.08$	$176^{b} \pm 1.15$
72	$69^{a} \pm 1.53$	$148.73^{b} \pm 1.76$	$173.67^{b} \pm 2.60$
Least Square Mean (LSM)	71.11^{C}	140.67^{B}	170.33^{A}

In a column values with different small super scripts differed significantly (P<0.01).
In a row LSM, values with different capital superscripts differed significantly (P<0.01).
The values are mean of six trials and sample in each trial was run in duplicate.

Table 2. Mean± SE of conc. of albumin (mg/dl) in caprine hepatocytes culture

Duration of incubation (Hours)	Temperature (°C)		
	37	40	42
24	$0.74^{a} \pm 0.02$	$0.66^{a} \pm 0.01$	$0.83^{b} \pm 0.02$
48	$0.74^{a} \pm 0.02$	$0.69^{a} \pm 0.01$	$0.56^{a} \pm 0.03$
72	$1.33^{b} \pm 0.02$	$0.65^{a} \pm 0.02$	$0.5^{a} \pm 0.01$
LSM	0.94^{A}	0.66^{B}	0.63^{B}

In a column values with different small super scripts differed significantly (P<0.01).
In a row LSM, values with different capital superscripts differed significantly (P<0.01).
The values are mean of six trials and sample in each trial was run in duplicate.

Table 3. Mean± SE of concentration of urea (mg/dl) in caprine hepatocytes culture

Duration of incubation (Hours)	Temperature °C		
	37	40	42
24	$9.86^{a} \pm 0.29$	$9.75^{a} \pm 0.38$	$9.92^{a} \pm 0.50$
48	$10.43^{a} \pm 0.88$	$8.5^{a} \pm 0.58$	$8.57^{b} \pm 0.84$
72	$13.83^{b} \pm 0.82$	$12.62^{b} \pm 0.69$	$11.56^{b} \pm 0.58$
LSM	11.37^{A}	10.29^{AB}	10.01^{B}

In a column values with different small super scripts differed significantly (P<0.05).
In a row LSM, values with different capital superscripts differed significantly (P<0.05).
The values are mean of six trials and sample in each trial was run in duplicate.

Table 4. Mean± SE of concentration of TGF-β (ng/ml) in the supernatant of caprine hepatocytes culture

Duration of incubation (Hours)	Temperature °C		
	37	40	42
24	$21.56^{a} \pm 1.39$	$19.69^{a} \pm 0.79$	$29.63^{a} \pm 0.86$
48	$20.81^{a} \pm 0.66$	$20.84^{a} \pm 1.01$	$29.42^{a} \pm 1.74$
72	$20.2^{a} \pm 0.82$	$21.88^{a} \pm 0.34$	$35.04^{b} \pm 1.04$
LSM	20.86^{A}	20.80^{A}	28.42^{B}

In a column values with different small super scripts differed significantly (P<0.01).
In a row LSM, values with different capital superscripts differed significantly (P<0.01).
The values are mean of six trials and sample in each trial was run in duplicate.

Transforming growth factor beta

The LSM value for TGF β concentration was significantly greater at 42°C (P<0.01) when compared with LSM value at 37°C and 40°C. It was observed that TGF β secretion at 37°C and 40°C was not significantly different at different times of incubation. However, at 42°C, the concentration of TGF β was significantly greater at 72 hours than the concentrations at 24 and 48 hours, but no significant difference was observed between 24 and 48 hours time period of incubation (Table 4).

Discussion

Hepatocytes are useful experimentally to study many biochemical reactions undergoing in liver (Dvorak *et al*., 2007; Guillouzo, *et al*., 1990). Active hepatocytes synthesize albumin, which is an important maker for its functionality. They can metabolize ammonia to urea. Albumin and urea are important indicators for cellular functions of hepatocytes. It has been suggested that urea and albumin secretion and hepatocyte damage in terms of LDH activity can be used as markers for hepatocyte function (Dickens *et al*., 2008; Wu *et al*., 2009).The viability of the cells was maximum at 37°C, which decreased, at higher temperature. But the decrease was significant (P<0.05) only at 42°C at 72 hours of incubation.

Concentration of albumin and urea in the supernatants of hepatocytes culture increased temporally at 37°C indicating optimum hepatocyte function, but at higher temperatures, the concentration of these parameters decreased post 24 hours, which was significant with increase in time of incubation. This indicates that functionality of the hepatocytes decreased at higher temperatures (40°C and 42°C) with increase in time of incubation.

Yang *et al.* (2005) reported higher LDH leakage with lower viability or cell membrane integrity of the cattle. It reflects the cell membrane damage. Increase in the LDH activity and decrease in the secretion of albumin and urea in culture at 40°C and 42°C when compared with respective LSM values at 37°C, indicates that viability and function of hepatocytes was greater at 37°C. Even though the core temperature of the body is 40°C, *in vitro* experiments revealed, 37°C to be most suitable for conducting culture experiments with goat hepatocytes.The role of TGF-β with respect to hyperthermia has been conducted in human and rat species in different types of cell cultures *in vitro* (Flanders *et al*., 1993; Banh *et al*., 2007; Cai *et al*., 2011) and

it has been reported that under hyperthermic conditions its expression is induced and it is helpful in inducing production of heat shock proteins which have a protective effect on the cell culture system.

No literature is available on the effect of hyperthermic conditions on the cell functionality of goat hepatocytes. From the present study it can be stated that , temperatures higher than 37°C are stressful to hepatocytes *in vitro*. It is suggested that *in vivo* there may be some mechanism, that controls the physiology of the goat at a core body temperature of 40°C.

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