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ORIGINAL ARTICLE

Effect of Thermal Stress on Caprine Hepatocyte's Antioxidant Enzymes Activity and TGF β Secretion *In Vitro*

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Liver is involved in several vital functions such as synthesis, secretion, storage and metabolism of plasma proteins and in detoxifying functions. In the present study, hepatocytes were obtained by disaggregation of caprine liver caudate lobe. The cells were cultured under controlled conditions in a CO_2 incubator at different temperatures. The function of hepatocyte culture was evaluated in terms of secretion of albumin, urea and acitivity of Lactate dehydrogenase. Cells were cultured at three different temperatures, i.e. 37, 40 and 42° C, and at each temperature, the culture was propagated for 24,48 and 72h time interval respectively. At 37°C, the concentration of albumin and urea increased in the culture temporally where as activity of LDH decreased by 72h. The least square mean (LSM) concentration of albumin, urea in culture decreased and activity of LDH increased significantly (P<0.01) at 40 and 42°C, when compared with respective values at 37°C. Hence effect of thermal stress(40 and 42°C) on activity of hepatocyte antioxidant enzymes (super oxide dismutase, catalase and glutathione peroxidase) and TGF β secretion *in vitro* was further evaluated. It was observed that there was significant increase (P<0.01) in the activity of the antioxidant enzymes . The LSM activity of all the enzymes increased significantly at 40 and 42°C when compared with the enzyme activity at 37°C, but increase in the secretion of TGF β could be observed only at 42°C post 48h of incubation. TGF β is associated with apoptosis and cytotoxicity. It was observed that viability of the cells decreased significantly (P<0.05) only at 42°C post 48h of incubation. It can be concluded that hyperthermic conditions were observed to be stressful for hepatocytes during *in vitro* conditions, which proved to be more significant at 42°C post 48h of incubation. Further work has to be carried out with some supplements which can reduce the hyperthermic effect. In vivo studies in caprine species will give further insight on thermal stress. To the best of our knowledge no studies on hyperthermic effect on hepatocytes in caprine species *in vitro* or *in vivo* has been reported.

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The liver is an important organ for regulation of metabolism in animals and humans. Hepatocytes are therefore a useful model for research studies on metabolism, cytotoxicity etc. (Dvorak *et al.*, 2007). Heat stress is one of the wide varieties of factors which cause oxidative stress *in-vivo*. Reactive

oxygen species (ROS), the major culprits for causing oxidative stress, are constantly generated *in vivo* as an integral part of metabolism. ROS may cause oxidative stress when their level exceeds the threshold value. They trigger progressive destruction of polyunsaturated fatty acids (PUFA), ultimately leading to membrane destruction (Kumar *et al.*, 2011, review). Several studies indicate the involvement of heat-mediated oxidative stress in Heat-induced cytotoxicity. Two major classes (enzymic and non-enzymic) of endogenous protective mechanisms work together to reduce the harmful effects of oxidants in the cell. Primary antioxidant enzymes include superoxide dismutase GSH peroxidase, and catalase; these enzymes are responsible for removing superoxide radicals, H_2O_2 and organic hydroperoxides, and H_2O_2 respectively (Powers *et al.*, 1999). After exposure to heat, increased levels of superoxide anions, hydrogen peroxide and nitric oxide as well as increased lipid peroxidation products have been found in various cell lines and in tumor tissue.SOD (super oxide dismutase), CAT (catalase), and glutathione peroxidase (GPx) are the main cellular ROS (reactive oxygen species) degrading enzyme systems. SOD converts the superoxide radical (O) into hydrogen peroxide, which is metabolized further by CAT and glutathione peroxidase (Frank *et al.*, 1998). Oxidative process that is regularly going on in cell is essential for life and death of a cell. Beneficial biological functions such as apoptosis, necrosis, and phagocytosis are mediated by reactive oxygen species (Noori, 2012). TGF β is a pleiotropic growth factor that plays an important role in cell proliferation, cell growth arrest, senescence, apoptosis, and differentiation (Puceat, 2007). TGF β can cause liver disease by disrupting the normal homeostasis between cell proliferation and apoptotic cell death (Michalopoulos and De Frances, 1997). It is known that TGF β also induced oxidative stress accompanied by NADPH oxidase 4 (Nox4) mRNA up-regulation and decreased protein levels of antioxidant enzymes. Antioxidants inhibit both TGF-b-induced caspase 3 activity and Bmf up-regulation, revealing an oxidative stress-dependent Bmf regulation by TGF β (Martınez-Palacian *et al.*, 2013).

MATERIALS AND METHODS

The culture media used in the present study for the culture of caprine hepatocytes was tissue culture medium Dulbecco's modified eagle's medium nutrient mixture f-12 ham (DMEM/Ham F12), RPMI-1640, Hanks balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (DPBS). The supplements added to the culture media, which included insulin-transferrin sodium selenite, triiodo-thyronine sodium, and antibiotics (penicillin and streptomycin) and antifungal amphotericin B was purchased from Sigma Chemicals Pvt. Ltd., New Delhi. All the cell culture media was in the form of ready-to-use liquid media. Enzymes (Collagenase Type IV and Trypsin-EDTA), Trypan blue solution, and other chemicals used was also from Sigma chemical Co., unless otherwise indicated.

Most of the chemicals used was of cell culture grade. Fetal Bovine Serum (FBS) was purchased from Hyclone (Logan, Utah, USA). For the assay of LDH enzyme activity, estimation of albumin and urea in hepatocyte culture, kits were purchased from Span Diagnostics Ltd. Gujarat, INDIA. EIA Kit for the estimation of TGF-β, SOD, and GPx enzyme activity were purchased from Biotech Inc. (USA) and Catalase from USCNK Life sciences Inc. (China).

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

Hepatocyte 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 (500 ug 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 cell 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, Penicillin and Streptomycin were added as antibiotics.

Hepatocyte culture subjected 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 37°C, 5% CO_2 in a humidified atmosphere under controlled conditions in a CO_2 incubator. After 4-6 h of seeding, cells firmly attached to the bottom of the plate. It was observed that 85% of the cells were viable. Viability of the hepatocytes was estimated by Trypan blue exclusion test (Chesne *et al.*, 1993)

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 hepatocyte plate was subjected to different temperatures i.e. 37^0C, 40^0C and 42^0C 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 h, 48 h and 72 h 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, SOD, Catalase and GPx enzyme activity were also estimated by Enzyme Immuno Assay. All the parameters were estimated using commercially available kits and according to manufacturer's protocol and as described in detail (Ashok, 2013).

Evaluation of hepatocyte function

Lactate dehydrogenase release, urea and albumin production was used as indices of hepatocyte function (Puviani *et al*., 1998). Hepatocyte culture medium from six well plates was collected at the different time intervals and centrifuged at40C for 8 min (12,000 g). Supernatants was collected and stored at - 20^0C, until concentration of TGF β, albumin, urea and LDH activity were estimated.

Preparation of hepatocyte cell lysates

Keeping the temperature parameter constant at different time interval, the hepatocytes were harvested. The cell monolayer were washed three times with ice-cold Ca^{2+}/Mg^{2+} free phosphate-buffered saline and harvested by using 0.02 % EDTA. Hepatocyte lysates were prepared by using 1 ml. Cold Tris buffer (20 mM Tris–HCl, pH 7.5, 2 m M

EDTA and 0.1% peroxide-free Triton X-100) and ultrasonication for 30 second in ice water and centrifuged at 12,000×g for 15 min at 4°C. The supernatants were aliquoted and stored at -20°C for subsequent analysis of antioxidant enzymes. The same procedure was repeated for cultures at different temperatures.

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 least square mean value (LSM) for the LDH activity at 37° was significantly different (P<0.01) from the values at 40˚C and 42˚C respectively. The value for the enzyme activity was greatest at 42˚C. The LSM value for urea concentration in the supernatant of culture at 37˚C was significantly greater (P<0.01) than the values at 40 and 42˚C. The LSM concentration of albumin in the supernatant of culture was also observed to be significantly greater (P<0.01) at 37˚C than at rest of the incubated temperatures (Table 1).

After obtaining the results for LDH activity, concentration of urea and albumin in the supernatant of hepatocyte culture, It was observed that at 37°C the cells exhibited the normal trend for the parameters and hence experiments were repeated for estimation of concentration of TGF β in the supernatant of cell culture and hepatocyte antioxidant enzymes activity namely Super oxide dismutase, Catalase and Glutathione peroxidase at different time intervals for different temperatures of incubation. Group of hepatocytes incubated at 37˚C, served as control for the groups, which were subjected to hyperthermic conditions.

The LSM value for TGF β concentration in the supernatant of cell culture was observed to be significantly greater (P<0.01) at 42˚C when compared with the LSM values at 37 and 40˚C. The secretion/concentration of TGF β at 42˚C at an interval of 72h of incubation was greater when compared with the Mean±SE values at 37˚C with respect to any of the time of incubation (Table 2). The TGF β concentration was found to be inversely related to the percentage of viable sperms (Table 6).

The LSM value for hepatocyte SOD activity was greatest (P<0.01) at 42˚C, the enzyme activity increased with increase in temperature. Similarly LSM value for hepatocyte antioxidant enzymes catalase and glutathione peroxidase activity was observed to be significantly greater (P<0.01) at 42˚C, when compared with LSM value at 37 and 40˚C (Table 3, 4, 5). The Mean ± SE of SOD activity increased temporally from 24 h till 72h of incubation, which was observed to be significant (P<0.01). This trend was observed at all temperatures (Table 3). When the temporal activity of the enzymes at different temperatures was evaluated, it was observed that at 37˚C, catalase enzyme activity increased significantly (P<0.05) post 48h of incubation, whereas at 40 and 42˚C, the significant increase (P<0.05) in the activity of enzyme could be observed from 24h of incubation itself (Table 4). At 42˚C hepatocyte GPx enzyme activity increased from 24h till 72 h of incubation. However at 37 and 40ºC the Mean±SE value of GPx activity significantly increased at 48h and then

decreased at 72htime of incubation, which was significantly less (P<0.01) than the Mean±SE activity estimated at 48h. However the increase recorded was not less than that of the Mean±SE enzyme activity observed at 24h of incubation (Table 5). Percentage of hepatocytes viable at different temperatures and times of incubation is given in Table 6.

Table 1. Least Mean Square concentration/activity of parameters in the supernatant of hepatocyte culture

Parameter	Temperature °C	
Concentration/activity	37	40
	LSM	
Albumin (mg/ml)	0.94^{A}	0.66^{B}
Urea (mg/dl)	11.37^{A}	10.29^{AB}
LDH activity(IU/ml)	71.11^{C}	140.67^{B}

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 ± S E of conc. of TGF -β (ng/ml) in the supernatant of caprine hepatocyte culture

Duration of incubation	Temperature		
	37°C	40°C	42°C
24h	21.56^{a} ±1.39	19.69^{a}±0.79	29.63^{a}±0.86
48h	20.81^{a} ±0.66	20.84^{a}±1.01	29.42^{a}±1.74
72h	20.2^{a} ±0.82	21.88^{a}±0.34	35.21^{b}±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.

Table 3. Mean± SE activity (IU/ml) of SOD enzyme of hepatocyte lysates at different temperatures and times of incubation

Duration of incubation (h)	Temperature °C		
	37	40	42
24	67.8^{a}± 1.91	611.13^{a}± 0.81	910.15a± 0.33
48	95.25^{b}± 1.24	969.04^{b} ±1.81	955.59b± 0.66
72	142.21^{c}± 1.77	1039.8^{c}± 1.48	1042.59c± 1.79
LSM	101.75^{C}	873.32^{B}	969.45^{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 4. Mean± SE activity (IU/ml) of Catalase enzyme of hepatocyte lysates at different temperatures and times of incubation

Duration of incubation (h)	Temperature °C		
	37	40	42
24	111.92^{a}± 1.44	148.81^{a} ±2.05	207.92^{a}± 1.47
48	115.24^{a}± 0.33	206.57^{b}±1.45	229.56^{b}± 0.91
72	156.42^{b}± 1.47	231.45^{c}±1.19	236.27^{c}± 2.08
LSM	127.86^{A}	195.61^{B}	224.59^{C}

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 5. Mean±SE activity (IU/ml) of Glutathione peroxidase enzyme of hepatocyte lysates at different temperatures and times of incubation

Duration of incubation (h)	Temperature °C		
	37	40	42
24	127.86^{a}± 0.78	195.76^{a}± 1.18	181.98^{a}±1.44
48	229.59^{b}± 0.76	203.85^{b} ±0.95	255.28^{b}±1.46
72	205.39^{c} ±1.6	255.28^{c} ±1.46	267.51c±1.47
LSM	187.61^{C}	198.76 ^{B}	234.92 ^{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 6. Effect of different temperatures on percentage of viable hepatocytes *in vitro* at different times of incubation

Parameter	Temperature °C		
Time (h)	37	40	42
	Percentage of viable cells		
24	81^{Aa}	78^{Ba}	77^{Aa}
48	76^{Aa}	74^{Ba}	70^{Aa}
72	74^{Aa}	71^{Ba}	60^{Bc}

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.

DISCUSSION

Hepatocytes are highly differentiated cells. Albumin and urea are used as indicators of hepatocyte function in vitro (Wu *et al.*, 2009). Even LDH enzyme activity is used as marker for evaluating cell damage (Dickens *et al.*, 2008). From LSM values for enzyme activity and concentration of albumin and urea, it clearly indicated that LDH enzyme activity increased significantly in the supernatant when hepatocytes were exposed to 40 and 42˚C when compared with the estimated activity at 37˚C. So, also, the secretion of albumin and urea decreased significantly under hyperthermic conditions when compared with the values estimated at 37˚C. From this study one can say that, exposing hepatocytes to hyperthermic conditions for 72 h, could definitely decreases the activity of hepatocytes. Hence, it can be suggested that culture of hepatocytes were under thermal stress, when exposed to 40 and 42˚C for 72 h period of incubation. Normally oxidative process is regularly going on in the cell, which is essential for life and death of a cell. Molecular oxygen leaves unstable free radicals, and these radicals bring about biological functions like apoptosis, phagocytosis etc. (Noori, 2012). It has been postulated earlier that, heat mediated oxidative stress leads to increased level of superoxide anions, hydrogen peroxide and nitric oxide in various cell lines. SOD, CAT and Glutathione peroxidase are the main ROS degrading enzyme systems (Frank *et al.*, 1998; Matsumoto *et al.*, 1999). When a higher endogenous antioxidative capacity increases, there will be decrease in ROS generation, it is considered as a first line of defense against heat induced pathways. (Katschinski *et al.*, 2000; Wang *et al.*, 2013). With the increase in the activity of antioxidant enzymes, the cells try to counteract the acute oxidative stress for eliminating reactive oxygen species. It is known that GPx along with SOD and Catalase forms the main antioxidative system and is a cellular protector against oxidative damage

(Gupta, 2006; Salvi, 2007)

The significant increase (LSM value) in concentration of TGF-β in hepatocyte culture at 42°C suggests, that the increase might have been for cell growth arrest, apoptosis, as, this factor is known to mediate the effect of ROS in cell lines (Yoon *et al.* 2005). It is known that increased antioxidants activity inhibits TGF β as a method of counteract (Marlinezfalacia *et al.* 2013). In the present study, TGF β concentration had increased significantly only at 42^{0}C post 48h of incubation, which suggests that cells did not suffer apoptosis or mitochondrial dysfunction at 37^{0}C or 40^{0}C. May be post 48 h of incubation, cells might have undergone some physiological changes. In the present study, studies were not conducted on apoptosis or other factor, hence, further studies are required in this direction. But significant increase in the activity of antioxidant enzymes at all the three temperatures at 72 h when compared with the activity at 24 h of incubation indicates that exposure to acute hyperthermic condition is increasing the activity of antioxidant enzymes, possibly may be due to increased ROS production, as a normal physiological mode of regulation. Since percentage of viable hepatocytes estimated at different times of incubations at 37 and 40^{0}C temperatures were not significantly different. The significant decrease in the number of viable hepatocytes was observed only at 42^{0}C post 48 h or 72 h of incubation. The coincident significant increase in TGF- β concentration and decrease in percentage of viable cells post 48 h at 42^{0}C, suggests detrimental physiological process to set in culture with continuous exposure to hyperthermic condition. At 37^0 or 40^{0}C the endogenous antioxidant enzyme machinery was able to reduce the inflammatory effects of oxidants which did not cause increase in TGF- β secretion significantly (Konig *et al.*, 2001). Heat stress is one of the factors, which causes oxidative stress in vivo. A decrease in heat induced ROS generation via endogenous antioxidative capacity confers resistance against that induced apoptosis (Katschinski *et al.*, 2000; Kumar *et al.*, 2011). Studies on chondrocytes by Jallali *et al.* (2007) suggested that TGF- β secretion exhibits an acute pro oxidant effect, which may also be not cytotoxic. It appears that certain concentration of oxidants in the system should be present to have stimulatory effect on TGF- β secretion. At 37 and 40^{0}C, the level of oxidants in hepatocyte culture was able to stimulate the activity of hepatocyte antioxidant enzymes but not TGF β secretion in to supernatant. Only at higher temperature at 42^{0}C, increase in TGF β secretion and activity of antioxidant enzymes reflects that post 48h of incubation, may be cells were in a state of stress and cytotoxic conditions resulting in significant decrease ($P<0.05$) in percentage of viable hepatocytes (71%). Significant Increase in the temporal activity of antioxidant enzymes at different temperatures in the hepatocytes suggests production of reactive oxygen species to set in and counteraction by the antioxidant enzymes, but exposure at 42°C for a period of 72 h definitely could not counteract the hyperthermic conditions leading to significant decrease in the percentage of viable cells. To the best of our knowledge nobody has reported the effect of hyperthermic conditions on the antioxidant enzymes activity and TGF β secretion of caprine hepatocyte culture *in vitro*. It is suggests that further research can be carried out *in vivo* system also.

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