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Beneficial Effect of Mannan Oligosaccharide on Caprine Hepatocyte Functional Parameters in vitro under Hyperthermia

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A B S T R A C T

Liver is involved in several important physiological functions of the body. In the present study, effect of hyperthermia and effect of supplementation of yeast cell wall component mannan oligosaccharide (MOS) under hyperthermic conditions on some of the functions of hepatocytes has been evaluated. Hepatocytes were isolated by disaggregation of liver piece with collagenase solution. It was observed that caprine hepatocytes were under thermal stress at 42˚C as obtained with results on activity of LDH and antioxidant enzymes. The Least Square mean (LSM) values of the parameters were significantly higher when compared with the values estimated for hepatocyte culture at 37˚C which served as control. Whereas the concentration of albumin decreased and TGF β increased at 42˚C when compared with estimated values for 37˚C. On supplementation of MOS to hepatocyte culture at 42˚C the results were reversed ($P<0.01$) by 72 h indicating protective effect of MOS on hepatocyte function. The results reported are based on *in vitro* studies only and further suggests that supplementation of MOS in feed to caprines under hyperthermic conditions may have beneficial effect on liver function.

Introduction

Liver is an important organ for regulation of metabolism in animals and humans, and therefore hepatocyte system serves as a useful model for in vitro studies (Dvorak et al., 2007). Method devised by Spotorno et al. (2006) for obtaining hepatocytes was by non perfusion technique. The hepatocyte function is assessed by measuring LDH, albumin and urea level in the supernatant of cell culture (Zhang et al., 2012). As mentioned by Carvalho et al. (2001), when mouse hepatocytes are subjected to mild

hyperthermia, it leads to oxidative stress in cells and reduction in viability of cells. To counteract the oxidative stress, activity of antioxidant enzymes is stimulated (Salvi et al., 2007). It is well known that TGF-β induces ROS generation, which if secreted in high concentration leads to apoptosis of cells (Sanchez et al., 1996). Mannan oligosaccharide (MOS) represents 30-40% of the yeast cell wall of *Saccharomyces cerevisiae*. It has high water solubility and has a practical application as antioxidant (Suzuki et al., 1969). It also potentiates free radical scavenging activity. Some work has been reported in the mouse hepatocyte with respect to heat stress, but there is lack of literature with caprine hepatocyte.

The aim of the study was to observe the effect of heat stress on caprine hepatocyte cell culture system and to evaluate the ability of MOS supplementation to counteract the heat stress effects in terms of reversal of activity of antioxidant enzymes and TGF-β secretion.

Materials and Methods

Plastic ware and glassware

All the glassware used in the present investigation was made of high grade glass and was thoroughly cleaned, rinsed with ultrapure water and then sterilized at 121˚C for 20 minutes. The plastic ware including disposable 50 mm X 15 mm PS sterile external grip cell culture Petri dishes and 6 well culture dishes was purchased from Genetix Biotech Asia, Pvt. Ltd. New Delhi. Plastic syringes and Disposable 18-gauge hypodermic needles was from Dispovan Hindustan Syringes and Medical Devices Ltd., Faridabad, (India), the 0.22 µm filters was from Millipore Corporation, Bedford, MA, (USA), the autoclavable disposable tips for micropipettes, centrifuge tubes and nitrile gloves from Axiva Sichem Biotech, New Delhi (India) were purchased.

Chemicals, cell culture media and supplements

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, mannan oligosaccharide (Cat. No. M3640-1G) from *Saccharomyces cerevisiae* and antibiotics (penicillin and streptomycin) and antifungal amphotericin B was purchased from Sigma Chemicals Pvt. Ltd. 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 were also from Sigma Chemicals Pvt. Ltd. unless otherwise indicated. Most of the chemicals used were of cell culture grade. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, Utah, USA)

Statistical analysis

Data was statistically analyzed by SAS software, Version (9.1) of the SAS System for Windows. Copyright © (2011), SAS Institute Inc., Cary, NC, USA. Data is expressed as Mean ± SE and analyzed by ANOVA, considering the different temperatures, concentration of MOS, and time of incubation as the main variables. Results revealing a significant effect were compared by the least significant difference pair wise multiple comparison test. Differences were considered statistically significant at $P<0.01$.

Mean ± S.E. values of each parameter, has been compared for different time intervals considering different doses of MOS (Table 2 and 3) whereas Least Square mean (LSM) values for different parameters were considered for making comparisons between results obtained for different temperatures (Table 1) and MOS doses (Table 4).

Caprine hepatocyte culture and treatments

Hepatocyte isolation was performed by non perfusion technique with some modifications according to Vyankati and Anand Laxmi (2013) and Spotorno et al. (2006). Cell viability was determined by Trypan Blue exclusion method (Shull et al., 1986). Hepatocytes were seeded into six well plates at a density of 4.5 X 10^5 viable cells/cm^2/well in 2 mL of Dulbecco's minimum essential medium (DMEM/Ham's F12). Cells were cultured at 37˚C, under controlled conditions in a CO_2 incubator. Hepatocytes attached to the surface of plates by 4 h post seeding.

Optimization

Briefly the cells were incubated for different time intervals and supernatant from the culture was collected at respective time intervals for assay of LDH activity (cell membrane integrity test), albumin, urea and TGF-β secretion. At the same time intervals, cells were harvested, lysed and processed for determining the activity of enzymes, Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase as discussed elsewhere (Vyankati and Anand Laxmi 2014). At the same time, cultures were subjected to higher temperatures 40˚C and 42˚C respectively for the mentioned different time intervals. The temperature which affected the function of hepatocytes significantly was further selected for observation of effect of supplementation of MOS under hyperthermic conditions. MOS was supplemented to culture at 37˚C after attachment of hepatocytes to the base of the culture dish and change of media. The temperature was adjusted to 42˚C after supplementation of MOS to the culture. The different doses of MOS were 150 μg, 300 μg and 500μg.The supernatant and hepatocytes from the hepatocyte culture were harvested for estimation of activity of antioxidant enzymes and TGF-β secretion in vitro at 24, 48 and 72 h of incubation. The group without supplementation of MOS served as control. Supernatant and cells were harvested from hepatocyte culture at 24, 48, and 72 h time intervals post incubation for estimation of various parameters.

Results and Discussion

Optimization of hepatocyte cell culture

LDH release, urea and albumin production were used as indices of hepatocyte function. The optimization studies and the results obtained were similar as reported earlier in detail (Vyankati and Anand Laxmi, 2014). Briefly, when the hepatocytes were incubated at three different temperatures 37˚C, 40˚C and 42˚C for three different time intervals, increase in LS Mean LDH activity , decrease in albumin activity and no change in urea secretion was observed which was significant ($P< 0.05$) (Table 1). From this study, it was observed that 37˚C was optimum for carrying out hepatocyte cell culture.

The above study for 72h at different temperatures was carried out for estimation of antioxidant enzymes (SOD, GPx and catalase) activity. It was observed that LS Mean activity of all the antioxidant enzymes had significantly increased at 40 and 42˚C. Results in detail have been published (Vyankati and Anand Laxmi, 2014). The mean concentration of TGF β secretion in

the culture medium also significantly increased at 42˚C post 72h of incubation (Table 1).

Effect of supplementation of MOS on albumin secretion by hepatocytes *in vitro*

At 42˚C when MOS was supplemented to hepatocytes, it increased significantly (P< 0.01) by 72h of incubation, although first a decrease could be observed by 48h of incubation with 150 and 300 µg dose of MOS. In the control group, conversely, the secretion decreased significantly (P<0.01) at both 48h and 72h time intervals when compared with Mean ± SE albumin secretion at 24h interval (Table 2). LS mean values were also significantly (P< 0.01) higher for the supplemented groups when compared with the control group (Table 4).

Effect of supplementation of MOS on urea secretion by hepatocytes *in vitro*

The different doses of MOS supplemented could decrease the concentration of urea in the supernatant but was not significantly different from the Mean ± SE values estimated for 24h interval. In the control group, the concentration of urea increased significantly (P<0.01) by 72h (Table 2) whereas the LS mean values for the four groups when compared with each other, were not significantly different from each other (Table 4).

Effect of supplementation of MOS on TGF β secretion by hepatocytes *in vitro*

In the control group, it was observed that TGF β secretion increased at 48 and 72 h which was significant (P<0.01) at 72 h only. With 150 µg MOS supplementation, it was observed that TGF β secretion declined at 48 and 72 h but was significantly less (P<0.01) only at 72h. With 300 and 500 µg MOS supplementation the significant decrease in TGF β secretion was observed at both 48 and 72 h interval (Table 2). The LS mean values for TGF β concentration for all the supplemented groups was significantly less when compared with the control value (Table 4).

Effect of MOS supplementation to hepatocyte culture on activity of hepatocyte LDH, and antioxidant enzymes at different time periods of incubation under hyperthermic condition

Effect of MOS on LDH activity

Effect of MOS supplementation on LDH activity decreased significantly (P<0.01) at 48 h of post supplementation and still further decreased at 72 h of incubation for all the supplemented groups of MOS (Table 3). Significant difference was not observed in LS mean LDH activity when compared between different time intervals for the control group (Table 4).

Effect of MOS on SOD activity

In the control group, activity of SOD enzyme increased significantly at 24, 48 and 72 h when compared with the activity at 24 h interval. In the supplemented groups, the significant increase (P< 0.01) in the activity of the enzymes was observed till 48 h, the Mean ± SE enzyme activity was observed to be greater at 24 and 48 h (P<0.01), when compared with enzyme activity at 72 h (Table 3). The LS mean values for enzyme activity for all the supplemented groups were significantly less (P<0.01) when compared with the control (Table 4).

Effect of MOS on Catalase activity

The Mean ± SE catalase enzyme activity was significantly (P<0.01) more at 48 and

72 h when compared with its activity at 24 h in the control group. In the treatment group, increase in the enzyme activity was observed till 48 h interval but reduced significantly at 72 h ($P<0.01$) when compared with its activity at 24 h (Table 3). The LS mean value for the enzyme activity were significantly less in the 150 and 300 µg supplemented groups, when compared with control but was higher for the 500 µg supplemented group (Table 4).

Effect of MOS on GPx activity

In the control group, GPx enzyme activity at 48 and 72 h time interval was significantly greater when compared with its activity at 24 h. For all the three supplemented groups, the activity of the enzyme was observed to be greater at 48 h interval but then decreased at 72 h which was significant ($P<0.01$) (Table 3).The LS mean values were greater for control and 500 µg supplemented group when compared with LS mean value for 150 and 300 µg supplemented groups (Table 4).

Morphology of hepatocytes

Under phase contrast microscopy hepatocytes appear bright, translucent and spherical in shape when incubated *in vitro.* Isolated hepatocytes try to reestablish cell to cell contact and cytoplasmic polarity as observed at 48h and 72h. When the cells were incubated at 37˚C, such cell to cell contact was not observed in cells when incubated at 42˚C. Under supplementation of MOS however studies were not performed in detail with regard to cell to cell contact of hepatocytes.

As stated by the Dickens et al. (2008) , Wu et al. (2009) and Vyankati and Anand laxmi, (2014) estimation of urea and albumin in supernatant and hepatocyte damage in terms of LDH activity can be used as markers for assessing the quality of hepatocytes cultured *in vitro*. In the present experiment also, first culture was optimized at 37˚C in terms of quantitative analysis of these parameters. The viability of the hepatocytes was maximum at 37˚C, when compared with the viability at 40 and 42˚C. Under hyperthermic conditions, (40 and 42˚C) the functional capacity of hepatocytes decreased as reflected by decrease in albumin and urea secretion and increase in LDH enzyme activity. It is in accordance with studies of Carvalho et al. (2001). When either dose of MOS was supplemented, at 42˚C, temperature which was selected for hyperthermic conditions, LDH activity could be reduced indicating damage to the hepatocytes reduced and similarly albumin concentration increased by 72 h when compared with its control indicating MOS supplementation has beneficial effect on hepatocyte culture by 72 h on these parameters.

Reports regarding effect of supplementation of any material under hyperthermic conditions on antioxidant enzyme activity in hepatocytes/ caprine hepatocytes are lacking. It has been suggested by Huber et al. (2006) that under oxidative stress conditions, when there is increase in production of ROS in cells *in vitro*, antioxidant enzyme activity increased.

As stated by Zhao et al. (2006), under heat stress/ hyperthermic conditions, there can be overproduction of ROS or free radicals. In hepatocytes, it has been shown that temperature stress leads to influx of cellular free radicals (Flanagan et al., 1998).
In the present study also similar phenomena might have taken place. Under such stress conditions, upregulation of catalase and other antioxidant enzymes might have taken place (Gupta, 2006; Salvi et al., 2007).

Mannan is an oligosaccharide which is component of yeast cell wall and has the potency to scavenge free radicals (Krizkova et al., 2001). Hence, in the present study when different doses of MOS was supplemented, it might have led to decrease in the activity of antioxidant enzymes when compared with the control group, which may be due to the fall in the concentration of ROS, although ROS and free radicals were not estimated in the present study.

Table.1 Effect of different temperatures on hepatocyte enzymes activity and albumin, urea, TGF β secretion in culture medium

GROUPS	LDH (IU/L)	SOD (IU/L)	CATALASE (IU/L)	GPx (IU/L)	ALBUMIN (g/dl)	UREA (mg/dl)	TGF β (ng/dl)
Group -I (37˚C) (Control)	71.11^{a}	101.75^{a}	127.86^{a}	187.61^{a}	0.94^{a}	11.37^{a}	20.86^{a}
Group -II (40˚C)	140.67^{b}	873.32^{b}	195.61^{b}	198.76^{b}	0.66^{b}	10.29^{a}	20.80^{a}
Group-III (42˚C)	170.33^{c}	969.45^{c}	224.59^{c}	234.92^{c}	0.63^{b}	10.01^{a}	28.42^{b}

GPx - Glutathione peroxidase; SOD - Superoxide dismutase; LDH- Lactate dehydrogenase; TGF β - Transforming growth factor β. Values are expressed as LS Mean for a period of 72 h incubation. Means having different superscripts in the same column differ significantly at P<0.01.

Table.2 Effect of MOS on secretion of different parameters in cell culture medium

Parameters	Duration of incubation	TREATMENTS			
		C (Control)	T_1(150 µg/ml)	T_2(300 µg/ml)	T_3 (500 µg/ml)
Albumin (g/dl)	24h	0.83^{b} ±0.02	0.71^{a} ±0.01	0.83^{b} ±0.03	0.75^{a} ±0.02
	48h	0.56^{a} ±0.03	0.67^{a} ±0.03	0.75^{a} ±0.02	0.82^{b} ±0.01
	72h	0.5^{a} ±0.01	0.84^{b} ±0.02	0.84^{b} ±0.01	0.83^{b} ±0.01
Urea (mg/dl)	24h	9.92^{ab} ±0.50	9.19^{a} ±0.66	9.64^{a} ±1.12	9.88^{ab} ±0.30
	48h	8.57^{a} ±0.84	8.4^{a} ±0.65	9.72^{a} ±0.19	11.23^{b} ±0.60
	72h	11.56^{b} ±0.50	8.17^{a} ±0.47	9.24^{a} ±0.86	8.18^{a} ±0.74
Transforming growth factor β (ng/dl)	24h	29.63^{a} ±0.86	29.39^{b} ±1.29	23.56^{c} ±0.33	28.49^{c} ±0.90
	48h	29.43^{a} ±1.74	26.95^{b} ±0.68	21.82^{b} ±0.27	21.45^{b} ±0.58
	72h	35.21^{b} ±1.04	11.16^{a} ±0.79	11.25^{a} ±0.17	16.98^{a} ±0.90

Values are expressed as Mean ± SE. Values with different superscripts in a column differ significantly from each other atleast at P<0.01

Table.3 Effect of MOS on hepatocyte enzymes activity

Hepatic Enzymes	Duration of incubation	TREATMENTS			
		C (Control)	T_1(150 µg/ml)	T_2(300 µg/ml)	T_3 (500 µg/ml)
Lactate dehydrogenase (IU/L)	24h	161.33^{a} ±1.45	131.33^{c} ±1.86	183^{b} ± 2.08	166^{b} ± 1.00
	48h	176^{b} ±1.15	91.33^{b} ±1.86	138^{a} ± 2.08	206^{c} ± 1.15
	72h	173.66^{b} ±2.60	81^{a} ±1.15	134^{a} ± 2.65	146^{a} ± 1.53
Superoxide dismutase (IU/L)	24h	910.15^{a} ±0.33	760.89^{a} ±1.01	791.28^{a} ±1.60	815.3^{a} ±1.12
	48h	955.59^{b} ±0.66	900.84^{b} ±1.00	802.29^{b} ±1.56	975.44^{c} ±1.55
	72h	1042.59^{c} ±1.79	899.12^{b} ±0.55	892.54^{c} ±1.25	918.48^{b} ±3.27
Catalase (IU/L)	24h	148.81^{a} ±2.05	166.69^{b} ±1.42	164.77^{b} ±2.00	177.83^{b} ±1.79
	48h	206.57^{b} ±1.45	248.45^{c} ±3.04	178.73^{c} ±2.03	263.94^{c} ±1.74
	72h	231.45^{c} ±1.19	151.85^{a} ±1.44	112.45^{a} ±1.74	260.61^{a} ±1.45
Glutathione peroxidase (IU/L)	24h	181.98^{a} ±1.44	174.22^{a} ±1.45	172.4^{a} ±1.91	182.26^{a} ±1.28
	48h	255.28^{b} ±1.46	244.13^{c} ±2.81	246.29^{c} ±1.19	273.28^{b} ±1.28
	72h	267.51^{c} ±1.47	234.54^{b} ±0.95	234.58^{b} ±0.92	270.12^{b} ±0.82

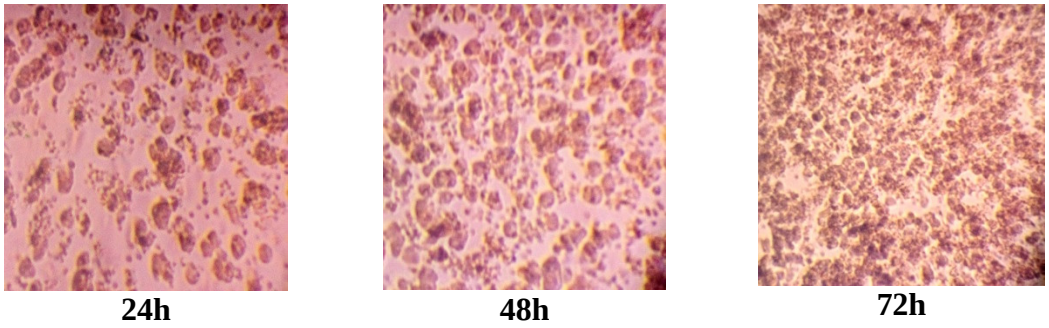
Values are expressed as Mean ± SE. Values with different superscripts in a column differ significantly from each other atleast at P<0.01

Table.4 Effect of MOS on hepatocyte enzymes activity and albumin, urea, TGF β secretion in culture medium

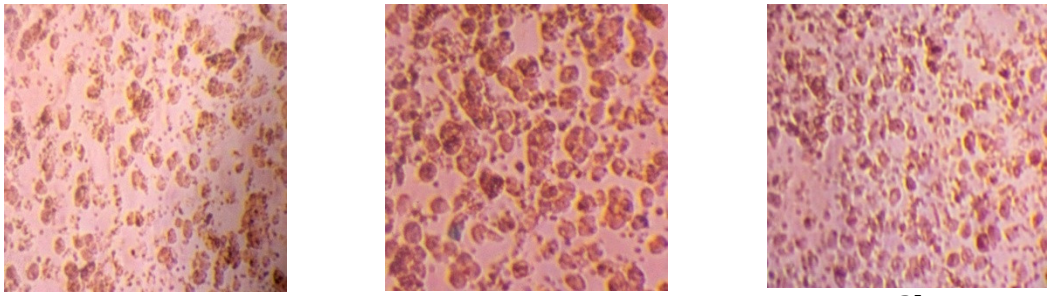
TREATMENT	LDH (IU/L)	SOD (IU/L)	CATALASE (IU/L)	GPx (IU/L)	ALBUMIN (g/dl)	UREA (mg/dl)	TGF β (ng/dl)
C (Control) (non-supplemented)	170.33^{a}	969.45^{a}	195.61^{a}	234.92^{a}	0.63^{a}	10.01^{a}	28.42^{a}
T_1 (150 µg/ml)	101.22^{b}	853.62^{b}	189^{b}	217.63^{b}	0.74^{b}	8.59^{a}	22.5^{b}
T_2 (300 µg/ml)	151.67^{c}	828.7^{c}	151.98^{c}	217.76^{b}	0.8^{c}	9.53^{a}	18.88^{c}
T_3 (500 µg/ml)	172.67^{a}	903.07^{d}	234.13^{d}	241.88^{c}	0.8^{c}	9.76^{a}	22.31^{b}

GPx - Glutathione peroxidase; SOD - Super oxide dismutase; LDH - Lactate dehydrogenase; TGF β - Transforming growth factor β.Values are expressed as LS Mean for a period of 72 h incubation. The means having different superscripts in the same column differ significantly atleast at P<0.05

Morphology of caprine hepatocytes cultured at I. 37˚C and II. 42˚C and different times of incubation (100X)



24h **48h** **72h**



24h **48h** **72h**

The reduction in the activity of the antioxidant enzymes except catalase at 72h was still higher than what was prevailing at 24h. This also indicates that alone MOS supplementation cannot reduce the activity of antioxidant enzymes to a greater level. Supplementation of MOS did not lead to similar effect on all the three antioxidant enzymes. Under hyperthermic conditions, transcription level of TGF β in cardiac cell was affected. TGF β is known to be a mediator in wound healing and tissue repair (Roberts et al., 1990). TGF β is known to inhibit proliferation of hepatocytes and can mediate hepatocyte apoptosis (Oberhammer et al., 1991). Similar reports are available that under variety of physiological stress conditions, TGF β secretion increases in tissues of brain, heart, lung, kidney and liver (Roberts and Sporn, 1993). It is also known that the increase in TGF β secretion is to establish thermotolerance and protect the cells from injury. Supplementation of either dose of MOS, reduced TGF β secretion by 72 h when compared with its concentration at 24 h interval. In the present study it was observed that addition of MOS could reverse the concentration of metabolic parameters, TGF β and antioxidant enzyme activity observed under hyperthermic conditions. In some cases, MOS could not totally reverse the result. A concentration of 150 and 300 µg of MOS were found to be beneficial in reversing the results obtained under hyperthermic conditions.

Supplementation of MOS to heat stressed broilers decreased antioxidant concentrations (Sohail et al., 2011). Tan et al. (2010) reported that *in vivo* there is increase in production of free radicals in relation with the increase in the activity of antioxidant enzymes during heat stress. This is the first report indicating the effect of

MOS in decreasing the heat stress effects on caprine hepatocytes *in vitro* and suggests that MOS supplementation as feed additive to caprines may have beneficial effects on liver function. Mannan oligosaccharides have been used as prebiotics in livestock feed (LeMieux et al., 2003). It has been reported that when used as feed supplement it has beneficial effects in ruminants and non ruminants (Zhao et al., 2012; Mehdi and Hasan, 2012).

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