



Redox disequilibrium *vis-a-vis* inflammatory cascade mediation of lymphocyte dysfunction, apoptosis, cytokine expression and activation of NF- κ B in subclinical diabetic goats

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ABSTRACT

Molecular basis of diabetes induced oxidative stress and immune dysfunction have not been reported in animal science. The present study envisages same in subclinical diabetic (SCD) goats (6) diagnosed on biochemical and histopathological basis in reference to non-diabetic (NSCD) goats (6). Oxidative stress indices were analyzed by manual methods. The concentration of reduced glutathione (GSH) and the activity of superoxide dismutase (SOD) was significantly lower in SCD goats than in NSCD goats; whereas the lipid peroxide (LPO) was higher in SCD. Catalase (CAT) activity was nonsignificantly lower in SCD goats than NSCD goats. SCD goats had significantly lower lymphocyte stimulation index by cell culture and higher apoptotic cell percentage by flow cytometry than NSCD goats. The concentration of the transforming growth factor beta 1 (TGF- β 1) by ELISA was significantly higher in SCD goats than in NSCD. The expressions of tumour necrosis factor alpha (TNF- α) and interleukin 8 (IL 8) by RT-PCR were higher in SCD goats than in non-diabetic ones. Expression of transcription factor (NF- κ B) by western blot was significantly higher in SCD goats than NSCD goats. Fall of antioxidants (GSH, SOD, catalase) and rise of oxidants (LPO) suggest oxidative stress. Decrease of immune cell function, rise of inflammatory cytokines and transcription factors suggest immune dysfunction. Hence it was concluded that SCD induced oxidative stress and impairment of immunity in goats, which was most likely associated with depletion of antioxidants, increase of oxidants and inflammatory mediators. NF- κ B, most likely have played a mediatory role in coordinating these intricate responses.

Key words: Diabetes, Goat, Immunity, NF- κ B, Oxidative stress

Diabetes mellitus (herein diabetes) is a disease in which affected humans or animals have high blood glucose (hyperglycemia), either because the body does not produce enough insulin (diabetes type I), or because body cells become refractory to insulin and eventually fell to produce sufficient amount of insulin (diabetes type II) (ADA 2018). In humans numerous etiologies have been described for diabetes (ADA 2018, Gao *et al.* 2018). In ruminants, cases of clinical diabetes are rare and results from primary disease that destroyed the normal function of the pancreas (Meier 1960, Gould 1981, Taniyama *et al.* 1993, Tajima *et al.* 1999,

Clark 2003). Yattoo *et al.* (2015) found that ~ 6% of the goats screened for diabetes were sub-clinically diabetic. In another study, among 256 cattle screened for diabetes, 5 (~ 2%) were found positive (Deepa *et al.* 2015). It can be inferred that the occurrence of diabetes in ruminant animals is a regular phenomenon though rare one but can have deleterious effects on health and production of valuable animals (Deepa *et al.* 2015, Yattoo *et al.* 2015) because of the diabetic complications yet to be explored.

In humans, complications from diabetes results from alteration of redox equilibrium and initiation of inflammatory cascade, which are mediated by activation of different transcription factors of signaling pathway (Graves and Kayal 2011, Vidigal *et al.* 2012, Reis *et al.* 2018). The outcome of these events is alteration of redox equilibrium and initiation of inflammatory cascade that induces oxidative stress and immune dysfunction and ultimately lead to apoptosis and diabetes-related complication, such as wounding, kidney failure and other cardiovascular complications (Giugliano *et al.* 1996, Ceriello 1998, Giacco and Brownlee 2010, Graves and Kayal 2011, Smulders and Serné 2018). These diabetic alterations, redox disequilibrium and inflammatory cascade

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Table 1. Diabetic biomarkers in SCD and NSCD goats

Diabetic biomarker	SCD goats	NSCD goats
Random glucose (mg/dl)	87–132 (Yatoo <i>et al.</i> 2015)	50–75 (Kaneko <i>et al.</i> 1997)
Fasting glucose (mg/dl)	84–129 (Yatoo <i>et al.</i> 2015)	45–72 (Yatoo <i>et al.</i> 2015, Alayash <i>et al.</i> 1988)
IVGTT/postprandial glucose (mg/dl)	92–137 (Yatoo <i>et al.</i> 2015)	67–74 (Todd <i>et al.</i> 2009)
Insulin (μ IU/ml)	3–21 (Yatoo <i>et al.</i> 2015)	33 (Celi <i>et al.</i> 2008, Yatoo <i>et al.</i> 2015)
GHb (%)	3.6–13.7 (Yatoo <i>et al.</i> 2015)	3.2 (Shahbazkia and Nazifi 2008, Alayash <i>et al.</i> 1988, Yatoo <i>et al.</i> 2015)
Fructosamine (mmol/l)	2.3–7.5 (Kaymaz <i>et al.</i> 2001, Yatoo <i>et al.</i> 2015)	2 (Filipoviæ <i>et al.</i> 2011, Deepa <i>et al.</i> 2015)
Cholesterol (mg/dl)	63.81–148.49 (Yatoo <i>et al.</i> 2015)	0.83–21.95 (Di Trana <i>et al.</i> 2006)
Triglycerides (mg/dl)	8.69–16.32 (Yatoo <i>et al.</i> 2015)	97.19–120.87 (Di Trana <i>et al.</i> 2006, Radostits <i>et al.</i> 2000)
Benedict's test	Positive (++) (Yatoo <i>et al.</i> 2015)	Negative (-)
Rothera's test	Positive (+) (Yatoo <i>et al.</i> 2015)	Negative (-)

related changes are governed by intricately linked pathways involving various intermediates or factors and regulating these pathways can have therapeutic implications (Pickering *et al.* 2018, Sharma *et al.* 2018, Volpe *et al.* 2018).

The recent study on SCD goats has shown that subclinical diabetes in goats was associated with oxidative stress and metabolic disturbance (Yatoo *et al.* 2015). In diabetic cattle also metabolic and oxidative disturbance has been noted (Deepa *et al.* 2015). This suggests possibility of alteration in other systems like oxidative balance and/or immunity but has not been yet worked out.

India has the second largest goat population in the world (with 135.17 millions heads), which play essential role in providing food for hundreds of millions inhabitants. Thus, in view that very little is known on the subject on ruminants, it was considered timely and important to highlight more deeply the etiology of these complications in goats. The aim of the study was to define more precisely this metabolic complication and to understand the chain of cause-effects responses that underlie the development of this subclinical diabetes. Hence we compared: oxidative stress markers, enzymatic activity of antioxidant enzymes (reduced glutathione; GSH, and superoxide dismutase; SOD and catalase) in blood cells, and the expression of anti-inflammatory (TGF- β 1) and pro-inflammatory (TNF- α , IL8) cytokines, through involvement of transcription factors [nuclear factor-kappa B (NF- κ B)], which is a key regulator of inflammatory responses and humoral and cellular immune responses.

MATERIALS AND METHODS

Present study was conducted at experimental Goat Farm of the institute. Geoclimatic features of the area have been described earlier (Yatoo *et al.* 2015). Six subclinical diabetic crossbred Black Bengal goats were compared with six non-diabetic goats. All animals were above 2 years of age in their 3rd–5th lactation and at 7th week of lactation. Diagnosis indices of subclinical diabetes have been described previously (Yatoo *et al.* 2015) and are presented in Table 1. Selected animals were managed under similar managemental conditions with equal amounts of same feed having uniform

composition, being provided to both the groups at same frequency during the study as described previously (Yatoo *et al.* 2015). Animals had free access to drinking water. This study was approved by the Institutional Animal Ethics Committee (IAEC) under Institute Project IVRI/MED/12-15/008. Blood samples (~7 ml) for the study were collected by jugular venipuncture from each animal using a 10 ml disposable syringe. Immediately blood samples were transferred into heparinized (20 IU/ml) tubes for further processing and analysis of various oxidative and immune indices.

Oxidative stress indices: About 3 ml of heparinized blood was centrifuged at $3,000 \times g$ for 10 min to separate plasma and packed erythrocytes. Packed erythrocytes were washed three times with normal saline solution. A portion (500 μ l) of washed erythrocytes was mixed with chilled distilled water (~4°C) and shaken vigorously to prepare 10% haemolysate. Another 500 μ l of washed erythrocytes were mixed with 500 μ l of normal saline solution to prepare red blood cell (RBC) suspension for the estimation of reduced glutathione. Haemoglobin concentration in the 10% haemolysate and RBC suspension was estimated by cyanomethaemoglobin method (Tentori and Salvati 1981). Reduced glutathione was measured by the method of Prins and Loos (1969), superoxide dismutase by Madesh and Balasubramanian (1998), catalase by Bergmeyer (1983) and lipid peroxides by Placer *et al.* (1966).

Immune indices: Cellular immune indices were evaluated by *in vitro* lymphocyte proliferation assay (Dar *et al.* 2015). The stimulation index (SI) was calculated using formula of Mosmann (1983). Apoptosis assay was done by flow cytometry (Van Oostveldt *et al.* 2001) on the isolated peripheral blood mononuclear cells (PBMCs) from subclinical diabetic and non-diabetic goats and the total counts were calculated from quadrants of apoptotic figures on flow cytometry. Humoral immune indices were evaluated by *in vitro* cytokine release assay including TGF β 1 by goat TGF β 1 ELISA kit (CSB-E12104G, CUSABIO Life Sciences, China) and TNF- α and IL8 by quantitative real time polymerase chain reaction (qRT-PCR) and expressed as fold expression (Dar *et al.* 2015). Primer sequence for caprine cytokines used were F5'-TCTTCTCAAGCC-

TCAAGTAACAAGC-3', F5'-CCATGAGGGCATTGG-CATAC-3' for TNF- α (Accession number, AY304502.1, Product size: 706 bp); F5'-CACTGTGAAAATTCA-GAAATCATTGTTA-3', F5'-CTTCACAAATACTT-GCACAACCTTC-3' for IL8 (Accession number, XM_005681749.1; Product size, 1481 bp) and F5'-GGCGTGAACCACGAGAAGTATAA-3', F5'-CCCTC-CACGATGCCAAAGT-3' for GAPDH (Accession number, XM_005680968.1; Product size, 1290 bp).

For relative quantification of cytokine mRNA, the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used. Expression of nuclear factor-kappa B (NF- κ B) protein was done by western blotting (Saxena *et al.* 2015).

Statistical analysis: Independent sample *t* test was used to compare oxidative stress indices and immune indices between subclinical diabetic and non-diabetic goats using SPSS.

RESULTS AND DISCUSSION

GSH levels and SOD activity were significantly lower ($P < 0.05$) in subclinical diabetic goats than non-diabetic goats indicating oxidative stress in subclinical diabetic goats and hence consumption of antioxidants due to oxidants (Table 2). CAT activity was nonsignificantly ($P \geq 0.05$) lower in SCD than in NSCD which may suggest its nonsignificant role. LPO levels were significantly ($P < 0.05$) higher in SCD goats than in NSCD ones. This might be due to rise of free radical species in subclinical diabetic goats.

Lymphocyte stimulation index was significantly ($P < 0.05$) lower in SCD goats than NSCD goats (Table 2) indicating decrease in immune competence of lymphocytes in SCD goats. Average early and late apoptotic cells in subclinical diabetic goats were significantly higher ($P < 0.05$) in comparison to NSCD ones (Table 2). This might be due to lowering of survivability of lymphocytes in subclinical diabetic goats. TGF- β 1 levels were significantly higher ($P < 0.05$) in SCD than in NSCD ones (Table 2). Expression of TNF- α was significantly higher ($P < 0.05$) and that of IL8 was nonsignificantly ($P \geq 0.05$) higher in SCD goats than in NSCD goats. Increase in proinflammatory cytokines in subclinical diabetic goats could be due to proinflammatory reaction by metabolic and oxidative disturbance. The expression of NF- κ B was significantly higher ($P < 0.05$) in SCD goats than in NSCD goats (Fig. 1). This might be due to role of NF- κ B in mediating metabolic alteration induced oxidative and inflammatory disturbances.

Many studies on human suffering from diabetes mellitus highlighted the intricacies among development of oxidative stress and immune dysfunction and that the disruption of these functions are related to redox disequilibrium and inflammatory cascade, which are reflected in modified interlinked molecular mechanisms (Turina *et al.* 2005, Luo *et al.* 2007, Liu *et al.* 2015). Recent studies in diabetic and SCD goats have shown that the affected animals were exposed to metabolic disturbance and oxidative stress (Deepa *et al.* 2015, Yattoo *et al.* 2015). However, unlike the situation with humans, very little is known about the cause-

effect interrelationships, which are leading to these modifications in ruminants. Detailed analysis of the biochemical changes and underlying mechanisms would help in better understanding of the entire process and to define more precisely SCD in goats, treatment strategies and prevalence of the phenomena.

Hyperglycemia is the most definite hallmark of diabetes. One of the consequences of hyperglycemia is glycation of various organic constituents that in turn induces increase of pro-inflammatory cytokine expression and induces inflammatory cascade (Guha *et al.* 2000, de Carvalho *et al.* 2012, Fiorentino *et al.* 2013). Further complication of hyperglycemia includes glycated-protein cross-linking, which cause structural and functional changes and loss of enzyme activities (Nowotny *et al.* 2015). The metabolic imbalance that characterized hyperglycemia can also generate superoxide radicals (Bernabucci *et al.* 2005, Al-Qudah 2011). The present results and those of our previous studies (Deepa *et al.* 2015, Yattoo *et al.* 2015) indicate that the above-mentioned factors are relevant to the development of sub-clinical and clinical diabetes in ruminants.

In humans, it is well established that hyperglycemia,

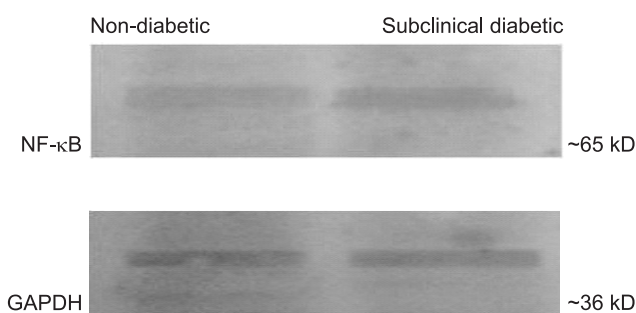


Fig. 1. Western blot banding patterns of NF- κ B protein in the subclinical diabetic and non-diabetic goats. GAPDH was used as internal control.

Table 2. Variation (Mean $\pm$ SE) of oxidative stress and immune indices between subclinical diabetic and non-diabetic (n=6) goats

Parameter	SCD	NSCD
GSH (nmol/ml)	104.8 $\pm$ 0.00049 ^a	114.0 $\pm$ 0.00052 ^b
SOD (nmol/ml)	425.0 $\pm$ 0.01039 ^a	535.6 $\pm$ 0.00682 ^b
CAT (μ mol H ₂ O ₂ decomposed/min/mg Hb)	10.2350 $\pm$ 0.29885	10.7933 $\pm$ 0.36616
LPO (nmol MDA/mg Hb)	3.6650 $\pm$ 0.17654 ^a	2.3678 $\pm$ 0.02277 ^b
Lymphocyte stimulation index	1.5961 $\pm$ 0.01346 ^a	1.8350 $\pm$ 0.01327 ^b
Early apoptotic cell percentage	3.0600 $\pm$ 0.05857 ^a	0.9956 $\pm$ 0.17179 ^b
Late apoptotic cell percentage	2.5667 $\pm$ 0.16415 ^a	0.5967 $\pm$ 0.11173 ^b
TGF- β 1 (ng/ml)	30.6522 $\pm$ 0.38599 ^a	26.5411 $\pm$ 0.40477 ^b

^{ab}Values with different superscript differ significantly between subclinical diabetic and non-diabetic group ($P < 0.05$). SE is standard error.

ketonemia and the above-described pro-inflammatory responses are associated with oxidative stress (Giugliano *et al.* 1996, Ceriello 1998, Giacco and Brownlee 2010, Graves and Kayal 2011, Vidigal *et al.* 2012). The increase in MDA and LPO levels in blood plasma in the SCD goats in present study was consistent with the recent findings (Yatoo *et al.* 2015) and clearly indicates that subclinical diabetes in goats is associated with oxidative stress. These metabolites are products of peroxidative breakdown of phospholipids, fatty acids, resulting in release of MDA from lipids and their accumulation in blood plasma (Jain *et al.* 1989, Morohoshi *et al.* 1996). The non-significant change of oxidative stress indices in NSCD goats indicates lack of appreciable oxidative stress, hence retaining normal levels of antioxidants and oxidants and as suggested (Deepa *et al.* 2015, Yatoo *et al.* 2015) their value may be used to establish normoglycemic reference value in goats. NF- κ B is a protein complex that controls transcription of DNA, cytokine production and cell survival and is known to be deeply involved in ruling the inflammatory reaction induced by metabolic disturbance and oxidative stress in humans (Guha *et al.* 2000, Graves and Kayal 2011, Liu *et al.* 2015). The increased expression of NF- κ B in present experiment strongly suggests that it plays similar role in controlling the event evoked by subclinical hyperglycemia and ketonemia in goats.

GSH level is a major mechanism that is involved in cellular scavenging of free radicals (Graber *et al.* 1999, Mytilineou *et al.* 2002, Giacco and Brownlee 2010). Therefore, the lowering its levels in red blood cells are likely by excessive consumption. Under persistent hyperglycemia there is increased vascular expression of thioredoxin-interacting protein (Txnip) which impairs thiol reductase activity (Schulze *et al.* 2004) hence lowering GSH levels (Liu *et al.* 2015).

SOD is a major antioxidant mechanism responsible for disintegration of superoxide into hydrogen peroxide that can be further disintegrated to water by GSH and catalase (Kashiwagi *et al.* 1996, Deepa *et al.* 2015). Enhanced autooxidation of glucose that occur during hyperglycemia and activation of protein kinase C, resulting in overproduction of superoxide and increased glycosylated SOD leading to inactivation of this enzyme (Kashiwagi *et al.* 1996, Guha *et al.* 2000). Thus, decreased SOD activity in the red blood cells of the SCD goats in present experiment may also be related to the induction of oxidative stress in those goats. The level of catalase in red blood cells of SCD goats also tended to decrease, but didn't reach significant level. Red blood cells, unlike other cells, lack synthetic activity. Our results suggest that red blood cells are appropriate source to trace intracellular responses to oxidative stress.

In humans, it is well established that diabetes affects both cellular and humoral immunity through lymphocyte dysfunction, apoptosis, alteration in cytokine expression and activation of transcription factors (Turina *et al.* 2005, Luo *et al.* 2007, Graves and Kayal 2011). Thus lower

lymphocyte stimulation index in subclinical diabetic goats may be due to impairment in functioning of lymphocytes due to hyperglycemia (Delamaire *et al.* 1997, Turina *et al.* 2005), glycation and elevated levels of advanced glycation end products (Kashiwagi *et al.* 1996, de Arriba *et al.* 2003, Pertyńska-Marczewska *et al.* 2004, Stirban *et al.* 2013). Impairment in functioning of PBMCs in diabetes and hyperglycemia had been reported in human patients (Delamaire *et al.* 1997, Turina *et al.* 2005). Ketoneemia, as reported in these subclinical diabetic goats (Yatoo *et al.* 2015) can also affect immune cell function (Grinberg *et al.* 2008). Hyperglycemia induces mitochondrial dysfunction and endoplasmic reticulum stress, promoting accumulation of reactive oxygen species that, in turn, promote cellular damage (Graber *et al.* 1999, Fiorentino *et al.* 2013, Liu *et al.* 2015, Mozzini *et al.* 2015). It also induces expression of receptors that trigger apoptosis. Chronic hyperglycemia induce the activation of the unfolded protein response (UPR) and endoplasmic reticulum (ER) apoptosis in PBMCs through activation of NF- κ B resulting in augmentation of inflammation and oxidative stress causing apoptosis of PBMCs (Liu *et al.* 2015, Mozzini *et al.* 2015). This is in corroboration with our finding of higher expression of NF- κ B in subclinical diabetic goats.

Higher TGF- β 1 levels in the subclinical diabetic goats may be as a result of immunosuppressive response induced by hyperglycemia, elevated glycated products or oxidative stress, causing rise in levels of immunosuppressive and anti-inflammatory cytokines. Hyperglycemia induced increase in TGF- β 1 in cardiac and renal diseases have been noted in hyperglycemic mice (Smoak 2004) and diabetic human patients (Ibrahim and Rashed 2007).

Higher expression of TNF- α and IL8 in the subclinical diabetic goats is related to inflammatory response to metabolic disturbance particularly persistent hyperglycemia and elevated glycated products causing increase in levels of pro-inflammatory cytokines. Rise in inflammatory cytokines TNF- α and IL8 due to hyperglycemia had also been reported by Esposito *et al.* (2002) in humans.

Subclinical diabetes results in slow but persistent metabolic alteration causing disturbance in biochemical and hormonal status. Of the importance is persistent hyperglycemia which results in increased glycation of organic substances like haemoglobin and albumin and ketonemia. These metabolic alterations and glycated end products in turn lead to redox disequilibrium causing oxidative stress by depletion of antioxidants and elevation of oxidants. Disturbance in metabolic status and oxidative stress induces inflammatory cascade aggravating oxidative stress and resulting in immune dysfunction. The metabolic disturbance cum oxidative stress induced depletion of antioxidants and elevation of oxidants are associated with lymphocyte dysfunction, apoptosis and release of pro-inflammatory cytokines. These disturbances follow intricate pathways involving an array of intermediates. Transcription factor NF- κ B appears to play a key role in ruling these events. Further research is needed to clarify the

etiopathogenic mechanisms and prevalence of sub-clinical diabetes in different production systems and what are the consequences in term of health, welfare and productivity in affected animals besides elucidating therapeutic implications in future.

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