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RESEARCH ARTICLE



Aquaporin 9 regulates Leydig cell steroidogenesis in diabetes

Arun Kannan^a, Lezy Flora Mariajoseph-Antony^a, Antojenifer Panneerselvam^a, Chithra Loganathan^a, Diwakar Kiduva Jothiraman^a, Kumarasamy Anbarasu^b, and Chidambaram Prahalathan^a 

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ABSTRACT

Diabetes mellitus induced hyperglycemia increases oxidative stress, which contributes to impairment of male reproductive function. Aquaporins (AQPs) belong to a transmembrane protein superfamily containing 13 isoforms (AQP0–12), differentially expressed in various organs, and play a pivotal role in male reproductive function. In the current study, we investigated the relationship between AQPs and testicular steroidogenesis under hyperglycemia *in vivo* and *in vitro*. The effect of high glucose on the role of AQPs in Leydig cell steroidogenesis was analyzed in diabetic rats (*in-vivo*) and LC540 rat Leydig cells (*in vitro*) via enzyme assays, quantitative RT-PCR, siRNA knock down and western blotting. AQP 9 was significantly up-regulated in STZ-induced diabetic rat testis and high glucose treated LC540 cells. Further, oxidative stress marker nuclear factor erythroid 2-related factor 2 (Nrf2) expression was decreased with impaired testicular steroidogenesis under hyperglycemia. Knock-down of AQP 9 resulted in increased Nrf2 expression and thus increased testicular steroidogenesis in hyperglycemia. Diabetes-associated hyperglycemia induced oxidative stress is a widely proven cause for diabetes-related male infertility. Our results collectively suggest that AQP 9 impairs testicular steroidogenesis *via* the regulation of oxidative stress in diabetes.

Abbreviations: AQPs: Aquaporins; RT-PCR: Real Time Polymerase Chain Reaction; STZ: Streptozotocin; Nrf2: Nuclear factor erythroid 2-related factor 2; DM: Diabetes Mellitus; SOD: Superoxide dismutase; CAT: Catalase; LPO: Lipid Peroxidation; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; 17 β -HSD: 17 β -hydroxysteroid dehydrogenase; StAR: Steroidogenic Acute Regulatory Protein

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Introduction

Diabetes mellitus (DM) is a multifactorial metabolic disorder characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association 2013). The prevalence of diabetes globally was 425 million people in 2017 and is estimated to rise by 629 million in 2045 (Saeedi et al. 2019). In United States, one in five adults and one in four young adults are prediabetic, providing an unfavorable condition for the development of diabetic complications, either macrovascular or microvascular such as diabetic nephropathy, retinopathy, neuropathy, cardiovascular diseases and sexual dysfunction, which can be prevented if diabetes is managed at early stages (Ahmad 2016; Papatheodorou et al. 2016; Khalil 2017; Maresch et al. 2018; Andes et al. 2020; Barkabi-Zanjani et al. 2020; Song et al.

2020). The widespread increase in DM worldwide affects young individuals in the reproductive age group as there is a rise in the number of childhood and adolescent males with DM (Nagarathna et al. 2020). Hyperglycemia increases oxidative stress, which contributes to the impairment of insulin function/secretion and further, plays a pivotal role in the impairment of male reproductive function (Newsholme et al. 2019; Lei et al. 2020). Recently, the diffusion of H₂O₂ across the plasma membrane to the extracellular fluid has also been regarded as a potential ROS elimination pathway (Sies 2014).

Aquaporins (AQPs) belong to a transmembrane protein superfamily containing 13 isoforms (AQP0–12), differentially expressed in various organs and tissues involved in fluid absorption and/or excretion, energy metabolism, solute diffusion across transmembrane, cellular trafficking, biological functions and

plays a vital role in preserving the cellular environment (Azad et al. 2021). Alteration in the expression, function and/or regulation of AQPs affects pathophysiology of various clinical conditions *viz* edema, cataract, obesity, cell mobility, wound healing, Sjögren's syndrome, cancer, diabetes, metabolic disorders and reproductive function (Wang et al. 2015; da Silva and Soveral 2017; Dajani et al. 2018; Ala et al. 2021; Mirabella et al. 2021; Ribeiro et al. 2021). AQPs differential expression and distribution in the efferent ductules and in the epididymal segments of cryptorchid canines suggests their involvement in luminal microenvironment modifications (Squillacioti et al. 2021). Recent studies suggest that, AQPs have a great potential for diagnostics and a target for therapy in diseases of altered water homeostasis (Dajani et al. 2018; Ichiyama et al. 2018; Meli et al. 2018; Su et al. 2020; Clarke-Bland et al. 2022).

Alterations in plasma osmolality and fluid body volume disturbs the water metabolism, which may give rise to adverse pathological conditions. AQPs expression is susceptible to modification, since stress response involves changes in cell volume and/or movement of specific solutes. Oxidative stress promotes detrimental changes during spermatogenesis, epididymal maturation, and sperm capacitation leading to infertility is also strongly associated with altered expression of AQPs in the male reproductive physiology and function (Calamita et al. 2001; Huang et al. 2006; Chauvigne et al. 2015; Pellavio and Laforenza 2021). Moreover, AQPs are also involved in the regulation of steroidogenesis and regulates steroid hormones in developing epididymis, maintaining male fertility (Oliveira et al. 2005; Shannonhouse et al. 2014).

Despite many treatment strategies available, patients with diabetes still develop associated complications; thus, it is necessary to understand the molecular mechanisms to prevent diabetic complications. The wide distribution of AQPs in male reproductive tissues emphasizes that water permeability is crucial in maintaining male fertility; hence we intended to investigate the role of AQPs in testicular steroidogenesis in diabetes.

Results

Effect of hyperglycemia on oxidative stress

STZ is an antibiotic extracted from *Streptomyces achromogenes* is a diabetogenic tool to induce hyperglycaemia in laboratory studies. In the present study, STZ administered diabetic animals showed a remarkable decrease in body weight along with a significant increase in serum glucose levels compared to control animals. However, body weight was significantly increased ($p < 0.01$), and glucose levels were dramatically decreased ($p < 0.0001$) on insulin treatment compared to diabetic animals (Figure 1A,B). Hyperglycemia determines an imbalance in the consumption of antioxidant enzymes, which are predominantly represented by the enzymes superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO). The activities of enzymatic antioxidants, ROS levels and LPO of control and experimental groups of rat testis are represented in Figure 2A–D. The activities of SOD and CAT were significantly decreased in the testis of STZ-induced diabetic rats with a significant increase in ROS. The LPO levels were found to be significantly increased in diabetic rats and the addition of exogenous inducers such as

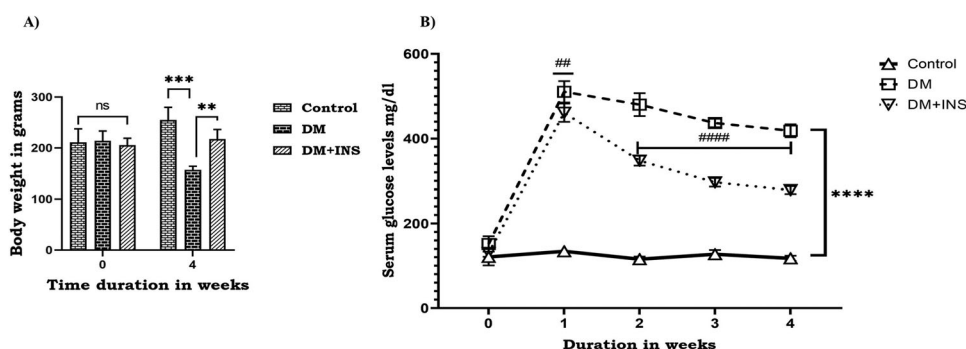


Figure 1. Effect of hyperglycemia. (A) Body weight of rats received citrate buffer (Control), diabetes (DM) and diabetes rats with insulin treatment (DM + INS) were recorded at 0th day and after 4 weeks; (B) Effect of hyperglycemia on serum glucose levels of control rats (Control), diabetes (DM) and diabetes treated with insulin (DM + INS) were measured before the start of experiment (0th day and after administration of STZ at 1st, 2nd, 3rd and 4th weeks). Values represent mean $\pm$ S.D. ($n = 6$). Values are statistically significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ and # indicates comparison between DM + INS and DM $##p < 0.01$, $####p < 0.0001$.

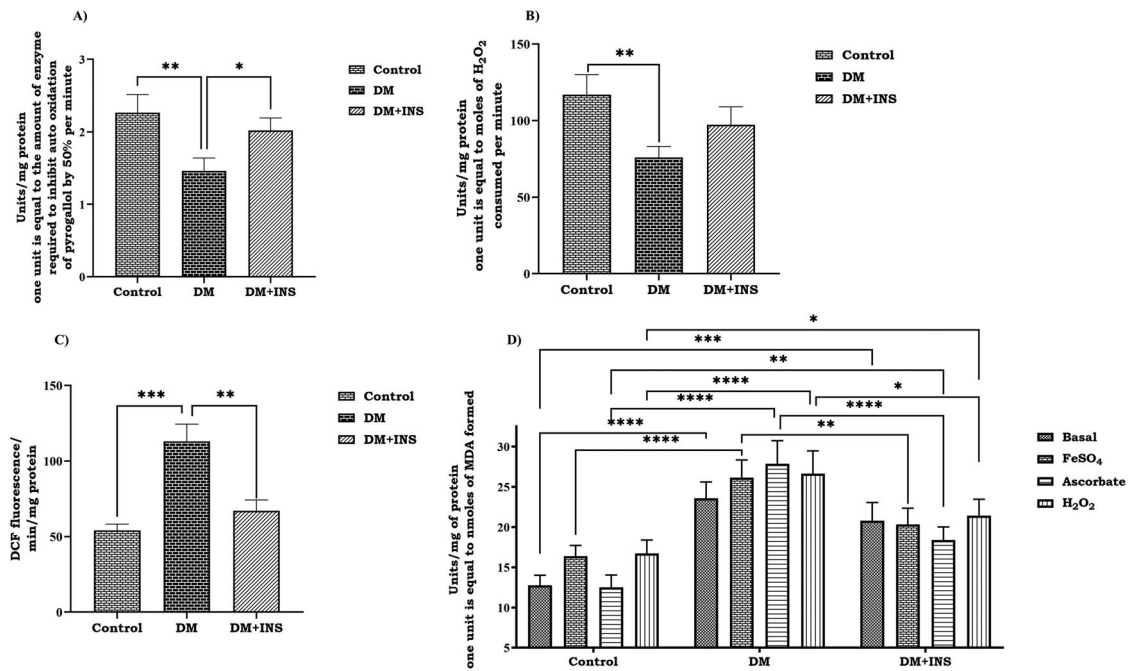


Figure 2. Effect of hyperglycemia on oxidative stress markers. (A) SOD, (B) CAT, (C) ROS and (D) LPO activity in rat testis (LPO was tested at basal level and also with the inducers FeSO₄, Ascorbate and H₂O₂). Enzyme activity for SOD is expressed as unit/mg protein; where one unit is equal to the amount of enzyme required to inhibit auto oxidation of pyrogallol by 50% per minute; CAT is expressed as unit/mg protein; where one unit is equal to moles of H₂O₂ consumed per minute; ROS is expressed as DCF fluorescence/min/mg protein; lipid peroxidation is expressed as unit/mg protein; where one unit is equal to nmoles of MDA formed. Values represent mean $\pm$ S.D ($n=6$). Values are statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

H₂O₂, ascorbate and FeSO₄ further increased LPO levels than basal in diabetic animals. Further, on insulin treatment SOD and CAT activity were increased with a significant decrease in LPO and ROS compared to the DM group.

Effect of hyperglycemia on expression of aquaporins (AQPs) *in vivo* and *in vitro*

The expression of various AQP isoforms in the testis and Leydig cells were examined using RT-PCR. The total RNA extracted from the testis and Leydig cells contained gene transcripts for a variety of AQPs, including AQP 0, 1, 3, 4, 5, 6, 7, 8, 9, 11, and 12 (Figure 3A–K). The transcript for AQP 2 was not detected either in the testis or Leydig cells and AQP10 was not analyzed as it is a pseudo gene. To determine whether experimental hyperglycemia *in vivo* or *in vitro* alters the expression of AQPs in the diabetic testis and Leydig cells, we compared the levels of gene transcripts for AQPs with diabetes and diabetes with insulin treated age-matched control animals. AQP 0 did not have any expression changes in diabetes or insulin-treated testis compared to controls further, AQP 3 and 4 were significantly decreased in diabetic testis compared to controls with a p -value less than

0.01. However, on insulin treatment, AQP3 and 4 were increased compared to DM. Our experimental results indicate a significant increase of AQPs 1, 7, 8, 9, 11, 12 in diabetic testis compared to controls and a significant decrease on insulin treatment compared to DM. AQPs 5 and 6 were significantly increased in diabetic testis compared to controls but on insulin treatment, AQP 5 remained unaltered with AQP 6 being downregulated. Specifically, AQP 9 was increased 36-fold compared to controls, on insulin treatment significantly reduced to DM with p -values less than 0.0001.

In our experiment, we did not find any expression of AQP 0 in LC540 rat Leydig cells and AQP isoforms 1, 3, 4, 7, 8, 9, 11, 12 were significantly increased with different glucose concentrations compared to normal glucose (5.5 mM). However, AQPs 5 and 6 did not show any significant changes with different glucose concentrations and also with mannitol, but AQP 6 was significantly decreased in 1 mM glucose compared to 5.5 mM glucose (Figure 4A–J). Moreover, AQPs 1, 4, 9, 12 did not show any significant expression changes on treatment with 30 mM mannitol compared to 5.5 mM glucose, surprisingly AQP 3 was found to be significantly downregulated on mannitol treatment compared to control 5.5 mM glucose. Furthermore,

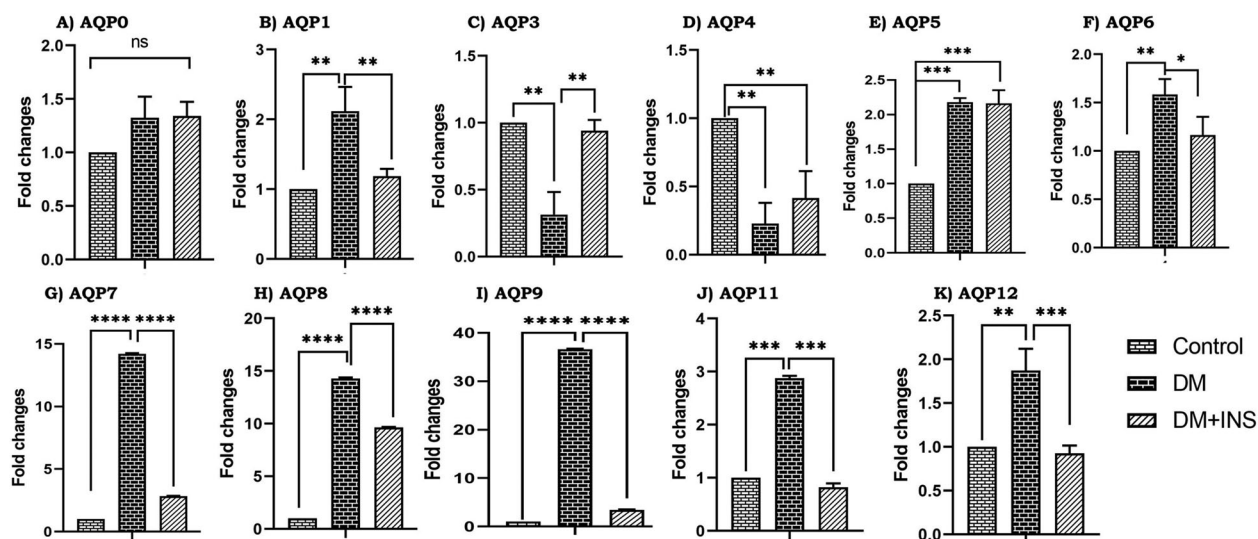


Figure 3. Effect of hyperglycemia on aquaporins gene expression. RNA levels in diabetic rat testis and also with insulin treatment (*in vivo*) were measured by RT-PCR, (A–K). All the results were normalized with β -actin. Values represent mean $\pm$ SD ($n = 6$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

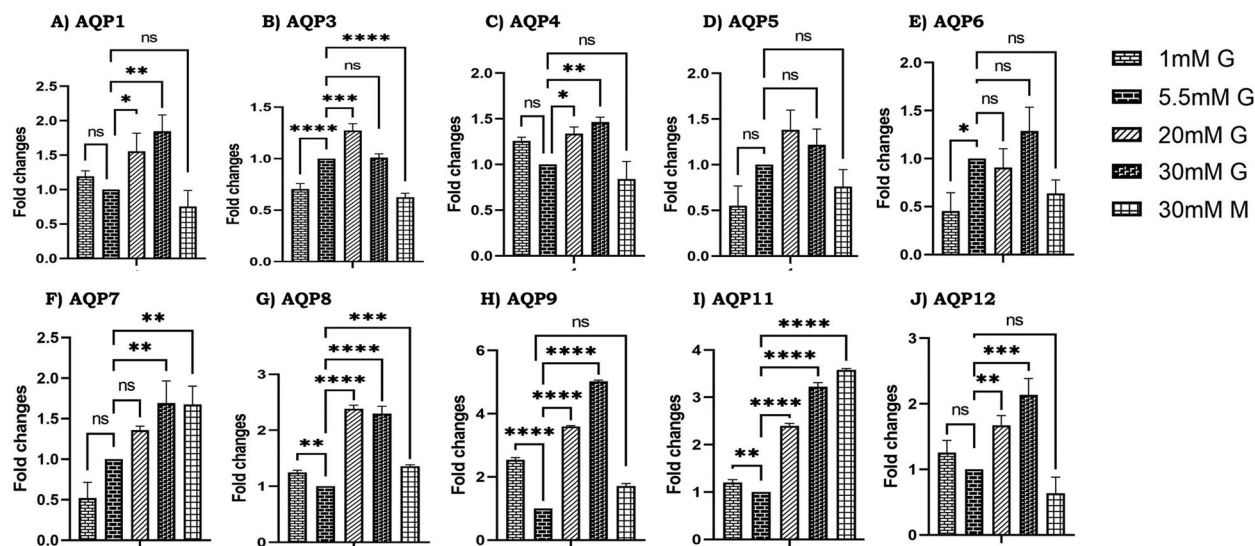


Figure 4. Effect of glucose on aquaporins gene expression. RNA levels in LC540 Leydig cells (*in-vitro*) treated with different concentrations of glucose (1 mM glucose, 5.5 mM glucose, 20 mM glucose, 30 mM glucose, 30 mM mannitol) were measured by RT-PCR (A–J). All the results were normalized with β -actin. Values represent mean $\pm$ SD ($n = 3$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

AQPs 7, 8 and 11 were significantly upregulated on mannitol treatment compared to normal glucose. Although AQP 9 was significantly increased to five-fold in high glucose (30 mM glucose) compared to controls, we didn't observe any significant alteration on mannitol treatment which suggests that high glucose induces the expression of AQP 9. Overall, our results indicate that AQP 9 is significantly increased *in-vivo* and *in-vitro* compared to other isoforms under hyperglycemia. Hence, we intended to study the role of AQP 9 in testicular steroidogenesis under hyperglycemia.

Effect of hyperglycemia on *in-vivo* and *in-vitro* expression of AQP9, nrf-2 and testicular steroidogenesis

Steroid hormones regulate essential physiological processes and are associated with various pathological conditions, finely regulated by rate-limiting steroidogenic enzymes 3β -HSD and 17β -HSD. We analyzed the steroidogenic enzymatic activity of both 3β -HSD and 17β -HSD, which were significantly decreased in diabetes testis compared to controls, however, on insulin treatment (DM + INS), enzyme activities were

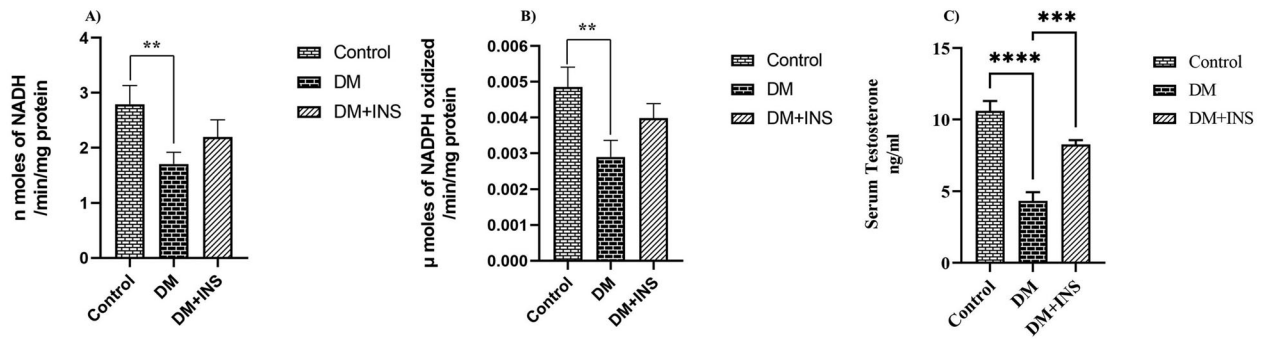


Figure 5. Effect of hyperglycemia on 3β -HSD and 17β -HSD enzyme activity and serum testosterone levels. (Control, DM, DM + INS) enzyme activity expressed as units/mg protein, whereas for (A) 3β -HSD one unit is equal to nmoles of NADH formed per minute and for (B) 17β -HSD one unit is equal to nmoles of NADPH oxidized per minute. (C) Effect of hyperglycemia on serum testosterone levels of diabetes rats (Control, DM, DM + INS). Values represent mean $\pm$ SD ($n = 6$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

increased compared to DM group but were not significant as shown in Figure 5A,B. The testosterone levels of the rats observed were dramatically decreased in diabetes group compared with those of the control group and insulin treatment significantly increased the serum testosterone levels (Figure 5C). Our results indicate that insulin treatment promotes the enzyme activity of 3β -HSD and 17β -HSD increasing serum testosterone. In the present study, the expressions of StAR, 3β -HSD and 17β -HSD were markedly downregulated in the DM group as compared with those in the control group. Surprisingly, insulin treatment resulted in significant upregulation in the expressions of StAR, 3β -HSD and 17β -HSD compared to the DM group (Figure 6A). Furthermore, *in vitro* expression of these key enzymes were studied in LC540 rat Leydig cells treated with different concentrations of glucose. Our results indicated that on treatment with 20 mM and 30 mM glucose StAR, the enzymes 3β -HSD and 17β -HSD were significantly decreased compared to 5.5 mM treated control. There were no significant changes on treatment with 30 mM mannitol as osmotic stress control compared to 5.5 mM glucose treated normal control as shown in the Figure 6B.

AQP 9 protein expression was significantly increased *in-vivo* in response to hyperglycemia compared to controls, however insulin treatment was significantly decreased compared to the DM group as shown in the Figure 6C. Moreover, *in-vitro* results also confirmed the same with significant increase in AQP 9 protein expression on high glucose treatment as shown in Figure 6D compared with 5.5 mM glucose.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor regulating antioxidant response elements, decrease oxidative stress response associated with hyperglycemia and increases insulin sensitivity (Rahimi et al. 2021). Our *in vivo* results show a

significant decrease of Nrf2 protein expression in the DM group compared to controls but on insulin treatment the expression was found to increase significantly with $p < 0.01$ and $p < 0.001$, respectively, as shown in the Figure 6C. *In vitro* Nrf2 protein expression was also analyzed with different concentrations of glucose and mannitol as an osmotic control. The expression was significantly decreased at 30 mM glucose concentration and mannitol did not show any significant changes compared to 5.5 mM glucose concentration with p -value ($p < 0.01$), as shown in Figure 6D. Our results indicate significant changes in enzymatic activity and gene expression of 3β -HSD and 17β -HSD, the key players in testosterone synthesis. We did investigate the protein expression of the key enzymes in testosterone secretion and the steroidogenic genes (3β -HSD and 17β -HSD) were found to be decreased significantly in the DM group along with steroid transport protein StAR compared to control. On treatment with insulin, StAR, 3β -HSD and 17β -HSD were significantly increased compared to DM group rats as shown in the Figure 6C with p values ($p < 0.01$). *In vitro* experiments with different concentrations of glucose treatment on LC540 Leydig cells significantly decreased the protein expression of StAR, 3β -HSD and 17β -HSD compared to 5.5 mM normal glucose but on treatment with mannitol did not have any significant changes compared to normal 5.5 mM glucose as shown in the Figure 6D.

Effect of AQP 9 knockdown on testicular steroidogenesis under hyperglycemia

We investigated the effect of AQP 9 knockdown on steroidogenic protein expression. The transfection of siRNA caused an almost 60% reduction in AQP 9 mRNA and protein levels in LC540 cells compared to

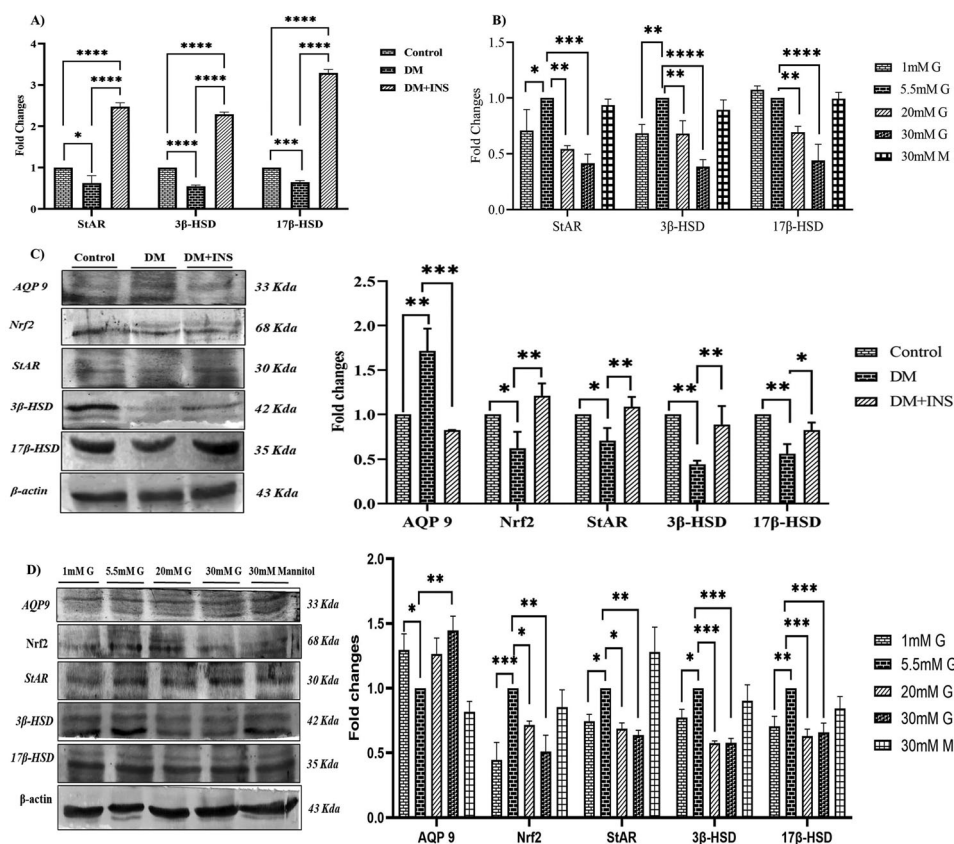


Figure 6. Effect of hyperglycemia on steroidogenesis. (A) *In-vivo* gene expression of StAR, steroidogenic genes 3β-HSD and 17β-HSD in diabetic rat testis was assessed using Real time PCR. (B) Effect of hyperglycemia on *in vitro* gene expression of StAR, steroidogenic genes 3β-HSD and 17β-HSD on LC540 rat Leydig cells treated with different concentrations of glucose using Real time PCR. (C) Effect of hyperglycemia on *in vivo* protein expression of AQP9; Nrf-2, StAR and steroidogenic genes (3β-HSD and 17β-HSD) in diabetic rat testis. (D) Effect of hyperglycemia on *in-vitro* protein expression of AQP9; Nrf2, StAR and steroidogenic genes (3β-HSD and 17β-HSD) in LC540 rat Leydig cells treated with different concentrations of glucose. All the results were normalized with β-actin (*In vivo* $n = 6$ & *In vitro* $n = 3$). Values represent mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

their respective controls as shown (Figure 7A,B). To confirm the role of AQP 9 on steroidogenesis, AQP 9 and NC siRNA transfected LC540 rat Leydig cells were treated with high glucose (30 mM) and the expression of Nrf2, StAR and steroidogenic proteins were determined by western blotting. Our results demonstrated that AQP 9 knockdown significantly upregulated the protein levels of Nrf2, StAR, 3β-HSD and 17β-HSD in high glucose treated Leydig cells (Figure 7C). Thus, our results confirm that AQP 9 knockdown restores the expression of Nrf2, StAR and steroidogenic proteins (3β-HSD and 17β-HSD) in high glucose treated Leydig cells.

Discussion

Chronic hyperglycemia arising from glucose dysmetabolism owing to insulin resistance and pancreatic dysfunction has been linked to the complications associated with diabetes (Wolpin et al. 2013). Moreover,

oxidative stress, hormonal modulation, impaired cellular pathways and altered testicular glucose metabolism have been implicated in the pathophysiology of testicular dysfunction in diabetes (Alves et al. 2013; Rato et al. 2013; Maresch et al. 2017). In the present study, body weight of diabetic rats was reduced along with a significant increase in serum glucose levels indicating severe hyperglycemia on STZ exposure. Further, treatment with insulin significantly increased body weight and decreased serum glucose levels indicating this effect is only due to hyperglycemia and our results were consistent with previous studies (Wang-Fischer and Garyantes 2018; Faisal Lutfi et al. 2021; Guven et al. 2021). A causative link among hyperglycemia, mitochondrial ROS generation, oxidative stress and the development of complications has been suggested to play a key role in the pathogenesis of diabetes (Kassab and Piowar 2012). Moreover, the primary cause of hyperglycemia is due to excessive formation of ROS and simultaneously deteriorating antioxidant

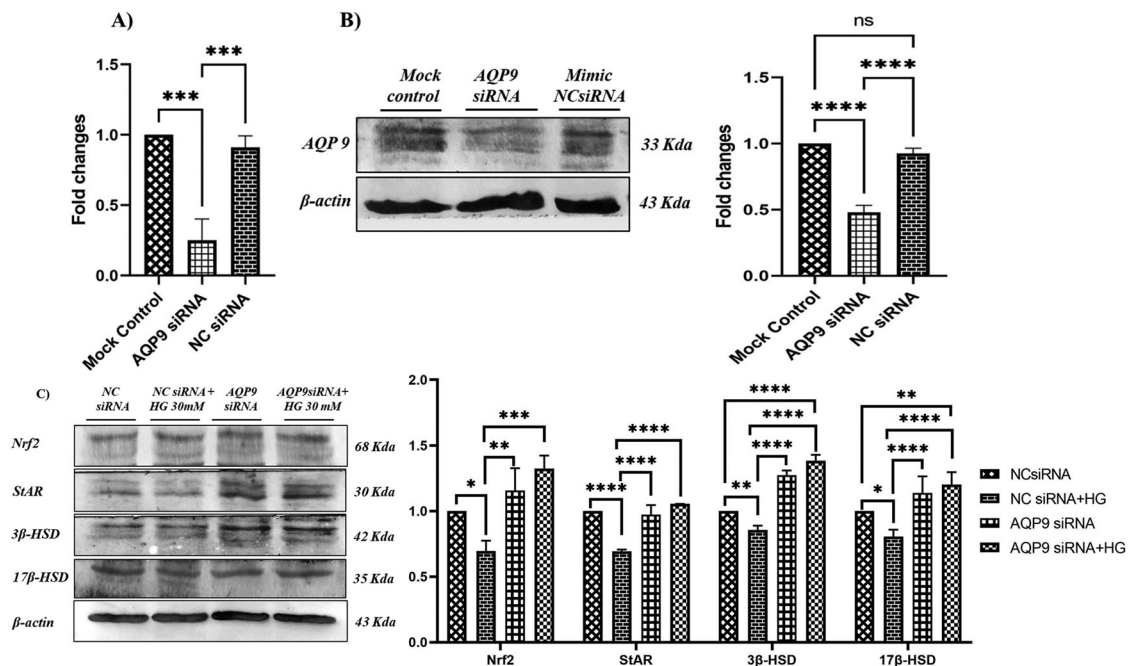


Figure 7. Effect of AQP 9 knockdown on Leydig cell steroidogenesis. Figures (A) and (B) are representative image to show AQP 9 knockdown in LC540 cells. (C) Effect of AQP 9 knockdown under hyperglycemia on protein expression of AQP 9, Nrf2, StAR and steroidogenic genes (3β-HSD and 17β-HSD) normalized with β-actin as internal control. Values represent mean ± SD ($n = 3$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

defense mechanism which leads to development of insulin resistance, increased lipid peroxidation, and cellular damage (Rehman and Akash 2017).

On the other hand, hyperglycemia induces testicular damage, impairs spermatogenesis, decreases sperm count by inducing large quantities of ROS including in the sperms, epididymis, and testes, leading to apoptosis induction. Earlier studies have shown that, hyperglycemia associated oxidative stress affecting SOD, CAT, LPO and GSH induced diabetic complications in different organs like liver, heart, kidney, pancreas, brain and testis (Singh et al. 2013; Yu and Huang 2015). Following severe hyperglycemia in our study, major radical scavenging enzymes SOD and catalase were reduced and increased ROS formation with enhanced lipid peroxidation. These changes lead to antioxidant imbalance; thereby affects steroidogenesis in diabetic rats and our results were consistent with the earlier studies (Singh et al. 2013; Yu and Huang 2015; Dlodla et al. 2017; Malekiyan et al. 2019; Nna et al. 2019; Shoorei et al. 2019).

AQPs functional involvement in the transport of ROS is the key factor in dysfunctions underlying oxidative stress related disease physiology and pathophysiology (Yusupov et al. 2019). Recent discoveries suggest that, AQPs may play an important role in fat accumulation and regulation of oxidative stress, the two crucial aspects of diabetes and metabolic

syndrome (Galli et al. 2021). AQP 3 and 5 expressed in cancerous tissues in response to oxidative stress plays an important role in cell survival and cancer progression (Milkovic and Gasparovic 2021; Wang et al. 2021). Moreover, under hyperglycemia, AQP 5 in eye lens controls ROS accumulation prevents cataractogenesis and also regulates diabetes induced dry mouth, hyposalivation, or xerostomia altering the antioxidant imbalance (Lee et al. 2020; Varadaraj and Kumari 2020). Increased AQP 8 expression induced by extracellular oxidative stress in retinal pigment epithelial cells is involved in pathology of diabetic retinopathy and age-related macular degeneration (Schnabel et al. 2020). In obese patients, AQP 9 prevents excessive lipid accumulation and the subsequent aggravation of hyperglycemia in response to AQP 3 glycerol reflux (Hou et al. 2018). Earlier studies have shown that, AQP 9 knockout mice showed decreased plasma glucose levels (Rojek et al. 2007; Jelen et al. 2011; Spegel et al. 2015). In liver, AQP 9 expression was found to be increased in both type 1 and type 2 diabetes but the expression was decreased with the circulating insulin levels (Carbrey et al. 2003; Hou et al. 2018). Following oxidative stress in our studies, we did analyze the expression changes of AQP isoforms under hyperglycemia. To the best of our knowledge, our study is the first to report differential expression of AQPs 0–12 in diabetes rats (*in vivo*) and

LC540 Leydig cells (*in vitro*). AQP 9 was found to be significantly upregulated both *in vivo* and *in vitro* indicating that hyperglycemia regulates AQP 9 expression. AQPs 1, 8 and 9 expression in testis is found to be cell specific and their expression appear to be regulated by androgens (Badran and Hermo 2002). Oxidative stress induced AQP 9 and 12 in retina aids in lactic acidosis, preventing subretinal edema (Hollborn et al. 2012). Aqp9 Gene Deletion Enhances Retinal Ganglion Cell (RGC) death and dysfunction induced by optic nerve crush (Carbrey et al. 2003; Mori et al. 2020). It is clear from the earlier reports that, AQPs are involved in maintaining oxidative stress and involved in the pathophysiology of diabetic complications. Furthermore, elimination of hydrogen peroxide and ROS accumulation affects oxidative stress and in turn regulates AQPs (3, 7, 8 and 11) involved in sperm volume regulation, swelling and tail bending affecting motility (Pellavio and Laforenza 2021). Aquaporin water channels expressed in the male reproductive tract plays an important role in lumen fluid secretion of seminiferous tubules occurring during testis development and the fluid movements during spermatogenesis, sperm concentration and maturation.

Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is the key regulatory process maintaining oxidative stress and prevents diabetic complications of multiple organs regulating antioxidant imbalance (Guo et al. 2021; Li et al. 2021; Mathur et al. 2021; Zhang et al. 2021). A recent study has shown that early brain injury after subarachnoid hemorrhage in rats, via activation of Nrf2 and inhibition of AQP4 reduced oxidative stress preventing injury (Zhang et al. 2020, 2021). Nrf2 plays a critical role in the long-term recovery of permanent cerebral ischemic damage and contribute to neuro protection. The reactive gliosis and regulation of transmembrane water channel AQP 4 confers neuro protection via Nrf2 (Liu et al. 2019). Upregulation of Nrf2 in testis suppressed oxidative stress and lowered lipid peroxidation. This in turn is shown to ameliorate antioxidant imbalance preventing the impairment of spermatogenesis and steroidogenesis (Nna et al. 2020; Arab et al. 2021; Khalil et al. 2021; Nasiri et al. 2021). We observed a significant decrease in Nrf2 expression in STZ exposed diabetic rats as shown in earlier studies (Zhao et al. 2016; Pan et al. 2017). Similarly, a significant decrease in Nrf2 expression was observed in high glucose treated LC540 Leydig cells and this could be due to oxidative stress following hyperglycemia. Nrf2 knockout mice exhibited significant diabetes-induced loss in testicular weight and sperm count, as

well as increased testicular apoptotic cell death compared to wild-type mice indicating its crucial role in maintaining fertility (Pan et al. 2017).

A proven feature of DM is the reduction in male fertility, in terms of semen parameters, sperm structures and testosterone deficiency (Pitteloud et al. 2005; Condorelli et al. 2018). The steroidogenic enzyme activities 3β -HSD and 17β -HSD were found to be decreased in diabetic rat testis. Steroid acute regulatory protein (StAR) transfers cholesterol from the outer to the inner mitochondrial membrane, which in turn mediates synthesis of key testosterone regulating enzymes 3β -HSD and 17β -HSD. 3β -HSD is a steroidogenic enzyme in rat Leydig cells that catalyses the oxidative conversion of pregnenolone to progesterone and 17β -HSD aids in the conversion of androstenedione to testosterone, the main rate-limiting enzymes. Further, *in vivo* and *in vitro* gene and protein expression analysis indicated a significant decrease in key steroidogenic enzymes 3β -HSD and 17β -HSD along with StAR. This in turn, decreased testosterone in diabetic rats in concurrence with earlier studies (Nna et al. 2019; Shoorei et al. 2019). It has also been reported earlier that silencing AQP 11 increased 3β -HSD gene expression and other spermatogenesis related genes (Shannonhouse et al. 2014). However, the involvement of AQPs and Nrf2 in regulation of male fertility cannot be denied. We hypothesize that impairment of testicular steroidogenesis could be due to AQP 9 regulating Nrf2 therefore, we knocked down AQP 9 and interestingly we observed a significant increase in Nrf2, StAR 3β -HSD and 17β -HSD protein expression. Thus, we conclude that hyperglycaemia induced oxidative stress upregulates AQP 9 expression and in turn downregulates Nrf2 leading to impaired testicular steroidogenesis.

Conclusion

Diabetes associated hyperglycemia induced oxidative stress is a widely proven cause for male infertility. Although studies address the role of AQPs in spermatogenesis, only handful of studies are available to show the role of AQPs in steroidogenesis. Our study showed that, hyperglycemia induced oxidative stress and, in turn, upregulated AQP 9 resulted in impaired testicular steroidogenesis. Further, the knock down of AQP 9 significantly upregulated Nrf2 thereby reducing oxidative stress and upregulating the expression of steroidogenic proteins. However, more studies are required to further understand the molecular

mechanisms behind AQP 9 mediated regulation of steroidogenesis under hyperglycemia.

Materials and methods

Chemicals

Streptozotocin (STZ) and cell culture reagents were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals used were of analytical grade obtained from Sigma Chemicals Company, Saint Louis, MO, USA, HiMedia Laboratories Pvt. Ltd., and Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

Experimental animals

Adult male albino rats of Wistar strain weighing 200 ± 25 g (10–12 weeks old) were used in this study. The animals were maintained under standard conditions of humidity, temperature ($25 \pm 2^\circ\text{C}$) and light (12 h light/dark). The animals were housed in large spacious cages bedded with husk and fed with a standard pellet diet with free access to water. The animals were randomly divided into three groups with six animals each and citrate buffer treated animals served as controls (Group I; control), Group II (DM) animals received intraperitoneal (i.p) injection of 60 mg/kg body weight Streptozotocin (STZ) dissolved in 0.1 M Citrate buffer. Group III (DM + INS) animals received intraperitoneal (i.p) injection with 60 mg/kg body weight STZ dissolved in 0.1 M Citrate buffer along with 2 U/kg b.w of Insulin subcutaneously (Lantus; Sanofi-Aventis, France) after 48 h on alternate days for 4 weeks. The serum glucose levels of control, DM and DM + INS were measured before the start of experiment (0th day) and after administration of STZ at 1st, 2nd, 3rd and 4th weeks by tail vein bleed, using ONE TOUCH Select glucometer (Life Scan, Europe). The serum glucose levels higher than 200 mg/dl were considered diabetic (Qinna and Badwan 2015). The animals were sacrificed with overdose of ketamine intramuscularly after the study period of 4 weeks and the organs isolated were stored at -80°C until the experiment. The serum testosterone levels were estimated by using enzyme immunoassay test kit (Alpco Diagnostics, USA) as per manufacturer protocol.

Cell culture and transfection

The LC-540 rat Leydig cells were purchased from National Centre for Cell Science (NCCS, Pune). Rat testis Leydig cells (LC-540) were cultured in a

humidified atmosphere containing 5% CO_2 in a complete medium composed of MEM Eagle (Earle's BSS) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (Sadasivam et al. 2015). To determine the effect of experimental hyperglycaemia on AQPs expression in LC540 rat Leydig cells, the growth medium was replaced by serum free medium and cells were exposed to different concentrations of glucose (1 mM, 5.5 mM, 20 mM and 30 mM) of which 5.5 mM served as control and 30 mM mannitol as an osmotic control for 24 h. For the AQP 9 knockdown experiment, LC540 cells were transfected with AQP 9 siRNA (Eurogentec, Belgium) or Silencer negative control (Sigma Aldrich, USA). siRNAs were obtained as annealed oligos and transfected at a final concentration of 60 nM. The transfection of siRNA was performed using lipofectamine RNAiMAX reagent according to the manufacturer's protocol (Invitrogen, Thermo Fisher Scientific, Inc.) (Hattori et al. 2017).

Preparation of tissue homogenate and cell extract

Briefly, cell extracts from LC540 cells and the tissue homogenate from testes were prepared in lysis buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 5 mM MgCl_2 , 0.2 mM EDTA, 0.5% NonidetP-40, 20% glycerol, 1 mM phenylmethylsulfonylfluoride, protease and phosphatases inhibitors). The cell extract/tissue homogenate was centrifuged and the supernatant was used for further analyses.

Estimation of protein

The total protein from the tissue extracts was estimated according to Lowry et al. (Lowry et al. 1951). Briefly, 0.1 mL of the diluted tissue homogenate was made up to 1 mL with water. To this, 4.5 mL of alkaline copper reagent (2% Na_2CO_3 in 0.1 N NaOH was mixed with 0.5% CuSO_4 containing 1% sodium potassium tartrate in the ratio of 50:1) was added, mixed and allowed to stand at room temperature for 20 min. Later, 0.5 mL of Folin's Ciocalteu reagent was added and shaken well. The blue colour complex formed was measured at 640 nm after 15 min against the blank with biospectrometer basic (Eppendorf, Germany).

Assay of enzymatic antioxidant - superoxide dismutase (SOD)

The enzyme was assayed according to the method of Marklund and Marklund (Marklund and Marklund

1974). The degree of inhibition of auto-oxidation of pyrogallol, in an alkaline pH by SOD was used as a measure of the enzyme activity. The enzyme activity is defined as units/mg protein, where one unit is equal to the amount of enzyme required to inhibit auto oxidation of pyrogallol by 50%. The assay was performed and the absorbance was read at 470 nm at different time intervals in an ELISA iMARKTM microplate reader (Bio-Rad, USA).

Assay of enzymatic Antioxidant - Catalase (CAT)

The activity of catalase was assayed by the method of Sinha (Sinha 1972). The assay is based on the fact that chromic acetate is formed reducing dichromate in acetic acid in the presence of H₂O₂. The chromic acetate thus formed was measured at 610 nm in an ELISA iMARKTM microplate reader (Bio-Rad, USA). The activity of catalase was expressed as μ moles of H₂O₂ consumed/min/mg protein.

Assay of lipid peroxidation (LPO)

Thiobarbituric acid (TBA) was used to estimate LPO following the procedure of Hogberg et al. (Hogberg et al. 1974). The intensity of oxidative stress is proportional to the malondialdehyde (MDA) formed, an end product of peroxidation of lipids. MDA reacts with TBA to generate a coloured product that absorbs at 532 nm was read in an ELISA iMARKTM microplate reader (Bio-Rad, USA). The ferrous sulphate and ascorbate induced LPO system contained 10 mM ferrous sulphate and 0.2 mM ascorbate as inducers (Devasagayam and Tarachand 1987). The lipid peroxides were expressed as nmoles of MDA formed/mg protein.

Estimation of 3 β -hydroxysteroid dehydrogenase (3 β -HSD)

The activity of testicular 3 β -HSD was measured by the method of Shivanandappa and Venkatesh (Shivanandappa and Venkatesh 1997). The assay buffer containing 0.1 M Tris HCl (pH 7.8), 500 μ M NAD⁺, 0.08% iodonitrotetrazolium chloride in 1% Tween 20 and 0.1 mM pregnenolone was made up to a final volume of 3 mL. To which 50 μ L of the enzyme was added and incubated at 37 °C for 60 min. And then, 2 mL of phthalate buffer was added to stop the reaction. The mixture was centrifuged at 3000 rpm for 20 min and the supernatant was read at 490 nm with Biospectrometer basic (Eppendorf, Germany). The

activity of 3 β -HSD was calculated from the standard curve of NADH and expressed as nmoles of NADH formed/min/mg protein.

Estimation of 17 β -hydroxy steroid dehydrogenase (17 β -HSD)

The activity of testicular 17 β -HSD was determined by the method described previously (HU 1974). Briefly, the reaction mixture was prepared with 100 μ L of LC540 cell lysate, 200 μ L of 0.5 μ M NADPH, to a final volume of 3 mL 100 μ M phosphate buffer (pH 7.4). The reaction was initiated by the addition of 100 μ L of 0.8 μ M androsten-3,17-dione and the decrease in absorbance of NADPH was followed at 340 nm at 20 sec intervals for 5 min with Biospectrometer basic (Eppendorf, Germany). The enzymatic activity of 17 β -HSD was given as nmoles of NADPH oxidized/min/mg protein.

Extraction of RNA and analysis of the gene expression by quantitative real time polymerase chain reaction (qRT-PCR)

Briefly, the total RNA from testes and LC540 cells were isolated according to instructions from the RNA isolation kit (One step RNA TRIzol Reagent; Biobasic Inc., Markham Ontario, Canada). First-strand cDNA synthesis was performed using the iScript cDNA synthesis kit Bio-Rad Laboratories, Inc., USA) following the manufacturer's protocol. The real-time amplification of the cDNA was achieved using SSO Advanced Universal SYBR Green Supermix according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., USA). The Insta Q96 Himedia Real-Time PCR (RT-PCR) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) was used to analyse the gene expression with specific sets of primers used for target of primers genes are given in Table 1. Fold differences in target genes expression were calculated using the formula $2^{\Delta\Delta Ct}$. The expression of target genes were normalized with respective internal controls.

Western blot analysis

Proteins from cell extract/tissue homogenate were separated by SDS-PAGE and subsequently electroblotted onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in 1 $\times$ TTBS (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) supplemented with 5% non-fat dry milk. After blocking, membranes were incubated overnight at

Table 1. Primer sequences for target gene – real time PCR.

Gene	Sense (5'-3')	Antisense (5'-3')
AQP 0	GCTCTGCTATCCTCACCAG	CAGCTTTACAGGCGCTGAG
AQP 1	CTTACTCCAGGACCTTCC	TAGTCATCCACAGTGCTC
AQP 2	GCTGTCAATGCTCTCCACAA	GGAGCAACCGGTGAAATAGA
AQP 3	AGCAGATCTGAGTGGGCACT	CTTGGGCTTAAGAGGGGAAC
AQP 4	CGGTTTCATGGAAACCTCACT	CATGCTGGCTCCGGTATAAT
AQP 5	TCTGGGTAGGGCTATTGTG	CAGCTCGATGGTCTTCTTCC
AQP 6	GTCAACGTGGTCCACAACAG	TGCAAACTTCCCAACAATGA
AQP 7	GCAGGTGGAGAACTGTTGGT	TGTGTTTCATGCCTAGGGACA
AQP 8	TGGAACCTGGAACTCCTTTG	AGTACGCATGCGACTGGGTTT
AQP 9	CTCAGTCCCAGGCTCTTAC	ATGGCTCTGCCTTCATGTCT
AQP 11	TTTGGGCACCTTTCAAACCT	GAATGAGCCTTTTCCAGCAG
AQP 12	GGGAGCTCAGCGAACTACAC	AGGATTGAAGAAGGCAGACG
Steroidogenic Acute Regulatory Protein (StAR)	AGCCAGCAGGAGAAATGGAGAT	CACCTCCAGTCGGAAACACCTT
3 β - Hydroxysteroid Dehydrogenase (3 β -HSD)	AACTGGTCTTCAGGTCACCAAGAA	GTCCCTGCACCTTGTTC
17 β - Hydroxysteroid Dehydrogenase (17 β -HSD)	CCTTTGGCTTTGCCATGAGA	CAATCCATCTGCTCCAACCT
β -Actin	AAGATCATTGCTCTCTCTG	AAAGAAAGGGGTGAAAACGC

Table 2. Primary and secondary antibodies used for western blotting (source and dilutions).

Antibody details	Cat.no	Dilution
AQP 9 Antibody (H-40); Rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)	SC-28623	1:500
Anti-StAR Antibody (StAR) (D-2); Mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)	SC-166821	1:200
Anti-3 β -HSD Antibody (3 β -HSD) (37-2); Mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)	SC-100466	1:500
Anti-17 β -HSD Antibody (17 β -HSD) (A-5); Mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)	SC-376719	1:200
Anti- β -Actin Antibody (β -actin) (AC-15); Mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)	SC-69879	1:500
Rabbit Anti-Nrf2, NFE2L2; polyclonal antibody (Bioassay Technology Laboratory, China)	BT-AP02152	1:200
Goat Anti-Mouse IgG H&L Alkaline Phosphatase; Secondary antibody (Abcam, Cambridge, UK)	ab97020	1:5000
Goat Anti-Rabbit IgG H&L Alkaline Phosphatase; Secondary antibody (Abcam, Cambridge, UK)	ab6722	1:2000

4°C, with specific primary antibodies listed in Table 2. Membranes were washed four times for 10 min in TTBS and subsequently incubated for 3 h at room temperature with a secondary alkaline phosphatase-conjugated antibody and the proteins were visualized using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) chromogen substrate (Sigma Chemicals Company, Saint Louis, MO Inc., USA). Lab image platform ver 2.1 software by Kapelan Bio-Imaging GmbH was used for densitometric analysis. The expression of each target protein were normalized with corresponding internal control.

Data analysis

The values are expressed as mean $\pm$ standard deviation (SD). Graph pad Prism 9.0 software was used to determine the differences between the groups assessed by one-way ANOVA. Inter-group comparisons were performed using Tukey's multiple comparisons test. Values were considered significant at $p < 0.05$.

Ethics approval for animal study

Experimental animals were handled according to university and institution legislation, regulated by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of

Social justice and Empowerment, Government of India (BDU/IAEC/P04/2018).

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Disclosure statement

No potential conflict of interest was reported by the authors.

Authors' contributions

The study concept, experimental design and execution, data acquisition, analysis, interpretation and manuscript preparation: AK; Supervision, data interpretation and manuscript revision: CP; Critical comments and suggestions: CP, KA,

LFMA, AP, CL and DKJ. All authors discussed the content and contributed to the final manuscript.

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