Influence of antifertility agents Dutasteride and Nifedipine on *CatSper* gene level in epididymis during sperm maturation in BALB/c mice

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Abstract

Calcium (Ca^{2+}) signaling is critical for successful fertilization. In spermatozoa, capacitation, hyperactivation of motility and the acrosome reaction are all mediated by increases in intracellular Ca^{2+} through CatSper (sperm-specific cation channel). The CatSper channel complex contains four pore-forming α subunits (CatSper1–4) and five accessory subunits called β , δ , ϵ , γ and ζ . Genetic deletion of any of the four *CatSper* genes in mice results in loss of hyperactivated motility and male infertility. Despite their vital role in male fertility, almost very little is known about influence of antifertility agents on *CatSper* gene expression in epididymis and epididymal spermatozoa. Therefore, we performed quantitative real-time qPCR analysis for *CatSper* expression in the epididymis and epididymal sperm of BALB/c mice after treatment with Dutasteride (DS), a dual 5- α reductase inhibitor and Nifedipine (NF) a calcium channel blocker as positive control. We observed that treatment with antifertility agents Dutasteride and Nifedipine induced significant decreases in the caput and cauda epididymal sperm counts, motility and fertility which could partly be attributed to alteration in the normal morphology of the sperm associated with downregulation/upregulation of *CatSper* mRNAs in epididymis and epididymal spermatozoa of male BALB/c mice. These can be explained on the basis of interference with mechanisms affecting calcium ion signaling resulting in changes in intracellular calcium required for sperm activity, finally affecting sperm maturation and fertility of male BALB/c mice. These studies provide some novel avenues for developing new male contraceptives in future.

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Introduction

The mammalian epididymis provides a specific intra-luminal environment for the morphological and biochemical modifications needed to produce functionally mature spermatozoa (reviewed by Cooper 2007, Cornwall 2009, Dacheux & Dacheux 2014). Mammalian spermatozoa undergo a series of morphological, physiological and biochemical post-translational changes during sperm maturation in the epididymis, which result in their acquisition of motility and fertilizing capability (Phelps *et al.* 1990, Jones 1998, Srivastav 2000). The sperm plasma membrane undergoes extensive structural and biochemical modifications within the epididymis that lead to the acquisition of progressive motility, capacitation, zona pellucida binding and penetration and fertilizing capability. In particular, sperm must undergo a process known as capacitation that leads to hyperactivated motility and prepares the sperm for acrosomal exocytosis (Visconti *et al.* 2011).

Calcium signaling is critical for successful fertilization. Calcium channels play important roles in the process

of fertilization and control sperm motility, chemotaxis toward the egg and the acrosome reaction (Chung *et al.* 2011, reviewed by Ren *et al.* 2010, Lishko & Kirichok 2010, Sun *et al.* 2017). In spermatozoa, capacitation, hyperactivation of motility and the acrosome reaction are all mediated by increases in intracellular Ca^{2+} through CatSper, which are expressed exclusively in spermatogenic cells in testis. These specifically traffic to membranes of the developing sperm flagellum and play important roles in sperm motility and male fertility (Ren *et al.* 2001, reviewed by Navarro *et al.* 2008, Sun *et al.* 2017). CatSper is a heterotetrameric Ca^{2+} channel required for the hyperactivated motility of spermatozoa and male fertility (Qi *et al.* 2007, reviewed by Navarro *et al.* 2008, Lishko *et al.* 2012, Sun *et al.* 2017). The CatSper channel complex contains four pore-forming α subunits (CatSper1–4) and five accessory subunits called β , δ , ϵ , γ and ζ (Chung *et al.* 2017, reviewed by Navarro *et al.* 2008, Sun *et al.* 2017). The involvement of several subunits makes the channel complex, which seems to be required for its functional co-ordination, localization to the flagella and sensitivity to intracellular pH, progesterone, other

proteins and cell-signaling molecules and activators (reviewed by Benoff *et al.* 2007, Navarro *et al.* 2008, Ren & Xia 2010, Darszon *et al.* 2011, Lishko *et al.* 2012, Sun *et al.* 2017).

Defects in the primary pH-sensitive cationic calcium channel of sperm (CatSper) were shown to result in complete male infertility without other apparent defects in phenotype. The absence of CatSper channels was shown to cause male infertility in mice (Qi *et al.* 2007, Hildebrand *et al.* 2010, Chung *et al.* 2011), which is primarily because of the inability of sperm to hyperactivate. Mutation or absence of some ion channels directly affects male fertility (Carlson 2003, Smith *et al.* 2013), and lack of expression/mutation or disruption of any of the four *CatSper* genes leads to complete male infertility in both human and mice. Targeted disruption of *CatSper1* (Ren *et al.* 2001), *CatSper2* (Quill *et al.* 2003), *CatSper3* or *CatSper4* (Jin *et al.* 2007) each produced male infertility in mice. Despite the restricted localization of CatSper to testicular, epididymal and ejaculated spermatozoa and their unique structure with vital role in male fertility, very little is known about the pharmacological targeting of the CatSper channel imposing a selective late-stage block to fertility (Carlson *et al.* 2009, Mannowetz *et al.* 2017, Wang *et al.* 2017). Carlson *et al.* (2009) have identified HC-056456 as a blocker of CatSper channel activity and further demonstrated that pharmacologic blockade of the CatSper channels not only prevents the development of sperm-hyperactivated motility but also causes a reversible loss of flagellar waveform asymmetry to pre-hyperactivated sperm.

The process of sperm maturation in the epididymis has been an advantageous post-testicular target for the development of safe, rapid and reversible male contraceptive. Some processes of epididymal sperm maturation, such as the initiation of progressive motility can be selectively interrupted, which induces dysfunction of sperm fertilization and male infertility. Several antifertility compounds are shown to interfere with epididymal function including a dual 5 α -reductases inhibitor Dutasteride (PNU157706) and Nifedipine, a calcium channel blocker. Dutasteride is a novel dual 5 α -reductase inhibitor used for the potential treatment of benign prostatic hyperplasia. This compound is of express interest as it inhibits both isoforms of 5 α -reductase enzyme (di Salle *et al.* 1998) and adversely affects sperm maturation and fertility (Henderson *et al.* 2004, 2006, Henderson & Robaire 2005, Robaire & Henderson 2006). Nifedipine, also known as calcium channel blocker (CCB), is an approved drug for high blood pressure and appears to have reversible antifertility effect on males showing functional defects in spermatozoa (Lee *et al.* 2006, Morakinyo *et al.* 2009). The antifertility effects of Nifedipine and similar CCBs are well understood in both *in vitro* experiments (Kanwar *et al.* 1993) and *in vivo* studies (Morakinyo *et al.* 2009,

Lee *et al.* 2011). However, little investigation on effect of male antifertility agents on *CatSper* gene expression in the epididymis and epididymal spermatozoa of mice has been conducted. Therefore, our objective in this study was to use real-time q-PCR analysis for *CatSper* gene expression in the epididymis and epididymal spermatozoa of BALB/c mice during the process of sperm maturation after treatment with antifertility agents such as dual 5- α reductase inhibitor Dutasteride and CCB Nifedipine.

Materials and methods

Materials

Dutasteride (AVODART) was obtained from GlaxoSmith Kline Pharmaceuticals, Mumbai, India. Nifedipine (N6792, purity 99%) was obtained from Sigma-Aldrich. TRIzol reagent was from Invitrogen, Superscript II reverse transcriptase from Gibco-BRL and Ex Taq DNA polymerase from TaKaRa. All other chemicals or reagents were obtained from Sigma-Aldrich, Invitrogen or otherwise mentioned in the text.

Animals

Young adult BALB/c male mice (2–3 months old, weighing 25–30 g) were bred and maintained in conditions with free access to water and food in the animal facility of CSIR-Central Drug Research Institute (CDRI), Lucknow, India. All protocols and experimental procedures for the use of animals in this study were performed with prior approval of the Institutional Animal Ethics Committee. The animals were housed in climate (23 $\pm$ 2°C, RH: 60%) and photoperiod (12-h light–12-h darkness cycles) controlled animal quarters.

Study design

Thirty mice were divided into three different groups with $n=10$ in each group. The doses were selected carefully from the available literature.

Control group

DS-treated group

Mice receiving Dutasteride 'AVODART' (5 mg/kg orally for 15 days).

NF treated group

Mice receiving Nifedipine dissolved in sesame oil (20 mg/kg orally for 15 days).

Tissue collection

After the last oral feeding on day 16, mice were rapidly killed by cervical dislocation and testes, epididymides, seminal vesicles (SV) and ventral prostate (VP) were dissected out. The organs were cleared free of fat and connective tissues by washing in ice-cold sterile physiological saline (0.9% w/v), dried

briefly and weighed on electronic balance (Afcoset, Mumbai, India; Model ER-120 A). The caput and cauda epididymides were divided and subjected to tissue fixation for histological examination or were immediately cryostored in liquid nitrogen for subsequent RNA extraction and RT-qPCR analysis. In another set of experiment, epididymal spermatozoa were collected from caput and cauda epididymides and subjected either to sperm motility, count and morphology examination or were immediately cryostored in liquid nitrogen for subsequent RNA extraction and RT-qPCR analysis.

Histological study

Testis, caput and cauda epididymides of control and treated mice were fixed in Bouin's fluid (2 h) and serial sections of 5 µm thickness were stained with Harris Hematoxylin and Eosin solution as described earlier (Srivastav *et al.* 2010). Images were analyzed and recorded using BX 60 Olympus microscope equipped with a digital imaging camera (DP-12) with the aid of the Micro Image Lite software 4.0 (Olympus).

Sperm preparation

Spermatozoa from the caput and cauda epididymides were obtained by mincing the segments in Tyrode's solution without sodium bicarbonate containing 1.0 mmol/L sodium pyruvate, 9.0 mmol/L sodium lactate (pH 7.6) and 5% (w/v) BSA. The sperm preparations were incubated at 37°C for 10 min in a CO₂ incubator. The sperm was gently filtered through nylon gauze, centrifuged at 500g for 10 min and were resuspended in 1 mL fresh Tyrode's solution. An aliquot of the sperm preparation was analyzed for sperm concentration, progressive motility, morphology and vitality, and the remaining fraction was processed for sperm mRNA isolation.

Sperm motility, count and morphology

Ten microliters of sperm fraction was placed on a pre-warmed slide with pre-warmed cover slip and examined quickly for sperm motility. The slides were visualized at 200× with a temperature-controlled (37°C) phase-contrast BX 60 Olympus microscope equipped with a digital imaging camera (DP-12) and Micro Image Lite software 4.0 (Olympus). At least four fields were evaluated per group. Spermatozoa from caput and cauda epididymides were counted using Neubauer's hemocytometer. For sperm morphology, spermatozoa were collected immediately after killing of mice by flushing the vas deferens with phosphate-buffered saline (pH 7.2), and a drop was smeared on a pre-warmed slide with pre-warmed cover slip. The slides were visualized at 1000× with a temperature-controlled (37°C) phase-contrast BX 60 Olympus microscope as described earlier.

Fertility study

Fertility testing of male mice from each group was performed during the last week of the treatment period on days 10–15. The male mice ($n=6$) were cohabitated overnight with proestrous females in a ratio of 1:2. Positive mating was confirmed by

vaginal plug and presence of sperm in the vaginal smear. Mated females were separated and maintained in individual cages to record the number of pregnant females for fertility index and the number of live implantations on day 15 *post coitum* in pregnant females.

RNA extraction

The sperm fractions were purified using a discontinuous Percoll gradient (2 layers comprising of 45% and 90% v/v) in Ham's F10 medium (Sigma) as described earlier (Srivastav *et al.* 2010) to rule out the possibility of contamination by residual cells. After centrifugation (20 min at 500g, 25°C), spermatozoa were collected from under layer of 90% Percoll, and the washed sperm pellet (>80% motile) was resuspended in ice-cold PBS and immediately frozen in liquid nitrogen.

For RNA extraction, the total RNA from the liquid nitrogen-frozen epididymal spermatozoa, frozen epididymides and testis were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The purity of total RNA was checked spectrophotometrically at 260 and 280 nm. A minimum optical density (OD 260/280) ratio 1.80 was selected for the following real-time reverse-transcriptase polymerase chain reaction (RT-PCR).

Real-time qPCR

Quantitative real-time polymerase chain reaction with SYBR green chemistry was used to perform quantitative determination of mRNA levels of *CatSper1–4* genes from Dutasteride- and Nifedipine-treated mice. Total RNA (1.0 mg) for epididymis and testis or 0.5 mg for spermatozoa was reverse transcribed to complementary DNA with the Revert Aid cDNA synthesis kit (Fermentas, Austin, TX, USA) using 2 µg of total RNA in 20 µL reaction volume. For quantitative real-time polymerase chain reaction (qPCR), the cDNA was amplified by PCR using selected primers in LightCycler 480 (Roche Molecular Biochemicals, Indianapolis, IN, USA) Real-Time PCR System with pre-installed software. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using the Universal Probe Library (Roche Diagnostics). To eliminate a possible contamination by genomic DNA, primers were chosen in different exons. Primer sequence of various genes used for qPCR is given in Table 1. Briefly, a 20 µL total PCR reaction was used that included, 1 µg of cDNA, 1 µL of forward primer, 1 µL of reverse primer, 10 µL SYBR Green and 7 µL RNAase-free water. The real-time-PCR cycling conditions were 95°C for 15 s, 60°C for 30 s, 72°C for 30 s followed by 40 cycles. The temperature for melting curve program was determined between 60°C and 95°C. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), was used as the internal control in this study. We studied the fold change in genes of interest as compared to control values normalized to reference gene *Gapdh*. GAPDH has consistently been used as an internal control to normalize many genes including *CatSper* gene expression (Kim *et al.* 2015, Jahromi *et al.* 2014, Gong *et al.* 2014, Park *et al.* 2014). Also, several previous studies have used GAPDH as house keeping control for normalization in

relative mRNA expression analysis while treating with DS (Schmidt *et al.* 2009) and NF (Iwai *et al.* 2011).

Relative quantification of the expression of genes was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001) where C_T is the cycle threshold. The ΔC_T was the difference between the PCR product and its internal control GAPDH.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{T_{\text{reference}}}}}{(E_{\text{target}})^{\Delta C_{T_{\text{reference}}}}}$$

$$C_{T_{\text{target=control}}} - C_{T_{\text{treatment}}}$$

$$\Delta C_{T_{\text{reference}}} = C_{T_{\text{control}}} - C_{T_{\text{treatment}}}$$

$$\text{Ratio} = 2^{-\Delta\Delta C_T} \text{Equation 2}$$

Whereas

$$\Delta\Delta C_T = \Delta C_{T_{\text{reference}}} - \Delta C_{T_{\text{target}}}$$

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by *post hoc* Newman–Keuls multiple comparison test of significance using GraphPad Prism 3.02 software. Qualitative observations were represented following assessments made by three individuals blinded to the experimental designs.

Results

Body and reproductive organ weights

In the present study, no significant change in final body weights of male mice treated with DS at 5 mg/kg for 15 days were observed compared to controls. NF at 20 mg/kg for 15 days slightly reduced the body weights of treated male mice (Table 2). In the present study, no significant changes were observed in testis weight of male BALB/c mice treated with DS or NF compared to controls (Table 2).

Total epididymis weight was significantly reduced by DS ($P < 0.001^{***}$) and reduced non-significantly by NF vs control mice in order of DS (-27.41%) > NF

(-8.66%) as shown in Table 2. However, significant ($P \leq 0.05^*$) differences were noted in total epididymis weights between intra-groups DS vs NF (Table 2). Both DS and NF reduced the weights of caput and cauda epididymides when compared with control mice (Table 2). Caput epididymis was reduced significantly by DS ($P < 0.001^{***}$) and less significantly by NF ($P \leq 0.05^*$) vs control in order of DS (-34.62%) > NF (-16.19%) while cauda epididymis weight was reduced significantly only by DS ($P < 0.01^{**}$) and non-significantly by NF (-8.73%). However, significant ($P < 0.01^{**}$) differences in caput epididymis weights and non-significant differences in cauda epididymis weights were observed between intra-groups DS vs NF.

SV and VP weights were reduced both by DS and NF in treated mice (Table 2). SV weight was highly significantly ($P < 0.001^{***}$) reduced by DS (-54.84%) and less significantly ($P < 0.01^{**}$) by NF (-24.44%). Similarly, VP weight was highly significantly ($P < 0.001^{***}$) reduced by DS (-52.40%) and non-significantly by NF (-18.31%). However, significant ($P < 0.01^{**}$) differences were noted in SV and VP weights between DS- and NF-treated mice.

Testis histology

Testis of control mice (Fig. 1A) showed normal seminiferous tubules with normal Sertoli and germ cells at various stages of spermatogenic cycle covering complete cycle. The Leydig cells in the interstitium showed normal morphology. Testis of mice treated with Dutasteride (5 mg/kg $\times$ 15 days) showed normal germ cells, Sertoli cells and Leydig cells (Fig. 1B). Testis of mice treated with Nifedipine (20 mg/kg $\times$ 15 days) showed reduced sperm formation in seminiferous tubules with normal Sertoli and Leydig cells (Fig. 1C).

Epididymal histology

The caput (Fig. 1D) and cauda (Fig. 1G) epididymides of control mice showed normal morphology, tubular diameter and high sperm density in epididymal lumen. DS treatment at 5 mg/kg for 15 days caused marked

Table 1 Primer sequence of various genes used for qPCR.

Target genes	Sequence (5'–3')	Amplicon size (bp)	GC (%)	Molecular weight (g/mol)	Accession number
CatSper1-LP	CTGAGCTAGAGATCCGAGGTG	93	57	6511	AF407332
CatSper1-RP	CAATTAGCTTGAGGACTGCTTCT		43	7029	
CatSper2-LP	TTTGTGCTGATGGTTGAAATAGA	106	35	7148	AF411816
CatSper2-RP	TGAAGCTAAGCAAGATGAACCA		41	6785	
CatSper3-LP	GCTTCTTTACTCTTTCAGTTTGG	91	40	7571	AK014942
CatSper3-RP	CCCGGCTCACAGTAAACTTC		55	6022	
CatSper4-LP	TCCGAGAGGAACTCAACATGA	92	48	6448	AK077145
CatSper4-RP	GGAGGTATACTTGTGTAGGATCACATT		41	8369	
Gapdh-LP	TTTGATGTTAGTGGGGTCTCG	62	47.6	6514.2	NM_017008
Gapdh-RP	AGCTTGTCATCAACGGGAAG		50	6166.1	

LP, left primer; RP, right primer.

Table 2 Effect of DS and NF treatments on the body and reproductive organ weights of adult male BALB/c mice treated for 15 days.

Groups	Body weights (g)			Absolute organ weights (mg)				
	Initial	Final	Testis weight	Epididymis		SV weight	VP weight	
				Total	Caput			Cauda
Control	27.23 ± 0.31	29.26 ± 0.50	110.58 ± 6.08	31.62 ± 1.18	15.67 ± 0.55	12.43 ± 0.53	188.08 ± 15.91	4.04 ± 0.27
DS (5 mg/kg × 15 days)	27.31 ± 0.32	24.97 ± 0.76***	101.22 ± 6.63	22.95 ± 0.66***	10.24 ± 0.44***	9.05 ± 0.45**	84.92 ± 4.64***	1.92 ± 0.19***
NF (20 mg/kg × 15 days)	27.32 ± 0.39	25.83 ± 0.38**	104.45 ± 5.11	28.88 ± 2.25	13.13 ± 0.89*	11.34 ± 0.87	142.1 ± 11.95	3.31 ± 0.28
DS vs NF		DS vs NF ns	DS vs NF ns	DS vs NF ⁺	DS vs NF ⁺⁺	DS vs NF ns	DS vs NF ⁺⁺	DS vs NF ⁺⁺⁺

Values are expressed as means±S.E.M. (n=10).

Levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$ compared with control group; DS vs NF levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$.

decline in epididymal tubular size and luminal sperm density in both caput (Fig. 1E) and cauda (Fig. 1H) epididymides. On the contrary, NF at 20 mg/kg for 15 days showed normal epididymal tubular size with slightly reduced sperm density in both caput (Fig. 1F) and cauda (Fig. 1I) epididymides.

Sperm morphology

Analysis of spermatozoa from the caput (Fig. 2A) and cauda (Fig. 2D) epididymides of control mice showed normal morphology depicting head, midpiece and long tail observed under phase-contrast microscope. As observed under phase-contrast microscopy, the number of abnormal sperm was higher in both DS-treated caput (Fig. 2B) and cauda (Fig. 2E) epididymides as compared to controls. Similar observations were recorded

in NF-treated caput (Fig. 2C) and cauda (Fig. 2F) epididymides. The percentage of highly agglutinated, tailless, decapitated and broken spermatozoa exhibiting other abnormalities like swollen heads and coiled tails with significant increase in flagellar angulation were higher in mice treated both with DS and NF.

Sperm count

Spermatozoa collected from caput and cauda epididymides of control mice showed normal sperm count and motility as shown in Table 3. Spermatozoa collected from caput epididymis of treated mice exhibited significant decline in sperm count by DS ($P<0.001$ ***) and non-significantly by NF as compared to controls in order of DS (−52.42%)>NF (−19.79%). Likewise, sperm count of cauda epididymis of treated

Figure 1 (A) Histology of testis of control mice showing normal spermatogenesis. A-spermatogonia, B-spermatogonia, P-pachytene spermatocytes, R-round spermatids, E-elongated spermatids/spermatozoa, SC-Sertoli cells, LC-Leydig cells and L-Lumen. Hematoxylin (HE)-stained ×400 magnification (scale bar=55 µm). (B) Histology of testis of mice treated with Dutasteride (DS) at 5 mg/kg orally for 15 days. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (C) Histology of testis of mice treated with Nifedipine (NF) at 20 mg/kg orally for 15 days. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (D) Histology of caput epididymis of control mice. EP, epididymal epithelium; ES, epididymal stroma; S, luminal spermatozoa. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (E) Histology of caput epididymis of mice treated with Dutasteride (DS) at 5 mg/kg orally for 15 days. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (F) Histology of caput epididymis of mice treated with Nifedipine (NF) at 20 mg/kg orally for 15 days. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (G) Histology of cauda epididymis of control mice. EP, epididymal epithelium; ES, epididymal stroma; S, luminal spermatozoa. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (H) Histology of cauda epididymis of mice treated with Dutasteride (DS) at 5 mg/kg orally for 15 days. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm). (I) Histology of cauda epididymis of mice treated with Nifedipine (NF) at 20 mg/kg orally for 15 days. Hematoxylin (HE) stained ×1000 magnification (scale bar=22 µm).

Figure 2 (A) Phase-contrast photograph showing morphology of sperm from caput epididymis of control mice. Eosin stained 1000 $\times$ magnification (scale bar=22 μ m). (B) Phase-contrast photograph showing morphology of sperm from caput epididymis of mice treated with Dutasteride (DS) at 5 mg/kg orally for 15 days (1000 $\times$) (scale bar=22 μ m). (C) Phase-contrast photograph showing morphology of sperm from caput epididymis of mice treated with Nifedipine (NF) at 20 mg/kg orally for 15 days (1000 $\times$) (scale bar=22 μ m). (D) Phase-contrast photograph showing morphology of sperm from cauda epididymis of mice treated with Dutasteride (DS) at 5 mg/kg orally for 15 days (1000 $\times$) (scale bar=22 μ m). (E) Phase-contrast photograph showing morphology of sperm from cauda epididymis of control mice. Eosin stained 1000 $\times$ magnification (scale bar=22 μ m). (F) Phase-contrast photograph showing morphology of sperm from cauda epididymis of mice treated with Nifedipine (NF) at 20 mg/kg orally for 15 days (1000 $\times$) (scale bar=22 μ m).

mice exhibited significant decline in sperm count by DS ($P<0.001^{***}$) and slightly less significantly by NF ($P\leq 0.05^*$) treatments as compared to controls in order of DS (–68.46%)>NF (–38.59%).

Sperm motility

As shown in Table 3, sperm motility of caudal epididymal spermatozoa was significantly reduced by DS ($P<0.001^{***}$) and less significantly by NF ($P<0.01^{**}$) vs control in order of DS (–75.17%)>NF (–36.17%). Thus, DS caused higher reduction than NF in motility of caudal epididymal spermatozoa of treated mice.

Fertility test and contraceptive efficacy

As shown in Table 4, fertility trial during 10–15 days of treatment schedule culminated in normal pregnancy outcome in control animals where 11 mice mated out of 12 mice put for mating (evidenced by vaginal plug) and became pregnant (mating index: 91.6%, fertility index 91.6%). The fertility of DS-treated mice (5 mg/kg/day $\times$ 15 days) was declined significantly as evidenced by

100% reduction in fertility of treated males. The ability of males to mate was severely affected as evidenced by the observation that out of 12 mice put for mating, none of the DS-treated males mated with cohabitated females (mating index 0%, fertility index 0%). The fertility of NF treated mice (20 mg/kg/day $\times$ 15 days) was also reduced where 8/12 mice mated and 5/12 became pregnant (mating index 66.6%, fertility index 42.31%). The mean number of live implantations observed on day 15 *post coitum* in pregnant females of NF-treated mice was reduced (1.54 ± 0.02) as compared to control mice (6.84 ± 0.650).

Downregulation of the CatSper mRNAs in epididymis and testis of DS and NF-treated mice

Caput epididymis

DS downregulated *CatSper4* non-significantly slightly (–8.25%) in caput epididymis of treated mice as compared to control mice (Fig. 3D). Similarly, NF caused significant downregulation of *CatSper1* (–95.60%, $P\leq 0.05^*$) and *CatSper4* (–93.76%, $P<0.0001^{***}$)

Table 3 Effect of DS and NF treatments on sperm motility and sperm count of adult male mice treated for 15 days.

Groups	Sperm motility (%)	Sperm count $\times 10^6$ /mL	
	Cauda epididymis	Caput epididymis	Cauda epididymis
Control	70.5 $\pm$ 6.16	5.36 $\pm$ 0.41	7.57 $\pm$ 0.62
DS (5 mg/kg $\times$ 15 days)	17.5 $\pm$ 3.09***	2.75 $\pm$ 0.22***	2.99 $\pm$ 0.26***
NF (20 mg/kg $\times$ 15 days)	45 $\pm$ 5.42**	4.65 $\pm$ 0.40	5.84 $\pm$ 0.68*
DS vs NF	DS vs NF ⁺⁺	DS vs NF ⁺⁺	DS vs NF ⁺⁺⁺

Values are expressed as means $\pm$ S.E.M. ($n=10$).

Levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$ compared with control group; DS vs NF levels of significance: + $P<0.05$; ++ $P<0.001$; +++ $P<0.0001$.

Table 4 Effect of DS and NF treatments on fertility of adult male mice treated for 5 days.

Treatment groups	No of males put for mating	No of females put for mating	Mating index (%)	Fertility index (%)	Implantation sites on day 15 pc
			No of females mated/no of females put for mating	No of pregnant females/no of cohabitated females	Implantation sites (mean $\pm$ S.E.M.)
Control	6	12	11/12 (91.6%)	11/12 (91.6%)	6.84 $\pm$ 0.650
DS (5 mg/kg $\times$ 15 days)	6	12	0	0	0
NF (20 mg/kg $\times$ 15 days)	6	12	8/12 (66.6%)	5/12 (42.31%)	1.54 $\pm$ 0.02

mRNAs (Fig. 3A and D). The downregulation of *CatSper2* (–98.72%) and *CatSper3* (–95.88%) mRNAs by NF was non-significant in caput epididymis of treated mice (Fig. 3B and C).

Cauda epididymis

Both DS and NF downregulated all four *CatSper* channels significantly ($P < 0.001$ ***) in cauda epididymis of treated mice. There was statistically significant downregulation of the *CatSper1* (–93.77%), *CatSper2* (–96.18%), *CatSper3* (–98.47%) and *CatSper4* (–89.69%) mRNAs in cauda epididymis of DS-treated mice as compared to control mice. Likewise, NF caused statistically significant ($P < 0.001$ ***) downregulation of the *CatSper1* (–99.77%), *CatSper2* (–99.79%), *CatSper3* (–99.81%) and *CatSper4* (–98.75%) mRNAs in cauda epididymis of treated mice (Fig. 3A, B, C and D).

Testis

DS caused slight non-significant downregulation (–18.38%) of *CatSper1* mRNA, while NF caused non-significant downregulation of *CatSper2* (–23.18%) and *CatSper3* (–5.70%) mRNAs (Fig. 3A, B and C) in testis tissue of treated mice as compared to control mice.

Caput vs cauda epididymis

All four *CatSper* mRNAs including *CatSper1*, *CatSper2*, *CatSper3* and *CatSper4* were downregulated more

severely in cauda epididymis by DS (–98.572, –98.81, –99.613 and –88.77%) as well as by NF (–94.781, –83.88, –95.77 and –80.27%) treatments respectively as compared to caput epididymis in treated mice (Fig. 3A, B, C and D).

Testis vs caput epididymis

DS caused higher expressions of *CatSper1* (4.35 times; 434.01%), *CatSper2* (2.079 times; 107.91%) and *CatSper3* (3.77 times; 277.81%) mRNAs in caput epididymis as compared to testis in treated mice (Fig. 3A, B and C). However, *CatSper4* mRNA was downregulated (–20.57%) more severely in caput epididymis as compared to testis in treated mice (Fig. 3D). Likewise, NF downregulated *CatSper1* (–97.66%), *CatSper2* (–98.28%), *CatSper3* (–95.34%) and *CatSper4* (–97.24%) mRNAs more severely in caput epididymis as compared to testis in treated mice (Fig. 3A, B, C and D).

Testis vs cauda epididymis

The expressions of all four *CatSper1–4* mRNAs including *CatSper1* (–92.35%), *CatSper2* (–97.54%), *CatSper3* (–98.54%) and *CatSper4* (–91.08%) were downregulated more severely by DS in cauda epididymis as compared to testis in treated mice (Fig. 3A, B, C and D). Likewise, NF downregulated more severely all four

Figure 3 (A) Fold change in mRNA level of *CatSper1* gene relative to GAPDH of caput, cauda epididymides and testis of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ compared with control group. (B) Fold change in mRNA level of *CatSper2* gene relative to GAPDH of caput, cauda epididymides and testis of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ compared with control group. (C) Fold change in mRNA level of *CatSper3* gene relative to GAPDH of caput, cauda epididymides and testis of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ compared with control group. (D) Fold change in mRNA level of *CatSper4* gene relative to GAPDH of caput, cauda epididymides and testis of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ compared with control group.

CatSper1 (−99.87%), *CatSper2* (−99.73%), *CatSper3* (−99.8%) and *CatSper4* (−99.46%) mRNAs in cauda epididymis in treated mice as compared to testis (Fig. 3A, B, C and D).

Upregulation of the *CatSper* mRNAs in epididymis and testis of DS and NF treated mice

Caput epididymis

CatSper1 mRNA was upregulated significantly ($P<0.001^{***}$) by DS 4 times (335.85%) in caput epididymis (Fig. 3A), *CatSper2* was upregulated 3 times (195.76%) significantly ($P<0.01^{**}$) by DS (Fig. 3B) and *CatSper3* was up-regulated 3.93 times (269.27%) in DS-treated mice (Fig. 3C) as compared to control mice, which was statistically highly significant ($P<0.001^{***}$).

Testis

DS caused non-significant upregulation of *CatSper2* (47.56%), *CatSper3* (4.23%) and *CatSper4* mRNAs (15.52%) in testis (Fig. 3B, C and D) of treated mice as compared to control mice. Likewise, NF caused significant ($P<0.01^{**}$) upregulation of *CatSper1* (87.790%) and *CatSper4* (128.71%) mRNAs highly significantly ($P<0.001^{***}$) in testis (Fig. 3A and D) of treated mice as compared to control mice.

CatSper mRNA expressions of caput and cauda spermatozoa are downregulated by male antifertility agents DS and NF, known to inhibit fertility in rodents

Downregulation of the *CatSper1*, 2 and 4 mRNAs by caput spermatozoa

We identified a remarkable downregulation of *CatSper*s in the epididymal spermatozoa of mice treated with male antifertility agents DS and NF, known

to inhibit fertility in rodents. *CatSper1* mRNA (Fig. 4A) was downregulated both by DS (−67.86%) and NF (−60.69%) treatments significantly ($P<0.001^{***}$), *CatSper2* mRNA (Fig. 4B) was downregulated by DS (−99.99%) and NF (−98.17%) highly significantly ($P<0.001^{***}$). Likewise, *CatSper4* mRNA (Fig. 4D) was downregulated both by DS (−99.99%) and NF (−98.17%) in caput spermatozoa, which was statistically significant ($P<0.001^{***}$).

Upregulation of the *CatSper3* mRNA in caput spermatozoa of DS- and NF-treated mice

CatSper3 mRNA (Fig. 4C) was upregulated by DS (316.36%) as well as by NF (440.02%) in caput spermatozoa, which was statistically significant ($P<0.01^{**}$).

Downregulation of the *CatSper1–4* mRNAs by cauda spermatozoa

Both DS and NF treatments downregulated all four *CatSper1–4* mRNAs of spermatozoa from cauda epididymis. DS downregulated *CatSper1* mRNA (−96.75%), *CatSper2* mRNA (−99.63%), *CatSper3* mRNA (−99.63%) and *CatSper4* mRNA (−89.82%) significantly ($P<0.001^{***}$). Likewise, NF treatment downregulated *CatSper1* mRNA (−86.19%), *CatSper2* mRNA (−98.17%), *CatSper3* mRNA (−98.17%) and *CatSper4* mRNA (−98.00%) significantly ($P<0.001^{***}$) as shown in Fig. 4A, B, C and D.

CatSper mRNA expressions are downregulated more severely in cauda sperm as compared to caput spermatozoa by antifertility agents

We further found that *CatSper* mRNA expressions were downregulated more severely in cauda sperm as

Figure 4 (A) Fold change in mRNA level of *CatSper1* gene relative to GAPDH of caput and cauda epididymal spermatozoa of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$ compared with control group. (B) Fold change in mRNA level of *CatSper2* gene relative to GAPDH of caput and cauda epididymal spermatozoa of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$ compared with control group. (C) Fold change in mRNA level of *CatSper3* gene relative to GAPDH of caput and cauda epididymal spermatozoa of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$ compared with control group. (D) Fold change in mRNA level of *CatSper4* gene relative to GAPDH of caput and cauda epididymal spermatozoa of control and treated BALB/c mice with Dutasteride (DS) at 5 mg/kg orally for 15 days and Nifedipine (NF) at 20 mg/kg orally for 15 days. Levels of significance: * $P<0.05$; ** $P<0.001$; *** $P<0.0001$ compared with control group.

compared to caput spermatozoa by antifertility agents. *CatSper1* mRNA was downregulated more severely in cauda sperm as compared to caput spermatozoa in both DS- (−89.913%) and NF (−64.86%)-treated mice (Fig. 4A). Likewise, *CatSper3* mRNA was downregulated more severely in cauda sperm by DS (−99.72%) as well as by NF (−98.05%)-treated mice (Fig. 4C). *CatSper4* mRNA was downregulated more severely in cauda sperm only by NF (−76.52%) as compared to caput spermatozoa (Fig. 4D).

Discussion

The research on sperm ion channels is now focusing on the identification and characterization of genes and proteins. Sperm ion channels are difficult to study using conventional electrophysiological methods, because of their small size, complex geometry and motile nature. Although spermatozoa are in principle transcriptionally silent cells, certain mRNAs have been detected in these cells. It has been proposed that these RNAs are long lived and can be delivered to the egg for later expression. During the last decade, very few investigations have revealed the presence of a complex population of mRNA in mature spermatozoa of mammals (Dadoune *et al.* 2005), including the transcripts of some ion channels (Li *et al.* 2007, Wang *et al.* 2013). The mRNA of ion channels might be a feasible target to study ion channels in spermatozoa. Since these transcripts represent the remnants of stored mRNA from genes activated during spermatogenesis, the study of mRNAs in spermatozoa could reflect past events of spermatogenesis and/or spermiogenesis (reviewed by Darszon *et al.* 2011).

The epididymis, an androgen dependent organ, is the site of sperm maturation and storage. Several *in vivo* and *in vitro* studies have confirmed that it is not testosterone, but rather dihydrotestosterone (DHT), that is the main androgen acting in this tissue (Robaire & Hamzeh 2011). 5 α -Reductases (types 1 and 2) are key enzymes in this tissue converting testosterone to dihydrotestosterone (DHT), the main androgen regulating epididymal functions. Both isoforms of 5 α -reductase, termed type 1 and type 2 (Andersson & Russell 1990) have been identified in the epididymis and are reported to be differentially expressed along the tubule (Viger & Robaire 1996).

In the present study, we performed quantitative real-time qPCR analysis for *CatSper1–4* gene expressions in epididymis and epididymal spermatozoa of BALB/c mice during the process of sperm maturation after treatment with antifertility compounds such as dual 5- α -reductase inhibitor DS and CCB NF as positive control. We duplicated a male mice infertility model using DS (5 mg/kg/day $\times$ 15 days) and NF (20 mg/kg/day $\times$ 15 days) orally and evaluated changes in epididymal and testicular histology, sperm morphology, sperm count, motility, fertility index and *CatSper* gene expression

in epididymis, testis and epididymal spermatozoa of BALB/c mice. The results showed that treatment with antifertility compounds DS and NF altered *CatSper* gene expression in epididymis and epididymal spermatozoa of mice during the process of sperm maturation.

The results of present study showed no significant changes in final body and testis weights with significant decreases in the weights of the DHT-dependent tissues such as epididymis, seminal vesicle or VP of male BALB/c mice treated with DS compared to controls. Our results are in agreement with findings of Henderson *et al.* (2004) and by Xie *et al.* (2016) in male rats after treatment with DS. The slight reduction in body weights of NF-treated mice is in accordance with results of Lee *et al.* (2006) in NF-treated ICR male mice. The less significant reduction by NF ($P \leq 0.05^*$) in caput epididymis weight and non-significant reduction in cauda epididymis weight in present study is similar to findings of Morakinyo *et al.* (2009) in NF-treated male rats.

Sperm functions such as count and motility are key indices of male fertility as these are prime markers in testicular spermatogenesis and epididymal sperm maturation. The significant decline in sperm count of caput and cauda epididymides of DS- and NF-treated mice in current study are in accordance with findings reported by Henderson and Robaire (2005) and by Xie *et al.* (2016) in DS-treated rats. The reduced sperm count of cauda epididymis in NF-treated mice is in accordance with findings of Morakinyo *et al.* (2009) in NF-treated rats.

Our results indicated that sperm motility of cauda epididymal spermatozoa was significantly reduced both by DS and NF in order of DS > NF. The results are in accordance with the results of Henderson & Robaire (2005) and Xie *et al.* (2016) who reported significant decrease in the percentage of motile and progressively motile sperm from the cauda epididymis of DS-treated rats. Likewise, Morakinyo *et al.* (2009) showed reduced sperm motility of cauda epididymal spermatozoa after NF treatment in rat. Previous reports showed that long-term treatment with the calcium channel blockers (CCBs), such as amlodipine and NF in hypertensive patients taking CCB not only inhibits spermatogenesis but also impairs sperm motility with reversible functional defects in sperm and inability to fertilize eggs (reviewed by Brezina *et al.* 2012).

In present study, the number of epididymal sperm showing abnormal morphology was higher in mice treated with DS and NF than in the controls. Majority of sperms exhibited abnormalities like swollen heads and coiled tails with significant increase in flagellar angulation. The percentage of highly agglutinated, tailless, decapitated, angulated and broken spermatozoa was common in treated mice both with DS and NF. Our findings parallel recent reports of Henderson & Robaire (2005) and Xie *et al.* (2016). It is interesting that male infertility of apparently healthy animals able to mate has,

in some instances, been correlated with spermatozoa displaying angulated flagella when released from the epididymis. Furthermore, several transgenic mice with normal sperm production that are infertile, also display flagellar angulation after epididymal transit, which is uncommon in normal mice. Angulation seems to result from permeability problems and often occurs at the end of the midpiece where the cytoplasmic droplet is located. The cytoplasmic droplet is a significant cytoplasm compartment and an important site for osmolyte and water permeation (reviewed by Darszon *et al.* 2011, Lishko *et al.* 2012). Henderson & Robaire (2005) observed adversely affected morphology of cauda epididymal spermatozoa of DS-treated rats, the most prominent effect was a markedly elevated proportion of sperm that retained their cytoplasmic droplet.

The fertility of DS-treated male mice was declined significantly (100% reduction) during fertility trial period (10–15 days of treatment schedule) in the current study. Likewise, the fertility of NF-treated mice (20 mg/kg $\times$ 15 days) was also reduced as evidenced by significant decrease in the mating index (66.6%) and fertility index (42.31%) of females paired with NF-treated males. In the present study, the ability of DS-treated males to mate was severely affected as evidenced by the observation that none of the males mated with cohabitated female mice ($n=12$) put for mating (mating index: 0%; fertility index 0%) suggesting that DS seemed to have an effect on the libido of treated mice. This is in contrast to no adverse effect reported on libido of adult Sprague–Dawley rats treated with DS either at 10 mg/kg dose (Henderson & Robaire 2005) or at 40 mg/kg dose (Xie *et al.* 2016) for 28 consecutive days. The reduction in sperm count and motility and the presence of normal germ cells, Sertoli and Leydig cells in testis of DS-treated mice suggests that the primary mechanism of action of DS could be due to a direct effect on epididymis, SV and VP. This is supported by previous findings of Xie *et al.* (2016) who showed that Dutasteride could inhibit male rat fertility by interfering with epididymal tight junctions, which could influence blood–epididymis barrier and change the composition of epididymal luminal fluids. This could probably be affecting 5 α -reductase activity causing deficit of dihydrotestosterone (DHT) in DS-treated mice resulting in loss of libido of treated male mice in present study. Further studies are needed to confirm this hypothesis by measuring levels of DHT in serum or in seminal fluid of DS-treated male mice.

The results of present studies showed that treatment of adult male mice with DS has a highly segment-specific effect on epididymal *CatSper1–4* gene expression as evidenced by significant ($P<0.001^{***}$) upregulation of the *CatSper1*, *CatSper2* and *CatSper3* mRNAs and non-significant marginal upregulation of *CatSper4* mRNA in caput epididymis of treated mice. All four *CatSper* mRNAs including *CatSper1*, *CatSper2*, *CatSper3* and *CatSper4* were downregulated more severely in

cauda epididymis by DS as well as by NF treatments respectively as compared to caput epididymis in treated mice. Similarly, Henderson *et al.* (2004, 2006) showed segment-specific effect of DS (PNU157706) on epididymal gene expression and demonstrated that the expression of various region-specific genes in the epididymis was differentially affected by 5 α -reductase inhibition. They further showed that DHT-dependent regulation of gene expression involved components of different signaling pathways potentially involved in sperm-related functions in the rat epididymis.

In the present study, DS and NF upregulated *CatSper3* mRNA significantly in caput spermatozoa of treated mice as compared to control mice. Likewise, NF caused significant upregulation of *CatSper1* and *CatSper4* mRNAs in testis of treated mice. The results are similar to those of Wang *et al.* (2013) who demonstrated statistically significant upregulation of the *CatSper1* channel associated with improved sperm motility in epididymal spermatozoa in cyclophosphamide (CP)-induced asthenozoospermic rats by Sheng–Jing–San (SJS), a traditional Chinese medicine.

Similar upregulation of *CatSper* genes have been reported by Mohammadi *et al.* (2009), who showed that selenium (Se) treatment in aging NMRI male mice could upregulate the expression of *CatSper1–4* genes and therefore resulted in elevation of sperm motility.

Most importantly, we identified remarkable downregulation of *CatSper1–4* gene expressions in the epididymal spermatozoa of mice treated with male antifertility agents DS and NF. Both DS and NF downregulated *CatSper1*, *CatSper2* and *CatSper4* mRNAs in caput and cauda spermatozoa along with significant reduction in sperm motility in treated mice. We further found that *CatSper1* and *CatSper3* mRNAs were downregulated more severely in cauda sperm of DS- and NF-treated mice while *CatSper4* mRNA was downregulated severely by NF treatment in cauda sperm as compared to caput spermatozoa. The downregulation of *CatSper1*, 2 and 4 mRNAs in caput and cauda spermatozoa may likely contribute to impairment of sperm motility in treated mice with DS and NF. Considerable evidence has accumulated that downregulation of the *CatSper1* channel in epididymal spermatozoa likely contributes to the pathogenesis of asthenozoospermia in rat (Wang *et al.* 2013). Male mice deficient in the *CatSper1* gene have been shown to be impaired both in sperm motility and hyperactivation and they are completely infertile (Ren 2001, Carlson 2003). Wang *et al.* (2017) showed decrease in the motility, viability and acrosome reaction of sperm exposed to cadmium *in vivo* and *in vitro*, which were correlated with reduction in *CatSper* (sperm-specific Ca²⁺ current) in cadmium-treated mice. A significant reduction in the level of *CatSper* gene expression has also been found among patients who lack sperm motility (Nikpoor *et al.* 2004). Mutations in the *CatSper1* channel protein have

also been identified as a cause of human male infertility, probably resulting from diminished sperm motility (Avenarius 2009). Repression of the CatSper1 channel in sperm cells impairs Ca^{2+} signaling, which in turn causes impairment of the sperm motility and hyperactivation associated with asthenozoospermia and male subfertility (Espino 2009, Tamburrino *et al.* 2015). The results from various studies suggested a direct mechanism in which NF blocked and reduced the sperm calcium ion channels, thereby reducing ion influx and subsequently inhibiting the capacitation process and acrosomal reaction in mouse (Feng *et al.* 2003) and rat (Morakinyo *et al.* 2011b).

Various mechanisms of action of both DS and NF have been deciphered previously by other investigators in other rodent species which may be directly or indirectly influencing/regulating the *CatSper* gene expression in both epididymal sperm and epididymis of BALB/c mice. It is well accepted that Ca^{2+} signaling serves as a central regulator in many key activities of sperm cells including capacitation, the acrosome reaction and fertilization (Ho & Suarez 2001, Costello 2009, Darszon *et al.* 2011). Ca^{2+} signaling affecting transmembrane fluxes of calcium ions leading to changes in intracellular calcium have been shown to be important for sperm motility initiation and regulation. DS has been shown to possibly affect essential processes such as luminal acidification, regulation of ion and fluid transport, protein processing and degradation, fatty acid and lipid metabolism and oxidative defense contributing to the formation of the optimal luminal microenvironment required for proper sperm maturation in epididymis (Henderson *et al.* 2004, 2006, Hamzeh & Robaire 2010, Xie *et al.* 2016). Morakinyo *et al.* (2011a,b) demonstrated that while calcium antagonists (CA) exert their sperm-immobilizing effect through calcium channel blockade, they may alternatively act through oxidative stress-induced cytotoxicity responsible for the adverse effects of decreased sperm count and motility ultimately leading to reduced fertility in rats. They further showed that Calcium channel blockers (CCBs) administration could cause inhibition of the acrosomal reaction in NF treated rats resulting from negative changes in the ionic environment and fluxes, especially of calcium ions, through the mammalian sperm membrane in rat epididymis resulting in loss of motility (Morakinyo *et al.* 2011a,b).

In conclusion, the antifertility changes induced by DS and NF in present study showed significant reduction in the epididymal sperm counts, motility and fertility of both DS and NF treated mice. These could partly be attributed to reduced weights of DHT-dependent organs like epididymis, seminal vesicle and VP accompanied by reduced sperm count and motility, abnormal sperm morphology exhibiting swollen heads, coiled tails, flagellar angulation, tail-less, decapitated, broken and agglutinated spermatozoa in treated mice.

These changes were associated with downregulation/upregulation of *CatSper* mRNAs in epididymal spermatozoa and epididymis of male BALB/c mice. These can be explained on the basis of interference with mechanisms affecting calcium ion signaling required for sperm activity ultimately resulting in changes in intracellular calcium levels finally affecting sperm maturation and fertility of male mice. Downregulation of *CatSper* genes by DS and by NF in caput and cauda epididymides and in caput spermatozoa observed in present studies needs to be studied in naïve mice to unravel whether it is via mutation or knockdown of the *CatSper* genes. Similarly, upregulation of *CatSper* genes through overexpression needs to be studied in depth to provide direct evidence that functional downregulation or upregulation of *CatSper* channels contributes to DS- and NF-induced antifertility in mice as suggested by Wang *et al.* (2013). Present studies provide new mechanisms of action of DS and NF for drug discovery of male antifertility compounds exploring novel avenues for identifying newer epididymal targets for developing new male contraceptives in future.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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