Article

Adverse Effects of a Clinically Relevant Dose of Hydroxyurea Used for the Treatment of Sickle Cell Disease on Male Fertility Endpoints

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Abstract: Two experiments were conducted to determine: 1) whether the adult male
transgenic sickle cell mouse (Tg58 × Tg98; TSCM), exhibits the patterns of reproductive
endpoints (hypogonadism) characteristic of men with sickle cell disease (SCD) and 2)
whether hydroxyurea (HU) exacerbates this condition. In Experiment 1, blood samples
were collected from adult age-matched TSCM and ICR mice (ICRM) (N = 10/group) for
plasma testosterone measurements. Subsequently, mice were sacrificed, testes excised and
weighed and stored spermatozoa recovered for the determination of sperm density,
progressive motility and percentage of spermatozoa with normal morphology. In
experiment 2, adult male TSCM were orally treated with 25 mg HU/kg body weight/day
for 28 or 56 days. Control mice received the vehicle for HU (saline) as described above. At
the end of the treatment periods, blood samples were collected for quantification of
circulating testosterone. Subsequently, mice were sacrificed, testes and epididymides were
recovered and weighed and one testis per mouse was subjected to histopathology. Stored

spermatozoa were recovered for the determination of indices of sperm quality mentioned in Experiment 1. Testis weight, stored sperm density, progressive motility, percentage of spermatozoa with normal morphology and plasma testosterone concentrations of TSCM were significantly lower by 40, 65, 40, 69 and 66%, respectively than those of ICRM. These data indicate that adult TSCM used in this study suffered from hypogonadism, characteristically observed among adult male SCD patients. In Experiment 2, HU treatment significantly decreased testis weight on day 28, (0.09 ± 0.004g) that was further decreased on day 56 (0.06 ± 0.003g; treatment x time interaction) compared with controls (day 28, 0.15 ± 0.01g; day 56, 2, 0.16 ± 0.01g). Concomitant with a 52% shrinkage (P<0.001) in area of testes in 56 days of HU treatment, testes from HU-treated TSCM exhibited significant atrophic degeneration in the seminiferous tubules compared with controls. Furthermore, treated TSCM had only Sertoli cells and cell debris remaining in most of the seminiferous tubules in comparison with controls. Leydig cell prominence and hyperplasia were more evident (P<0.05) in the steroidogenic compartments of testes of HU-treated TSCM compared with controls. However, plasma testosterone concentrations were reduced by HU treatment (P<0.05; treatment x time interaction) compared with controls on the two time periods studied. Epididymides from HU-treated TSCM sustained a 25% shrinkage (P<0.05), along with 69 (P<0.005) and 95% reduction (P<0.005), in stored sperm density and sperm progressive motility (treatment x time interaction P<0.05), respectively on day 56 of treatment compared with controls. These data demonstrate that TSCM used in this study exhibited SCD-induced hypogonadism, thus authenticating their use for studying the effect of HU on male reproductive endpoints observed in SCD patients. Secondarily, our data show that HU treatment exacerbated the already SCD-induced hypogonadism to gonadal failure.

Keywords: Hydroxyurea; Hypogonadism; testicular failure; transgenic sickle cell mouse; Sickle cell disease; spermatozoa, testosterone.

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1. Introduction

Sickle cell anemia is one of the most common human autosomal recessive disorders caused by a mutational substitution of thymine for adenine in the sixth codon (GAG to GTG) of the β globin gene on chromosome 11p [1]. This mutation results in the encoding of valine rather than glutamic acid at position 6 of the β globin chain [1]. Sickle cell disease (SCD) is characterized by a painful vaso occlusive crisis resulting from the blockage of capillaries by the interaction of sickle erythrocytes, leukocytes, platelets and plasma proteins with vascular endothelium [1]. Compared with children with normal hemoglobin, the onset of the adolescent growth spurt is usually delayed in SCD patients by 1.4 years, with no significant sex difference [2]. However, men carrying homozygous genes for SCD are more prone to delayed somatic and sexual development than their female counterparts [3]. Additionally, male patients suffer from episodes of priapism, a persistent painful erection of the penis unassociated with sexual stimulation or desire and detumescence does not occur following ejaculation [4]. Furthermore, male SCD patients also suffer from repeated testicular infarction [5] that can impair

testicular function. Testicular index, indices of semen quality and serum testosterone concentrations of male SCD patients are generally lower than those of men with normal hemoglobin even though circulating follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL) are comparable, indicating hypogonadism among SCD patients [6].

Currently there is no cure for SCD. However, the FDA has recently approved hydroxyurea (HU), an antineoplastic agent, for the treatment of SCD [7]. Hydroxyurea increases fetal hemoglobin which has a higher oxygen carrying capacity and does not undergo sickling under low oxygen tension, thus improving some aspects of quality of life in patients suffering from moderate to severe SCD [8,9]. It was only recently that Cokic *et al.* [10] presented results that provided the first explanation of how HU increases fetal hemoglobin levels in treated patients. Specifically, fetal hemoglobin increases in response to activation of soluble guanynyl cyclase (sGC) by HU-derived nitric oxide. As an antineoplastic agent, the specific action of HU is on ribonucleotide reductase whose action is to reduce ribonucleotides to deoxyribonucleotides. Hydroxyurea impedes the latter reaction and limits DNA biosynthesis. This makes HU an S-phase-specific cytotoxic and antineoplastic agent that interrupts the cell cycle at the G1 and S phases [11]. It is conceivable that HU-induced inhibition of DNA replication in treated SCD patients can compromise the function of organs like the testis and epididymis that rely on DNA transcription. Interestingly, studies with laboratory animals indicate that treatment with HU impairs spermatogenesis, resulting in testicular atrophy [12-14], a reversible decrease in sperm count, motility [12-16] and abnormal sperm morphology [13,15-17]. Furthermore, HU alters the chromatin structure of preleptotene spermatocytes [13,14] and increases apoptosis in spermatogonia and early spermatocytes with no apparent adverse effect on stem spermatogonia [18]. It could therefore be surmised that the possibility for the resumption of spermatogenesis exists after the withdrawal of HU treatment. The common denominator among the aforementioned animal studies is that the doses of HU used in these studies were several times the dose range used for treating patients with SCD.

A case-study reported follow-up semen analyses for a 29-year old SCD patient under HU treatment [19]. After a normal semen analysis at the beginning of therapy and one month later, the patient became azoospermic at six months, and remained in that state one month later. Ten months after the cessation of HU treatment, another semen analysis showed partial recovery of spermatogenesis. Thus an adverse effect of hydroxyurea was suspected in this case of transient and partially reversible azoospermia. In a retrospective review of four adult men who had semen analyses during HU therapy, three cases after cessation of treatment suggested that HU generally reduces sperm counts, motility and increased incidence of abnormal sperm morphology. Cessation of HU in one HU-induced azoospermia resulted in the resumption of spermatogenesis, however in two of the three cases with HU-induced altered sperm parameters, sperm morphology and motility remained impaired [20]. Despite the suggestion by these two human studies that HU could adversely impact fertility indices; they constitute case studies that lack sufficient numbers to warrant statistical scrutiny and inferences.

Recently, Berthaut *et al.* [21] showed in a retrospective study that SCD-induced hypogonadism was exacerbated by HU treatment. This is the first study in humans that attempted to study the effect of a clinically relevant dose of HU (20–30 mg/kg body weight/day) on fertility endpoints of male SCD patients. However, because of its retrospective nature, this study is plagued by a lack of design and powerful statistical tools that would have resolved some of the subtle differences missed. Kopsombut *et al.* [22] showed that treatment of adult normal male mice (ICR strain) with a clinically relevant dose

of HU (30 mg/kg) for 30 days, significantly reduced testis weight, stored sperm density and progressive motility. Testis weight and stored sperm parameters were not reversed even at the 4th month post withdrawal of HU treatment. This is the only designed study that showed that a clinically relevant dose of HU used for the treatment of SCD albeit conducted in normal mice, adversely affects testis and epididymal function. Consequently, the use of an appropriate transgenic SCD animal model for the determination of the link between SCD and HU treatment on male fertility indices is warranted.

The transgenic SCD mouse model used in this study is one that was developed by Popp *et al.* [23] from Tg58/β-thal and Tg98/β-thal mice, out of constructs of the human α (*h*α) and human β$^{S\ Antilles}$ (*h*β$^{S\ Antilles}$) genes [24]. The Hb S Antilles transgene insertions from Tg58/β-thal and Tg98/β-thal mice were bred into the genome of mice with high oxygen affinity referred to as MHOAH mice to produce M-Tg58Ru and M-Tg98Ru mice, respectively. Subsequently, these mice were mated to each other to produce a doubly homozygous Tg58 × Tg98 line of Hb S Antilles transgenic mice hereafter referred to as transgenic sickle cell mice (TSCM). These mice produce sickle shaped red blood cells (RBCs), exhibit reticulocytosis, elevated white blood cell count, lung and kidney pathologies commonly found in sickle cell patients [23]. However, the incidence of hypogonadism characteristic of men with SCD was not studied in these mice. The objectives of this study were: 1) to determine whether TSCM is a suitable transgenic mouse model for studying the link between SCD and hypogonadism; and 2) to determine whether a clinically relevant dose of HU used for treating SCD exacerbates SCD-induced hypogonadism.

2. Materials and Methods

2.1. Animals

Acclimatized adult male ICR mice (ICRM; 7-8 weeks of age) and age-matched TSCM were housed by strain in groups of four in polyethylene cages. Mice were maintained in an environmentally controlled room with 12 hour light: 12 hour dark cycle, 22°C and humidity range of 50 to 60% and allowed *ad libitum* access to commercial pelleted mouse chow and water.

Experiment 1

The rationale for conducting this experiment was to determine whether Tg58 × Tg98 line of Hb S Antilles transgenic mouse is a suitable model for studying the effect of a clinically relevant dose of HU on fertility of male SCD patients. To accomplish this, acclimated age-matched adult male ICRM and TSCM (N = 10/strain) were anesthetized with isoflurane between 1,400 and 1,600 hr, following which mid-ventral laparotomy was performed to permit blood collection via inferior vena cava puncture into heparinized tubes. Plasma was harvested from each sample by centrifugation at 1,500-x g for 10 minutes at 4°C and stored at -20°C until assayed for testosterone concentrations. Testes were subsequently recovered and weighed. Cauda epididymides excised and stored spermatozoa were recovered as detailed in Archibong *et al.* [25] in Whitten's medium [26] pre-equilibrated at 37°C in an atmosphere of 5% CO_2 in air under washed mineral oil. After 15 min incubation at the above

mentioned conditions, sperm density, progressive motility and morphology were determined in homogenous sperm suspensions.

2.2. Stored Sperm Density

The density of stored spermatozoa was determined by hemocytometric counting after sperm suspensions were each subjected to 20X dilution with an immobilizing diluent (50 g sodium bicarbonate, 10 mL of 40% formaldehyde solution and 0.25 g trypan blue dissolved in 1 liter of water [22]. Stored sperm density per sperm suspension was based on two counts of 100 cells each.

2.3. Progressive Sperm Motility

Progressive sperm motility was determined microscopically with a phase contrast microscope (magnification = 400X; Olympus BX 41). The percentage of progressively motile spermatozoa per sperm suspension was based on two counts of 100 cells each.

2.4. Sperm Morphology

Stored spermatozoa were stained with modified Papanicolaou staining kit (Spermac, Stain Enterprises, S. Africa) according to the manufacturer's instructions and sperm morphologies were assessed according to the criteria of Filler [27].

2.5. Testosterone Radioimmunoassay (RIA)

Plasma samples were analyzed for testosterone concentrations by RIA validated in our laboratory. The sensitivity of testosterone assay was 2 pg/tube and the intra- and inter-assay coefficients of variation were 8.5 and 8.7%, respectively [28].

Experiment 2

The rationale for conducting this experiment was to assess whether HU exacerbates SCD- induced hypogonadism. Adult TSCM were randomly assigned to a treatment or a control group (N = 12/group). Treatment consisted of 25 mg HU (Sigma Chemical Co., St. Louis, MO, USA)/kg body weight via oral gavage for 28 (N = 6) or 56 (N = 6) days, to assess HU toxicity to testis and epididymal function before or after completion of a spermatogenesis cycle, respectively. The maximum interval between type A spermatogonium and its release as mature spermatozoa from the seminiferous tubule is 35.5 days [29]. Transgenic sickle cell mice in the control group were administered with saline (vehicle for HU) as described for treated mice and equal numbers were sacrificed along with HU-treated mice on the respective days mice in the latter group were sacrificed. All mice were weighed monthly and examined daily for normal activity and behavior, coat condition and normal feed and water intake. At the end of the treatment periods, mice were anesthetized, blood samples collected and plasma recovered and assayed for testosterone content as described in

Experiment 1. Furthermore, testes and epididymides were recovered and weighed and one testis/mouse/treatment and control group (N = 5/group) sacrificed on day 56 of HU treatment was prepared for histopathology. Assessments of testes from control and HU-treated TSCM included the evaluation of gonadal dimensions (length × width [L × W] of largest step section; sq mm), integrity of the seminiferous tubules (normal or atrophic/degenerative) and Leydig cell hyperplasia and prominence. Cauda epididymides of control and treated TSCM were processed for the recovery of stored spermatozoa as detailed in Archibong *et al.* [25].

2.6. Histopathology

Testes were fixed in Bouin's solution for 24 to 72 hours prior to histological preparation according to the method of Lunstra *et al.* [30]. Briefly, fixed testis pieces from HU-treated and control TSCM were washed in PBS (1 × 1 h), dehydrated through graded ethanol (50, 70, 80, 90, 100; 2 × 1 hr each), cleared in xylene (2 × 1 h; Sigma, St. Louis, MO, USA), infiltrated with paraffin wax (60°C; 4 × 1 hr), and embedded in paraffin wax. Serial sections (5 μm) were subsequently cut from the middle of each testis preparation. Sections were dried overnight onto glass slides at $37^{\circ}C$ and stored at room temperature until processed for histology. Sections were deparaffinized in Microclear (2 × 5min; Micron Environmental Industries, Fairfax, VA, USA) and rehydrated through graded ethanol (2 × 100%, 2 × 95%, and 1 × 70%). Thereafter, sections were rinsed thoroughly in water, stained with hematoxylin, dehydrated, cleared in Microclear and mounted using DPX mounting media (Fluka). Stained histological preparations were evaluated at 50x magnification using a Leitz microscope coupled to a computerized morphometric planimetry system (Bioquant Nova 2000 Advanced Image Analysis, R&M Biometrics, Nashville, TN, USA) for structural alterations in the seminiferous tubules and Leydig cells. The stages of structural alterations in seminiferous tubules were classed as normal, minimally atrophic/degenerative, moderately atrophic/degenerative or severely atrophic/degenerative and assigned the following numerical scores: 1.1, 2.1, 3.1 or 4.1, respectively. Leydig cells were classed as normal, minimally prominent, moderately prominent, or severely prominent and assigned numerical scores similar to those of seminiferous tubules.

2.7. Stored Sperm Progressive Motility and Density

Spermatozoa were recovered from the cauda epididymides of each HU-treated and control TSCM on day 28 or 56 of treatment and the density and progressive motility of stored spermatozoa were evaluated as described in Experiment 1.

2.8. Statistical Analyses

Data on testis weight, stored sperm density and progressive motility, stored spermatozoa with normal morphology and plasma testosterone concentrations in Experiment 1 were compared by unpaired "t" test. Data collected in Experiment 2 (monthly body weight, testis and epididymal weight, stored sperm density and motility and plasma testosterone concentrations) were analyzed by Two-way analysis of variance and differences among means were tested for significance with unpaired "t" tests.

The scores for stages of structural damage to the seminiferous tubules and Leydig cell prominence were compared with unpaired "t" tests [31].

3. Results

The reproductive characteristics of ICRM and TSCM are presented in Table 1. The reproductive system of TSCM used in this study was mature and functional based on the presence of mature spermatozoa in the cauda epididymides. However, testis of TSCM weighed less (P<0.025) than those of ICRM. Similarly, stored spermatozoa recovered from TSCM were fewer (P<0.005), less progressively motile (P<0.005) and had fewer (P<0.005) spermatozoa with normal morphology than those recovered from their ICRM counterparts. Furthermore, plasma testosterone concentrations were lower (P<0.05) in TSCM compared with ICRM.

Table 1. Comparison of Fertility Indices (Mean ± SE) of TSCM (Tg58 x Tg98) and <u>ICRM.</u>

Item	TSCM	ICRM
Testis wt (g)	0.15 ± 0.01**	0.25 ± 0.01
Stored sperm density (x 10^6)	14 ± 3.6***	40.3 ± 3.6
Sperm progressive motility (%)	39.0 + 8.7***	65.13 + 2.3
Sperm with normal morphology (%)	14.7 ± 2.3***	48.0 ± 5.1
Plasma testosterone concentrations (ng/mL)	2.4 ± 0.72*	7.0 ± 0.75

* P<0.05; ** P<0.025; ***P<0.005

Monthly body weights of control and HU-treated TSCM were similar during the two months of this study, suggesting that treatment of TSCM for 28 to 56 days with the clinically relevant dose of HU used for treating patients with sickle cell disease did not affect body weight. Mean monthly weights (Mean + SE) of TSCM increased from 30.43 ± 0.99 to 32.7 ± 0.89 and 34.37 ± 0.88 during the first and second month of treatment, respectively among control TSCM, similar to increases in body weights that occurred among HU-treated TSCM from 30.88 ± 0.93 at the onset of treatment to 32.01 ± 0.99 and 33.76 ± 0.96 g during the first and second month of treatment, respectively. Even though the mean body weights of TSCM was not affected by the regimen of HU used in this study, it adversely affected the testis of treated compared with control mice. Hydroxyurea treatment cause a 40% decrease in testis weight on day 28, followed by a further 22% decrease in this organ by day 56 of treatment (treatment x time interaction; P<0.05) compared with control TSCM (Figure 1). Table 2 depicts the dimension and histological characteristics of testes from HU-treated versus control TSCM. The area of testes from HU-treated mice shrank to 5.2 ± 0.2 sq mm (range, 5.0 to 6.0 sq mm) in 56 days of treatment, approximately 52% reduction (P<0.001) compared to their control counterparts (10.8 ± 0.9; range, 7.5 to 12.0). Our results also show that testes from HU-treated TSCM exhibited atrophic/degenerative seminiferous tubules (P<0.001) with only Sertoli cells and cell debris remaining in most of the tubules in comparison with controls. Furthermore, Leydig cell prominence and hyperplasia were more evident (P<0.05) in the testes of HU-treated TSCM compared with controls. The above mentioned pathological changes are depicted in the photomicrographs of testis histologies of HU-treated and control mice (Figure 2). In comparison with controls, epididymides from HU-

treated TSCM weighed approximately 25% less (treatment x time interaction P<0.05) on day 56 of treatment (Figure 3). The HU-induced shrinkage in this organ was followed concomitantly with approximately 69% reduction in the ability of the cauda epididymis to store matured spermatozoa (Figure 4; treatment x time interaction; P<0.005) compared with controls. Furthermore, epididymides from HU-treated TSCM sustained about 95% reduction in their ability to impartment motility to matured spermatozoa (Figure 5; treatment x time interaction; P<0.005) compared with those of controls. Plasma concentrations of testosterone (ng/mL) were lower (treatment x time interaction; P<0.05) in HU-treated TSCM versus those of their control counterparts (Figure 6).

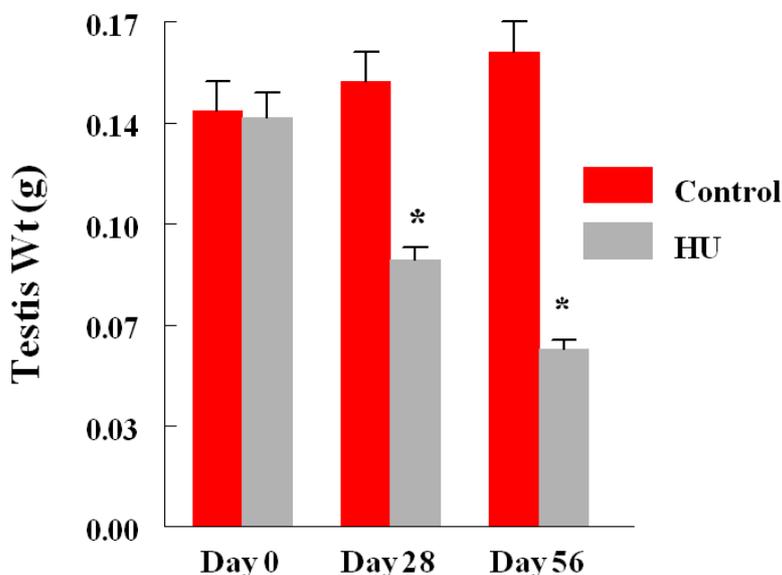**Figure 1.** Mean Testis Weight of HU-treated Versus Control TSCM.

Table 2. Adverse effects of HU (25 mg/kg)-treated TSCM after 56 days.

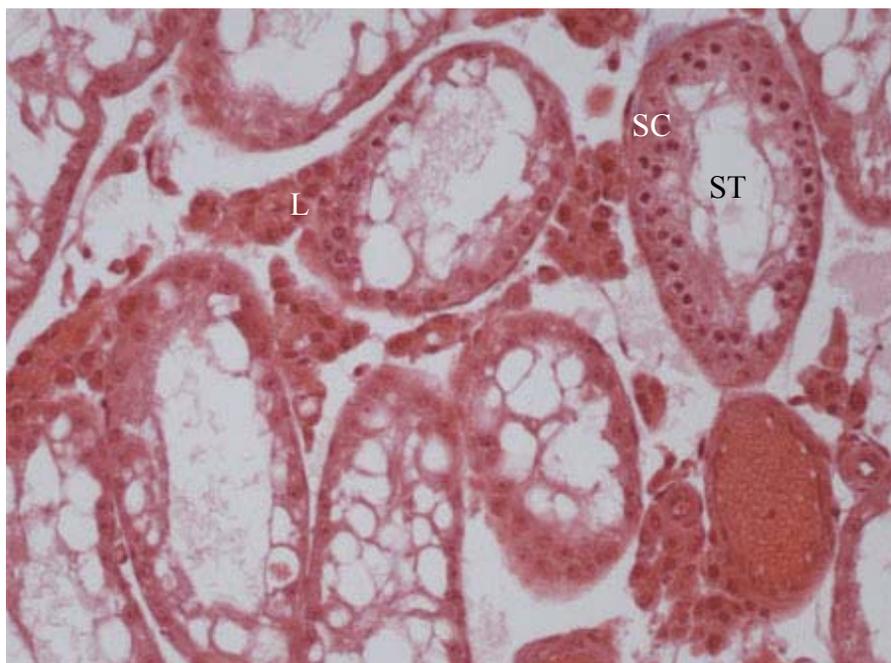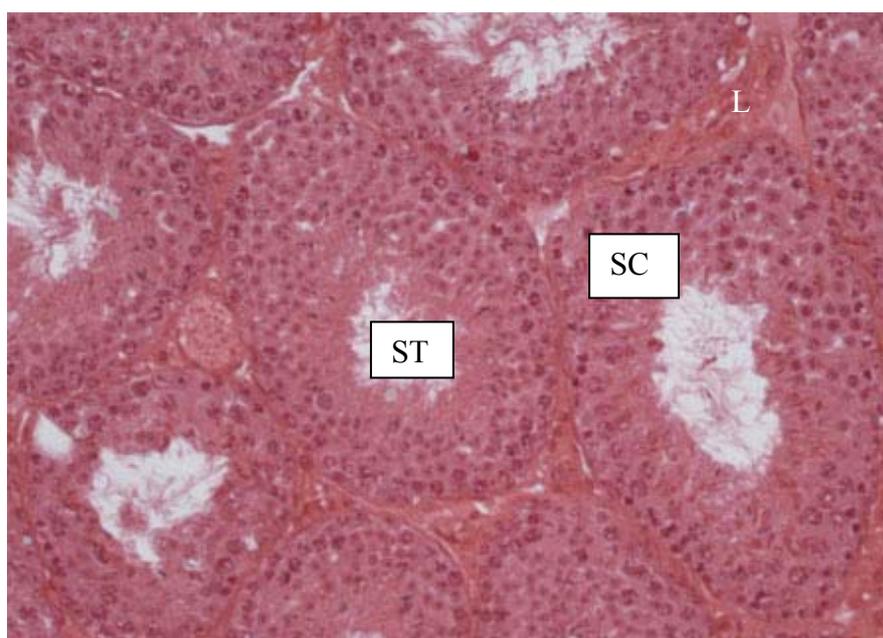| Findings	Control TSCM						HU Treated TSCM					
	#1	#2	#3	#4	#5	Mean	#1	#2	#3	#4	#5	Mean
Testis dimensions (L X W; sq mm)	12.0	7.5	12.0	10.5	12.0	10.8 ± 0.9	5.0	5.0	5.0	5.0	6.0	5.2 ± 0.2***
Seminiferous tubular atrophy/ degeneration (Score)	Min. (2.1)	None (1.1)	None (1.1)	None (1.1)	None (1.1)	1.3 ± 0.2	Mod. (3.1)	Mod. (3.1)	Mod. (3.1)	Mod. (3.1)	Min. (2.1)	2.9 ± 0.2***
Leydig cell prominence (Score)	Min. (2.1)	WNL (1.1)	WNL (1.1)	WNL (1.1)	WNL (1.1)	1.3 + 0.2	Mod. (3.1)	Mod. (3.1)	Min. (2.1)	Min. (2.1)	WNL (1.1)	2.3 + 0.4*

*P<0.05; ***P<0.001

Mod. = Moderate

Min. = Minimum

WNL = Within Normal Limits

Figure 2. Photomicrographs of testes histologies from: A) TSCM treated with 25 mg HU/kg; B) Control TSCM (Magnification bar = 200X); C) TSCM treated with 25 mg HU/kg; D) Control TSCM (Magnification bar = 400X). The TSCM treated with HU have degenerative/atrophic seminiferous tubules (ST) with only Sertoli cells (SC) and cellular debris remaining in the tubules compared with controls. Associated with the HU-induced changes in the seminiferous tubules are hyperplasia and cell prominence in the Leydig cell (L) compartment compared with controls. This indicates a reduction in spermatogenic activity and loss of fluid in the seminiferous tubules due to decreased testosterone production, hence the decrease in testis size.

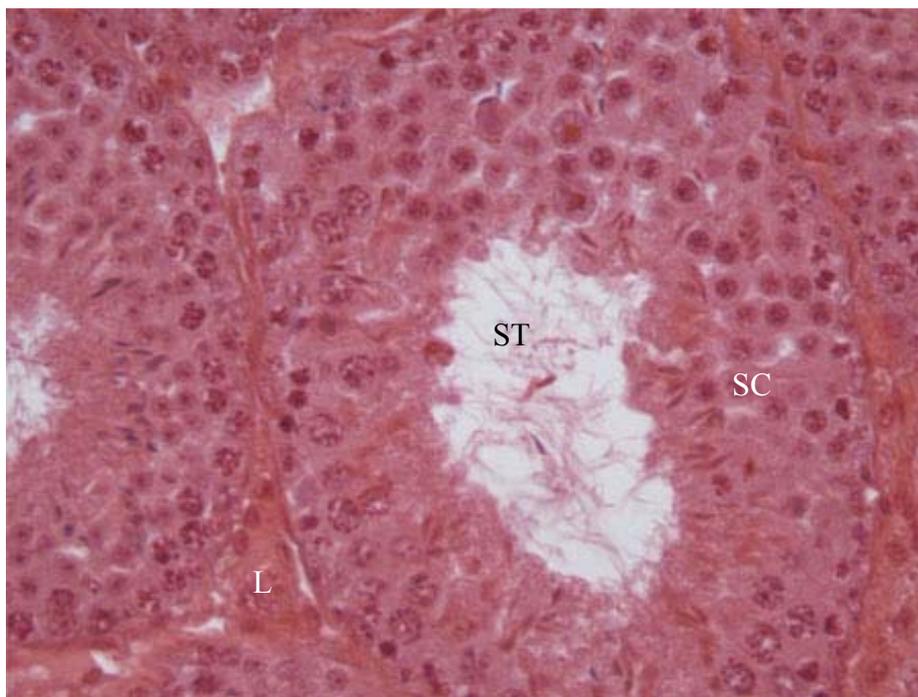A. HU-treated TSCM (200X magnification)

B. Control TSCM (200X magnification)

Figure 2. Cont.

C. HU-treated TSCM (400X magnification)

D. Control TSCM (400X magnification)

Figure 3. Effect of 25 mg HU/kg administered via oral gavage for 28 or 56 days on mean epididymal weight of TSCM; n = 6 per treatment or control group during the two time periods studied. Results are expressed as mean ± SE (HU = treated TSCM; Control = untreated TSCM). Asterisks indicate a significant difference from controls ($P < 0.05$).
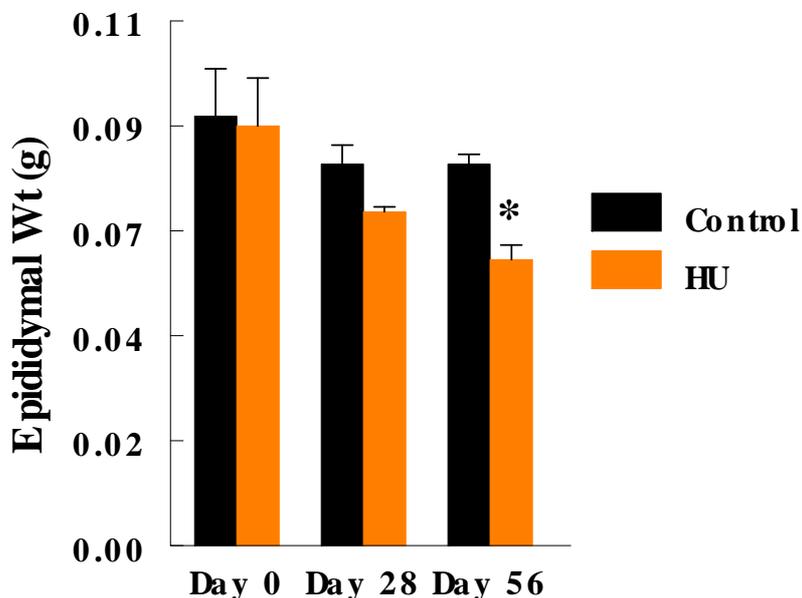

Figure 4. Effect of HU (25 mg/kg, administered via oral gavage for 28 or 56 days) on mean stored sperm progressive motility of TSCM; n = 6 per treatment or control group during the two time period studied. Results are expressed as mean ± SE (HU = treated TSCM; Control = untreated TSCM). Asterisks indicate a significant difference from controls ($P < 0.005$).

Figure 5. Effect of HU (25 mg/kg, administered via oral gavage for 28 or 56 days) on mean stored sperm progressive motility of TSCM; n = 6 per treatment or control group during the two time periods studied. Results are expressed as mean ± SE (HU = treated TSCM; Control = untreated TSCM). Asterisks indicate a significant difference from controls ($P < 0.005$).
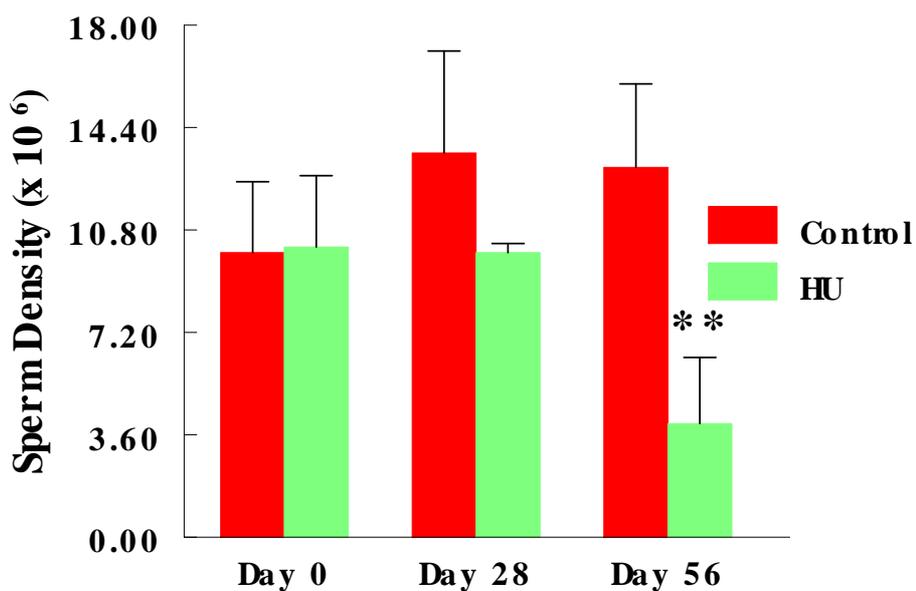

Figure 6. Effect of HU on plasma testosterone concentrations in male TSCM treated by oral gavage with 25 mg HU/kg for 28 or 56 days; n = 6 per treatment or control group during the two time periods studied. Results are expressed as mean ± SE (HU = treated TSCM; Control = untreated TSCM). Asterisks indicate a significant difference from controls ($P < 0.05$).
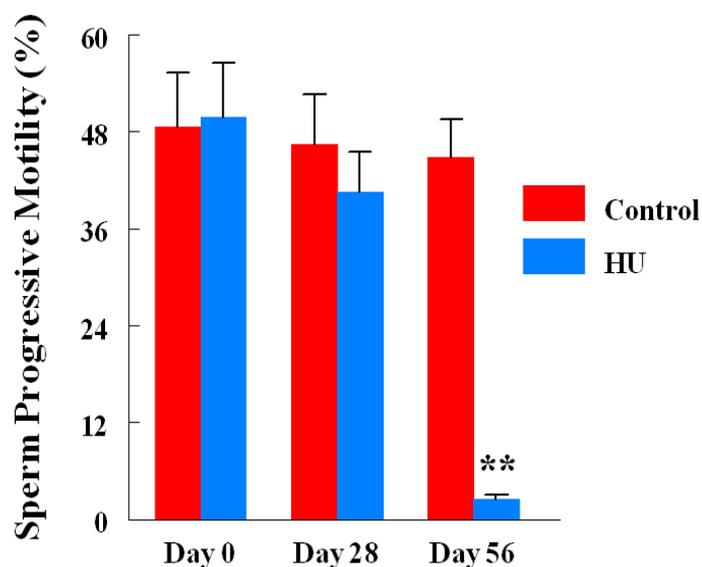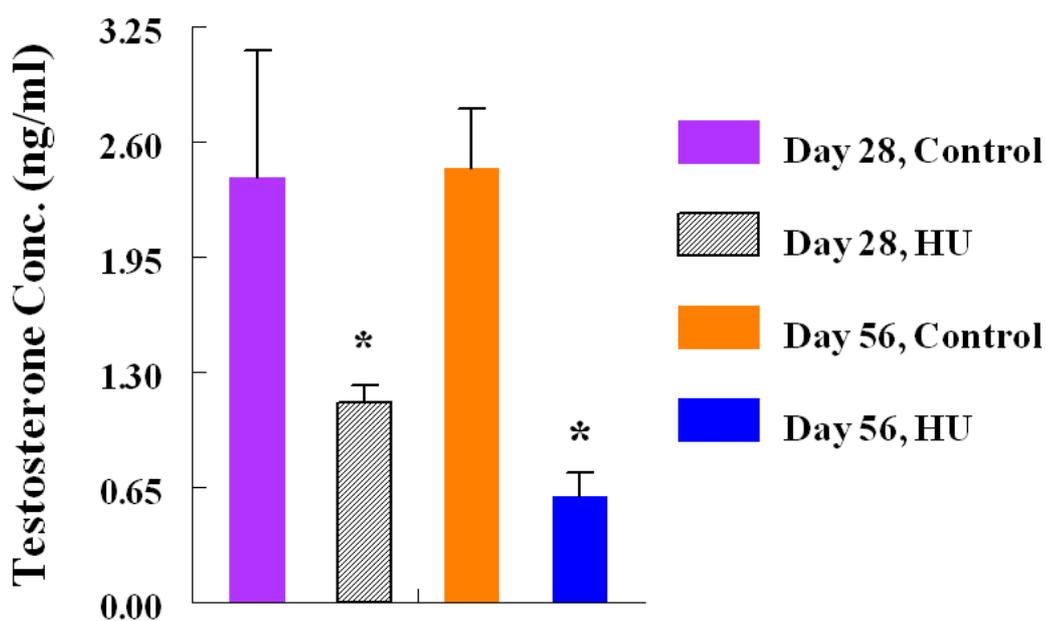
4. Discussion

In Experiment 1, we wanted to determine if adult male TCSM exhibit the pattern of reproductive endpoints that are characteristic of adult men with homozygous genes for SCD. In this experiment, we observed that testis weight was significantly reduced in TSCM compared with ICRM, indicating impairment in testicular exocrine function. In this experiment, we observed a significant reduction in the key spermatogenesis regulating hormone, testosterone, in TSCM versus ICRM, similar to the observation made by Modebe and Eze [6] among men with SCD compared with controls. Therefore the significant reduction in testis weight among TSCM versus ICRM can be explained by a reduction in spermatogenesis due to reduced testosterone synthesis and release [25] in as much as about 85% of the testis bulk is involved in spermatogenesis [25,32]. The observed reduction in testis weight and plasma testosterone concentrations among TSCM versus ICRM is indicative of hypogonadism, a condition that is inducible by SCD [6].

Additionally, we observed a significant decrease in mean stored sperm density among TSCM versus ICRM, in agreement with the data reported by Modebe and Eze [6] in men with SCD compared with controls. This is likely due to SCD-induced reduction in testosterone-regulated spermatogenic and epididymal function of TSCM versus ICRM [6,25,32-33] resulting from the impairment of Leydig cell function by reactive oxygen species (ROS). The intermittent vascular occlusion occurring in SCD [34-36] creates peripheral vascular insufficiency accompanied by periodic restoration of blood flow. This scenario places ischemic organs such as the testis and epididymis at risk of additional injury by inducing a proinflammatory state reflected by excessive generation of reactive oxygen species (ROS; super oxide, hydroxyl, nitric oxide, peroxide, peroxynitrile [37]). The latter has been shown to contribute to idiopathic male infertility [37].

Interestingly, the presence of homozygous SCD genes was associated with a significant reduction in progressive motility among stored spermatozoa harvested from TSCM compared with ICRM and similar to observations made in patients with SCD [6]. Sperm progressive motility is positively correlated with fertilization of oocytes [38] and pregnancy rates [39], the decline of which is an indicator for impaired male gonadal function [40], a condition that is prevalent among patients with homozygous genes for SCD. Sickle cell disease-induced ROS [41] have been localized to several organs including the epididymis [42-46], the functions of which are compromised by these reactive molecules [47].

Mature spermatozoa acquire the ability to move progressively during epididymal transit, a process that is regulated by testosterone and dihydrotestosterone (DHT; [48]). In Experiment 1, we observed a significant reduction in total plasma testosterone concentrations in TSCM compared with ICRM. This observation is significant because it suggests that the reduced sperm motility observed among TSCM is secondary to reduced epididymal exposure to physiological concentrations of androgens. It is also conceivable that excessive presence of ROS in the epididymides of TSCM compared with ICRM contributes to reduced sperm motility [49].

Additionally, we observed a disproportionately higher percentage of spermatozoa with abnormal morphologies among stored spermatozoa recovered from TSCM versus ICRM, similar to the observation made in men with SCD compared with men with normal hemoglobin [6]. The reduction in the percentage of normal morphologic forms of spermatozoa in TSCM is attributable to ROS-induced

testosterone reduction [37]. If intra-testicular steroidogenesis is reduced substantially in TSCM, the level of testosterone reaching the epididymides from general circulation and testicular fluid may not be sufficient to optimally regulate the epididymides. This scenario could lead to a significant reduction in sperm maturation, survival and ultimately to the presence of degenerated spermatozoa in the cauda epididymides and vas deferens [33]. The increased percentage of abnormally shaped spermatozoa in the cauda epididymides of TSCM versus ICRM could be likened to the degenerating conditions of stored spermatozoa due to chronic deprivation of the epididymides of adequate testosterone. This is comparable to the situation found in seasonal-breeding animals at the time of testicular regression, when large numbers of semi-condensed spermatids and decapitated spermatozoa are present in the epididymis [50]. The results in this experiment on TSCM and those of others on SCD patients [6,51] suggest that inheritance of homozygous sickle cell genes results in inadequate functioning of the steroidogenic and spermatogenic compartments of the testis. Taken together, TSCM constitutes an authentic mouse model for studying the effect of HU on fertility indices of men with SCD.

Hydroxyurea, at a dose range between 25 to 30 mg/kg used for the treatment of SCD [21], reduces the frequency of painful crisis in SCD patients by increasing the levels of fetal hemoglobin, which has a higher oxygen carrying capacity and does not undergo polymerization at low oxygen tension [7]. This SCD therapeutic agent is a potent inhibitor of the enzyme ribonucleotide reductase and inhibits DNA replication in a wide variety of cells [11] and therefore, has the ability to affect growth and function of organs that rely on DNA transcription, negatively. Interestingly, the mean body weight of HU-treated TSCM versus controls were comparable during the two time periods studied, in agreement with the finding that HU, at least at the dose range used for the treatment of SCD, does not limit weight gain among SCD patients [52,53]. However, we observed a significant reduction in testis weight among treated versus control mice at the two time periods studied. This observation is similar to that made by Kopsombut *et al.* [22] in adult ICRM treated with a clinically relevant dose of HU normally used for treating patients with SCD and indicates HU toxicity to the testis. The most vulnerable compartment of the testis to the toxic effects of xenobiotic agents is the spermatogenic compartment [25] that makes up approximately 85% of the testis bulk [32]. Consequently, a reduction in testis weight among treated TSCM suggests the suppression and or inhibition of spermatogenesis by HU. Based on available research data, HU can suppress spermatogenesis directly by arresting mitotic activity in spermatogonia through the inhibition of DNA synthesis [11,54]. However, it can also suppress spermatogenesis indirectly by repressing testosterone synthesis and release by Leydig cells [55]. For this to occur, HU must be metabolized by the liver to nitric oxide [56], a radical that has important consequences for the pathophysiology and treatment of SCD [57-60] albeit with a detrimental effect on a major spermatogenesis regulatory hormone, testosterone. The process of spermatogenesis is a testosterone-regulated process that occurs in waves along the length of the seminiferous tubules. It consists of mitotic proliferation and maintenance of spermatogonia (spermatocytogenesis) followed subsequently by meiotic divisions to insure genetic diversity; and spermiogenesis for morphologic transformation of spermatids by the Sertoli cells to spermatozoa. Eventually, mature spermatozoa are detached from the Sertoli cells into the adluminal compartment of the seminiferous tubules during spermiation. Because HU has antimitotic property, the 40% reduction in testis weight among treated versus control TSCM on day 28 of treatment can be attributed to the suppression of spermatocytogenesis by HU in the sperm cycle that was initiated at the onset of HU

treatment. The additional 20% reduction in testis weight among treated versus control TSCM on day 56 of treatment is attributable to the migration of spermatozoa whose development was initiated before and after HU treatment. It is noteworthy that the 56 days of HU treatment is greater than one sperm cycle (35.5 days in the mouse [29]). Therefore, it could be hypothesized that spermatids will not be apparent in the seminiferous tubules of treated TSCM versus their control counterparts if spermatogenesis is suppressed by HU from the onset of HU treatment. This hypothesis was proven to be true based on the absence of spermatids in most of the seminiferous tubules of HU-treated mice, in contrast with the presence of spermatids in the tubules of control mice (Figure 2).

The 62-63% reduction in testis weight of treated versus control TSCM on day 56 of HU treatment was also contributed to by seminiferous tubular atrophy/degeneration which ultimately resulted in reduced testis dimensions (shrinkage). These observations are similar to those reported by Fiscor and Ginsberg [12], Evenson and Jost [13] and Wiger *et al.* [14] in HU-treated rodents and suggest that HU suppresses the spermatogenic process in the seminiferous tubules and causes degeneration of these tubules.

Essentially, the mean epididymal weights of treated and control TSCM on day 28 of HU treatment were comparable due to the presence in this organ of mature spermatozoa whose development was initiated prior to HU treatment. However, due to HU-induced suppression of spermatogenesis during the 56 days of treatment (longer than one sperm cycle in the mouse [39]), it is conceivable that the reduced epididymal weight in treated TSCM versus controls may have resulted from fewer spermatozoa migrating into the epididymis.

The decrease in epididymal weight among HU-treated versus control mice on day 56 of treatment was accompanied concomitantly by decreased stored sperm density and due to HU-induced reduction in sperm numbers transported into the epididymides post spermeation [61,62]. According to Plant and Marshall [63] and Archibong *et al.* [25], high intra-testicular testosterone concentrations are required for the regulation of spermatogenesis, the reduction of which results in fewer matured sperm produced for migration into the epididymis. Furthermore, the reduced concentrations of plasma testosterone among HU-treated TSCM compared with controls may have caused a rapid regression in the epididymal epithelium, especially in the initial segment and the proximal caput epididymal region [33]. Consequently, epididymal sperm maturation and survival were adversely affected and during the ensuing weeks, only a few degenerated spermatozoa remain in the cauda epididymides and vas deferens [33]. Thus, the reduced stored sperm density observed in this study may have been due to reduced spermatogenesis resulting from nitric oxide-induced reduction in testosterone synthesis and release [55].

Interestingly, treatment of TSCM with HU significantly reduced progressive motility among stored spermatozoa compared with that of their control counterparts in the second month of treatment, most likely due to the direct action of HU-induced nitric oxide. A correlation exists between high concentrations of nitric oxide in semen and reduced sperm motility. According to Balercia *et al.* [64], the concentrations of nitric oxide in semen samples of normozoospermic fertile men are significantly lower than those of their asthenozoospermic infertile counterparts.

Mature spermatozoa acquire the ability to move progressively during epididymal transit, a process that is regulated by testosterone and dihydrotestosterone (DHT; [48]). In this study, we observed a significant reduction in total plasma testosterone in HU-treated compared with control TSCM during

the two time periods studied. However, reduced sperm motility was significantly observed only on day 56 of the study in HU-treated TSCM versus controls. This is probably due to epididymal exposure to significantly high concentrations of nitric oxide and reduced testosterone by day 56 of testament in HU-treated TSCM.

Altered morphological change (increased prominence) and hyperplasia were observed in Leydig cells of HU-treated TSCM compared with their control counterparts; however, it is not known whether the changes observed in Leydig cells from treated TSCM constitute a cause or effect of low testosterone synthesis in these mice.

The interpretation of the significance of the changes observed in the Leydig cells of HU-treated TSCM is beset by the lack of knowledge of the precise stage at which HU-induced morphological changes occurred in relation to reduced testosterone biosynthesis. It is conceivable that the Leydig cell hyperplasia and altered prominence of this cell type in HU-treated TSCM are secondary to reduced testosterone biosynthesis. Similar changes in Leydig cells were observed in low testosterone producing transgenic mice overexpressing human aromatase by Li *et al.* [65]. Although the specific mechanism involved in the regulation of testosterone synthesis by HU is not examined in the present study, data generated by Lamanna *et al.* [55] indicate that nitric oxide, a metabolite of HU [56], is a suppressor of testosterone biosynthesis. The suppression of testosterone synthesis and release by the Leydig cells of HU-treated TSCM was progressive probably due to increased production of nitric oxide over the experimental time periods. It has been established that the inhibition of Leydig cell testosterone synthesis by nitric oxide is at select segments of the steroidogenesis pathway that does not involve the inhibition of LH receptor binding and induction steroidogenic acute regulatory protein (StAR) mRNA [66]. Rather, nitric oxide is effective in inhibiting testosterone biosynthesis after the synthesis of the first pro-steroid hormone (pregnenolone). It inhibits 3β-hydroxysteroid dehydrogenase (3β-HSD; [67]) and 17α-hydroxylase/c17-20 lyase (P450c17; [67,68]); required for the conversion of pregnenolone to progesterone and the latter to androstenedione, respectively. Arguably, it calls to question whether the reversal of the inhibition of 3β-HSD and P450c17 in vivo can restore normal testosterone production by the Leydig cells of HU-treated TSCM and subsequently, the resumption of normal spermatogenesis. Kopsombut *et al.* [22] demonstrated that sperm motility was fully restored while spermatogenesis was partially restored (69%) in HU-treated ICRM compared with controls after four months treatment withdrawal. These data suggest that the activities of 3β-HSD and P450c17 were restored to permit the production of enough testosterone to fully restore sperm motility during epididymal transit but not enough to restore full spermatogenic activity. The partial restoration of spermatogenesis in HU-treated male mice was accompanied by significant fetal losses in females mated with these males compared to those mated to control males [22]. This observation indicates that the resumption of spermatogenesis four months post HU withdrawal does not necessary translate to the production of normal spermatozoa. It is conceivable that the fertilizing spermatozoa produced at the above mentioned period post HU withdrawal carry defective genes that contribute the high fetal mortality. Until research establishes a time-line for the production of normal spermatozoa post HU withdrawal, men anticipating a family should cryo-store their spermatozoa before submitting to HU treatment.

The findings of our study strongly suggest that HU treatment exacerbated the condition of hypogonadism that is prevalent in patients with SCD. As a consequence, HU-treated TSCM suffered

testicular failure and reduced stored sperm density and motility compared with their control counterparts. Data presented in this study should raise awareness among adult male SCD patients considering a family to cryo-store their gametes prior to undergoing HU treatment due to the inconsistent data on return to normal reproductive function after treatment withdrawal.

Acknowledgements

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