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Gestational-onset Hypothyroidism Affects Genes Controlling Epididymal Sperm Maturation in F_1 Progeny Rats

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Abstract

Purpose: Hypothyroidism is associated with infertility. We have reported that gestational-onset hypothyroidism impairs post-testicular sperm maturation in F_1 progeny rats, whereas the underlying mechanism remains obscure. In this study, we tested the hypothesis "transient gestational-onset hypothyroidism affects post-testicular sperm maturation by inducing oxidative stress and modifying the expression of specific genes controlling epididymal function in F_1 progeny rats". **Methods:** Hypothyroidism was induced by providing 0.05% methimazole in drinking water to pregnant rats during specific periods of foetal differentiation of testis and epididymis. On the postnatal day 120, epididymes were dissected out and used for various analyses. Sperm parameters and activities of antioxidants and pro-oxidants were assayed using standard protocols. qRT-PCR and western blot were carried out to assess the expression of epididymal functional genes and their respective proteins. **Results:** Gestational-onset hypothyroidism produced decrease of sperm motility and membrane integrity, and increase of abnormal sperm morphologies. While the concentration of reduced glutathione and specific activities of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase decreased, levels of pro-oxidants hydrogen peroxide and lipid peroxidation increased. Expression levels of androgen and thyroid hormone receptors α/β, aquaporin 9, and glutathione peroxidise 5 decreased, whereas estrogen receptors α/β increased in rats with gestational-onset hypothyroidism. **Conclusion:** Our results support our hypothesis and we conclude that gestational-onset hypothyroidism impairs post-testicular sperm maturation due to oxidative stress and modified expression of nuclear hormone receptors and aquaporin 9 in the epididymis of F_1 progeny.

Keywords: Androgen Receptor, Antioxidants, Aquaporin 9, Estrogen Receptors, Thyroid Hormone Receptor

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1. Introduction

Post-testicular sperm maturation in the epididymis plays an important role in determining sperm quality, and any pathological situation that interferes with hormones/receptors and their target genes controlling this physiological event may affect the fertility of the subject[1,2]. The spermatozoa acquire progressive motility and fertilizing ability during their sojourn through the specialized environment in the epididymal conduit by interacting with specific secretory proteins, sugars, and lipids[1,3]). Reabsorption of testicular fluid and constitution of a specific epididymal environment required for sperm maturation and storage are important physiological events occurring in the excurrent duct[1,4]. Aquaporin 9 (APQ9), a major apical water channel protein, which plays a crucial role in maintaining the specific epididymal fluid milieu, is a target for androgen receptor (Ar)[5,6], and is expressed in the principal cells[7].

Sperm membrane contains a high concentration of polyunsaturated fatty acids, which are susceptible to peroxidative damage[8]. Glutathione peroxidase (GPx) and catalase (Cat) neutralize the reactive oxygen species (ROS) and thus, prevent oxidative stress[9]. GPx5 is an androgen-dependent specific epididymal secretory protein which scavenges ROS to protect sperm cells from oxidative damage[10,11].

Recent evidence attests the importance of estradiol (E_2) in epididymal development, structure, and function[12–15]. E_2 may influence fluid reabsorption by modulating the expression of ion transporter proteins, water channel proteins such as aquaporins[16], and sodium-hydrogen exchanger-3[17]. In mouse and humans, estrogens promote sperm capacitation and acrosome reaction[18,19]. Another hormone which has come to stay as an important factor controlling male fertility is thyroid hormone[20–22]. Hypothyroidism during childhood delays sexual maturation[23]. A series of reports which emanated from our laboratory proposed that mammalian testis may be a target organ for direct action of thyroid hormones; these studies on experimental induction of hypo- and hyperthyroidism in pre-puberal, puberal, and adult rats showed altered intermediary metabolism in the testis, which became normal with reversion to euthyroidism, irrespective of changes in gonadotropins titre[24–27]. Subsequent reports from other laboratories underscored the essential role of thyroid hormones in testicular development in rats and piglets, and established Sertoli Cells (SCs) as the testicular target for tri-iodothyronine (T_3) in young animals[28–34]. Gestational hypothyroidism, the prevalence of which is relatively high in India, was shown to be associated with maternal-fetal complications[35,36]. One of the reports from our laboratory has shown that adult male rats with transient gestational-onset hypothyroidism experienced sub-fertility by decreasing sperm forward motility and fertilizing ability[37]. It is well established that post testicular sperm maturation in the epididymis plays a vital role in determining male fertility. The above report attributed subfertility and reduced fertilizing ability of sperm in rats with transient gestational hypothyroidism to the subnormal level of androgens 5α-reductase expression/activity, and Ar ligand-binding activity in the epididymis of such rats[37]. However, the exact molecular mechanism underlying impaired post-testicular sperm maturation due to gestational-onset hypothyroidism remains obscure. In the light of the above background information, the present study was designed to test the hypothesis that transient gestational-onset hypothyroidism affects post-testicular sperm maturation in F_1 progeny rats by inducing oxidative stress and modifying the expression of specific genes controlling epididymal function.

2. Materials and Methods

2.1 Animals

The animal experiments in the present study were approved by the Institutional Animal Ethics Committee (IAEC No: 01/23/2013). Male and female albino Wistar rats (*Rattus norvegicus)* were maintained at the Central Animal House facility under controlled temperature (24±2°C) and humidity (60–70%) with constant photoperiod of 12 h light/dark cycle as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Rats were fed with standard rat pellet diet (Lipton India, Mumbai, India) and water *ad libitum* throughout the study. Proven-fertile female rats at late proestrus phase were allowed to mate with proven fertile male rats in 2:1 ratio. Presence of vaginal plugs in the morning confirmed successful mating and the day was counted as "0" Day Post-Coitum (DPC), and the following day as Gestational Day (GD) 1. The day of parturition was counted as Postnatal Day (PND) 1.

2.2 Induction of Hypothyroidism

Hypothyroidism was induced by feeding the rats with the goitrogen methimazole (MMI, Sigma-Aldrich, St. Louis, MO, USA; 0.05% w/v in drinking water) as described in our previous reports[37–40]. The study consisted of three Groups of rats with six pregnant dams each- Group I: control pregnant dams which were not exposed to MMI; Group II: MMI treatment to pregnant dams from GD 9 to 14, covering the specific period of fetal testicular differentiation from bipotential gonad[39]; and Group III: MMI treatment from GD 9 to 18, covering the period of foetal differentiation and proliferation of testicular cell types as well as differentiation of epididymis from Wolffian duct[41]. MMI treatment commenced on GD 9, the crucial period of completion of implantation[42], to minimize fetal loss. On completion of MMI treatment, the rats were given MMI-free drinking water. At birth, the litter size was culled to the maximum of six male pups per mother after recording the total number of pups. On PND 120, progenies were sacrificed under sodium thiopentone (40 mg/Kg bw) anesthesia and the epididymes were dissected out.

2.3 Sperm Count

The caput (including initial segment), corpus, and cauda epididymides were separated and placed in petri dishes containing physiological saline at pH 7.4. The cauda epididymidis was minced well with fine scissors and centrifuged at 600x g for 10 min, and the sperm content in the pellet was diluted (1:20) in physiological saline. The total sperm count in the cauda epididymidis was calculated[43] by counting the number of spermatozoa in the appropriate squares of the haemocytometer in a phase-contrast microscope (Nikon Eclipse 80i,Tokyo, Japan).

2.4 Forward Sperm Motility

Immediately after sacrifice, the epididymis on one side was dissected out and an incision was made at the junction between vas deferens and cauda epididymidis, and the fluid was allowed to ooze out onto a new clean dry glass slide and mixed with physiological saline. One drop of sperm suspension was placed on a new clean and dry glass slide pre-warmed at 37°C. Sperm motility was assessed by visual examination of 100 spermatozoa per animal, in triplicate, in a phase-contrast microscope (Nikon Eclipse 80i, Tokyo, Japan) in different fields at x200 magnification. The number of progressive, slow progressive, non-progressive and immotile spermatozoa were counted and expressed in percentage[44].

2.5 Sperm Morphology

One mL of sperm suspension was mixed with one drop of 1% Eosin-Y and kept for 30 min. Smears were prepared on clean glass slides and dried. One hundred spermatozoa per animal in triplicate were examined at x200 magnification in a phase-contrast microscope (Nikon Eclipse 80i, Tokyo, Japan, and classified as normal or abnormal based on the description reported by Narayana *et al.*[45].

2.6 Sperm Membrane Integrity

Sperm membrane integrity was assessed by the Hypo-Osmotic Swelling Test (HOST), which is considered as a valid procedure, even though other sophisticated techniques such as cell sorting are available[46]. An aliquot of 0.1 mL cauda epididymidal sperm suspension was mixed with 1 mL hypo-osmotic solution (1.5 mM sodium citrate and 1.5 mM fructose in 1000 mL distilled water) and kept in a water bath at 37°C for an hour. One hundred spermatozoa per sample were counted in different fields at x400 magnification using phase-contrast microscope (Nikon Eclipse 80i, Tokyo, Japan). HOST-positive sperm with coiled tails were considered to have normal membrane integrity and expressed as a percentage of total sperm.

2.7 Determination of Total Protein, Antioxidants, Hydrogen peroxide (H_2O_2) and Lipid Peroxidation (LPO)

The total protein in the epididymis was determined by standard Lowry's Folin phenol method. Specific activities of enzymatic antioxidants, namely superoxide dismutase (SOD) [E.C. No. 1.15.11][47], catalase (Cat) [E.C. No. 1.15.1.6][48] and glutathione peroxidase (GPx) [EC 1.11.1.9] [49], and the level of non-enzymatic antioxidant, reduced glutathione (GSH)[50], H_2O_2 [51] and LPO [52] were determined by standard colorimetric methods indicated in parentheses.

2.8 Quantitative Real-time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from caput, corpus and cauda epididymides tissue, free from spermatozoa, by using TRIzol (Invitrogen, Carlsbad, California, USA) reagent as per the manufacturer's instructions. Total RNA was dissolved in RNAase-free water (Qiagen, Hilden, Germany) and the concentration and purity were quantified spectrophotometrically (Pharmacia Biotech, Ultrospec 4000, Ontario, Canada) by measuring the absorbance at 260/280 nm. RNA with an absorbance between 1.8 and 2.0, considered as good quality (Fourney, 1988), alone was used for the subsequent cDNA conversion. 200 ng of total RNA was used for the cDNA conversion as per the manufacturer's instructions (Qiagen, Hilden, Germany). After cDNA conversion, 2.5 µL template mixed with 12.5 µL master mix 2x reaction buffer, dNTP mix, Hot Gold Star® DNA polymerase, $MgCl_2$ and dye (Eurogentec, Seraing, Belgium), sense and anti-sense primers for *Ar*, *Erα*/β, *Trα*/β, *Aqp9*, *Gpx5*, miRNA 124a, U6 and β-actin (Sigma-Aldrich, St. Louis, MO, USA) were added and the volume was made up to 25 µL with RNase-free water. The reaction cycles were as follows: PCR enzyme activation at 95°C for 5 min; denaturation at 95°C for 15 sec, annealing at 60°C for 45 sec and elongation at 72°C for 1 min followed by melt curve analysis. Primer sequence and annealing temperature used for each gene are listed in table 1. All reactions were run in triplicate. Non-template control and non-primer control were run simultaneously. The reactions were carried out in CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, California, CA, USA). The fold change in the expression of the target gene was normalized to β-actin and calculated by using $2^{-\Delta\Delta CT}$ [53].

2.9 Western Blot Detection of Proteins

The expression levels of Aqp9, Gpx5, Ar, Erα/β and Trα/β protein were detected in the caput, corpus and cauda epididymides by western blotting as reported earlier[37,54] using specific antibodies for Aqp9 (G-3): sc-74409, Gpx5 (D-3): sc-390092, Ar (C-19): sc-815, Erα (H-184): sc-7207, Erβ (1531): sc-53494, Trα (FL-408): sc-772, Trβ (J52): sc-738 and β-actin (C4): sc-47778 (Santa Cruz Biotechnology Inc., Dallas, Texas, USA). The band intensity of protein was normalized against the loading control β-actin and quantified, using Quantity One software (Bio-Rad Laboratories, Hercules, California, CA, USA).

2.10 Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) and Newman–Keul's test to assess the significance of individual variations between the control and treatment Groups with a computer-based GraphPad Prism software version 3.0 (GraphPad Software Inc., San Diego, CA, USA).

Table 1. Sequence of primers used for the qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Accession number
Aqp9	AGGACCTCCTGCAAAGGAGAA	TGGTTGTGTTGCCAAACCAG	60	NM_022960.2
Gpx5	CTCGTTCCACTTCTTCTAGCC	AGACAGAGCCTCGTAGTTGT	60	NM_001105738.1
Ar	TGAAGTGTGGTATCCTGGTG	GTAAAACGTGGTCCCTGGTA	59	NM_012502.1
Erα	AACTTGCTCTTGGACAGGAA	CATCATGCGGAATCGACTTG	59	NM_012689.1
Erβ	AAGTGCAAAAATGTGGTCCC	TACTGTCCTCTGTTGAGCTG	60	NM_012754.1
Trα	ACAGCTGCTGCGTCATCGACAA	TTGGCCACCCGCTTTGAATCGT	59	NM_001017960.1
Trβ	GACAAGGATGAGCTCTGCGTA	CTCTGGTGTGAGAAGGCTTGC	60	NM_012672.3
mir124a	TCCGTGTTCACAGCGGAC	CATTCACCGCGTGCCTTA	60	NR_031866.1
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT	60	XR_005498700.1
β-actin	CCCGCGAGTACAACCTTCTTG	TCATCCATGGCGAACTGGTGG	59/60	NM_031144.3

3. Results

3.1 Epididymal Weight, Litter Size and Sperm Characteristics

The body weight of adult F_1 progeny of mothers with gestational exposure to the goitrogen MMI was comparable to coeval control rats, whereas the relative weight of epididymis, testis, seminal vesicles and ventral prostate decreased significantly from the level of control rats (Table 2). The litter size decreased significantly in both experimental Groups when compared with coeval control rats (Table 2). There was a significant reduction in sperm forward motility (Table 2) and sperm membrane integrity in F_1 progeny of mothers with transient gestational-onset hypothyroidism, though there was no obvious change in sperm count. An increase in abnormal sperm morphology (amorphous head, banana head and bent at cephalocaudal junction) was observed in F_1 progeny of mothers subjected to MMI-induced gestational hypothyroidism (Table 2). Comparison among gestational hypothyroidism during fetal testicular differentiation (Group II) and/or testicular and epididymis differentiation (Group III) produced similar results in the F_1 progeny rats except for sperm membrane integrity.

3.2 Enzymatic and Non-enzymatic Antioxidants, H_2O_2 and LPO

A significant decrease in specific activities of SOD, Cat and GPx, and in the level of GSH was evident in the epididymis of experimental rats in Group II and even more in Group III, when compared with Group I control rats (Table 3). On the other hand, H_2O_2 and LPO levels increased in experimental rats belonging to Groups II and III, when compared to coeval controls (Table 3). Experimental Group III has more H_2O_2 and LPO levels compared to Group II.

3.3 Expression of Epididymal Functional Genes and their Regulators

qRT-PCR data showed a significant decrease in the expression of *Aqp9* in all three regions of the epididymis of F_1 progeny rats belonging to Groups II and III, when compared to coeval control rats belonging to Group I. Western blot detection revealed a consistent decrease in *Aqp9* protein in the caput epididymidis of Group II and III rats, and in the corpus and cauda epididymides of Group III rats, when compared with coeval control rats (Figure 1A). In general, expression of both mRNA and protein of *Aqp9* showed a consistent decrease in all three regions of epididymis of Group III rats, compared to coeval controls.

qRT-PCR and western blot detections showed a significant reduction in the expression of *Gpx5* mRNA in all three regions of the epididymis (Figure 1B) of experimental Groups II and III, whereas *Gpx5* protein decreased only in caput and cauda regions, corpus remaining unaltered (Figure 1B).

Table 2. Impact of transient gestational-onset hypothyroidism on body weight, relative organs weight of caput/corpus/cauda epididymidis, testis, seminal vesicles and ventral prostate, litter size, sperm count, sperm membrane integrity and sperm abnormalities of F_1 progeny rats.

Group	Body weight (g) n=6	Relative organs weight (mg/100g body weight) n=6						Litter size/ mother n=6	Cauda epididymal sperm count ($X10^6$ sperm) n=6	Percentage of sperm motility n=6				Sperm membrane integrity (in percentage) n=6	Sperm abnormalities (in percentage) n=6
		Caput epididymis	Corpus epididymis	Cauda epididymis	Testis	Seminal vesicles	Ventral prostate			Progressive	Slow progressive	Non-progressive	Immotile		
I	185.5± 11.2[a]	149.6±3.9a	65.4± 4.2[a]	146.8±6.7[a]	512± 11[a]	317± 4.2[a]	111.7± 4.5[a]	8.6± 0.5[a]	306± 10[a]	88± 6.5[a]	6.0± 3.1[a]	4.2± 3.7[a]	1.8± 2.9[a]	54.3± 5.77a	3.33± 0.57[a]
II	204.2± 20.4[a]	113.4±5.7[b]	56.6± 3.5[b]	110.6±5.7[b]	430± 15[b]	288± 6.1[b]	92.2± 5.2[b]	4.2± 0.4[b]	298± 12[a]	75± 5.0[b]	10.0± 3.5[a]	8.4± 3.2[a]	6.6± 2.2[b]	26.3± 3.04[b]	7.00± 1.00[b]
III	191.3± 17.6[a]	118.8±4.0[b]	55.0± 3.9[b]	124.4±3.1[c]	408± 17[b]	286± 5.3[b]	93.3± 4.5[b]	4.2± 0.8[b]	296± 18[a]	73± 6.7[b]	11.0± 2.2[a]	7.4± 3.1[a]	8.6± 2.1[b]	16.6± 2.08[c]	7.66± 0.57[b]

Group I: Control F_1 progeny rats not exposed to MMI; Group II: MMI treatment during gestation period GD 9 to GD 14 (foetal testicular differentiation window); Group III: MMI treatment during gestation period GD 9 to GD 18 (Embryonic window encompassing testis and epididymis differentiation). Each bar represents mean ± SEM of six observations (n=6). Bars with the same letters indicate that the difference between the two means is not statistically significant, whereas those with different letters are statistically significant at the $p \leq 0.05$ level. Comparisons are between coeval control and experimental rats.

Table 3. Impact of transient gestational-onset hypothyroidism on enzymatic and non-enzymatic antioxidants, and levels of H_2O_2 and LPO in the epididymis of F_1 progeny rats

	SOD (units/mg protein)			Cat (H_2O_2 utilized/min/mg protein)			GPx (µg of GSH consumed / mg protein)			GSH (µg/g wet tissue)			H_2O_2 (µmol H_2O_2 generated/ min/ mg protein)			LPO (nmoles of MDA / mg tissue)		
Group	Caput	Corpus	Cauda	Caput	Corpus	Cauda	Caput	Corpus	Cauda	Caput	Corpus	Cauda	Caput	Corpus	Cauda	Caput	Corpus	Cauda
I	2.34 ±0.15^a	1.96 ± 0.10^a	2.06 ±0.21^a	23.59 ±3.34^a	21.72 ±1.09^a	23.73 ±2.92^a	0.78 ±0.09^a	0.66 ±0.05^a	0.76 ±0.09^a	0.78 ±0.09^a	0.74 ± 0.03^a	0.45 ±0.02^a	15.76 ±1.40^a	23.88 ± 3.03^a	33.39 ±2.58^a	7.24 ±0.56^a	6.67 ± 0.58^a	6.24 ±0.07^a
II	2.05 ±0.09^b	1.76 ± 0.14^b	1.65 ±0.04^b	17.33 ±1.27^b	16.36 ± 1.91^b	18.32 ±2.07^b	0.47 ±0.03^b	0.66 ± 0.04^a	0.56 ±0.06^b	0.54 ±0.03^b	0.53 ± 0.07^b	0.30 ±0.01^b	22.25 ±3.80^b	36.67 ± 5.02^b	38.16 ±1.87^b	9.10 ±0.66^b	8.01 ± 0.50^a	7.11 ±0.17^b
III	1.51 ±0.12^c	1.57 ± 0.08^c	1.34 ±0.07^c	12.17 ±1.30^c	13.11 ± 1.93^c	11.28 ±1.09^c	0.32 ±0.07^c	0.53 ± 0.07^a	0.40 ±0.06^c	0.46 ±0.04^c	0.43 ± 0.04^c	0.27 ±0.01^c	43.19 ±8.24^c	48.67 ± 3.89^c	42.12 ±2.68^c	10.99 ±0.85^c	10.79 ± 1.24^c	8.06 ±0.11^c

Superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (GPx), reduced glutathione (GSH), hydrogen peroxide (H_2O_2) and lipid peroxidation (LPO).

Group I: Control F_1 progeny rats not exposed to MMI; Group II: MMI treatment during gestation period GD 9 to GD 14 (fetal testicular differentiation window); Group III: MMI treatment during gestation period GD 9 to GD 18 (Embryonic window encompassing testicular and epididymis differentiation). Each bar represents mean ± SEM from six observations (n=6). Bars with the same letters indicate that difference between the two means is not statistically significant, whereas those with different letters are statistically significant at the p≤0.05 level. Comparisons are between coeval control and experimental rats.

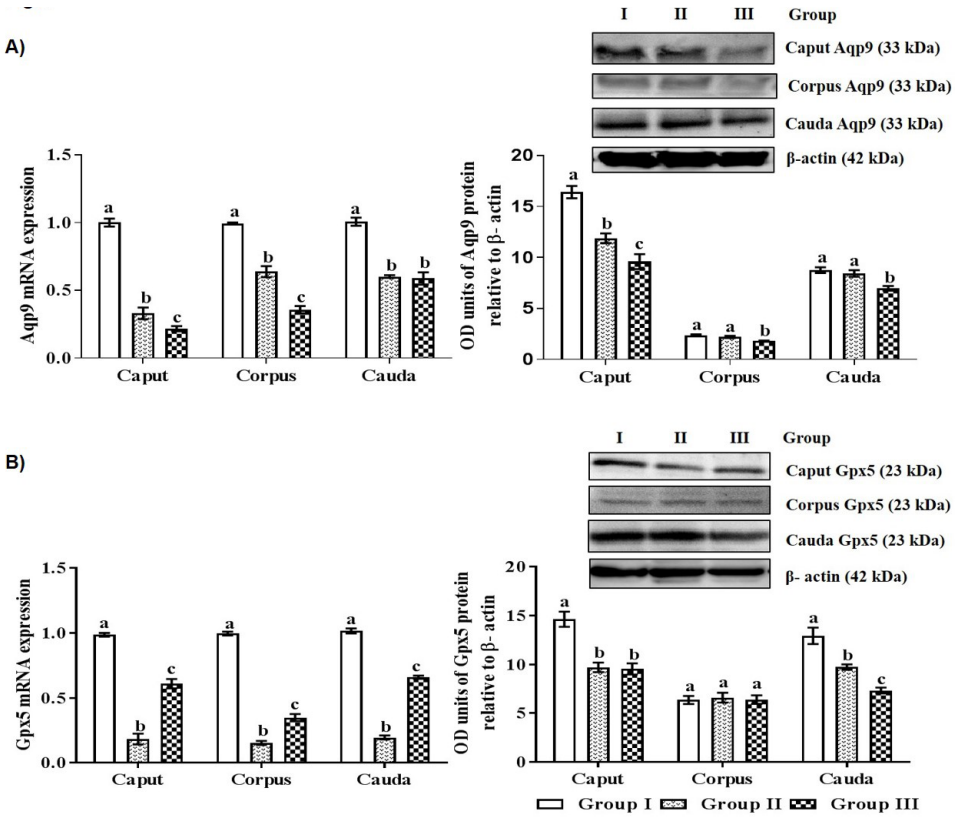

Figure 1. Impact of transient gestational-onset hypothyroidism on **(A)** *Aqp9,* and **(B)** *Gpx5* gene expression in the epididymis of F_1 progeny rats on PND120. Group I: Control F_1 progeny rats which were not exposed to MMI; Group II: MMI treatment during GD 9 to GD 14 (fetal testicular differentiation window); Group III: MMI treatment during gestation period GD 9 to GD 18 (testicular and epididymis differentiation window). Each bar represents mean ± SD of three observations; each sample had pooled tissues from two animals. Bars with the same alphabet indicate that the difference between the respective means is not statistically significant, whereas those with different alphabets are statistically significant at the $p \leq 0.05$ level. Comparisons are between coeval control and experimental rats. *Aqp9* -aquaporin 9; *Gpx5*- Glutathione peroxidase 5. qRT-PCR data revealed increased *Ar* mRNA level in the caput, corpus and cauda epididymides of experimental rats belonging to Group II and III, whereas corpus epididymidis of Group II rats alone showed a decrease in Ar mRNA. On the contrary, western blot detection revealed decreased Ar protein in all the three epididymal regions of experimental Groups (Figure 2A). qPCR detection of miRNA124a expression showed a significant increase in all the three regions of the epididymis of F_1 progeny rats belonging to Groups II and III when compared to coeval control rats belonging to Group I (Figure 2B).

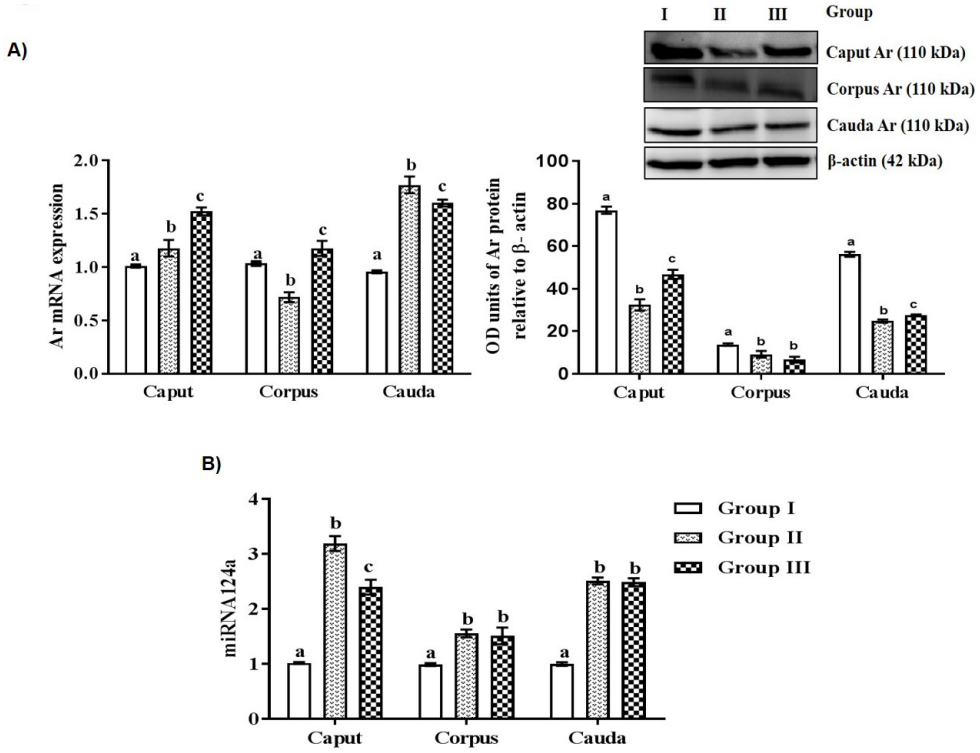

Figure 2. Impact of transient gestational-onset hypothyroidism on *Ar* **(A)** and miRNA 124a **(B)** gene expression in the epididymis of F_1 progeny rats on PND 120. Group I: Control F_1 progeny rats which were not exposed to MMI; Group II: MMI treatment during GD 9 to GD 14 (fetal testicular differentiation window); Group III: MMI treatment during gestation period GD 9 to GD 18 (testicular and epididymis differentiation window). Each bar represents mean ± SD of three observations; each sample had pooled tissues from two animals. Bars with the same alphabet indicate that the difference between the respective mean is not statistically significant, whereas those with different alphabets are statistically significant at the $p \leq 0.05$ level. Comparisons are between coeval control and experimental rats. Comparisons are between coeval control and experimental rats. Ar- Androgen receptor; miR124a- microRNA124a.

Both qRT-PCR and western blot detections showed the increased expression level of *Erα* and *Erβ* genes in experimental rats, except *Erα* mRNA in the cauda epididymidis of Group III rats, and Erα protein in the cauda epididymidis of Group II rats (Figures 3A, B).

4. Discussion

Data on relative organs weight point out that transient gestational-onset hypothyroidism may affect the growth of male reproductive tract organs in the F_1 progeny. It is well established that growth of most of the male accessory sex organs depend on androgens, whereas testis growth is under the control of gonadotropins[55–57]. Previous reports from our laboratory on the progeny of mothers with transient gestational-onset hypothyroidism pointed out hypoandrogenism in peripheral circulation and in the testicular interstitial fluid at puberal and adult ages[37,54]. The present study found subnormal level of Ar protein in the epididymis of F_1 progeny of mothers with gestational-onset hypothyroidism of both Groups II and III suggesting subnormal androgenic action in epididymis. Interestingly, qPCR shows increased Ar mRNA expression which is inconsistent with the protein level suggesting that there may be interference in the translation of Ar mRNA or an augmented rate of metabolic degradation of Ar protein. Increased level of miRNA124a was reported to decreases GR and AR protein in myeloma cells and human thyroid cancer cell lines[58,59]. This may be another mechanism underlying the decrease in Ar protein since experimental rats of the present study recorded a significant increase in the miRNA124a in all three regions of epididymis of

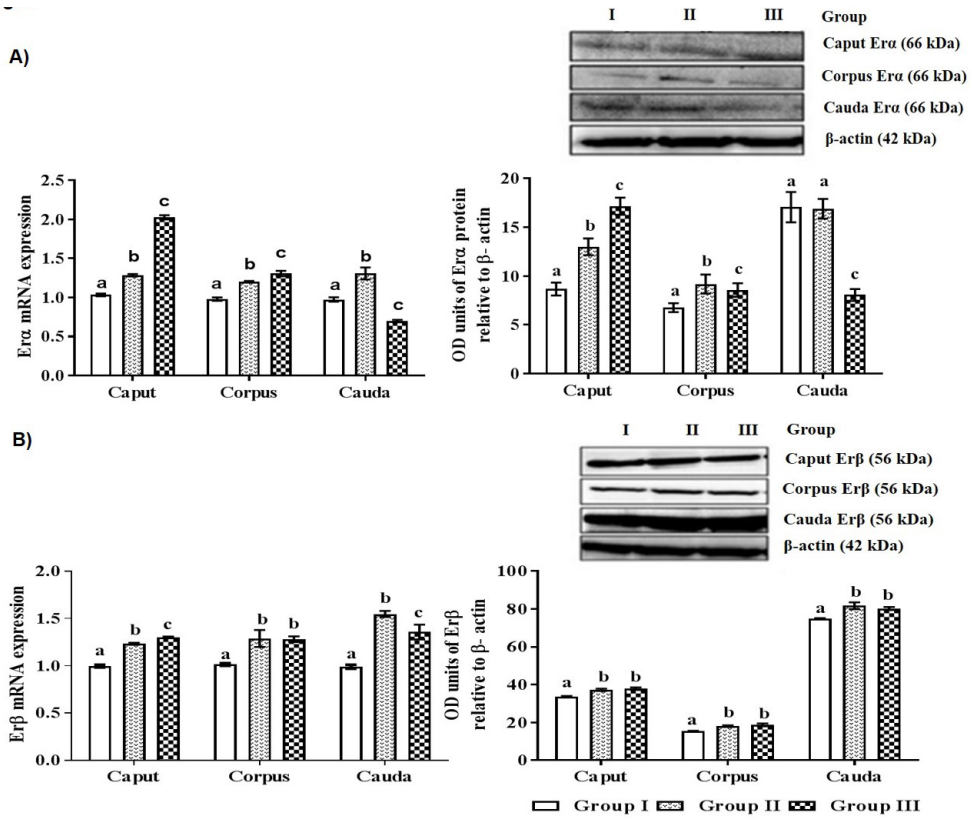

Figure 3. Impact of transient gestational-onset hypothyroidism on **(A)** *Erα* and **(B)** *Erβ* gene expression in the epididymis of F_1 progeny rats on PND 120. Group I: Control F_1 progeny rats which were not exposed to MMI; Group II: MMI treatment during GD 9 to GD 14 (fetal testicular differentiation window); Group III:MMI treatment during gestation period GD 9 to GD 18 (testicular and epididymis differentiation window). Each bar represents mean ± SD of three observations; each sample had pooled tissues from two animals. Bars with the same alphabet indicate that the difference between the respective mean is not statistically significant, whereas those with different alphabets are statistically significant at the $p \leq 0.05$ level. Comparisons are between coeval control and experimental rats. Erα- Estrogen receptor alpha; Erβ- Estrogen receptor beta.

Unlike *Ar* and *Er*, *Trα and Trβ* genes recorded decreased expression in the epididymis of experimental rats in general, except for unaltered Trα protein in the corpus epididymidis of Group II rats (Figure 4A, B).

F_1 progeny. We observed a decrease in Ar mRNA and a significant increase in miRNA124a expression in the corpus epididymidis of Group II rats alone, suggesting a two-pronged negative effect on Ar protein expression; in all other Groups increased level of miRNA 124a expression seems to be the major mechanism behind diminution of AR protein. Overall, gestational-onset hypothyroidism induced during the crucial period of fetal testicular differentiation from the bipotential gonad (Group II) and an extended period encompassing differentiation of testicular cell types and initiation of epididymis differentiation (Group III) has similar effect on Ar protein expression.

Male fertility evaluation begins with assessment of sperm characteristics[60]. Functional sperm plasma membrane, an important component of spermatozoa, plays a vital role during sperm capacitation, acrosome reaction and binding of the spermatozoon to the surface of the ovum[61,62]. Therefore, the findings of increased abnormalities in sperm morphology coupled with decreased sperm forward motility and sperm membrane integrity in the experimental rats of the present study suggest that hypothyroidism induces increased sperm abnormalities; this is consistent with findings in hypothyroid men (22). In experimental rats of the present study there was no appreciable change in the histoarchitecture of the epididymis (Supplementary data, Figure 1). Presumably, there is interference during spermiogenesis and, hence, a detailed investigation on spermiogenesis may throw additional light on the issue.

Oxidative stress is one of the major causes of male infertility[63]. The high content of polyunsaturated fatty acid within the plasma membrane of spermatozoa can lead to cellular and plasma membrane damage through the induction of peroxidation[64]. It is known that an imbalance between cellular antioxidant defence systems and ROS production may lead to oxidative stress[65]. Hence, our findings of decreased enzymatic SOD, Cat, GPx and non-enzymatic antioxidants GSH, accompanied by enhanced level of pro-oxidants, like H_2O_2 and LPO, in the epididymis of F_1 progeny rats of mothers with transient gestational exposure to MMI point out the possibility of oxidative stress. This might have adversely affected the sperm membrane integrity in hypothyroid rats.

Developmental defects and fetal loss were reported in normal mice mated with *Gpx5*$^{-/-}$ males[66]. Overexpression of epididymal specific *GPx5* decreased the DNA damage, increased the resistance to oxidative stress and decreased the levels of lipid peroxidation in CHO-K1 cell line *in-vitro* (11). Hence, sub-normal expression of *Gpx-5* observed in the present study may be an additional factor contributing to the probable increase in free radical toxicity and, thus, another mechanism by which gestational exposure to MMI interferes with post-testicular sperm maturation in the epididymis of F_1 progeny. Loss of *Gpx-5* transcript in the epididymis after castration and restoration of the same with subsequent T supplementation suggested that it is an androgen-dependent protein involved in maintaining sperm DNA integrity[67]. Data from the present study points out repression of *Ar* in the epididymis of experimental rats. We previously reported decreased Ar ligand binding activity[37,54] and subnormal level of Ar protein in the epididymis of F_1 progeny of mothers with transient gestational exposure to MMI. might have affected the expression of *Gpx5*. Therefore, decrease of androgenic control seems to be a plausible mechanism by which transient gestational-onset hypothyroidism decreases the expression of epididymis-specific *Gpx5*$^{-/-}$ in the progeny.

One of the important functions of the initial segment and proximal caput epididymidis is fluid reabsorption to help concentrate sperm[16,68]. Decreased expression level of *Aqp9,* the water channel protein involved in reabsorption of testicular fluids in the epididymis[16] might have interfered with the post-testicular sperm maturation process by altering epididymal luminal milieu in hypothyroid rats of the present study. Immunolocalization studies revealed decreased *Aqp9* in proximal cauda epididymidis of castrated rats, and in rats treated with the anti-androgen flutamide, which was restored by T supplementation[7]. Therefore, subnormal level/ligand binding activity of Ar[37,54], may be attributed to decreased *Aqp9* expression. Another dimension of the issue is the stimulatory effect of E_2 on fluid reabsorption in the excurrent duct[69]. In the present study, there was increased expression of *Erα* and *Erβ* in experimental rats, which is inconsistent with the findings of decreased expression of *Aqp9*. Probably, Ar is a more potent transcriptional regulator of *Aqp9* than Er. Therefore, the impact of increased levels of *Erα* and *Erβ*, if any, on the expression of *Aqp9* might have been obliterated by the decreased expression level of *Ar* gene and its protein, the primary regulator of the gene.

We have previously reported that transient gestational exposure to MMI induces hypothyroidism in the offspring, which persists till the pre-puberal age of 7 weeks as indicated by elevated serum Thyroid Stimulating Hormone (TSH) level accompanied by decreased T_4 and T_3 titres[37]. This shall indicate that the F_1 progeny of mothers with gestational exposure to goitrogens remained in hypothyroid state during critical windows of post-natal differentiation and maturation of male reproductive tract organs, including the epididymis[42]. The decreased expression of *Trα* and β observed in all the three regions of epididymis of progeny rats belonging to Groups II and III indicates the subnormal action of thyroid hormone at the level of epididymal tissue. This might have affected epididymal function, directly at the level of the organ, including the induction of oxidative stress and its impact on sperm characteristics[9]. The present study ascertains that distorted post-testicular sperm maturation in the epididymis of adult rats subjected to transient gestational-onset hypothyroidism is the result of attenuated action of androgens and thyroid hormones, despite a boost in estrogenic action.

Taken together, the findings of the present study and our earlier reports[37,54] point out that men whose mothers had taken antithyroid therapy during pregnancy may be susceptible to impaired fertility. The findings support the proposed hypothesis, and it is concluded that transient gestational-onset hypothyroidism might affect fertility of F_1 progeny by interrupting post-testicular sperm maturation and augmenting sperm abnormalities. Free radical toxicity and decreased expression of *Ar* and *Tr α /β* in the epididymis appear to be the plausible mechanism underlying the adverse effect of hypothyroidism on post-testicular sperm maturation. Our findings emphasize that euthyroid state during pre- and post-natal life is one of the key factor that determine male fertility.

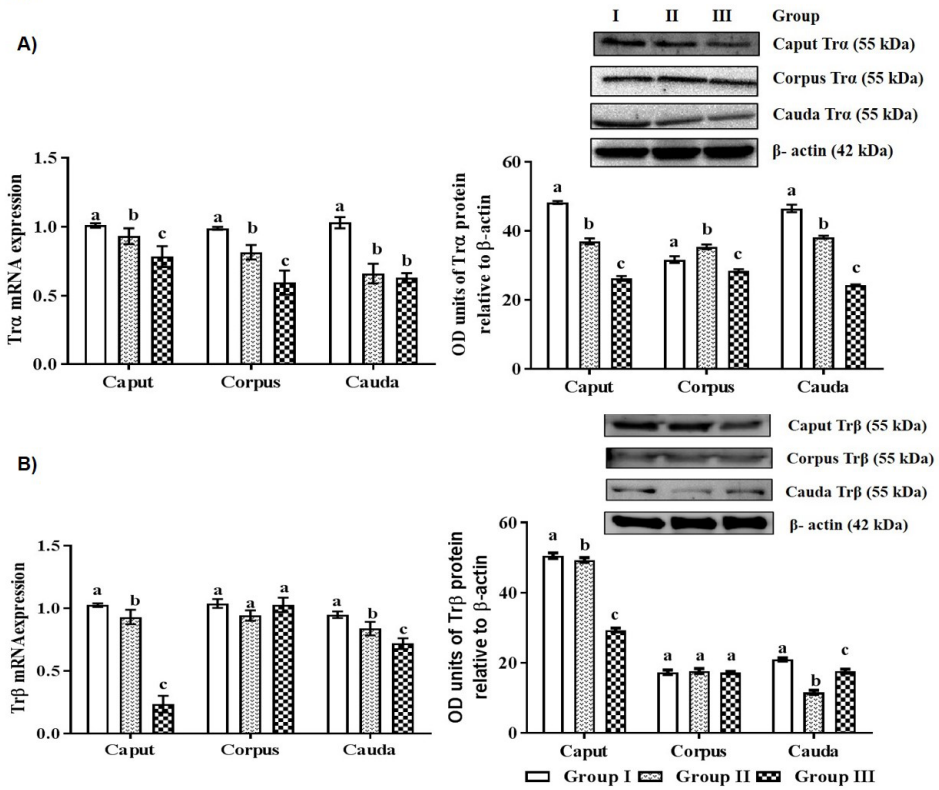

Figure 4. Impact of transient gestational-onset hypothyroidism on **(A)** *Trα* and **(B)** *Trβ* gene expression in the epididymis of F_1 progeny rats on PND 120. Group I: Control F_1 progeny rats, which were not exposed to MMI; Group II: MMI treatment during GD 9 to GD 14 (foetal testicular differentiation window); Group III: MMI treatment during gestation period GD 9 to GD 18 (testicular and epididymis differentiation window). Each bar represents mean ± SD of three observations; each sample had pooled tissues from two animals. Bars with the same alphabet indicate that the difference between the respective mean is not statistically significant, whereas those with different alphabets are statistically significant at the $p \leq 0.05$ level. Comparisons are between coeval control and experimental rats. Trα- Thyroid hormone receptor alpha; Trβ- Thyroid hormone receptor beta.

5. References

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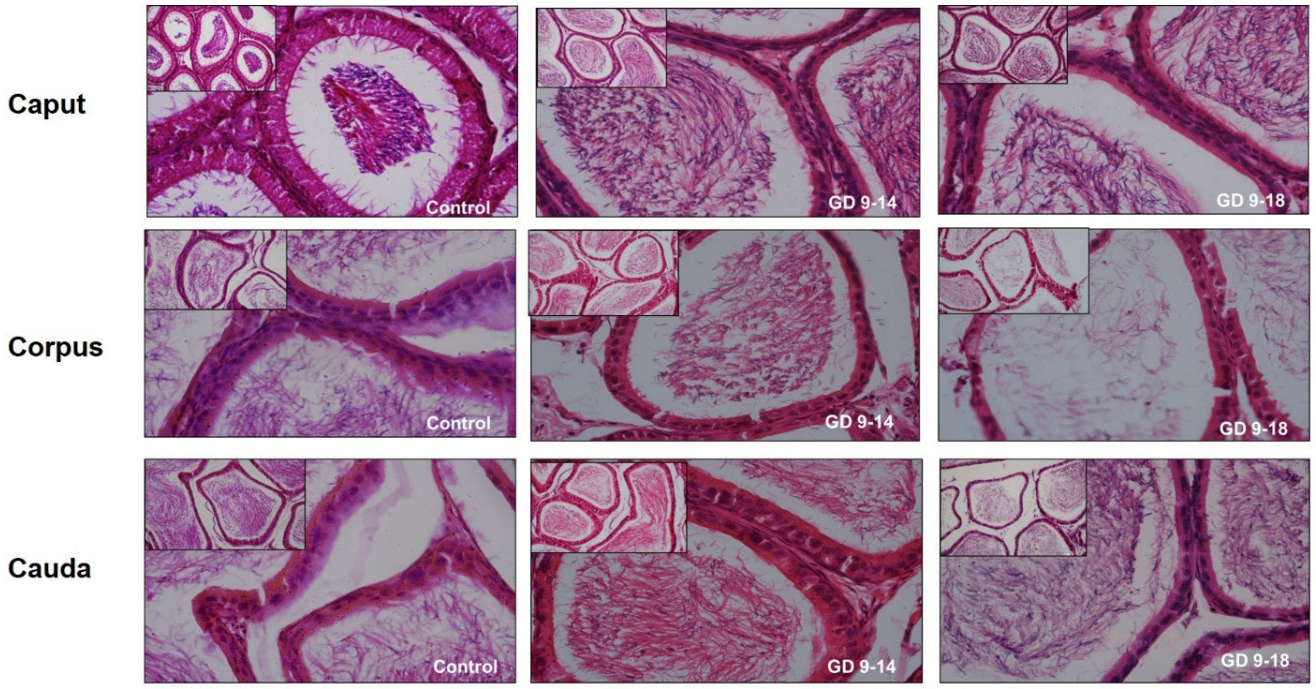
Supplementary Figure 1. Histological observation of epididymis of F_1 progeny exposed to gestational-onset



Supplementary data 1c Histological observation (H&E staining) of epididymidis of F_1 progeny subjected to transient gestational-onset hypothyroidism. Magnification: X200 (Left Upper End), X400