Heme oxygenase gene expression in human azoospermic testicular tissue

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ABSTRACT

Heme Oxygenase (HO), a microsomal enzyme, catalyzes the rate-limiting step in heme catabolism. To assess the expression of HO-1 and HO-2 genes in human azoospermic testicular tissues, forty seven non-obstructive azoospermic (NOA) men were studied. They were divided according to the results of their testicular sperm extraction (TESE) into; successful positive TESE (n=19) and unsuccessful negative TESE (n=28) compared to 12 obstructive azoospermia cases with matched age. Serum FSH hormone was carried out by ELISA method. Testicular biopsies obtained from azoospermic men were subjected to histopathology, testicular sperm extraction (TESE) and detection of HO-1 and HO-2 gene expression by RT-PCR. The results showed that all azoospermic testicular tissue specimens expressed HO-2 mRNA cases. HO-1 mRNA expression was evident in all obstructive azoospermia and successful TESE positive NOA cases (n=19) as well as in 7/28 unsuccessful TESE negative NOA cases. HO-1 mRNA expression was negative in 21/28 negative TESE cases. It is concluded that HO-1 may play a role in the spermatogenic process.

RIASSUNTO

Espressione del gene della eme ossigenasi nel tessuto testicolare umano azoospermico

L'enzima microsomiale eme ossigenasi (HO) catalizza il passaggio limitante nel catabolismo dell'eme. Per valutare l'espressione dei geni HO-1 e HO-2 nel tessuto testicolare umano azoospermico sono stati studiati 47 uomini con azoospermia non-ostruttiva (NOA). In base ai risultati di una prova di estrazione testicolare dello sperma (TESE) sono stati divisi in TESE-positivi (n=19) e TESE-negativi (n=28), e confrontati con 12 casi di azoospermia ostruttiva di età comparabile. La determinazione dell'ormone FSH è stata fatta con un metodo ELISA. Le biopsie testicolari ottenute da uomini azoospermici erano sottoposte a esame istopatologico, a prova di estrazione testicolare dello sperma (TESE) e alla determinazione dell'espressione dei geni HO-1 e HO-2 mediante RT-PCR. I risultati hanno mostrato che tutti i campioni di tessuto testicolare azoospermico esprimevano il mRNA del HO-2. L'espressione del mRNA dell'HO-1 era evidente in tutti i casi di azoospermia ostruttiva e di NOA TESE-positivi (n=19) come pure in 7/28 casi di NOA TESE-negativi; risultava invece negativa in 21/28 casi TESE-negativi. Si conclude che il gene HO-1 può giocare un ruolo nel processo del spermatogenesi.

INTRODUCTION

Heme oxygenase (HO); is a microsomal enzyme that catalyzes the rate-limiting step in heme catabolism: the oxidative cleavage of heme molecules to biliverdin, carbon monoxide (CO), and iron. HO consists of three isoforms as products of separate genes: an inducible form (HO-1), a constitutive form (HO-2), and a third isoform (HO-3), with properties similar to HO-2 (1). HO-1, formerly known as heat shock protein-32, is inducible by diverse stimuli causing oxidative stress (2). HO-2, in contrast, is expressed and induced only by adrenal glucocorticoids. The differences in regulation of HO-1 and HO-2 are reflected in the presence of regulatory elements in the promoter regions, HO-1 promoter contains sequences necessary for binding multiple regulatory factors, as nuclear factor and heat shock factor (3), whereas HO-2 gene contains a single glucocorticoid response element (4). The heme-heme oxygenase system has been recognized to possess important regulatory properties. It is tightly involved in both physiological as well as pathophysiological processes, such as cytoprotection, apoptosis, and inflammation (5).

Trakshel et al. (6) detected 2 isoforms of HO enzyme in the rat testis, the predominant one was HO-2 with minute HO-1. Trakshel and Maines, (7) identified prevalent HO-2 in human testis. McCoubrey et al., (8) reported that HO-2 expression is regulated by developmental and cell type-specific factors in the testis. Although abundant HO-2 mRNA could be detected in spermatogonia, spermatocytes and spermatids, HO-2 protein was detected, only in spermatids demonstrating that HO-2 expression is regulated at both transcriptional and translational levels. The exclusive use of HO-2 by the mature testis is consistent with the possibility of playing a role in male reproduction (9). Also, Leydig cells appear to use HO-1-derived CO to trigger apoptosis of pre-meiotic germ cells and thereby modulate spermatogenesis under conditions of stress (10).

In some infertile non-obstructive azoospermic (NOA) men, minute foci of spermatogenesis may be found but the importance of such finding was not apparent until the era of intracytoplasmic sperm injection (ICSI) (11). Actually, testicular sperm extraction and retrieval may not always be successful in all NOA cases that have both an emotional and financial implications because the procedure involves treatment for both partners as the husband undergoes testicular sperm recovery and his wife undergoes ovarian stimulation and possibly oocyte retrieval (12). However, there are no certain means for predicting the presence of such foci of spermatogenesis, other than surgical extraction of testicular tissue. Different clinical, biochemical and technical predictive parameters were suggested but without conclusive results (13). Hence, predicting a successful recovery procedure can offer realistic expectations for both the couple and the physician.

MATERIALS AND METHODS

Sample selection

Forty seven NOA men were included in this study compared to 12 obstructive azoospermia cases with matched age. NOA cases were further subdivided according to testicular sperm extraction (TESE) results into successful TESE positive and unsuccessful TESE negative cases. All subjects were subjected after consent to history taking in addition to general and genital examinations. Men who had received hormonal medications, smokers and those with leukocytospermia were excluded. Testicular size was considered normal if ≥15 ml, moderate 10-15 ml and small <10 ml (14). Azoospermia was verified after 3 different analyses and centrifugation (15). The men were subjected to serum FSH estimation by ELISA method, testicular tissue biopsy for both histopathology and diagnostic TESE, and then a part was preserved in lysis buffer for detecting HO mRNA in testicular tissues by RT-PCR technique (16).

Testicular Sperm Extraction

Under anesthesia, a small incision was made in the scrotal skin and carried through the tunica vaginalis. The extruded testicular tissue was excised and placed in a Petri dish containing Ham's F_{10} (Gibco, Scotland) solution with 8% (w/v) human serum albumin (Pharmacia, Sweden) and 2% HEPES buffer (Gibco, Scotland). Each testicular biopsy was minced, separated under stereo microscope and then examined under an inverted microscope (Olympus, Japan) at 400 X using the Hoffman modulation contrast system. If sperms were retrieved, no further tissue was excised. If not, repeated sampling was done before exploring the other testis. In all patients a small piece of testicular tissue was taken in Bouin's fixative for histopathology. NOA specimens were classified histopathologically into; mixed pathology, spermatid maturation arrest, primary spermatocyte arrest, Sertoli cell only (SCO) and tubular hyalinization (15).

RNA extraction

RNA was extracted from the testicular tissue using QIAamp^R RNA blood mini-kit (Qiagen, Grawlay, UK).

RT-PCR for HO-1 and HO-2

Primers for HO-1 were prepared using the oligo-1000 DNA synthesizer (Beckman, California, USA), with sequence: Sense 5' CAGGCAGAGAATGCTGAGTTC 3' and Reverse

3' GATGTTGAGCAGGAACGCAGT 5' .RNA was reverse transcribed using 12.5 μl oligo (dT)18 primer (final concentration 0.2 μM) and was denatured at 70 °C for 2 min. The denatured DNA was placed on ice and 6.5 μl of reverse transcription mixture (containing 50 mM Tris-HCl pH 8.3, 50 mM KCl_2, 1.5 mM Mg Cl_2, 0.5 mM of each dNTP,1 μ/μl RNase inhibitor and 200 μ of MMLV reverse transcriptase) was added. The tube was placed at 42 oC for 1hour followed by heating to 92 °C to stop the reaction then placed on ice. PCR reaction was performed by adding, to the reverse transcription tube,the PCR mix to a final volume of 100μl.The PCR mix contained 10 mmol/L Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin, 250 μM dNTPs mix, 2.5U Taq polymerase, and 100 μM of each primer[17].The reaction mixture was subjected to 40 cycles of PCR amplification as follows: denaturation at 95 °C for 1minute, annealing at 55 °C for 1 minute and extension at 72 °C for 2 minute. After the last cycle, a final extension at 72 °C for 10 minute was done. Negative controls containing nuclease free water instead of RNA were included in every RT-PCR run to avoid false results. HO-2 primers sequence: sense 5' GCGAGACGAGCAAGAACCAC 3', antisense 5' TCACGGTGTGGCATGATGGGG 3'. The RT-PCR for HO-2 was as HO-1 except using the specific primers for amplification of HO-2 gene. PCR cycling conditions were as HO-1 except the annealing temperature was 60°C (17).

Agarose gel electrophoresis

Ten μl of the PCR product were used. Agarose used was molecular biology grade (Sigma) at concentration 1.5% in 1x Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide (18). Resulting bands were visualized by UV rays and compared to a standard PCR marker (Promega, Madison, WI, USA). Positive cases for HO-1 expression gave bands at 555 bp (base pairs) and for HO-2 at 1004 bp.

RESULTS

- All testicular tissues specimens studied (n=59) gave positive HO-2 mRNA expression (100%) (Figure 1).
- In obstructive azoospermia group (n=12), all testicular

Figure 1
An agarose gel electrophoresis (2%), showing positive expression of HO-2 by PCR at 1004 bp
M: Molecular DNA marker (1000,750,500,300,150,50 bp). Lanes 1-6,8 positive expression and lane7: negative control

specimens yield successful TESE positive result with normal histopathology. Mean age of this group was 33.7 ± 3.5 years (range 26-46 years), mean serum FSH was 4.3 ± 2.3 mIU/ml (range 3.5-9.8 mIU/ml) and normal testis size. All testicular specimens expressed HO-1 mRNA (Figure 2).

- In NOA group, TESE result was successful (positive) in 19 cases and unsuccessful (negative) in 21 cases, respectively.
- All successful positive TESE cases (n=19) gave positive testicular HO-1 expression in testicular tissue. Mean age of this subgroup was 32.8 ± 6.6 years (range 23-43 years), mean serum FSH was 8.3 ± 5.3 mIU/ml (range 1.5 - 21.8 mIU/ml). Testicular size was; normal in 9 cases and moderate in 10 cases. Scrotal varicocele was diagnosed 6 cases. Histopathological patterns were; spermatid maturation arrest in 7 cases, primary spermatocyte arrest in 2 cases, mixed pathology in 4 cases and SCO in 6 cases (Table 1).
- Unsuccessful TESE negative NOA cases (n=21) demonstrated absent HO-1 expression in testicular tissues (Figure 3). Mean age of this subgroup was 32.2 ± 5.7 years (range 24-42 years), mean serum FSH was 15.4 ± 4.3 mIU/ml (range 6.5-23 mIU/ml).Testis size was normal in 6 cases, moderate in 13 cases and small in 2 cases. Varicocele was diagnosed in 5 cases. Histopathology was; primary spermatocyte arrest in 2 cases, SCO in 19 cases (Table 2).
- Seven unsuccessful negative TESE cases expressed positive testicular HO-1. Mean age was 37.7 ± 5.6 years (range 25-42 years), mean serum FSH was 21.3 ± 16.2 mIU/ml (range 1 - 49.5 mIU/ml). Testicular size was moderate in 3 cases, small in 4 cases with no varicocele. Histopathology was SCO in 4 cases, tubular hyalinization in 3 cases (Table 3).
- No case demonstrated absent HO-1 expression with successful positive TESE.
- Scrotal varicocele was associated with HO-1 positive expression in 6/26 NOA cases (23.1%) and with HO-1 negative expression (23.8%) 5/21 NOA cases (Fig 3).

Figure 2
An agarose gel electrophoresis (2%) stained with ethidium chloride, showing expression of HO-1 by PCR at 555 bp of testicular sperm extraction positive cases. M: Molecular DNA marker (100 bp each). Lanes 1-8: positive expression and lane 9: negative control.

Figure 3
An agarose gel electrophoresis, showing expression of HO-1 by PCR at 555 bp of testicular sperm extraction negative cases. M: Molecular DNA marker (100 bp each). Lanes 1, 2: negative cases. Lane 3- 5: positive cases. Lanes 6: negative control

Table 1
Non obstructive azospermic (NOA) cases (n=19) with positive testicular sperm extraction (TESE) and positive HO expression.

No	Age years	Size	Vx	FSH mIU/ml	Pthology	TESE	HO-1 Testis	HO-2 Testis
1	40	M	-	15.9	Mixed	+	+	+
2	40	M	-	15.9	Mixed	+	+	+
3	41	M	+	4.2	Spd arrest	+	+	+
4	30	M	+	2.8	Mixed	+	+	+
5	40	N	-	14.5	SCO	+	+	+
6	40	N	-	6.7	Spd arrest	+	+	+
7	32	N	-	4.2	C1 arrest	+	+	+
8	29	N	-	6.7	SCO	+	+	+
9	31	M	-	6.5	SCO	+	+	+
10	23	N	-	6.5	Spd arrest	+	+	+
11	23	N	-	6.5	Spd arrest	+	+	+
12	36	N	-	1.5	Spd arrest	+	+	+
13	30	M	-	3.8	Spd arrest	+	+	+
14	43	M	+	11.4	Mixed SCO	+	+	+
15	37	M	-	6.5	C1 arrest	+	+	+
16	24	M	+	21.8	SCO	+	+	+
17	29	N	+	4.6	Spd arrest	+	+	+
18	25	N	+	7.8	SCO	+	+	+
19	31	M	-	9	SCO	+	+	+
Mean	32.8			8.3				
SD ±	6.6			5.3				

Vx: varicocele M: Moderate size Spd: Spermatid SD: Standard Deviation
N: Normal size C1: primary spermatocyte SCO: Sertoli Cell Only S: Small size

DISCUSSION

Spermatogenesis is a complex developmental process that depends on pituitary gonadotrophins and testosterone, exerting indirect effects on spermatogenic cells by locally produced autocrine, paracrine and juxtacrine regulators of testicular functions (19). Many investigators made many trials to establish a link between testicular specific parameters and the event of spermatogenesis. Such relation had been suggested for at least three testicular proteins with varied intensities; lactate dehydrogenase, inhibin, Müllerian inhibiting substance (20).

Regarding NOA azoospermic cases in the current study, TESE results were not correlated with either the age of the patient, testicular size, serum FSH, presence or absence of varicocele. Eckel et al., (21) and Amer et al., (22), found no correlation between testis size and TESE results. Menashe et al., (23) found that neither the age, testicular size, serum FSH nor LH levels have a predictive value for successful positive TESE. Hauser et al., (14) found no correlation between serum FSH, LH, PRL, Testosterone, testicular size, consistency and TESE or histopathology in NOA. These data are in discipline that there are no certain means for predicting the presence of such foci of spermatogenesis, other than performing surgical extraction of testicular tissue. Therefore, objective counseling based on understanding the process of spermatogenesis and the determination of those factors which may predict a successful TESE procedure can offer realistic expectations for both the patient and the physician.

RT-PCR for HO-2 was expressed in all testicular tissue samples studied (n=59) going with Trakshel et al., (6); Trakshel and Maines, (7). Ewing and Maines, (24) concluded that HO-2 mRNA translation is linked to the maturation and expression of a factor (s) that regulates sperm development in rats. Lui et al., (10) found that the highest levels of HO-2 in the testis are controlled by glucocorticoids and that there are developmental and tissue-specific factor (s) which determine the generation of transcripts unique to the organ. The exclusive use of HO-2 by the mature testis is consistent with the possibility that HO-2 may play a role in male reproduction.

Expressed HO-1 mRNA was evident in all obstructive azoospermic cases (n=12) and in 26/47 NOA testicular specimens with different histopathological patterns. Since increased temperature is documented as HO-1 inducers, scrotal varicocele has been expected to be associated with HO-1 positive expression, as one of its leading theories affecting spermatogenesis is testicular hyperthermia (25&26).

In the current study, HO-1 positive expression was associated with varicocele in 6/26 cases (23.1%) while HO-1 negative expression was associated with it in 5/21 cases (23.8%). This denotes that testicular varicocele did not affect HO-1 induced expression inside the human

Table 2
Non obstructive azospermic (NOA) cases (n=21) with negative testicular sperm extraction (TESE) and negative HO expression.

No	Age years	Size	Vx	FSH mIU/ml	Pthology	TESE	HO-1 Testis	HO-2 Testis
1	36	M	+	13.8	SCO	-	-	+
2	36	M	+	13.8	SCO	-	-	+
3	36	M	-	6.5	C1	-	-	+
4	36	M	-	6.5	C1	-	-	+
5	25	N	-	18.6	SCO	-	-	+
6	30	N	-	14.4	SCO	-	-	+
7	26	S	-	17	SCO	-	-	+
8	40	N	-	11.6	SCO	-	-	+
9	40	N	-	12.0	SCO	-	-	+
10	40	M	-	16.5	SCO	-	-	+
11	26	M	-	12	SCO	-	-	+
12	32	M	-	19	SCO	-	-	+
13	42	M	-	18	SCO	-	-	+
14	42	M	-	18	SCO	-	-	+
15	30	M	-	15.7	SCO	-	-	+
16	30	M	-	15.7	SCO	-	-	+
17	29	S	+	23	SCO	-	-	+
18	29	S	-	23	SCO	-	-	+
19	31	N	+	17.3	SCO	-	-	+
20	31	N	+	17.3	SCO	-	-	+
21	24	M	-	13	SCO	-	-	+
Mean	32.8			15.4				
SD ±	5.7			4.3				

Vx: varicocele M: Moderate size Spd: Spermatid SD: Standard Deviation
N: Normal size C1: primary spermatocyte SCO: Sertoli Cell Only S: Small size

Table 3
Non obstructive azospermic (NOA) cases (n=7) with negative testicular sperm extraction (TESE) and positive HO expression.

No	Age years	Size	Vx	FSH mIU/ml	Pathology	TESE	HO-1 Testis	HO-2 Testis
1	35	S	-	11.9	SCO	-	+	+
2	42	S	-	13.8	Hyalinization	-	+	+
3	32	M	-	49.5	SCO	-	+	+
4	38	M	-	27.7	SCO	-	+	+
5	29	S	-	13.4	Hyalinization	-	+	+
6	40	M	-	32	SCO	-	+	+
7	27	S	-	1	Hyalinization	-	+	+
Mean	37.7							
SD ±	5.6							

testicular tissues. In addition, HO-1 expression in testicular tissues could not be attributed to hormonal treatment or smoking that elevates reactive oxygen species because these conditions were excluded beforehand. It is expected that HO-1 expression must have a role to be searched for in relation to spermatogenesis and/or steroidogenesis as the complexity of the testicular cell types and architecture mandates a variety of local control and regulatory mechanisms.

HO-1 mRNA was positive in all NOA TESE positive cases (n=19). In some cases during our work, many biopsy specimens from different sites in the same testis gave different histopathological patterns but showed the same positive expression of HO-1. This suggests that HO-1 gene represents the whole testicular tissue condition irrespective of the histopathological profile in a precise area. Skakkebaek et al.; (27) defined the "heterogeneous picture" of the testis where some tubules were involved in spermatogenesis while others are not. Giwercman and Skakkebaek (28) added that focal existence of spermato-

genesis might be missed unless more tissue slices are accessed. This could be confirmed in unsuccessful TESE negative cases showing absent HO-1 expression (n=21). In addition, there were no false negative cases. The explanation for false positive cases (n=7) could be either the actual absence of sperms, a hidden tiny spermatogenic focus in another site or due to the effect of a pathological process taking place as most of these cases demonstrated severe testicular pathology.

Actually, the reported issues about HO relation to male reproductive organs and spermatogenesis are few and most of them were carried out experimentally. Kutty and Maines (29)suggested that inducibility of HO-1 extends to the testis, supporting the possibility that HO-1 is the only inducible form of HO. Kurata et al., (30) showed that HO gene was expressed during spermatogenesis to spermatocytes. Middendorff et al., (31) found that the CO-generating enzyme, HO-1, was localized to Sertoli cells and adluminal cell compartments to demonstrate a link between HO-1 activity in Sertoli cells and soluble guanylate cyclase (sGC)-dependent cGMP production in seminiferous tubules, suggesting a functional role of CO in the human testis.

HO-1 might have different functions inside the testis affecting male reproduction. Ewing and Maines, (24) found that HO-1 activity may have a role in maintenance of the conditions required for spermatogenesis. Navarra et al., (32) found that HO activity is involved in controlling release of endocrine factors. Petrache et al., (33) demonstrated HO-1 over-expression role in prevention of programmed cell death. Durante, (34) proposed a role for HO-1 in growth regulation as it influences indirectly the delivery of oxygen or nutrients to specific target tissues. Also, HO-1 exerts important effects on tissue size, including cell proliferation, apoptosis and hypertrophy in other organs. Indeed, all these processes take place throughout the spermatogenesis process. Therefore, this work represents a pilot study about HO-1 mRNA expression relationship with testicular sperm retrieval in NOA infertile men. It may be one of the items to be used in future to foretell about the TESE probability in NOA cases, especially TESE-ve cases. Hopefully, a gate may be opened in the future to think about HO as a gene therapy in NOA cases, if any.

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