Levels of the retinoic acid synthesizing enzyme aldehyde dehydrogenase-1A2 are lower in testicular tissue from men with infertility

John K. Amory, M.D., M.P.H.,[a] Samuel Arnold, M.S.,[b] María C. Lardone, M.Sc.,[c] Antonio Piottante, M.D.,[d] Mauricio Ebensperger, M.D.,[e] Nina Isoherranen, Ph.D.,[b] Charles H. Muller, Ph.D.,[f] Thomas Walsh, M.D.,[f] and Andrea Castro, M.Sc.[c]

[a] Department of Medicine, and [b] Department of Pharmaceutics, University of Washington, Seattle, Washington; [c] Institute of Maternal and Child Research, School of Medicine, University of Chile, [d] Andrés Bello University, and [e] San Borja Arriarán Clinical Hospital, Santiago, Chile; and [f] Department of Urology, University of Washington, Seattle, Washington

Objective: To determine whether decreased testicular levels of enzymes necessary for retinoic acid biosynthesis were associated with male infertility, as retinoic acid is known to be necessary for spermatogenesis.
Design: Observational analysis of testicular tissue samples, sperm indices, and serum hormone concentrations.
Setting: Two infertility centers in Chile.
Patient(s): 32 infertile men and 11 control men.
Intervention(s): Measurement of the three enzymes necessary for retinoic acid biosynthesis, aldehyde dehydrogenase (ALDH) 1A1, 1A2, and 1A3, in testicular tissue by a novel liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) peptide assay.
Main Outcome Measure(s): ALDH isozyme levels compared by type of infertility and correlated with testicular germ cell numbers, sperm parameters, and serum and intratesticular hormone concentrations.
Result(s): Men with infertility had statistically significantly reduced levels of ALDH1A2 but not ALDH1A1 or ALDH1A3 in their testicular tissue compared with men with normal spermatogenesis. The ALDH1A2 protein levels were strongly correlated with the number of germ cells found via testicular biopsy.
Conclusion(s): These findings suggest that ALDH1A2 is the enzyme involved in retinoic acid biosynthesis in human germ cells. Further study of the relationship between intratesticular ALDH1A2 and male infertility is warranted to determine whether men with infertility have a reduced ability to synthesize retinoic acid within their germ cells that could impair spermatogenesis. (Fertil Steril® 2014;101:960–6. ©2014 by American Society for Reproductive Medicine.)
Key Words: Germ cells, retinaldehyde, retinol, Sertoli cell-only syndrome, spermatogenesis

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/amoryjk-retinol-spermatogenesis-germ-cells-sertoli/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Infertility attributable to the male partner accounts for 30% to 40% of all cases of infertility (1). The most common form of male infertility involves some type of impairment in spermatogenesis. Unfortunately, over 75% of men with infertility from impaired spermatogenesis do not have a medically treatable cause such as a gonadotropin deficiency (2). In such men, surgical sperm extraction from the testes coupled with in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) offers some hope of fertility (3). However, these procedures are invasive, expensive, and unsuccessful in some cases, and they do not address the underlying cause of infertility. Therefore, new insights into the etiology of male infertility are needed before more effective treatments can be developed.

Received November 6, 2013; revised and accepted December 30, 2013; published online February 10, 2014.
J.K.A. has nothing to disclose. S.A. has nothing to disclose. M.C.L. has nothing to disclose. A.P. has nothing to disclose. M.E. has nothing to disclose. N.I. has nothing to disclose. C.H.M. has nothing to disclose. T.W. has nothing to disclose. A.C. has nothing to disclose.
The *Eunice Kennedy Shriver* National Institute of Child Health and Human Development supported this work through cooperative agreement U54 HD42454 as part of the Cooperative Contraceptive Research Centers Program and the Chilean National Fund for Scientific and Technological Development [Grant 1120176].
Reprint requests: John K. Amory, M.D., M.P.H., Professor of Medicine, University of Washington, Box 356429, 1959 NE Pacific Street, Seattle, Washington 98195 (E-mail: jamory@u.washington.edu).

Fertility and Sterility® Vol. 101, No. 4, April 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2013.12.053

The essential role of vitamin A (retinol) in spermatogenesis has been long appreciated, as vitamin A deficiency induces sterility secondary to a cessation of spermatogenesis (4). In vitamin A–deficient rodents, the conversion of undifferentiated to differentiated spermatogonia is arrested. Spermatogenesis can be reinitiated in vitamin A–deficient animals by the administration of either vitamin A (5) or retinoic acid (6), its active metabolite. Vitamin A is converted to retinoic acid in the germ and Sertoli cells via the activity of retinol and retinal dehydrogenases (7, 8). Retinoic acid then binds retinoic acid receptors to regulate gene transcription (9). In the testes, two retinoic acid receptors, α and γ, are present in Sertoli cells and developing germ cells (10–12), and deletion of either of these receptors in mice results in male infertility (13, 14). In addition, spermatogenesis is completely and reversibly suppressed by pharmacologic inhibition of retinoic acid biosynthesis (15) or by the administration of retinoic acid receptor antagonists (16, 17), demonstrating that intratesticular retinoic acid biosynthesis and function are necessary for spermatogenesis.

Given the crucial role of retinoic acid in spermatogenesis, it seems possible that some men with "idiopathic" infertility may have intratesticular concentrations of retinoic acid below those necessary to initiate or maintain spermatogenesis. However, whether deficiencies of intratesticular retinoic acid contribute to infertility in men is unknown. In theory, poor dietary intake of vitamin A could lead to male infertility, but a nutritional cause of infertility seems highly unlikely for most men. More feasibly, a low intratesticular concentration of retinoic acid could occur in an infertile man due to impaired retinoic acid biosynthesis from vitamin A or increased catabolism of retinoic acid to inactive metabolites. Recently, our group observed that concentrations of intratesticular retinoic acid were significantly lower in men with subnormal sperm quality as compared with men with normal sperm quality (18). However, the direct measurement of testicular retinoic acid concentrations is challenging, given its low concentration in tissue, the corresponding need for large samples of testicular tissue, and the specialized collection techniques required given the photosensitivity of retinoic acid, which rapidly degrades with light exposure.

An alternative to the direct measurement of intratesticular retinoic acid is to measure the relative levels of the enzymes involved in retinoic acid biosynthesis. These proteins are more stable in frozen tissue than retinoic acid and provide insight into the capacity of the tissue to synthesize retinoic acid. The three isozymes of aldehyde dehydrogenase (ALDH) that are responsible for synthesizing retinoic acid are ALDH1A1, ALDH1A2, and ALDH1A3 (7, 19). These ALDH enzymes catalyze the final step of retinoic acid biosynthesis, from retinaldehyde to retinoic acid. Retinoic acid then induces the expression of *Stra8*, whose function is unknown, but it is essential for the differentiation of A spermatogonia to A1 spermatogonia, as *Stra8* knockout mice are infertile, exhibiting only type A spermatogonia in testicular biopsies (20). In particular, ALDH1A2, which was first cloned and characterized in testicular extracts in 1996 (21), appears to be specifically expressed in the testes, with much lower levels of expression in other tissues (19, 22). Indeed, work in mice suggests that ALDH1A2 localizes to germ cells within the seminiferous tubules, while ALDH1A1 and ALDH1A3 localize to Leydig and Sertoli cells (23).

Therefore, to better understand the synthesis of retinoic acid in the human testes in fertile and infertile men, we used a novel liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) peptide assay to measure the level of ALDH1A1, 1A2, and 1A3 in testicular tissue from a well-characterized cohort of men with infertility and controls (24). This cohort had frozen testicular tissue available as well as extensive information regarding testicular germ cell numbers and serum and intratesticular hormones concentrations, which allowed for correlation between these measurements and the levels of ALDH isozymes in the testes. As ALDH1A2 seemed most likely to be responsible for retinoic acid biosynthesis in developing germ cells, we hypothesized that testicular tissue from infertile men would have reduced levels of ALDH1A2 when compared with testicular tissue from men with normal spermatogenesis.

MATERIALS AND METHODS

Patients

A subset of 43 men, from our original cohort (24), in whom testicular tissue was still available for measurement of ALDH1A1, 1A2, and 1A3 were included in this study. The inclusion criteria included azoospermia or oligozoospermia and an indication for testicular biopsy for sperm retrieval or diagnosis. The original workup and biopsies were performed at the Institute of Maternal and Child Research and at the Jose Joaquin Aguirre Hospital of the University of Chile during a 12-year period between 1999 and 2011. All infertile men underwent a complete physical examination, karyotype analysis, and evaluation for Y-chromosome microdeletions. Men with abnormal karyotypes, hypogonadotropic hypogonadism, or chronic disease, or those undergoing hormone treatments were not included. The study was approved by the institutional review boards of both hospitals in Chile where the biopsy samples were obtained, and the samples were anonymized before analysis of the ALDH protein levels at the University of Washington, which also approved this study.

Measurements

The seminal fluid analyses and hormone measurements were performed as previously described elsewhere (24). Histologic analysis of testicular biopsy material was performed on a small piece of tissue fixed in Bouin's solution and stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Masson's (trichrome), or orcein staining. Each biopsy was classified into a definite histopathologic type (normal, Sertoli cell-only, maturation arrest, or hypospermatogenesis) as previously described elsewhere (24). Quantitation of germ cell numbers was performed on 20 seminiferous tubules, allowing for enumeration of the number of a given type of germ cell per tubule cross-section (25, 26).

The relative expression of ALDH1A1, 1A2, and 1A3 in testicular tissue was measured by LC-MS/MS using an AB-Sciex 5500 qTrap Q-LIT mass spectrometer coupled to Agilent

1290 ultra-high performance liquid chromatography. To identify unique peptides for each ALDH isoform, ALDH1A1, ALDH1A2, and ALDH1A3 were trypsin digested in silico using Skyline software. All of the resulting peptides were screened against peptides from the human proteome downloaded from uniprot.org using Skyline, and the peptides used for relative quantification of each ALDH1a protein were chosen based on their predicted selectivity and solubility. Selectivity was determined using the SRM Collider to decrease redundancy with peptides that share similar fragmentation and retention characteristics. The peptides with reported sites of posttranslational modifications or mutations were excluded (27). For each peptide, two fragments were required for quantification.

For the ALDH1A protein analysis, testicular tissues were homogenized using a Precellys 24-bead homogenizer and were kept cold with liquid nitrogen using an advanced temperature controller (Cryolys; Bertin Technologies). Each tissue sample was homogenized in 500 μL 0.1% NaCl using 2-mL ceramic bead tubes (Omni International) and was centrifuged at 10,000 $\times$ *g* for 60 minutes to pellet the cellular membranes, nuclei, and organelles. The supernatant was collected and used for ALDH1a analysis. The samples of testicular supernatant were prepared for LC-MS/MS analysis as follows: 4 μL dithiothreitol (100 mM) and 10 μL of ammonium bicarbonate buffer (100 mM, pH 7.8) were added to 20 μL of sample at total protein concentration of 2 mg/ mL followed by 10 μL of 0.4% Rapigest (Waters). The samples were incubated at 56°C for 30 minutes and cooled to room temperature, and 4 μL of iodoacetamide (200 mM) was added. The samples were then incubated at room temperature in the dark for 20 minutes, trypsin was added at a 1:25 trypsin/protein ratio, and the proteins were digested at 37°C for 24 hours. The digestion was quenched by the addition of 30 μL of chilled 50:50 acetonitrile/H_2O containing 0.1% formic acid and a peptide labeled at the N terminal lysine or arginine with C^{13} and N^{15} for ALDH1A1, ALDH1A2, and ALDH1A3 as internal standards. The samples were centrifuged at 3,000 $\times$ *g* for 25 minutes at 4°C before analysis.

The peptides were separated using a Supelco express C18 column (150 $\times$ 2.1 mm) with a 5 μM particle size at 40°C using a 16-minute gradient between H_2O + 0.1% formic acid and acrylonitrile + 0.1% formic acid. The peptides were quantified using the peak area of the main MS/MS fragment of the analyte peptide and the ratio between the target peptide, and the heavy labeled internal standard was calculated. The mean expression level of each ALDH1a enzyme in the control samples was calculated, and the fold difference in the ALDH1A expression was determined from the peak area ratios of the individuals in each group. The relative expression levels in each group are reported as the fold difference in comparison with the group of men with normal spermatogenesis.

Statistical Analysis

Due to non-normal distributions, the relative protein levels of ALDH1A1, 1A2, and 1A3 in the testicular tissue are presented as medians and interquartile ranges. For comparison of ALDH isozymes levels between men with proven fertility and those with Sertoli cell-only, maturation arrest, and hypospermatogenesis, a Kruskal-Wallis (nonparametric) analysis of variance (ANOVA) with a Wilcoxon rank-sum post hoc test was used. Correlations between ALDH isozymes level and germ cell numbers were performed using Spearman's technique. Linear regression was used to examine the relationship between ALDH levels, germ cell number, intratesticular and serum hormone levels, serum gonadotropin levels, and age for hypothesis generation. Statistical analyses were performed using STATA software (StataCorp). For all comparisons, an alpha of .05 was considered statistically significant.

RESULTS

Patient Characteristics and Hormone Concentrations

There was sufficient testicular tissue for measurement of ALDH isozymes levels on 43 men of the original cohort who also had undergone quantitation of testicular germ cell numbers as well as serum and intratesticular hormone measurements (Table 1). Of these 43 men, there were 11 normal men, 9 of whom had obstructive azoospermia as their indication for testicular biopsy, 20 men with Sertoli cell-only syndrome, 8 men with maturation arrest, and 4 men with hypospermatogenesis. There were no statistically significant differences between the groups in terms of age, but a statistically significant difference in ejaculate volume was noted, with the normal men being statistically significantly reduced compared with the other groups, which was consistent with the fact that most of these men had obstruction as the cause of their infertility.

The germ cell populations between the groups were markedly different, with the Sertoli cell-only group being statistically significantly different from all the other groups because of their lack of germ cells. The maturation arrest group had statistically significantly larger numbers of spermatogonia and spermatocytes compared with the other groups (see Table 1). The serum and intratesticular hormone data are presented in Table 2. The normal group had a statistically significantly greater level of serum testosterone, whereas the Sertoli cell-only group had a statistically significantly greater level of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH). There were no other statistically significant differences between the groups in terms of serum or intratesticular hormones.

ALDH Protein Levels

The protein levels in the men stratified by subtype of infertility are shown in Figure 1. There were no statistically significant differences in ALDH1A1 or ALDH1A3 protein levels between the groups. However, there were statistically significant relative reductions in ALDH1A2 protein level observed in both the Sertoli cell-only group [0.53 (0.42, 0.67) units, P=.0001] and the hypospermatogenesis group [0.64 (0.42, 0.87) units, P=.03], compared with the control value 1.0 (0.95, 1.3) units, corresponding to a 36% to 47% reduction in this protein in the testes of the infertile men.

There were no statistically significant differences in ALDH1A2 protein level between the normal men and the

TABLE 1

Demographics, ejaculated sperm characteristics, and testicular germ cell populations by testicular histology type of the 43 men included in the analysis.

Characteristic	Testicular histology: Normal (n = 11)[a]	Sertoli-cell only (n = 20)	Maturation arrest (n = 8)	Hypospermatogenesis (n = 4)	*P* value
Age (y)	30 (29, 37)	35 (32, 39)	31 (29, 33)	35 (31, 37)	.32
Sperm characteristics					
Semen volume (mL)	1.8 (0.5, 2.7)[b]	3 (2.5, 3.0)	4 (3.8, 4.8)	2.5 (0.7, 6)	.01
Sperm concentration (10^6/mL ejaculate)	0 (0, 0.2)	0 (0, 0)	0 (0, 0.01)	0 (0, 0)	.25
Total sperm count (10^6/ejaculate)	0 (0, 0.3)	0 (0, 0)	0 (0, 0.04)	0 (0, 0)	.15
Germ cell populations (per tubule cross-section)					
Spermatogonia	11 (9, 14)	0 (0, 0)[b]	16 (10, 18)[c]	6.8 (6.7, 7.0)	.001
Spermatocytes	14 (13, 15)	0 (0, 0)[b]	19 (14, 29)[c]	5.2 (4.9, 8.6)	.001
Spermatids (type a+ type b)	9 (5, 13)[c]	0 (0, 0)[b]	1.5 (0.3, 2.5)	3.1 (0.8, 4.4)	.001
Spermatids (type c+ type d)	8 (7, 9)[c]	0 (0, 0)[b]	0 (0, 0.9)	0.8 (0.7, 1.6)	.001

Note: All values are expressed as median (25th, 75th percentiles).
[a] Nine of these men had obstructive azoospermia.
[b] *P*< .01 compared with other groups.
[c] *P*< .05 compared with other groups.

Amory. Testicular ALDH1A2 in infertile men. Fertil Steril 2014.

men with maturation arrest, or among the subtypes of infertility. When all 32 infertile men were grouped together, the ALDH1A2 protein levels in the infertile men were statistically significantly reduced by 36% compared with those in the 11 fertile men [normal 1.0 (0.95, 1.3) units vs. all infertile men 0.64 (0.46, 0.73) units, *P*=.0004].

The ALDH1A2 protein levels were highly significantly correlated with the number of each type of germ cell seen in the testicular biopsy samples (Fig. 2A–2C). This correlation was similar for spermatogonia and spermatocytes, and was strongest between spermatids and ALDH1A2 protein levels (see Fig. 2C). When the men with Sertoli cell-only were excluded in a sensitivity analysis, the correlation between germ cell number and ALDH1A2 was no longer statistically significant between ALDH1A2 and spermatogonia and spermatocytes, but remained statistically significant between ALDH1A2 and both early ($r = 0.52$, *P*=.02) and advanced spermatids ($r = 0.64$, *P*=.0003). Testicular protein levels of ALDH1A1 and ALDH1A3 were not statistically significantly correlated with germ cell numbers (not shown).

Predictors of ALDH1A2 Protein Level

As ALDH1A2 was the ALDH isozymes most strongly associated with an increased number of germ cells on testicular histology, we sought to determine whether any demographic or hormone factors appeared to correlate with testicular ALDH1A2 protein levels. Of the examined factors, only serum LH and FSH were statistically significantly correlated with testicular ALDH1A2 protein levels in linear regression analysis [FSH: −0.02 (95% CI, −0.03 to −0.01); *P*=.01; and LH: −0.05 (95% CI, −0.08 to −0.02); *P*=.007], suggesting that higher gonadotropins were associated with lower levels of testicular ALDH1A2 in the group as a whole. When the men with Sertoli cell-only syndrome were excluded from this analysis, the associations between FSH and LH and testicular ALDH1A2 protein level were no longer statistically significant.

DISCUSSION

Despite the demonstrated importance of intratesticular retinoic acid for spermatogenesis in experimental animals, there

TABLE 2

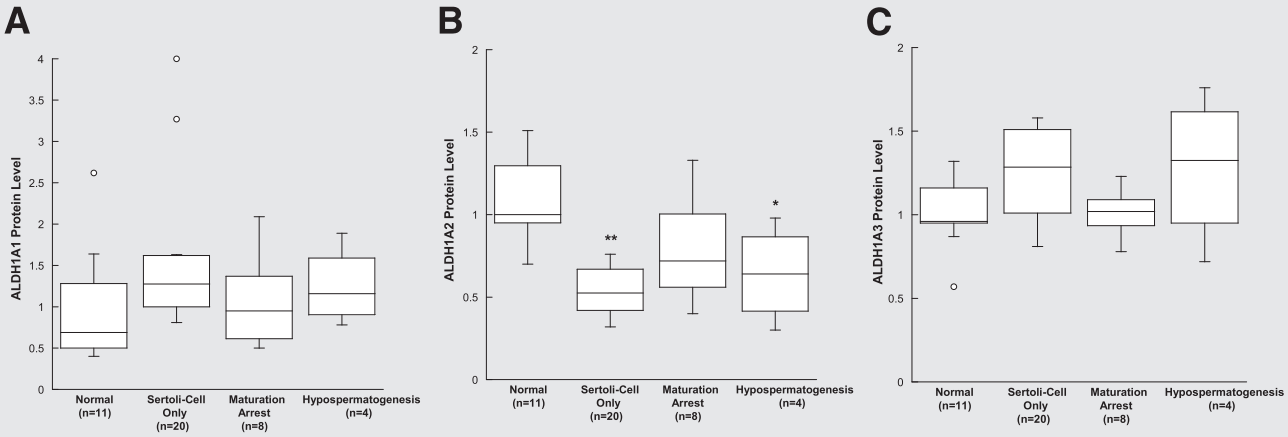
Serum and intratesticular hormones by testicular histology type of the 43 men included in the analysis.

Hormone	Testicular histology: Normal (n = 11)	Sertoli-cell only (n = 20)	Maturation arrest (n = 8)	Hypospermatogenesis (n = 4)	*P* value
Testosterone (ng/mL)	4.5 (2.4, 4.9)[a]	2.5 (2.0, 3.2)	2.8 (2.6, 3.6)	2.3 (2.2, 3.0)	.03
FSH (IU/L)	3.8 (2.9, 4.7)	18 (13.5, 24)[b]	6.7 (3.6, 13)	2.9 (2.0, 6.6)	.001
LH (IU/L)	2.2 (1.3, 3.3)	6 (4.3, 8.3)[b]	4.1 (2.5, 5.9)	4.6 (2.7, 4.9)	.005
SHBG (nmol/L)	24 (14, 45)	26 (19, 31)	25 (18, 31)	22 (12, 23)	.76
Estradiol (pg/mL)	47 (42, 53)	33 (28, 40)	39 (29, 51)	42 (19, 61)	.31
Intratesticular testosterone (ng/mg protein)	24 (13, 63)	41 (25, 88)	35 (19, 50)	18 (13, 24)	.30
Intratesticular estradiol (ng/mg protein)	167 (103, 269)	136 (67, 359)	214 (158, 270)	81 (73, 110)	.39

Note: All values are expressed as median (25th, 75th percentiles). FSH = follicle-stimulating hormone; LH = luteinizing hormone; SHBG = sex hormone-binding hormone.
[a] *P*< .05 compared with other groups.
[b] *P*< .01 compared with other groups.

Amory. Testicular ALDH1A2 in infertile men. Fertil Steril 2014.

FIGURE 1



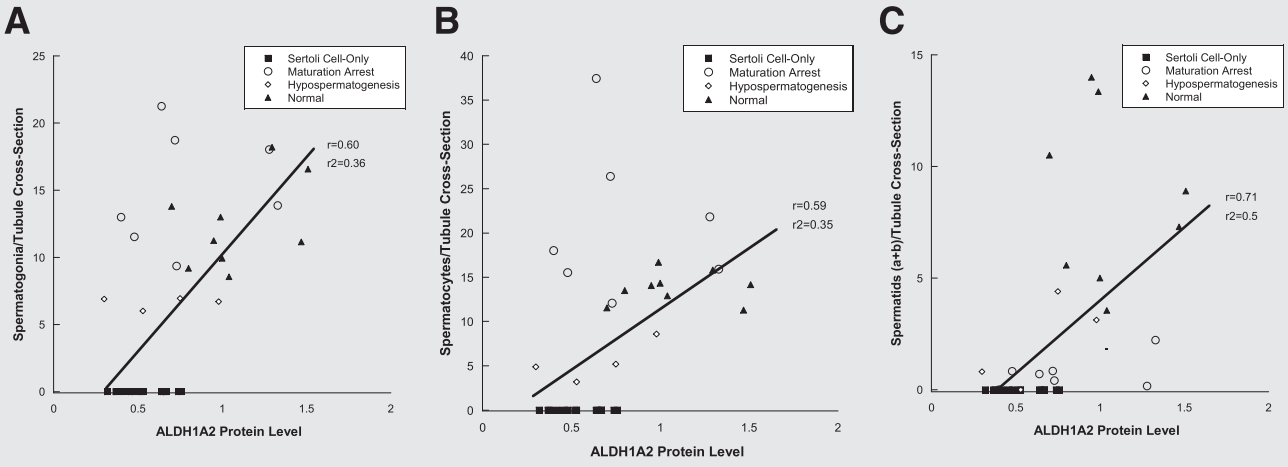
Box and whiskers plots of (A) ALDH1A1, (B) ALDH1A2, and (C) ALDH1A3 from testicular tissue of the men by histologic subtype. Each box represents the median, 25th and 75th percentiles, with the upper and lower values represented by the bars and outliers represented as open circles. The values are expressed relative to the mean of the group of normal men. Therefore, amounts of protein are comparable between the groups of subjects within an assay but not between proteins. $^{**}P<.001$ compared with normal men; $^{*}P<.05$ compared with normal men.

Amory. Testicular ALDH1A2 in infertile men. Fertil Steril 2014.

has been little work investigating the role of intratesticular retinoic acid in human spermatogenesis. Our study, for the first time, demonstrates in a cohort of men with infertility a statistically significant association between the protein levels of one of the three enzymes known to synthesize retinoic acid and germ cell numbers. Using a novel mass spectrometric peptide assay, we found strong associations between ALDH1A2 protein level and populations of germ cells in testicular tissue. In particular, men with Sertoli cell-only syndrome had statistically significantly reduced levels of ALDH1A2 compared with men with normal spermatogenesis. Men with hypospermatogenesis also appear to have reduced testicular levels of ALDH1A2, but this reduction was not as great as that seen in Sertoli cell-only syndrome, possibly due to the presence of some germ cells in the men with hypospermatogenesis. Notably, protein concentrations of ALDH1A1 and ALDH1A3 were not different between normal men and men with infertility and did not correlate with germ cell populations from the testicular biopsy samples.

These findings suggest that the reduction of germ cells observed in the testicular tissue from the infertile men is driving the observed difference in ALDH1A2 protein levels between the groups. From these observations, it seems likely that ALDH1A2 is the major isozyme of ALDH involved in retinoic acid biosynthesis within germ cells in the human testes. This conclusion is consistent with the data from mice, which

FIGURE 2



Scatter plots of (A) spermatogonia, (B) spermatocytes, and (C) early spermatids versus the ALDH1A2 protein level from testicular tissue of the 43 study participants. Correlations were performed using Spearman's technique.

Amory. Testicular ALDH1A2 in infertile men. Fertil Steril 2014.

localizes ALDH1A2 to murine germ cells using immunohistochemical staining (23).

It is interesting that the relationship between ALDH1A2 protein level and germ cells was stronger for the more advanced germ cell types. Indeed, the correlation between ALDH1A2 protein level and spermatids was the only one to persist when the men with Sertoli cell-only syndrome were omitted from the analysis. This suggests a role for retinoic acid in spermiogenesis in humans, in addition to its likely role in spermatogonial differentiation. Such a role has been demonstrated in mice in elegant studies performed by Chung et al. (11, 12). In one such study, germ cell stem cells from mice deficient for retinoic acid receptor-α were transplanted into germ-cell depleted wild-type testes. Although the transplanted germ cells were able to initiate spermatogenesis, probably via retinoic acid receptor-γ signaling, they failed to develop proper cellular associations during spermiogenesis, and abnormal sperm formation was observed, demonstrating the need for retinoic acid signaling during spermiogenesis.

The absence of a correlation between serum and intratesticular hormones suggests that ALDH1A2 expression and function are not regulated by serum or testicular hormones. Although some investigators have suggested an interrelationship between androgens and retinoids (28, 29), the factors controlling testicular retinoic acid biosynthesis are not well understood. It is known that the effect of retinoic acid appears to be mediated in part by the induction of the 45 kD protein STRA8 (stimulated by retinoic acid-8) within the developing germ cells (20), but the molecular function of this protein remains to be elucidated. The signals regulating retinoic acid biosynthesis in humans will be the focus of future work.

It is unknown whether deficiencies in retinoic acid production or function are a cause of human infertility. Recently, a case-control study identified polymorphisms in retinoic acid pathways that were associated with male infertility. It is interesting that a polymorphism that decreased STRA8 function significantly increased the risk of infertility by a factor of twofold. In contrast, a polymorphism that decreased the activity of Cyp26, the enzyme that catabolizes retinoic acid to inactive metabolites (thereby increasing intratesticular retinoic acid), decreased the risk of infertility by 50% (30). Clearly, further study examining other associations between polymorphisms and mutations in proteins in the retinoic acid pathway and infertility is warranted.

Our study has several important limitations. First, we cannot determine whether the reduced protein levels of ALDH1A2 caused the reduction in germ cell number or whether reduced germ cell numbers lead to reduced ALDH1A2 protein levels, although the latter possibility may be more likely. Second, our sample size was only moderate. Ideally, larger studies examining the ALDH concentrations in infertile men can be performed in the future to determine the strength of this association. However, obtaining sufficient testicular tissue for this type of analysis is extremely challenging. The specimens used in this study were obtained over a 12-year period, and they represent a unique and valuable cohort for this type of analysis, given the extensive information available on germ cell population number and serum and intratesticular hormone concentrations. Another limitation of our study is that the control samples of testicular tissue came from men with obstructive azoospermia. Chronic obstruction can lead to vacuolations of Sertoli cells and may have affected the results of our study (31). Ultimately, as an observational study, the significance of our findings is unknown. Additional study of the molecular mechanisms underlying testicular ALDH expression and retinoic acid synthesis will be required before the role of intratesticular retinoic acid in human infertility is understood.

In conclusion, for the first time, we have measured the levels of ALDH1A2 protein in normal human testicular tissue and tissue from men with infertility. We have demonstrated that the ALDH1A2 protein level is significantly reduced in testicular tissue from infertile men and is strongly correlated with testicular germ cell populations but not with serum or intratesticular hormone concentrations. Significantly, testicular levels of ALDH1A1 and ALDH1A3 did not differ in the infertile men and were not associated with germ cell populations in testicular tissue. Taken together, these findings suggest that ALDH1A2 is the most important ALDH isozyme for retinoic acid biosynthesis in human germ cells. Given the importance of retinoic acid in spermatogenesis in animal models, further study of intratesticular retinoic acid biosynthesis and ALDH1A2 levels in human spermatogenesis and male infertility is warranted.

Acknowledgment: The authors thank the study participants and Dr. William J. Bremner, Dr. Michael Griswold, and Dr. David W. Amory Sr. for critical review of the manuscript.

REFERENCES

1. deKretser DM. Male infertility. Lancet 1997;349:787–90.
2. Anawalt BD. Approach to male infertility and induction of spermatogenesis. J Clin Endocrinol Metab 2013;98:3532–42.
3. Schlegel PN. Nonobstructive azoospermia: a revolutionary surgical approach and results. Semin Reprod Med 2009;27:165–70.
4. Wolbach SB, Howe PR. Tissue changes following deprivation of fat soluble A Vitamin. J Exp Med 1925;42:753–7.
5. Van Pelt AMM, de Rooij DG. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A deficient mice. Biol Reprod 1990; 43:363–7.
6. van Pelt AMM, de Rooij DG. Retinoic acid is able to reinitiate spermatogenesis in vitamin A deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology 1991;128:697–704.
7. Napoli J. Retinoic acid: its biosynthesis and metabolism. In: Moldave K, editor. Progress in nucleic acid research and molecular biology, vol. 63. San Diego: Academic Press; 2000:139–88.
8. Koubova J, Menke D, Zhou Q, Capel B, Griswold MD, Page DC. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci USA 2006;103:2472–9.
9. Dufour JM, Kim KH. Cellular and subcellular localization of six retinoid receptors in rat testis during postnatal development: identification of potential heterodimeric receptors. Biol Reprod 1999;61:1300–8.
10. Doyle TJ, Braun KW, McLean DJ, Wright RW, Griswold MD, Kim KH. Potential functions of retinoic acid receptor α in Sertoli cells and germ cells during spermatogenesis. Ann NY Acad Sci 2007;1120:114–30.
11. Chung SS, Sung W, Wang X, Wolgemuth DJ. Retinoic acid receptor alpha is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. Dev Dyn 2004; 230:754–66.
12. Chung SS, Wang X, Wolgemuth DJ. Expression of retinoic acid receptor alpha in the germline is essential for proper cellular association and spermiogenesis during spermatogenesis. Development 2009;136:2091–100.

13. Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor gamma in the mouse. Cell 1993;73:643–58.
14. Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, et al. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc Natl Acad Sci USA 1993;90:7225–9.
15. Amory JK, Muller CH, Shimshoni AJ, Isoherranen N, Paik J, Moreb JS, et al. Suppression of spermatogenesis by bisdichloroacetyldiamines is mediated by inhibition of testicular retinoic acid biosynthesis. J Androl 2011;32:111–9.
16. Schulze GE, Clay RJ, Mezza LE, Bregman CL, Buroker RA, Frantz JD. BMS-189453, a novel retinoid receptor antagonist, is a potent testicular toxin. Toxicol Sci 2001;59:297–308.
17. Chung SS, Wang X, Roberts SS, Griffey SM, Reczek PR, Wolgemuth DJ. Oral administration of a retinoic acid receptor antagonist reversibly inhibits spermatogenesis in mice. Endocrinology 2011;152:2492–502.
18. Nya-Ngatchou JJ, Arnold SLM, Walsh TJ, Muller CH, Page ST, Isoherranen N, et al. Intratesticular 13-cis retinoic acid is reduced in men with abnormal semen analyses. Andrology 2013;1:325–31.
19. Alnouti Y, Klaassen CD. Tissue distribution, ontogeny and regulation of aldehyde dehydrogenase (ALDH) enzymes mRNA by prototypical microsomal enzyme inducers in mice. Toxicol Sci 2008;101:51–64.
20. Anderson EL, Baltus AE, Roepers-Gajadein HL, Hassold TJ, de Rooij DG, van Pelt AMM, et al. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci USA 2008;105:14976–80.
21. Wang X, Penzes P, Napoli J. Cloning of a cDNA encoding an aldehyde dehydrogenase and its expression in Escherichia coli. J Biol Chem 1996;27:16288–93.
22. Hsu LH, Chang W-C, Yoshida A. Mouse type-2 retinaldehyde dehydrogenase (RALDH2): genomic organization, tissue-dependent expression, chromosome assignment and comparison to other types. Biochem Biophys Acta 2000;1492:289–93.
23. Vernet N, Dennefeld C, Rochett-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, et al. Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. Endocrinology 2006;147:96–110.
24. Lardone MC, Piottante A, Valdevenito R, Ebensperger M, Castro A. Histological and hormonal testicular function in azo/oligozoospermic infertile men. Published online September 26 . Andrologia 2012.
25. Johnsen SG. Testicular biopsy score count-a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones 1970;1:2–25.
26. Jezek D, Knuth UA, Schulze W. Successful testicular sperm extraction (TESE) in spite of high serum follicle stimulation hormone and azoospermia: correlation between testicular morphology, TESE results, semen analysis and serum hormone values in 103 infertile men. Hum Reprod 1998;13:1230–4.
27. Röst H, Malmström L, Aebersold R. A computational tool to detect and avoid redundancy in selected reaction monitoring. Mol Cell Proteomics 2012;11:540–9.
28. Chaudhary LR, Hutson JC, Stocco DM. Effect of retinol and retinoic acid on testosterone production by rat Leydig cells in primary culture. Biochem Biophys Res Comm 1989;158:400–6.
29. Appling DR, Chytil F. Evidence of a role for retinoic acid (vitamin A-acid) in the maintenance of testosterone production in male rats. Endocrinology 1981;108:2120–3.
30. Lu C, Xu M, Wang Y, Qin Y, Du G, Wu W, et al. Genetic variants in meiotic program initiation pathway genes are associated with spermatogenic impairment in Han Chinese population. PloS One 2013;8:e52443.
31. Levin HS. Testicular biopsy in the study of male infertility: its current usefulness, histological techniques, and prospects for the future. Hum Pathol 1979;10:569–84.