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LETTER TO THE EDITOR

Male Infertility

Simultaneous expression analysis of deleted in azoospermia-family genes and *CDC25A*: their potential as a predictor for successful testicular sperm extraction

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Dear Editor,

Infertility is a major health problem affecting 10%–15% of couples seeking to have children, and a male factor can be identified in about half of these cases.[1] Nonobstructive azoospermia is one of the causes of male infertility (10%), resulting from testicular failure.[2] The most common histological patterns in these patients are hypospermatogenesis (HS), maturation arrest (MA), and Sertoli cell-only syndrome (SCO).[3]

The search for sensitive and specific markers of spermatogenesis that could better predict the sperm retrieval rates in patients with nonobstructive azoospermia can lead to improved management of male infertility. *DAZ* (Deleted in Azoospermia) gene family has been extensively studied because the microdeletions containing *DAZ* genes in the Y chromosome are associated with a variety of testicular failures and impaired spermatogenesis.[4–6] *DAZ* gene family consists of two autosomal genes, *BOULE* and *DAZ-L* (*DAZ-like*), and the *DAZ* gene cluster on chromosome Y. These genetic factors encode for RNA-binding proteins that are mainly expressed in germ cells and are considered essential for male fertility.[5] Recently, the members of the cell cycle regulators *CDC25* family were recognized as potential substrates for DAZ family proteins. Particularly, *CDC25A* is abundantly expressed in the testis and functions in the G1-S transition and M-phase exit, suggesting a role in mitotic or meiotic regulation of spermatogenesis.[7–9] The analysis of single *DAZ* gene has shown that its dysfunction leads to abnormal spermatogenesis and may cause infertility. They were, however, poorly associated to sperm recovery during assisted reproductive treatments. Whether the simultaneous expression of the members of the *DAZ* gene family and its substrates may provide better information of testicular damage and success of sperm recovery in infertile patients has not been yet analyzed.

We evaluated eight men (29–38-year-old) with idiopathic infertility and nonobstructive azoospermia diagnosed by open testicular biopsy. None of these patients showed genitourinary infections, varicocele, hypogonadotropic hypogonadism, chromosome abnormalities, and obstruction or agenesia of the seminal ducts. The study was designed in accordance with the Helsinki Declaration and its last modification (Tokyo 2004) on human experimentation, and it was approved by the Ethics Committees from Universidad Maimónides and the Centro de Estudios en Genética y Reproducción. Informed Consent was obtained from all patients. The diagnosis of azoospermia was established on the basis of the independent analysis of, at least, two semen samples collected 1 week apart. The serum concentrations of FSH (normal range: 1.5–7 mIU ml^{-1}), LH (normal range: 1.1–9 mIU ml^{-1}), and testosterone (normal range: 10–30 pmol ml^{-1}) were measured and fell into the normal range in all patients. Azoospermic patients underwent a diagnostic testicular biopsy and sperm retrieval (TESE) by microsurgery and agreed to provide a small piece of testicular tissue (5 mm in diameter) for research purposes. The testicular histopathology was categorized according to the most advanced degree of spermatogenesis, and the biopsies were classified either as HS ($n = 5$) or MA ($n = 3$), according to McLachlan *et al*. (2007).[10] DAB (3,3'-diaminobenzidine) immunohistochemistry was performed to localize DAZ family proteins and CDC25A in each biopsy. Negative controls were processed simultaneously by omitting the primary antibody or preabsorbing the primary antibody with specific synthetic peptides. Relative quantitation of gene expression by Real-time PCR of the *DAZ* gene family and *CDC25A* was calculated using standard curves and normalized to actin in each sample.

Immunohistochemical analysis showed that in all biopsies with HS and MA, expression of DAZ was mainly detectable in the cytoplasm of spermatogonia clusters, near the basal lamina of the seminiferous tubules (**Figure 1a**), and DAZL was detectable in the cytoplasm of some spermatogonia and spermatocytes (**Figure 1b**). BOULE and its downstream substrate CDC25A shared a similar pattern of expression in germ cell cytoplasm (**Figure 1c** and **1d**). Interestingly, in one biopsy diagnosed with MA, no immunoexpression of both proteins was detected, but we were able to detect *BOULE* mRNA in this particular biopsy, suggesting that the translation of *BOULE* might be regulated by another RNA-binding protein.

We did not found an association between the expression levels of the *DAZ* family genes analyzed by real-time PCR with the diagnosis of

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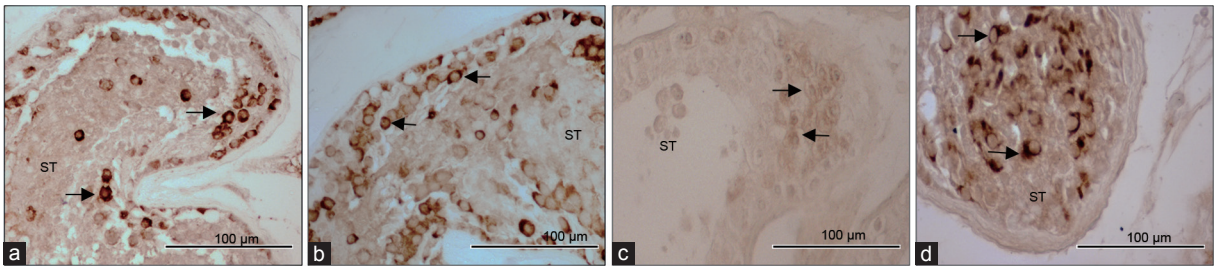

Figure 1: Immunolocalization of DAZ (**a**), DAZL (**b**), BOULE (**c**), and the cell-cycle regulator CDC25A (**d**) in pathological testicular biopsies from patients with hypospermatogenesis (**a** and **b**) and maturation arrest (**c** and **d**). Note, DAZ cytoplasmic staining in the spermatogonia clusters (**a**) and DAZL staining in some spermatogonia and spermatocytes (**b**). BOULE was detected in the cytoplasm of the germ cells (**c**) in all biopsies and paralleled CDC25A immunostaining pattern (**d**). The arrows indicate immunopositive cells. ST: seminiferous tubules.

Table 1: Results of TESE and mRNA expression ratios of *DAZ* gene family and *CDC25A* of the infertile patients

Sperm retrieval group	*n*	*DAZ mRNA ratio*	*BOULE mRNA ratio*	*DAZ-L mRNA ratio*	*CDC25A RNA ratio*
Success	5 (HS=3, MA=2)	1.76±0.08[a]	12.43±6.09[a]	0.74±0.25	1.06±0.30[a]
Failure	3 (HS=2, MA=1)	0.01±0.006[b]	0.37±0.10[b]	0.03±0.02	0.01±0.001[b]

Values represent the mean±s.e.m. [a,b]Significant differences between groups (Student's *t*-test, $P<0.05$). HS: hypospermatogenesis; MA: maturation arrest; s.e.m.: standard error of mean; TESE: testicular sperm extraction; *DAZ*: deleted in azoospermia

the testicular pathology. No statistically significant differences between HS and MA pathologies were found when the *DAZ* family genes were analyzed individually; however, a positive correlation between the mRNA transcript ratios of *DAZ* gene family members and *CDC25A* was detected, irrespective of the testicular pathology. We found a statistically significant positive correlation for mRNA transcript ratios between *DAZ* and *DAZ-L* ($r = 0.9131$ Pearson; $P = 0.0002$), *DAZ-L* and *BOULE* ($r = 0.8163$ Pearson; $P = 0.0021$), and *DAZ* and *BOULE* ($r = 0.8484$ Pearson; $P = 0.0078$). We also observed a positive correlation in the expression of *DAZ-L* and *BOULE* with their testicular target *CDC25A* (*DAZ-L/CDC25A* $r = 0.8990$ Pearson; $P = 0.0024$; *BOULE/CDC25A* $r = 0.9120$ Pearson; $P = 0.0016$).

Finally, the infertile patients with testicular failure were divided into two groups according to the presence (success) or absence (failure) of sperm retrieval during TESE (**Table 1**). We found that irrespective of the testicular pathology, patients with success in sperm retrieval showed a statistically significant higher expression of *DAZ*, *BOULE*, and *CDC25A*, compared to patients with failure in sperm retrieval. These results suggest that *DAZ* family genes would be collectively rather than individually altered in patients with HS and MA. Since TESE is considered an invasive procedure with only 40%–60% success, the identification of molecular markers that can predict the presence of mature spermatozoa becomes a useful clinical tool. Although *BOULE* but its own shows conspicuous expression, our preliminary results pinpoint that the analysis of the expression level of *BOULE* together with *DAZ*, *DAZ-L*, and their molecular target *CDC25A* could be used as a confident measure of the testicular damage and a more certain predictor of successful recovery of spermatozoa by TESE in pathological testicular biopsies.

AUTHOR CONTRIBUTIONS

CRG performed the experiments, prepared the figures and tables, and contributed to writing the manuscript; CAS, FN, and SP contributed to interpreting the data and preparation of the manuscript; and ADV contributed to the study design, analysis, and interpretation of the results, and writing and final editing of the manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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