ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

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Keywords:
androgen receptor, azoospermia, Sertoli cell

Received: 13-Mar-2014
Revised: 3-May-2014
Accepted: 12-May-2014

doi: 10.1111/j.2047-2927.2014.00240.x

Expression of testicular androgen receptor in non-obstructive azoospermia and its change after hormonal therapy

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SUMMARY

Several trials aimed at improving the sperm retrieval from men with non-obstructive azoospermia (NOA) by optimizing intratesticular testosterone (ITT) have reported partial responses, however, an appropriate level of ITT has not been identified. In this study, we examined the expression of the testicular androgen receptor (AR) in NOA and investigated its correlation with clinical and pathological parameters. Expression of the testicular AR was investigated in 52 men with NOA and 22 men with obstructive azoospermia (OA). Twenty-two patients for whom sperm retrieval failed during microdissection testicular sperm extraction (micro-TESE) were enrolled in hormonal therapy using hCG with or without recombinant human follicle stimulating hormone (rhFSH) prior to a second micro-TESE. Sertoli cells were identified by vimentin immunostaining, and positivity in Sertoli cells was used as the AR index. AR immunostaining was robust in the nuclei of Sertoli cells [Sertoli cell androgen receptor (SCAR)] in both OA and NOA. The mean AR index in NOA was significantly higher than that in OA ($p < 0.05$). In NOA patients, there was no correlation between the AR index and the clinical parameters, whereas the AR index of early maturation arrest (MA) was significantly lower than that of Sertoli cell only, late MA and hypospermatogenesis ($p < 0.05$). A significant increase in the AR index after salvage hormonal therapy was shown, particularly when using rhFSH. The AR index in patients from whom spermatozoa could be retrieved at the second micro-TESE increased significantly after hormonal therapy. In human testes, the expression of AR is dominant in Sertoli cells, and the expression of SCAR is upregulated by FSH. Germ cell maturation, especially during spermatogonia to spermatocyte stage, has been shown to be SCAR-dependent. Taken together, the results indicate that SCAR elevation is closely associated with sperm retrieval after hormonal therapy and that FSH-based hormonal therapy is potentially effective in NOA men with MA.

INTRODUCTION

Non-obstructive azoospermia (NOA) is a failure in primary spermatogenesis characterized by a lack of spermatozoa in the ejaculate, and microdissection testicular sperm extraction (micro-TESE) is a suitable surgical option to for retrieving spermatozoa for intracytoplasmic sperm injection. However, most causes of NOA remain unknown. Testosterone is one of the most essential factors for spermatogenesis (Quigley *et al.*, 1995; McLachlan *et al.*, 2002); however, the mechanism by which testosterone regulates the spermatogenic process is not fully understood. Testosterone levels are more than 100-fold greater in the testes as compared than in the serum (Jarow *et al.*, 2001), and intratesticular testosterone (ITT) acts via a paracrine mechanism on androgen receptors (ARs) expressed on target cells; however, the appropriate ITT concentration for spermatogenesis using intratesticular fluid obtained during micro-TESE remains unknown (Shinjo *et al.*, 2013).

The AR is a member of a large family of ligand-activated nuclear receptors and is highly expressed in the testes (Takeda *et al.*, 1989; Ruizeveld de Winter *et al.*, 1991; Kimura *et al.*, 1993; Iwamura *et al.*, 1994; Van Roijen *et al.*, 1995; Guillaume *et al.*, 2001), particularly in Sertoli cells (Suarez-Quian *et al.*, 1999). Sertoli cells are thought to be the major cellular target for the testosterone signalling that is required to support male germ cell development and survival (Griswold, 2005). Total AR knockout mice exhibit a severe defect in reproductive development and spermatogenesis (Wang *et al.*, 2009), and male mice lacking Sertoli cell ARs (SCARs) are infertile because spermatogenesis rarely progresses beyond meiosis (Chang *et al.*, 2004; De Gendt *et al.*, 2004; Holdcraft & Braun, 2004). In addition, in humans, the

SCAR plays important roles in the induction of spermatogenesis (Boukari *et al.*, 2009). Several spermatogenic disorders are associated with impaired androgenic stimulation of Sertoli cells; thus, low levels of the SCAR are likely to be involved in spermatogenic disorders (Verhoeven *et al.*, 2010).

In addition to the ITT concentration, information regarding the expression level of the testicular AR in NOA men is important for investigating testosterone action. This study was designed to retrospectively investigate AR immunoexpression in human testes in relation to the quality of spermatogenesis and to investigate the changes in SCAR expression before and after hCG-based hormonal therapy for NOA (Shiraishi *et al.*, 2012).

MATERIALS AND METHODS

We retrospectively reviewed the records of consecutive NOA patients who underwent micro-TESE. The procedures were performed by the same surgeon (K.S.) between April 2010 and December 2013. Azoospermia was confirmed by analysing at least two semen samples that were concentrated by centrifugation. Of the 187 micro-TESE procedures performed during this period, sperm retrieval was successful in 76 cases (40%). Of the men in whom sperm retrieval failed, those with chromosomal abnormities, such as Klinefelter syndrome and Y chromosome microdeletion; extremely small testes (<4 mL); low levels of serum testosterone (250 ng/dL) because of pre-operative hormonal therapy; any history of reproductive disorders, such as varicocoele and cryptorchidism; and any history of life-threatening diseases were excluded. The study protocol was approved by the Ethics Committee of the Ube Industries Central Hospital, and all patients provided written informed consent before their hormonal treatments. After being informed of the results and the purpose of this study, immunohistological studies were performed on samples from 52 NOA patients, and 22 NOA patients were enrolled in salvage hormonal therapy (Shiraishi *et al.*, 2012). Twenty-two obstructive azoospermia (OA) cases were included as controls for the histological study. At least 6 months after the first micro-TESE, the patients began performing thrice weekly subcutaneous self-injections of 5000 IU of hCG (Gonadotropin; ASKA Pharmaceutical, Tokyo, Japan) to fully stimulate the Leydig cells; this therapy was performed for 3 months. The patients who continued to show high plasma follicle-stimulating hormone (FSH) values were maintained on the hCG treatment for 1–2 months prior to the second micro-TESE. The patients whose gonadotropin levels decreased to <2 mIU/L received additional recombinant human FSH (rhFSH) (GONAL-F; Merck Serono, Geneva, Switzerland) at 150 IU thrice weekly for 2 months prior to the second micro-TESE. Testis volume is expressed as the mean testis volume. The levels of serum testosterone, luteinizing hormone and FSH were measured monthly. Oestradiol (E2) was measured just before the second micro-TESE.

Micro-TESE was performed according to the procedure described by Schlegel (1999) with some modifications. The tunica albuginea of the testis was opened with a wide incision in an equatorial plane at approximately 300° of the circumference of the organ, and the testicular parenchyma was directly examined under an operative microscope. When no spermatozoa were identified in the initial sample, subsequent samples were obtained from the same testis and from the contralateral testis, as needed. Three testicular samples were obtained from a bilateral site with a representative appearance and sent for histological analyses.

Diagnostic testicular biopsy samples obtained during micro-TESE were sent for histopathological analysis by experienced pathologists. Three testicular samples were obtained, and the biopsy specimens were fixed in Bouin's solution for 2 h, embedded in paraffin, and stained with haematoxylin and eosin. Potential histopathological diagnoses based on testicular biopsies from men with NOA include Sertoli cell only (SCO), maturation arrest (MA) and hypospermatogenesis. MA is subcategorized into early MA, in which only spermatogonia or spermatocytes are found, and late MA, in which only spermatids are detected without spermatozoa (Weedin *et al.*, 2011). If mixed patterns were observed, the most advanced histopathological diagnosis from all specimens was used.

After deparaffinization, the 5 μm sections were incubated with hydrogen peroxide to inhibit endogenous peroxidases. The antigen was retrieved by heating in citrate buffer (pH 6) at 98 °C. After non-specific binding was blocked with rabbit serum, the slides were incubated overnight with antibodies against the AR (1 : 100 dilution; SC-816; Santa Cruz, Dallas, TX, USA) for 24 h at room temperature. We used an antibody against vimentin (1 : 100 dilution; sc-6260; Santa Cruz) as a molecular marker of the Sertoli cell, including the periphery and junctions (Aumuller *et al.*, 1988). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was used as a secondary antibody (1 : 100 dilution; Dako, Kyoto, Japan), and antibody binding was visualized using the avidin–biotin complex method. The sections were then counterstained with haematoxylin. The primary antibody was omitted in the negative control slides. At least 20 seminiferous tubules with round cross sections were randomly selected, and the AR index was calculated for each tubule as follows: AR-positive Sertoli cells × 100/total Sertoli cells (vimentin-positive cells). The mean AR index was calculated in combination with the bilateral side. The delta AR index [(AR index at the second micro-TESE) − (AR index at the initial micro-TESE)] was also calculated for each patient.

The obtained data were transferred to a software program (GraphPad InStat 3, La Jolla, CA, USA) for statistical analysis using paired *t*-tests, unpaired *t*-tests and the Mann–Whitney *U*-test. Correlations between the AR index and clinical parameters were performed using the non-parametric Spearman technique and graphed using best linear fit lines. A p value <0.05 was considered significant.

RESULTS

Of the 52 NOA patients, the means ± standard deviations (SDs) for the age of the patients, the age of the patients' wives, body mass index (BMI), testicular volume, LH, FSH and testosterone were 34.9 ± 5.2, 33.2 ± 4.8, 23.5 ± 3.2, 9.8 ± 3.6, 8.7 ± 2.5, 18.2 ± 7.2 and 3.6 ± 1.0 respectively. Significant differences were noted compared with OA cases, except for the parameters of patient age and BMI (Table 1). No spermatozoa were detected in the ejaculates of any of the 22 patients with NOA during the hormonal therapy prior to the second micro-TESE. The mean period between the first and second micro-TESEs was 14 months, and spermatozoa were successfully retrieved at the second micro-TESE from four of the 22 men (18%) treated with salvage hormonal therapy. The 12 patients whose gonadotropin levels decreased during the hCG treatment period were supplemented with rhFSH. There were no serious adverse effects during the hormonal therapy.

Table 1 Patients' background

	Obstructive azoospermia (n = 22)	Non-obstructive azoospermia (n = 52)	p-value
Patients' age	35.1 ± 6.4	34.9 ± 5.2	n.s.
Wives' age	29.2 ± 3.6	33.2 ± 4.8	<0.01
Body mass index	23.1 ± 3.0	23.5 ± 3.2	n.s.
Testicular volume (mL)	20.6 ± 3.2	9.8 ± 3.6	<0.0001
LH (IU/L)	3.1 ± 1.5	8.7 ± 2.5	<0.0001
FSH (IU/L)	3.9 ± 1.4	18.2 ± 7.2	<0.0001
Testosterone (ng/dL)	5.2 ± 1.7	3.6 ± 1.0	<0.0001

AR immunostaining was observed in the nuclei of Sertoli and peritubular myoid cells (Fig. 1A and C). The intensity of AR immunostaining was more robust in the Sertoli cells (SCAR) than in the peritubular cells. No AR immunostaining was detected in germ cells of any type or in Leydig cells. Sertoli cells are characterized by their ample fibrillar and/or microvacuolated cytoplasm with either an overall round or triangular-shaped nucleus, and they exhibit an irregular surface with occasional deep fissures. These features were clearly demonstrated by vimentin immunostaining in both OA and NOA (Fig. 1B and D). The mean AR index in NOA was significantly higher than that in OA (18.0 and 23.7%, respectively, $p < 0.05$) (Fig. 2). There was no significant difference in the number of Sertoli cells between OA and NOA (36.4 and 38.7 cells/tubule respectively), and the AR index was correlated with the absolute number of AR-positive Sertoli cells.

There was no correlation between the AR index and the clinical parameters (age, testicular volume, LH, FSH, and testosterone) in NOA, whereas there was a significant positive correlation between the AR index and the FSH level in OA ($p < 0.05$) (Table 2). In OA, the AR index tended to decrease with age, although this decrease was not statistically significant ($p = 0.06$). NOA cases were histopathologically diagnosed as SCO (28 cases, 54%), early MA (11 cases, 21%), late MA (5 cases, 10%) or hypospermatogenesis (8 cases, 15%) (Fig. 3A). The AR index of the early MA cases was significantly lower than that of the SCO, late MA and hypospermatogenesis cases ($p < 0.05$). There was no difference in FSH levels among the four pathological categories, and the presence of spermatozoa did not affect the AR index (Fig. 3B).

A significant increase in the AR index was observed after salvage hormonal therapy, particularly when using rhFSH; however, hCG alone did not cause an increase (Fig. 4). There was no change in the number of Sertoli cells before and after salvage hormonal therapy. There was no significant difference in the post-treatment AR index between the group in which sperm retrieval was successful and the group in which sperm retrieval was unsuccessful (data not shown); however, there was a significant increase in the ΔAR index in the group in which sperm retrieval was successful at the second micro-TESE (Fig. 5) (7.2 and 3.1, respectively, $p < 0.05$). There was no associations between ΔAR and the other clinical parameters as listed in Table 1.

DISCUSSION

In human testes, AR expression is dominant in Sertoli cells, and its expression level was higher in NOA vs. OA (Fig. 2). Based on the FSH-dependent increase in SCAR expression in OA (Table 2) and the FSH-stimulated increase after salvage

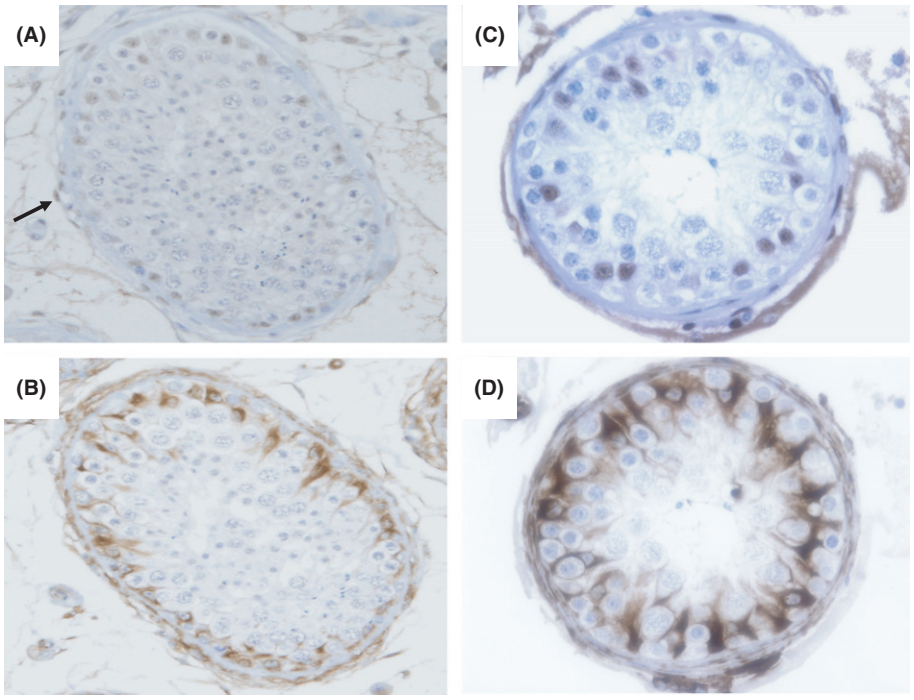
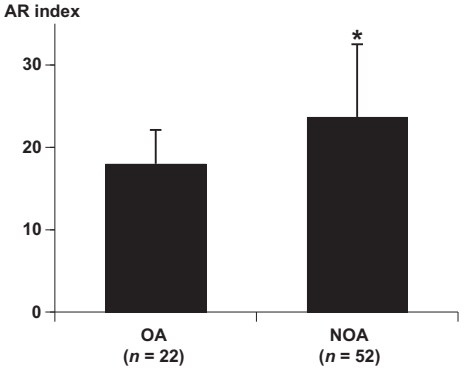


Figure 1 The immunostaining of the human seminiferous tubules with the anti-androgen receptor (AR) (A and C) and vimentin (B and D) antibodies in obstructive azoospermia (A and B, ×400) and non-obstructive azoospermia (C and D, ×200). AR is expressed mainly in the nuclei of Sertoli cells. The arrow indicates the positive nuclear staining in peritubular myoid cells.

Figure 2 Androgen receptor index in obstructive azoospermia and non-obstructive azoospermia. Data are expressed as mean ± standard deviation. *$p < 0.05$, unpaired *t*-test.



hormonal therapy (Fig. 4), the expression of SCAR is considered to be tightly regulated by FSH. When testosterone action is insufficient, spermatogenesis is mainly blocked at meiosis and spermiogenesis, as previously shown using AR knockout mice (Chang *et al.*, 2004; De Gendt *et al.*, 2004; Holdcraft & Braun, 2004). However, SCAR expression was decreased in early MA compared with late MA (Fig. 3A), indicating that SCAR may play roles during the development of spermatogonia and in primary spermatocyte levels. Our recent results showing that FSH stimulation increases DNA synthesis in spermatogonia (Shinjo *et al.*, 2013) indicate that FSH as well as hCG (Shiraishi *et al.*, 2012) stimulation may potentially promote spermatogenesis in NOA.

AR has been reported to localize to the nuclei of Sertoli cells, peritubular myoid cells, Leydig cells and fibroblasts (Takeda *et al.*, 1989; Ruizeveld de Winter *et al.*, 1991; Iwamura *et al.*, 1994; Van Roijen *et al.*, 1995). We confirmed the absence of AR immunoexpression in germ cells and intense staining in Sertoli cells (SCAR) (Fig. 1A and C), which has been a universal result among all laboratories. This finding supports the hypothesis that Sertoli cells are most likely mediator of androgen action in spermatogenesis because of their intimate anatomical and functional interactions with developing germ cells (O'Shaughnessy *et al.*, 2010). The SCAR intensities vary depending on the stage of the cycle (Bremner *et al.*, 1994), and the SCAR is normally localized in stages VII–VIII of the seminiferous epithelium in rats (Hill *et al.*, 2004). AR expression is similarly cyclical in men (Suarez-Quian *et al.*, 1999); however, we avoided the effect of germinal stage by using testicular samples with disturbed spermatogenesis, which were obtained from infertile men. On the other hand, Van Roijen *et al.* (1995) found no evidence supporting the stage-dependent immunoexpression of AR in human testes, and none of the publications that have investigated the localization of testicular AR identified specific variations in AR immunoexpression in the six stages of the human spermatogenic cycle (Takeda *et al.*, 1989; Ruizeveld de Winter *et al.*, 1991; Kimura *et al.*, 1993; Iwamura *et al.*, 1994). SCAR immunostaining is also dependent on the primary antibody concentration, which causes different rates of positive staining of Sertoli cells (Suarez-Quian *et al.*, 1999; Chemes *et al.*, 2008).

AR expression appears faintly in a low proportion of Sertoli cells in the second half of the first year of life, and a progressive increase is observed between the ages of 4 and 8 years (Rey *et al.*, 2009). By the age of pubertal onset (9–14 years), however, the factors that regulate SCAR expression remain unknown, although FSH may play an important role (Blok *et al.*, 1989; Verhoeven *et al.*, 2010). In men with oligozoospermia and normal serum gonadotropin levels, Van Roijen *et al.* (1995) reported that the gonadotropin levels and semen parameters were not correlated with SCAR expression and concluded that inappropriate SCAR expression was not an important causative factor for – or an accompanying phenomenon of – male infertility. In our study, serum gonadotropin and testosterone levels did not correlate with SCAR expression in NOA, whereas there was a significant positive correlation between the FSH level and SCAR expression in OA, which is usually observed in normal spermatogenesis (Table 2). Furthermore, combination therapy with rhFSH, but not hCG alone, significantly increased SCAR expression at the second micro-TESE in NOA (Fig. 4), supporting the notion that SCAR expression is FSH dependent. Testosterone also plays a role in the final maturation of Sertoli cells (Sharpe *et al.*, 2003), and both testosterone and FSH have additive effects on SCAR expression (Verhoeven *et al.*, 2010). Rather than FSH stimulation, SCAR expression has been shown to be primarily dependent on the ITT level (Hill *et al.*, 2004). Furthermore, there was no significant association between serum testosterone concentration or ITT and SCAR expression in our study (data not shown). There was no positive correlation between the FSH level and SCAR expression (Table 2), indicating that the stimulation of FSH on Sertoli cells is maximal in NOA. The exogenous administration of rhFSH had a positive effect on SCAR expression in NOA (Fig. 4). An animal study indicated that AR gene transcription and the number of AR increase after the treatment of Sertoli cells with FSH, and the number of FSH receptors in the rat testis also increases during the development and maturation of Sertoli cells (Tsutsui, 1991).

Thus, the effects of SCAR expression on spermatogenesis are the most concerning issue. SCAR signalling is generally recognized to be crucial for spermatogenesis in humans (Plant & Marshall, 2001; McLachlan *et al.*, 2002). SCAR knockout affects the ability of Sertoli cells to support maturing germ cells, resulting in

Table 2 Correlation of clinical parameters and androgen receptor expression

	Obstructive azoospermia ($n = 22$)	*p*-value	Non-obstructive azoospermia ($n = 52$)	*p*-value
Patients' age	$r = -0.40$	0.06	$r = -0.02$	n.s.
Testicular volume	$r = -0.10$	n.s.	$r = 0.12$	n.s.
LH	$r = 0.32$	n.s.	$r = 0.17$	n.s.
FSH	$r = 0.51$	<0.05	$r = -0.05$	n.s.
Testosterone	$r = -0.13$	n.s.	$r = 0.16$	n.s.

LH: luteinizing hormone. FSH: follicle stimulating hormone.

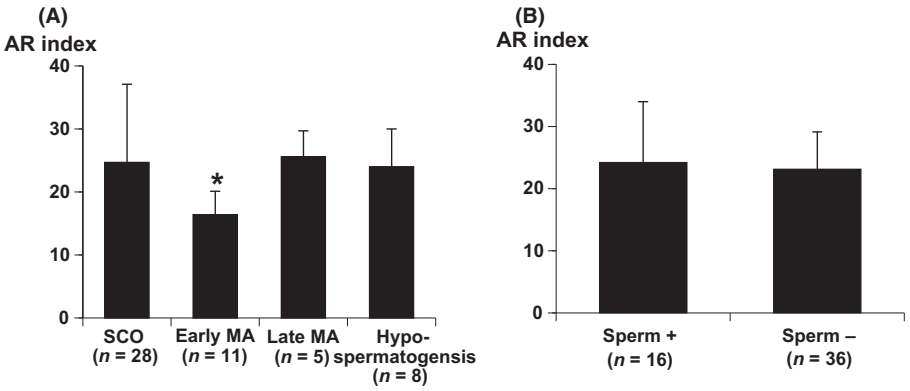


Figure 3 Androgen receptor (AR) index categorized by histological appearance (A) and sperm retrieval (B). Data are expressed as mean ± standard deviation. *: $p < 0.05$, Mann–Whitney *U*-test.

Figure 4 Androgen receptor index before and after the hormonal treatment in total and subdivided with or without recombinant human FSH. Data are expressed as mean ± standard deviation. *$p < 0.05$, **$p < 0.01$, paired *t*-test.

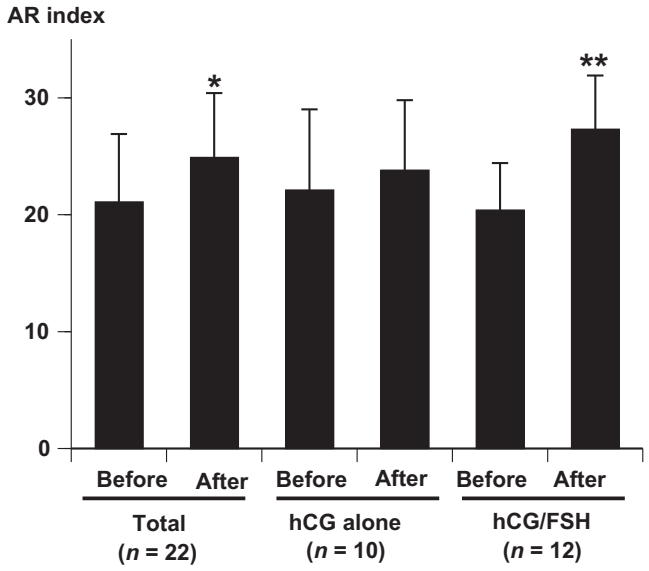
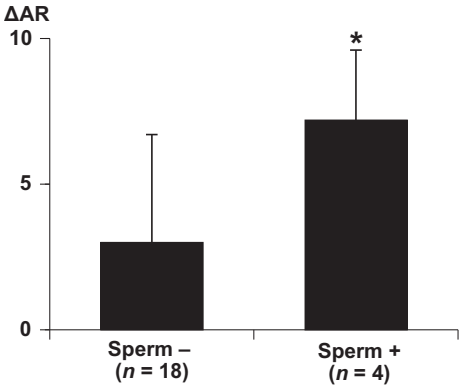


Figure 5 Changes in androgen receptor index before and after the hormonal treatment (ΔAR) categorized by the sperm retrieval at second microdissection testicular sperm extraction. Data are expressed as mean ± standard deviation. *$p < 0.05$, unpaired *t*-test.



spermatogenesis arrest before the first meiotic division and during the transition from round to elongated spermatids, causing a progressive loss of fertility (Chang *et al.*, 2004; De Gendt *et al.*, 2004; Holdcraft & Braun, 2004). In addition, testosterone withdrawal appears to induce the apoptosis of pachytene spermatocytes and round spermatids (Sharpe, 2005). Moreover, Hazra *et al.* (2013) developed transgenic Sertoli cell-specific AR (TgSCAR) gain-of-function mice and reported that TgSCAR induced pre-mature post-natal spermatogenic development, as shown by increased levels of meiotic and post-meiotic germ cells (Hazra *et al.*, 2013). These findings from animal models demonstrate that SCAR is particularly crucial for the development of pachytene spermatocytes into pre-meiotic spermatocytes, which histologically resembles early MA, and support the finding that AR expression was lower in early MA compared with late MA (Fig. 3A).

Variable histological patterns in different tubules, even in the same individual, may explain the poor correlation of TESE results with SCAR expression (Fig. 3B). However, the increase in AR expression in men from whom spermatozoa could be retrieved at the second micro-TESE was significantly greater than that observed in men from whom spermatozoa could not be retrieved (Fig. 5). We cannot determine whether the increased expression of the AR plays a major role in improving spermatogenesis or is a phenomenon that accompanies the improvement of spermatogenesis. In both scenarios, Sertoli cells that readily respond to FSH stimulation may have restored other factors to that support spermatogenesis (e.g. production of paracrine/juxtacrine factors, support of the blood–testis barrier). Because spermatogonial proliferation (Dwyer *et al.*, 2013) and spermatogonial DNA synthesis (Shinjo *et al.*, 2013) are accelerated by rhFSH, the Sertoli cell-mediated restoration of spermatogenesis can be expected through the use of rhFSH. In fact, hCG-based salvage hormonal therapy is effective in cases with late MA but not in cases of early MA (Shiraishi *et al.*, 2012). FSH-based salvage hormonal therapy may help to restore disrupted spermatogenesis in men with early MA.

The first limitation of this study is the small sample size. As mentioned above, we are revising our hormonal therapy protocol, and the recruitment for the current protocol has ended; thus, we analysed only the 22 available NOA cases. The new protocol involves the use of rhFSH from the beginning. Second, because of the limitation of clinical studies, we used OA cases for the controls. While seminal obstruction slightly impairs spermatogenesis, the main purpose of this study was to investigate the effect of hCG/rhFSH therapy on sperm retrieval and AR expression. Therefore, our conclusions were unaffected by the controls. Third, we did not investigate the intensity of AR

immunostaining in each Sertoli cell, and we did not examine the molecular structure of the AR, in which CAG repeats are closely related to spermatogenesis. Molecular biological and in vitro experimental approaches are currently being utilized to investigate the functional aspects and regulatory mechanisms of SCAR expression.

CONCLUSIONS

The expression of AR in Sertoli cells (SCAR) was shown to be FSH-dependent, and its expression level is significantly associated with spermatogenesis in humans. As additional molecular mechanisms of SCAR signalling continue to be revealed, we will gather the intellectual resources required to select a candidate for hormonal therapy and establish therapies for specific male infertility.

ACKNOWLEDGEMENTS

We thank Dr Shimabukuro for giving us the opportunity to perform this study at UBE Industries Central Hospital. This study was supported by a Grant-in-Aid for Scientific Research (C) (24592447) from the Japan Society for the Promotion of Science.

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