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Significant correlation between high motility and P450-aromatase expression in human spermatozoa - an immuno-chemistry based study

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ABSTRACT

Study was planned to evaluate the correlation between sperm motility, an important functional parameter of spermatozoa and aromatase P450 expression in human spermatozoa. Total 70 random seminal samples were included in the present study. Seminal samples from men undergoing seminal investigations for couple infertility were collected. Immunochemical localization with specific monoclonal antibodies against the P450- aromatase enzyme with washed seminal slides was carried out and the results correlated with spermatozoon motility evaluation retrospectively. Expression of aromatase P450 was assessed by assigning 1+ to least and 4+ to highest. The 94.6% cases of spermatozoa showing immunochemical positive grading >3+ for -aromatase P450, had motility greater than 70%. Similarly 94% cases show <2+ intensity of aromatase P450 expression on spermatozoa found with motility of less than 70%. The study suggests that the spermatozoa aromatase P450 produced estradiol might be play an important role in spermatozoa motility.

Introduction

Estradiol, normally considered as female hormone is present in low concentration in male blood but extraordinarily high in semen, where its concentration could be higher than in female (Hess et al. 1997). Aromatase enzyme complex (Cytochrome P450) also known as Estrogen synthetase, localized mainly in endoplasmic reticulum of various tissues, is responsible for irreversible conversion of androgens to estrogens and is highly conserved in the mammals. The main source of estrogen production lies in the testis, whereas in rodents aromatase activity is more in sertoli cells before sexual maturity but in adults mainly found in Leydig cells (Carreau et al. 2007). *In vitro* production of Estrogen by both sertoli cells as well as Leydig cells has been reported in human (Carreau et al. 2008). Aromatase activity has also been demonstrated in spermatogenic cells of several species including humans (Carreau et al. 2010). Aromatase has been immuno-histochemically localized in pachytene spermatocytes, round spermatids, elongated spermatids, flagella of late spermatids as well as in ejaculated spermatozoa (Kwon et al. 1995), which were further confirmed by Reverse Transcriptase Polymerase Chain Reaction (RT-

PCR) and western blotting (Aquila et al. 2003). It has been hypothesized that estradiol produced by testicular cells may be acting as a germ cell survival factor in testis (Pentikainen et al. 2000) and improving sperm fertilizing capability of the ejaculated spermatozoa (Aquila et al. 2003). Estradiol, or a phytoestrogen (Genistein) and environmental estrogens have been reported to enhance functions including capacitation, acrosome loss and fertilizing ability of human spermatozoa (Adeoya-Osiguwa et al. 2003; Fraser et al. 2006). The important role of the aromatase and estrogen in male fertility has also been established by the knockout studies of Aromatase (Fisher et al. 1998; Robertson et al. 1999) and estrogen receptors (ERα and ERβ) (Eddy et al. 1996; Krege et al. 1998; Sharma et al. 2017) that has provided direct evidence on the involvement of aromatase and estrogens in male fertility.

The short half-life of estradiol, the demonstrated presence of aromatase from the testis to ejaculated spermatozoa and the availability of the aromatase enzyme substrate androgens in the male reproductive tract indicate that aromatase through estrogens synthesis may be playing specific autocrine and/or intracrine roles in the spermatozoa physiology and motility. Investigations were undertaken with an objective to evaluate the relationship, if any, between the presences of aromatase in ejaculated spermatozoa with spermatozoa motility.

Materials and Methods

Chemicals and reagents

Mouse anti-Human Cytochrome P450 aromatase monoclonal antibody, Clone H4 (Turner et al. 2002) used in the immunochemical studies were procured from Bio-Rad. Vactastain Elite ABC-HRP Kit (Peroxidase, Mouse IgG) and ImmPACT DAB (Brown) from Vector Laboratories (USA). All the other chemicals and reagents, except where specified otherwise, were procured from Sigma Aldrich (USA).

Semen samples and spermatozoa preparation

Left over anonymous semen samples were used from individuals who were undergoing semen analysis for couple infertility. All the samples were collected in accordance to guidelines issued by Indian Council of Medical Research "ICMR ethical guidelines for biomedical research on human subject". Total 70 semen samples exhibited Normospernia, a sperm count of at least 40×10^6/mL spermatozoa and fulfilling the color, volume, spermatozoan morphology, viscosity, motility, vitality and falling in criteria recognized for fertile group as per WHO (WHO 2010) were included for the study. The samples with leukocytes and/or immature germ cell concentration greater than 10^6/mL were not included in the study. Semen samples were collected in polypropylene pre-sterilized graduated tube and incubated at 37 ℃ for 15 min. After liquefaction, routine semen analyses were performed (WHO 2010) within 45 min after sample collection. Seminal smears were prepared from the semen samples, air-dried and fixed for 20 min in 95% ethanol and stored till further analysis (WHO 2010).

Immunohistochemical staining

Slides were incubated in methanol containing 0.3% Hydrogen peroxide (H_2O_2) for 30 min to quench endogenous peroxidase activity. Immunohistochemical staining for localization of aromatase in human spermatozoa was conducted using primary antibody, Mouse anti-Human Cytochrome P450 aromatase monoclonal antibody, Clone H4 (Turner et al. 2002). The slides were incubated with primary antibody (dilution 1:50) for one hour at 37ºC in humid chamber. Vactastain Elite ABC-HRP Kit (Peroxidase, Mouse IgG) were used for detection of primary antibody (Hsu et al. 1981) in the specimen as per manufacturer's instructions for use given with the Vactastain Elite ABC-HRP Kit.

The peroxidase binding sites were then demonstrated by using ImmPACT DAB (Brown) as substrate and counter stained with

Haematoxylin. The Immunohistochemical localization of aromatase was performed regularly in batches consisting 10 slides each. Spermatozoa slides incubated without primary antibody and processed concurrently were used as negative control and slide of human placenta incubated with aromatase P450 monoclonal antibody were use as positive control. However, the microscopic evaluations of localizations were carried by two independent observers to remove biasness and in accordance to WHO laboratory manual for the examination and processing of human semen Positive slides were further graded 1+ (minimum), 2+. 3+ and 4+ (maximum).

Statistical Analysis

The motility of the spermatozoa in the semen samples were evaluated as per the WHO laboratory manual (WHO 2010) by two independent observers. Arithmetic mean of values was taken in account for the calculation of result. The data were analyzed, tabulated in 2 x 2 contingency table and was tested employing Fisher's exact test. Fisher's exact test is recommended for statistical application when total no of sample is less.

Results

The positivity grading of the aromatase expression was based on presence of color, pattern and intensity in term of 1+, 2+. 3+ and 4+ of brown color chromogen deposits on the spermatozoa. Negative staining was defined as lack of brown chromogen deposits and the presence of blue colour of counter stain Hematoxylin only. Samples meeting the required validity criteria were included in the study for result calculation purpose. Intensity grading was assigned only after counting at least 200+ spermatozoa and taking into account of not less than 95% population on single specimen. The localizations were seen mainly on the post-acrosome (nuclear) region of the head of spermatozoa. In some cases the aromatase expression was observed in the lower 2/3 head potion of the spermatozoa. Aromatase grading and motility in Human Spermatozoa were summarized in Table 1. Aromatase Expression in Human Spermatoza has been observed at various level (Fig 1). The Fisher Exact test is a test of significance that is used in the place of chi square test in 2 by 2 contingency tables, especially in cases of small samples size.

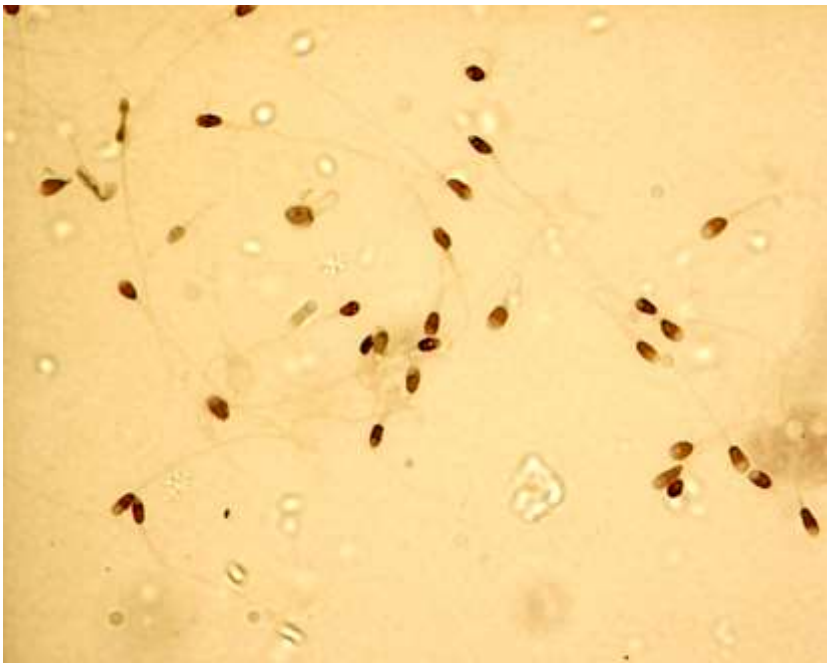

Fig 1. Aromatase Expression in Human Spermatoza

Table 1: Aromatase and motility in Human Spermatozoa

Aromatase grading	Spermatozoa Motility Percentage (%)		
	> 70%	< 70%	Total
>3+	35 (94.6%)	2 (5.4%)	37
<2+	2 (6.0%)	31 (94%)	33
Total	37	33	70

The two-tailed 'P' value is less than 0.0001. The association between rows (groups) and columns (outcomes) is considered to be extremely statistically significant.

Among aromatase positive >3+ cases, 94.6% belong to the group having motility greater than 70% (a better attribute for fertilizing capacity of the spermatozoa), while 94% of the aromatase positive <2+ cases showed motility below 70%. The difference in the proportion of subjects revealing spermatozoa aromatase positivity with the cut-off level of greater than and less than 70% motility was found to be statistically significant ($p< 0.0001$). Only 2 samples of the aromatase positive <2+ cases belong to the group having motility above 70%, while only 2 samples of aromatase positive >3+ cases showed a motility of 48% and 54%. All these observations clearly indicate the expression of Cytochrome P450 aromatase on human ejaculated spermatozoa. Statistically significant correlation between aromatase expression and in relation with sperm motility also suggests important role of aromatase in normal sperm function.

Discussion

To our knowledge, the results of the current investigation seem like the first reports to directly correlate the relationship between immunolocalization of aromatase on the spermatozoa and the high spermatozoa motility, wherein higher aromatase expression (>3+) was demonstrated in 94.6% cases with motility greater than 70%. The relevance of the observations become more significant as 94% of the spermatozoa with aromatase positivity moderate to low as described as <2+ cases showed a motility below 70%. The aromatase localization profiles observed during the current investigation was slightly different from the observation of (Aquila et al. 2002), where aromatase were found mainly in mid piece of spermatozoa. Further in the year 2007, data obtained using a confocal microscopy approach two immune-reactive sites have been demonstrated (Galeraud-Denis et al. 2007) one in tail as per earlier report and second one corresponding to high fluorescent zone of head upper postacrosomal region/equatorial region that is in similar with present study. It is important to mention here that antibody used in article (Aquila et al. 2002) were rabbit polyclonal antiserum directed against human placental aromatase P450 where in the current investigation monoclonal Mouse anti-Human Cytochrome P450 aromatase antibody, Clone H4 (Turner et al. 2002) were used that recognizes a conserve epitope within Cytochrome P450 aromatase corresponding to amino acids 376-390 of human aromatase.

The presence of aromatase mRNA in human spermatozoa (Lambard et al. 2003; Galeraud-Denis et al. 2007) that is in agreement with other documented reported (Aquila et al. 2002, 2003; Rago et al. 2003) apparently point towards crucial role in energy metabolism of spermatozoa. Further, in immotile spermatozoa the amount of aromatase P450 mRNA transcripts was 30% lesser as compared to motile spermatozoa (Lambard et al. 2003). In fact, aromatase expression was higher in the motile fraction as compared to immotile fraction of spermatozoa (Lambard et al. 2004). Interestingly, studies of a small group of teratospermic men aromatase localization at equatorial region (upper postacrosomal region) of human spermatozoa was twofold less than normospermic men (Galeraud-

Denis et al. 2007). The current observation also gets indirect support from the earlier observations that *in-vitro* addition of estradiol enhanced motility, oocyte penetration, longevity, oxygen intake, lactate production, and metabolization of several substrates increases spermatozoa motility and ATP utilization (Idaomar et al. 1989). The documented short half-life of estradiol also suggests the testicular and spermatozoa endogenous estradiol production in sperm survival. It is also reported that low estradiol concentrations have more effect as compare to androgens in the prevention of germ cell apoptosis in human adult (Pentikainen et al. 2000). Endogenous estradiol may be playing specified local (paracrine/autocrine) roles at all locations right from fetal testis (Gustafson and Donahoe 1994), adult testis (Lambard et al. 2003), epididymis (Janulis et al. 1998) to stimulation of spermatozoa motility as reported in the present study, following ejaculation. The reported possibilities on the existence of a link between spermatozoa aromatase produced estradiol and sperm capacitation and acrosome reaction in the absence of exogenous mediators (Aquila et al. 2003), also support the results obtained and the hypothesis made during the current investigations on the autocrine role of aromatase and spermatozoa motility.

Conclusion

The demonstration of significant correlation between the aromatase presence in the spermatozoa and the motility of the spermatozoa also opens newer possibilities for development of aromatase stimulators for treatment of idiopathic male infertility and aromatase inhibitors for appropriate use in the regulation of male fertility and contraception.

Conflicts of interest

The authors declare that they have no conflicts of interest in this study.

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