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Experimental Paper

Seminal Plasma Annexin A5 Concentration is not Associated with Male Subfertility and cannot be Influenced by Folic Acid and Zinc Sulfate Treatment

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ABSTRACT

Annexin A5 (anxA5) is abundantly present in seminal plasma, however, its endogenous function in seminal plasma is not known. Recently, we demonstrated that folic acid and zinc sulfate intervention increased sperm count. To explore the involvement of anxA5 in human (sub)fertility, we measured anxA5 concentrations in seminal plasma, using sandwich ELISA, before and after folic acid (5 mg/day) and zinc sulfate (66 mg/day) intervention in 86 fertile and 78 subfertile males participating in a randomized placebo controlled intervention study. Seminal plasma anxA5 concentrations at baseline were not significantly different between fertile and subfertile males, (median) 5.2 μg/mL (25th–75th percentile: 4.2–7.2), and 5.6 μg/mL (4.3–6.7), respectively. The various treatments did not affect seminal plasma anxA5 concentrations. In conclusion, seminal plasma anxA5 concentration is not associated with male factor subfertility and the observed increase in sperm count after folic acid and zinc sulfate treatment cannot be explained by a change in the seminal plasma anxA5 concentration. Further studies are needed to elucidate the mechanisms responsible for the beneficial effect of this intervention treatment on sperm count.

INTRODUCTION

Subfertility is a common disorder with a prevalence of about 15% in all couples in the Western world.[1-3] In about half the number of cases a male factor is identified, defined as male factor subfertility. In most cases, subfertility is regarded as idiopathic, however, gene-environment interactions are suggested to be involved.[4] The environmental causes are particularly of interest, because they are better amendable to curative and/or preventive measures than genetic factors. A significant but largely neglected environmental factor is nutrition. It is well known that nutrition plays an important role in reproduction.[5] The vitamin folate is known to contribute to the prevention of neural tube defects when taken periconceptionally.[6,7] Folate plays an important role in the synthesis of transport ribonucleic acid (tRNA) and deoxyribonucleic acid (DNA) and methylation of proteins.

Zinc is an essential nutritional compound, serving as a cofactor for more than 80 metallo-enzymes, and also as a cofactor in the synthesis of macromolecules such as DNA and tRNA. It has been shown that zinc is essential in testicular development.[8] Also, seminal plasma zinc concentrations influence the oxygen consumption of spermatozoa,[9,10] nuclear chromatin condensation,[11] acrosome reaction,[12] and acrosin activity.[13] Furthermore, the synthesis of testosterone in the Leydig cells and the conversion of testosterone to 5α-dihydro-testosterone by the 5α-reductase enzyme is dependent on zinc supply.[14]

Recently, we conducted an intervention study supplying both folic acid and zinc sulfate to fertile and subfertile men, and found that after 26 weeks of intervention treatment, subfertile men had a 74% increase in normal sperm count.[15] Despite the knowledge that zinc and folate are essential for the synthesis of genetic material, the precise underlying mechanism by which these micronutrients affect spermatogenesis is not clear.

AnxA5 is a member of the protein family of annexins, which contains more than ten members. These proteins (especially anxA5) are characterized by their high affinity for negatively charged phospholipids present in cell membranes.[16] AnxA5 is primarily known because of its world-wide use to detect apoptosis in vitro and also experimentally in vivo.[17,18] Due to the affinity to negatively charged phospholipids, anxA5 is a potent inhibitor of blood coagulation and inflammation.[19] It is also known that anxA5 is abundantly present in seminal plasma.[20]

Because our research group has not yet identified the underlying mechanism of the beneficial effect of folic acid and zinc sulfate on spermatogenesis, and because of the
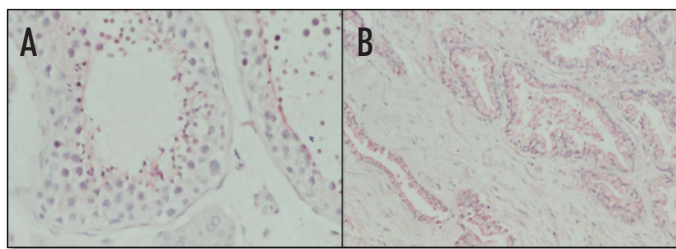
Figure 1. Immunohistochemical staining with a polyclonal antibody directed against Annexin A5 counterstained by Haematoxylin. Sections stained were (A) seminiferous tubules of the testis (x 40) and (B) prostate (x 20). Annexin A5 is stained red whereas the nucleus is blue. In the testis a gradual staining of anxA5 was observed. The highest intensity is nearby the spermatocytes. The spermatogonia are not stained. In the prostate mainly the glandular epithelium is stained.

observation that anxA5 is abundantly present in seminal plasma, we explored the possibility that endogenous anxA5 is directly involved in male factor subfertility. Furthermore, we evaluated the effect of intervention treatment with folic acid and zinc sulfate on anxA5 concentrations in seminal plasma.

MATERIALS AND METHODS

From the randomized, placebo controlled trial designed to study the effect of folic acid and zinc sulfate on semen parameters,[15,21] samples before and after intervention from 86 fertile and 78 subfertile males were available for the determination of endogenous anxA5 concentrations.

In the referred study, fertile males were recruited from nine midwifery practices in the surrounding areas of Nijmegen, in The Netherlands. These healthy men, without a history of fertility problems at the moment of enrollment, had a pregnant partner who conceived spontaneously within one year of regular, unprotected intercourse.

Subfertile males were recruited from the fertility clinics of the University Medical Centre Nijmegen and the Canisius Wilhelmina Hospital in Nijmegen. Subfertility was defined as failure of the female partner to conceive after one year of regular, unprotected intercourse and a sperm concentration between five and twenty million spermatozoa per mL on the first routine semen analysis after referral to the fertility clinic. The females of these subfertile males were not further evaluated, because the main focus was on the effect of folic acid and zinc sulfate treatment on semen parameters in subfertile males.

The fertile and subfertile males were included after having given their written informed consent and assigned to the four intervention groups by computer-generated random numbers. The interventions consisted of a daily dose of folic acid (5 mg) and placebo, zinc sulfate (66 mg) and placebo, or a combined dose of folic acid and zinc sulfate, or placebo/placebo throughout 26 weeks.

Before and after intervention one standardized semen sample was obtained from every participant for semen analysis according to World Health Organization (WHO) guidelines.[22] The semen samples were produced by the participants via masturbation after an abstinence period of at least three to five days. These samples were delivered within one hour after production to the fertility laboratory. In this hour the participants were advised to keep the sample at room temperature. After liquefaction, an aliquot of semen was centrifuged at 1,400 x g (Hettich 16A, 1323 rotor) for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at -80°C until assayed. Sperm concentration was determined with a Makler counting chamber.

The Medical Ethical Committee and the Institutional Review Board of the University Medical Centre Nijmegen approved of this trial.

AnxA5. The anxA5 concentration was investigated by sandwich enzyme-linked immunosorbent assay (ELISA) (ZYMUTEST anxA5, Hyphen BioMed, Andrésy, France) as described by van Heerde et al.[23] The antibodies used in this test are affinity purified rabbit polyclonal antibodies specific for human anxA5 (F(ab')$_2$ fragments) and an horse radish peroxidase coupled affinity purified rabbit polyclonal antibody against anxA5. The substrate used is ortho-phenylene diamine (OPD) in presence of hydrogen peroxide. After color development adsorption is measured in a micro ELISA plate reader at 492 nm (Easy reader, SLT Labinstruments Austria).

Quantitative real time AnxA5 RT-PCR. The seminal plasma anxA5 concentration may originate from different sites of synthesis, e.g., from testis, prostate or seminal vesicles. To determine whether the prostate or the testis is the main producer of anxA5, anxA5 messenger RNA (mRNA) was measured and the anxA5 antigen was stained in human prostate and testis sections. Complementary DNA (cDNA) was synthesized by using 1 µg RNA of prostate tissue and testis (Clontech, Palo Alto). Testis mRNA was isolated out of whole normal testes pooled from 45 Caucasians (age 14–64) who deceased suddenly. Prostate mRNA was isolated from 47 Caucasians (age 14–57) who also deceased suddenly. The mRNA was mixed with 625 µM dNTPs, 5 µg/ml random hexamer primer DTT, RNAsin (20 U) and M-MLV RT (200 U) in a total volume of 15 µl to obtain cDNA. The mixture was incubated for 10 min at 20°C, followed by 45 min at 42°C and 10 min at 95°C. Primer-probe combinations for the anxA5 cDNA were designed using PRIMER-EXPRESS software. De forward primer CCACAGTCTGGTCCTGCTTC, the reverse primer AGTCACAGTGCCTCTGAGAACCT, and the minor groove-binding probe CTGACCTGAGTAGTCGC were mixed with 50 ng cDNA, 1.25 U AmpliTaq Gold DNA polymerase with 250 µmol/l dNTPs. 1 X Taqman buffer A in a total volume of 50 µl. Samples were heated at 95°C for 10 min and amplified for 45 cycles of 15 sec at 95°C and 60 sec at 60°C (ABI/Prism 7700 Sequence detector, Applied Biosystems). The expression of porphobilinogendeaminase (PBGD), a low copy number housekeeping gene, was measured in duplicate onto each sample to normalize for PCR and cDNA input variations.[24] The anxA5 mRNA concentrations were measured in duplicate and analyzed with Taqman software. The results were expressed as delta cycle threshold (δCt) in which δCt = Ct (PBGD) minus Ct (AnxA5). The relative difference in expression is calculated by Comparative Ct method using the equation $2^{-\delta\delta Ct}$.

Immunohistochemistry. Post mortem human paraffin-embedded prostate and testis tissue sections were stained with a polyclonal antibody directed against human anxA5. The sections were macroscopically and microscopically checked by the pathologist as being normal prostate and testis tissues. The sections were routinely processed to remove the paraffin and to rehydrate the tissues. Next, the sections were blocked with human serum albumin (0.1%) containing 50mM tris-buffered salt buffer, pH 7.4 (TBS/HSA) to which 20% normal swine serum was added. After 30 min the sections were washed in TBS and incubated for another two hours with a polyclonal antibody against human anxA5 (1000 X diluted in TBS/HSA)(Hyphen Biomed, Andrésy, France). The sections were washed again and incubated for 90 min with a biotin-conjugated swine anti-rabbit polyclonal antibody (1000-fold diluted in TBS/HSA)(Dako, Glostrup, Denmark). Finally, after washing the sections were incubated with alkaline phosphatase-conjugated streptavidin-biotin complex (DakoCytomation, Glostrup, Denmark) for 1 hour. After extensive washing the sections were stained by using the alkaline-phosphatase substrate kit containing 2 mM levamisole (Sigma, St Louis, MI) to block the endogenous alkaline phosphatase activity, according to the manufacturers procedure (Vector, Burlingame, CA, USA). The presence of anxA5 is notified by a red color. The nucleus was counterstained blue with Mayer's Haematoxylin (Merck, Darmstadt, Germany).

Statistical analysis. The results were analyzed for statistical significance using nonparametric tests, because of the skewed distributions of the determinants. Concentrations of endogenous anxA5 are given as median and 25th–75th percentile. Baseline seminal plasma anxA5 concentrations were compared between fertile and subfertile males using the Mann-Whitney U test. The effect of the four interventions in fertile and subfertile males was investigated by comparison between the baseline and post-intervention seminal plasma anxA5 concentration by the Wilcoxon Signed Ranks test. We corrected for a possible placebo-effect by comparing the delta anxA5 concentration for males receiving placebo with the delta anxA5 concentration

Table 1A The effect of interventions on seminal plasma annexin A5 concentrations (μg/mL) in fertile males

	N	Pre-intervention AnxA5	Post-intervention AnxA5	Delta
All (baseline)	86	5.2 (4.2–7.2)	—	—
Placebo	21	5.2 (4.0–5.9)	5.2 (4.2–6.9)	-0.5 (-1.1–1.6)
Folic acid	20	5.6 (4.3–7.6)	5.4 (3.7–7.2)	-0.3 (-2.1–1.6)
Zinc sulfate	22	5.4 (4.3–8.1)	5.5 (3.2–6.9)	-0.7 (-2.1–0.8)
Folic acid and zinc sulfate	23	4.9 (3.6–7.1)	3.4 (1.8–6.6)	-1.3 (-2.3–1.0)

NB, data are the median (25th–75th percentile). Deltas are calculated as post-intervention-preintervention value.

Table 1B The effect of interventions on seminal plasma annexin A5 concentrations (μg/mL) in subfertile males

	N	Pre-intervention AnxA5	Post-intervention AnxA5	Delta
All (baseline)	78	5.6 (4.3–6.7)	—	—
Placebo	23	5.7 (4.3–7.6)	5.5 (2.6–8.9)	-0.5 (-2.6–2.9)
Folic acid	16	5.4 (3.8–6.6)	5.2 (3.0–6.4)	-0.01 (-1.7–1.2)
Zinc sulfate	18	5.2 (4.4–6.7)	4.9 (3.5–6.9)	-1.1 (-2.0–1.6)
Folic acid and zinc sulfate	21	5.6 (2.5–6.1)	3.7 (2.1–5.7)	-0.4 (-1.6–0.9)

NB, data are the median (25th–75th percentile). Deltas are calculated as post-intervention-preintervention value.

for males receiving the folic acid, zinc sulfate, or combined intervention treatment. The p values were two tailed and $p \leq 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 10.0 for Windows software (SPSS Inc, Chicago, IL, USA).

RESULTS

AnxA5 concentration in seminal plasma. Although anxA5 could be determined in seminal plasma, it did not show an association with male fertility. The median (25th–75th percentile) baseline seminal plasma anxA5 concentrations was 5.2 μg/mL (4.2–7.2) in fertile (Table 1A) and 5.6 μg/mL (4.3–6.7) in subfertile males (p = 0.96, (Table 1B).

Effect of intervention on seminal plasma AnxA5 concentration. Tables 1a and 1b show the effects of all interventions on seminal plasma anxA5 concentration in both fertile and subfertile males, respectively. We observed no effect of the combination treatment with folic acid and zinc sulfate on anxA5 concentration in both fertile and subfertile males (from 4.9 μg/mL (3.6–7.1) to 3.4 μg/mL (1.8–6.6) in fertile males and from 5.6 μg/mL (2.5–6.1) to 3.7 μg/mL (2.1–5.7) in subfertile males). All the other intervention types (placebo, folic acid, and zinc sulfate intervention) also did not significantly affect the anxA5 concentrations, neither in fertile, nor in subfertile males.

AnxA5 site of synthesis. To get some global insight in the synthesis of anxA5, the anxA5 mRNA concentrations and immunohistochemical localization of anxA5 protein were determined. AnxA5 mRNA concentrations appeared to be approximately twice as high in the prostate (δCt 5.0) as compared to the testis (δCt 3.8). Furthermore, as shown in Figure 1, immunohistochemical staining of anxA5 revealed strong staining in the prostate and some staining in the testis. In the prostate mainly the glandular epithelium is stained. In the testis a gradual staining of anxA5 was observed. The highest intensity is nearby the spermatocytes. The spermatogonia are not stained.

DISCUSSION

This study was performed to explore if anxA5 is associated with male factor subfertility and to find an underlying mechanism for the intriguing observation that folic acid and zinc sulfate intervention increases the sperm count in subfertile males.[15,21] Remarkable was the finding that the endogenous seminal plasma anxA5 concentrations are at least 1000-fold higher compared to the concentrations of anxA5 in blood plasma of healthy volunteers, in which anxA5 concentrations up to 5 ng/mL are found.[23,25]

The baseline seminal plasma anxA5 concentration, however, was not significantly different between fertile and subfertile males. This strongly suggests that it is not very likely that anxA5 is associated with sperm concentration. Therefore, anxA5 is probably not involved in male factor subfertility.

Our data suggest that anxA5 is not produced by the spermatozoa themselves, since the immunohistochemical results indicate that the spermatocytes themselves are not stained. Furthermore, the real time mRNA analysis shows that the prostate is the most important organ in the production of anxA5 in seminal plasma.

Only one other study could be found in which the authors investigated the concentration of anxA5 in seminal plasma. These authors obtained semen samples from normal or vasectomized patients and found anxA5 concentrations of approximately 20 μg/mL.[20] It is remarkable that these authors found an almost 4 times higher anxA5 concentration in seminal plasma compared to our findings. A possible explanation for this difference in anxA5 concentration is the method used to measure these concentrations. Christmas et al.[20] pooled all there seminal plasma, purified the annexins present in the seminal plasma using an affinity column coated with phospholipids to which the annexins bind, and thereafter used SDS-PAGE and immunoblot analysis to determine if anxA5 was present in seminal plasma. The concentrations of anxA5 in seminal plasma were estimated by comparing the immunostaining intensity of immunoreactive bands with known standards of placental anxA5. In comparison to our ELISA procedure the method used by Christmas et al.[20] is only semiquantitative which may explain the higher yields of anxA5 from seminal plasma. Christmas et al.[20] further state that the annexins in seminal plasma are actively secreted by the prostate, it being the main producer of seminal plasma anxA5, which is in agreement with our findings. They exclude the epididymis as a possible source of annexin because the seminal plasma annexin concentrations are not changed by vasectomy. This is consistent with our finding that the anxA5 concentrations were not significantly different between fertile and subfertile males at baseline.

The question remains what the possible link can be between increases in sperm count in subfertile males after folic acid and zinc sulfate intervention and the seminal plasma anxA5 concentration. An interesting hypothesis is the effect of both nutrients on the control of apoptosis. Spontaneous death of certain classes of germ cells has been shown to be a constant feature of normal spermatogenesis in a variety of mammalian species, including man.[26,27] Scarce information is available on the biological significance of apoptosis in spermatogenesis or its possible role in male fertility.[28] Since it is known that endogenous anxA5 binds to apoptotic cells in vivo, possibly the seminal plasma anxA5 concentration reflects the grade of apoptosis of spermatozoa and other cell types involved in spermatogenesis and seminal fluid production.

Another possible link between seminal plasma anxA5 concentration and sperm concentration is related to the function of anxA5 as an inhibitor of inflammation. It is well known that subfertile men have higher leukocyte numbers in their semen compared to their fertile counterparts, the frequency of leukocytospermia (>10[6] white blood cells/mL semen) being between 10–20% among infertile males.[29] Sperm damage by white blood cells can amongst others be mediated by proteases and cytokines, released during inflammation reactions. Since anxA5 inhibits inflammation it could have a protective effect in these situations, keeping sperm counts up.

In conclusion, the results presented in the present paper do not support that anxA5 is associated with male factor subfertility. Intervention with folic acid and zinc sulfate does not affect seminal plasma anxA5 concentration. Therefore, it is not very likely that the observed increase in sperm count after intervention can be attributed to a possible decrease in apoptosis rate of cells involved in spermatogenesis or protection to inflammation by endogenous seminal plasma anxA5 concentration. Further research is needed to clarify the underlying mechanisms responsible for the observed increase in sperm count after folic acid and zinc sulfate intervention.

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