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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...
calcium binding proteins 93

enzyme-linked immunosorbent assay (ELISA) (ZYMUTEST anxA5, Hyphen with a Makler counting chamber. 

and stored at -80˚C until assayed. Sperm concentration was determined 

10 minutes. The supernatant seminal plasma was frozen without preserva-

aliquot of semen was centrifuged at 1,400 x g (Hettich 16A, 1323 rotor) for 

production to the fertility laboratory. In this hour the participants were 

intervention treatment with folic acid and zinc sulfate on anxA5 

in male factor subfertility. Furthermore, we evaluated the effect of 

explored the possibility that endogenous anxA5 is directly involved 

observation that anxA5 is abundantly present in seminal plasma, we 

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 
to r ehydrate the tissues. Next, the sections were blocked with human 

serum albumin (0.1%) containing 50mM tris-buffered salt buffer, pH 7.4 

and to. The nucleus was counterstained blue with Mayer’s Haematoxylin (Merck, 

Burlingame, CA, USA). The presence of anxA5 is notified by a red color. 

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 seminal plasma anxA5 concentration was investigated by sandwich 

enzyme-linked immunosorbent assay (ELISA) (ZYMUTEST anxA5, Hyphen 

BioMed, Andrésy, France) as described by van Heerde et al. 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 
tests, prostate or seminal vesicles. To determine whether the prostate or the 
tests is the main producer of anxA5, anxA5 messenger RNA (mRNA) was 
measured and the anxA5 antigen was stained in human prostate and tests 
sections. Complementary DNA (cDNA) was synthesized by using 1 µg 
RNA of prostate tissue and tests (Clontech, Palo Alto). Tests 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 
and 10 min at 95˚C. Primer-probe combinations for the anxA5 cDNA were 
designed using PRIMER-EXPRESS software. De forward primer 
CCACCATGCTGTCATGTCC, the reverse primer AGTCACAGTGC-
CTCTGAGAACCT , and the minor groove-binding probe CTGACCT-
AGTAAGAACCT; and the minor groove-binding probe CTGACCT-
AGTAAGAACCT 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/Priism 7700 Sequence 
detector. Applied Biosystems). The expression of porphobilinogen deaminase 
(PBGD), a low copy number housekeeping gene, was measured in duplicate 
ono to each sample to normalize for PCR and cDNA input variations. The 
anxA5 mRNA concentrations were measured in duplicate and analyzed with 
Taqman software. The results were expressed as delta cycle threshold (ΔCt) 
in which 8Ct = Ct (PBGD) minus Ct (AnxA5). The relative difference in 
expression is calculated by Comparative Ct method using the equation 2-ΔΔCt. 

Immunohistochemistry. Po st mortem human paraffin-embedded 
prostate and tests 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 
tests 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 deter-

minants. 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
for males receiving the folic acid, zinc sulfate, or combined intervention treatment. The p values were two tailed and p ≤ 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, respectively. AnxA5 mRNA concentrations were not significantly different between fertile and 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 (8Ct 5.0) as compared to the testis (8Ct 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 tests 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 these 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 semi-quantitative which may explain the higher yields of anxA5 from seminal plasma. Christmas et al.20 further state that the annexins in seminal plasma were 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.

Table 1A The effect of interventions on seminal plasma anxA5 concentrations (µg/mL) in fertile males

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

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 anxA5 concentrations (µg/mL) in subfertile males

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

NB, data are the median (25th–75th percentile). Deltas are calculated as post-intervention-preintervention value.
Another possible link between seminal plasma annexin A5 concentration and sperm concentration is related to the function of annexin A5 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. Sperm damage by white blood cells can amongst others be mediated by proteases and cytokines, released during inflammation reactions. Since annexin A5 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 annexin A5 is associated with male factor subfertility. Intervention with folic acid and zinc sulfate does not affect seminal plasma annexin A5 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 annexin A5 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.

References


