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ORIGINAL RESEARCH

Feedback Regulation of sPLA2-COX/5-LOX-Ca2+ in Seminal Plasma and Its Impact on Sperm Quality Parameters

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Background: Male reproductive health is of growing concern. Sperm quality declines due to multiple factors. The role of AA metabolic network in sperm quality is unclear, and AA/COX's protection under heat stress needs study.

Aim: This study aimed to investigate the relationship between seminal plasma arachidonic acid (AA) metabolic network markers and sperm quality, as well as explore the protective effects of AA and cyclooxygenase (COX) on sperm under heat stress.

Methods: We analyzed 164 seminal plasma samples from 164 male infertility patients, categorized by sperm concentration and motility (PR < 32% vs PR ≥ 32%). A heat-stressed weak spermatogenesis model (37–42°C) was established, and AA/COX were added in vitro to assess their impact on sperm quality.

Results: Significant correlations were found between AA pathway markers (PL, sPLA2, AA, COX1/2, PGE1, PGF2α, 5-LOX, LTB4) and sperm parameters (motility, acrosome reaction, mitochondrial function, DNA fragmentation). A predictive model combining PL, AA, and COX1 effectively assessed sperm quality. In vitro, 100 pg AA ± 300 pg COX1 protected sperm at 42°C by upregulating COX2, PGE1, and PGF2α while reducing LOX/LTB4 and modulating Ca^{2+} levels, improving acrosome reactivity and reducing oxidative stress.

Conclusion: Seminal plasma AA metabolism strongly influences sperm quality, likely via sPLA2-COX/5-LOX-Ca^{2+} feedback mechanisms. The PL-AA-COX1 model may serve as a sperm quality predictor, and AA/COX1 supplementation could protect sperm under heat stress.

Keywords: seminal plasma, sPLA2-COX/5-LOX-Ca^{2+}, positive and negative feedback, spermatozoa, quality

Introduction

Sperm production initiates in the testicles, matures within the epididymis, and during sexual arousal, traverses the vas deferens before its release, combined with epididymal fluid, seminal vesicle fluid, and prostatic secretions, to form semen.[1,2] Sperm count regulation occurs within the testicles, while sperm motility is influenced by the composition of seminal plasma, which comprises multiple components impacting motility.[3]

Notably, the metabolism of phospholipids (PL), arachidonic acid (AA), and their derivatives significantly alters sperm membrane fluidity, membrane integrity, and overall motility.[4] In human sperm, polyunsaturated fatty acids (PUFAs) make up approximately 20–30% of the total fatty acids in the sperm membrane.[5] Although human sperm membrane has a lower PUFA content compared to that of livestock such as bull, boar, and stallion sperm, this relatively small amount of PUFA still renders the human sperm membrane susceptible to oxidative stress (OS). PUFAs contain multiple double bonds, which are vulnerable to attack by reactive oxygen species (ROS). When ROS react with PUFAs in the sperm membrane, lipid peroxidation occurs. This process leads to the formation of lipid peroxides, which can disrupt the membrane structure, alter membrane fluidity, and damage membrane-associated proteins and ion channels. As a result,

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sperm function is impaired, including reduced motility and viability, and an increased likelihood of DNA damage, all of which can compromise male fertility.[6] Secretory phospholipase A2 (sPLA2), 5-lipoxygenase (5-LOX), and cyclooxygenase (COX) function as rate-limiting enzymes within lipid metabolic pathways, making the investigation of sPLA2, 5-LOX, COX, and their metabolic products crucial for understanding the role of lipid metabolism in sperm function and for suggesting potential therapeutic targets for asthenozoospermia management.[7,8]

Fertility assessment is a complex process and should not be based solely on sperm motility. Other crucial parameters, such as the hypo-osmotic swelling (HOS) response, acrosome integrity, protamine deficiency, and DNA integrity, also play significant roles in determining sperm fertilizing potential.[9] The HOS response reflects the integrity of the sperm membrane and its ability to respond to osmotic stress, which is essential for sperm survival and function during fertilization. Acrosome integrity is necessary for the sperm to penetrate the zona pellucida of the egg. Protamine deficiency can lead to abnormal sperm chromatin packaging, affecting DNA stability and potentially leading to infertility. DNA integrity is fundamental as damaged sperm DNA can result in failed fertilization, early pregnancy loss, or genetic disorders in the offspring.

Previous studies have shown that COX and 5-LOX enzymes mediate sperm motility through the regulation of the CatSper1 channel.[10] However, it remains unclear whether these enzymes influence other fertility-related parameters. For instance, it is unknown if the activity of COX and 5-LOX is associated with the upregulation or downregulation of the HOS response, acrosome integrity, protamine levels, or DNA integrity. Understanding these potential relationships could provide a more comprehensive view of how lipid metabolism, regulated by sPLA2, 5-LOX, and COX, impacts sperm function and male fertility. This knowledge could also suggest potential therapeutic targets not only for asthenozoospermia management but also for other fertility-related issues associated with abnormal sperm function.[7,8]

PLA2, secreted by the prostate, seminal vesicles, and epididymis in males, encompasses two primary families with distinct roles: the cytoplasmic PLA2 (cPLA2) family, which primarily participates in the acrosome reaction, and the sPLA2 family, involved in seminal plasma lipid metabolism, both necessitating Ca^{2+} activation.[11,12] Anfuso et al reported that sPLA2 levels in sperm heads were significantly elevated in individuals with normal fertility compared to those in infertile subjects, with an inverse relationship observed between seminal plasma sPLA2 content and sperm motility.[13] Additionally, Sato et al demonstrated that Pla2g3 knockout in male mice led to reduced sperm motility and decreased tail whipping frequency, implicating sPLA2 as a potential regulator of sperm motility.[14]

In recent years, dietary shifts have led to a rise in overall fat intake, altering the body's polyunsaturated fatty acid (PUFA) profile, with imbalances notably observed in the proportions of linoleic acid, linolenic acid, and AA, extending to seminal composition.[15,16] AA, particularly sensitive among fatty acids, generates leukotriene B4 (LTB4) and other inflammatory mediators through 5-LOX activity. Adipose tissue contributes approximately 30% of the body's inflammatory mediators even under non-inflammatory states.[17] Seminal fat cells similarly produce LTB4 in non-inflammatory contexts, impacting physiological functions.[18,19] Yao et al[10] demonstrated that LTB4 modulated CatSper1 in the sperm midpiece by altering Ca2+ flux, which, along with the fact that CatSper1 modulates sperm motility by regulating Ca2+ influx as reported by Neuschäfer-Rube et al,[20] suggests that even under physiological conditions, 5-LOX and LTB4 modulate sperm motility via the CatSper1 channel.

Intrinsic COX1 and inducible COX2 represent two forms of COX, each playing distinct yet complementary roles in maintaining physiological stability and responding to inflammatory injury.[21] COX1 supports baseline physiological functions,[22] whereas COX2 is activated specifically in response to inflammatory stimuli,[23] with both pathways working together to support bodily defense mechanisms. Using AA as a substrate, COX produces various types of prostaglandins (PGs), including PGE and PGF2α, which interact within the midpiece of sperm.[24] Our team's preliminary findings suggest that COX1 levels may serve as a physiological marker for evaluating sperm retrieval status, with PGE and PGF2α positively correlated with sperm quality, highlighting their role in modulating sperm motility.

In this study, we formulate the scientific hypothesis that (Figure 1), the activation of seminal plasma sPLA2 by Ca^{2+}, initiating its interaction with glycerophospholipids to produce AA. Subsequently, AA served as a substrate for 5-LOX and COX enzymes, which catalyzed the formation of LTB4 and PGE, PGF2α. These compounds then targeted the sperm CatSper1 channel, inducing Ca^{2+} influx and enhancing sperm motility. Over time, extracellular Ca^{2+} levels gradually decreased, leading to a reduction in sPLA2 activity and a subsequent decline in sperm motility. This feedback mechanism

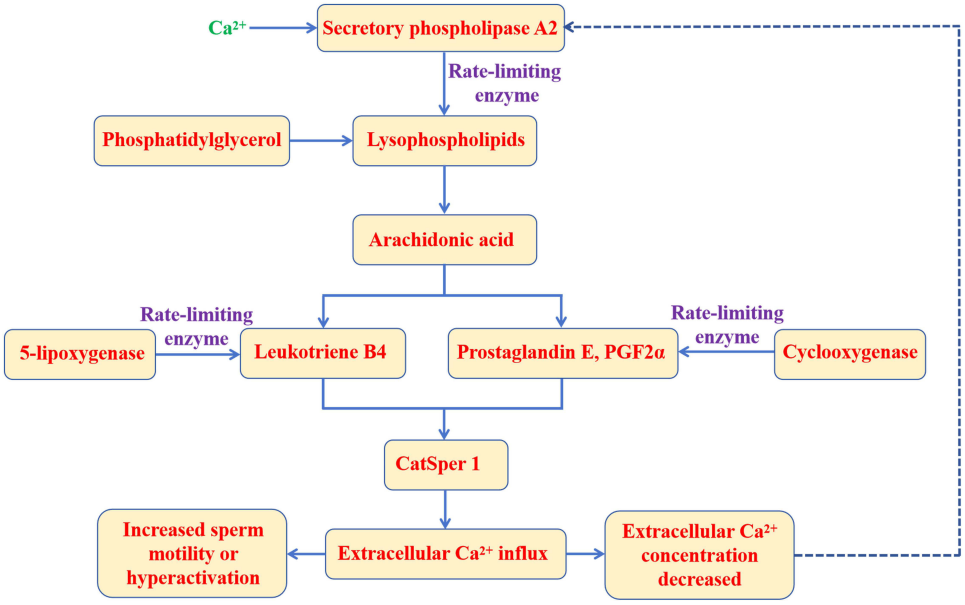

Figure 1 Schematic diagram of the team's scientific hypothesis.

within the seminal plasma sPLA2-COX/5-LOX-Ca^{2+} pathway thus orchestrates both the stimulation and attenuation of sperm motility.

This schematic illustrates the scientifically hypothesized mechanism that regulates sperm motility in semen. Ca^{2+} activates secretory phospholipase A2 (sPLA2), which acts as the rate-limiting enzyme on phosphatidylglycerol to produce lysophospholipids and arachidonic acid (AA), which then serve as substrates for 5-lipoxygenase (5-lipoxygenase) and 5-lipoxygenase (5-lipoxygenase) respectively. (sPLA2) acts as a rate-limiting enzyme on phosphatidylglycerol to produce lysophospholipids and arachidonic acid (AA), which then serves as a substrate for leukotriene B4 (Leukotriene B4), which is catalyzed by 5-lipoxygenase (5-LOX, the rate-limiting enzyme) and cyclic oxidase (Cyclooxygenase, COX, the rate-limiting enzyme) respectively. B4 (Leukotriene B4, LTB4) as well as Prostaglandin E (PGE) and Prostaglandin F2α (PGF2α) catalyzed by cyclooxygenase and cyclooxygenase. These compounds act on the CatSper1 channel in spermatozoa to induce the inward flow of extracellular Ca^{2+}, thereby enhancing sperm motility or hyperactivating them. Over time, the concentration of extracellular Ca^{2+} decreases, leading to a decrease in sPLA2 activity and, consequently, a decrease in sperm motility. This mechanism regulates the stimulation and reduction of sperm motility through a feedback mechanism in the sPLA2-COX/5-LOX-Ca^{2+} pathway in semen.

Materials and Methods

Introductory Paragraph

We have proposed a technical route design based on the above scientific hypotheses (Figure 2), we evaluated the AA metabolic network and sperm quality parameters in semen from both normal and asthenozoospermia groups, identifying the protective effect of AA and COX1 on sperm quality under heat stress through in vitro supplementation. Results confirmed that the seminal plasma sPLA2-COX/5-LOX-Ca^{2+} pathway operated under both positive and negative feedback, influencing sperm quality. The research was oriented toward addressing clinical needs, offering objective theoretical insights for diagnostic and therapeutic applications.

Collection of Semen Samples

Semen samples were collected from 164 male infertility patients who attended the male department of Yinchuan Maternity and Child Health Hospital from January 2021 to January 2024. All participants have signed a written informed

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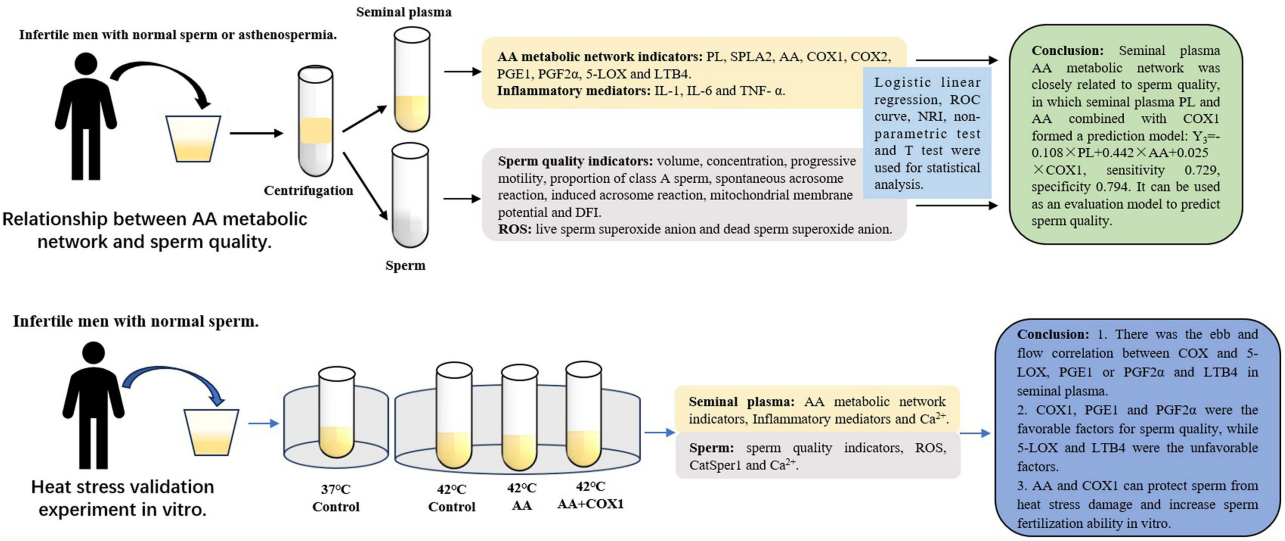

Figure 2 Technical roadmap.

consent. This study was approved by the Ethics Committee of Yinchuan Maternity and Child Health Hospital and complied with the Declaration of Helsinki.

Samples inclusion criteria: specified the selection of semen samples from infertile males with a body mass index (BMI) of 18.5–23.9 kg/m² and sperm concentrations of ≥ 15×10^6/mL. Based on progressive motility (PR), samples were categorized into an experimental group (< 32%) and a control group (≥ 32%).

Patient exclusion criteria: encompassed recent medication use, urogenital infections, presence of varicocele, chromosomal anomalies, and systemic illnesses including diabetes and hypertension.

Semen Sample Processing

Male infertility patients meeting eligibility criteria abstained for 2–7 days before providing semen samples via masturbation, which were then collected in sterile cups and liquefied in a 37°C incubator. Post-liquefaction, the samples underwent centrifugation at 800×g for 20 minutes, with seminal plasma recovered from the upper layer for further preparation. The sperm pellet was washed by re-suspending it in 5 mL physiological saline solution, thoroughly mixed, and by centrifugation at 800×g for 20 minutes, in triplicate. This wash step was repeated twice, after which saline was added to achieve a sperm concentration of 5×10^6/mL, readying the sample for subsequent use.

The sperm suspension (1 mL) was centrifuged at 800×g for 20 min, after which the supernatant was discarded. A cell extract (0.5 mL) was introduced, followed by sperm disruption using a non-contact ultrasonic processor (BILON-R500, Shanghai Bilon Instrument Manufacturing Co., Ltd., Shanghai) at 200W power, with a 3-second ultrasound duration and a 7-second interval repeated 30 times. The sample was then centrifuged at 4°C, 8000×g for 10 min, and the resulting supernatant was collected for subsequent analysis.

Detection of Sperm Quality Parameters

Semen analysis was conducted using a computer-assisted sperm dynamics analyzer (SAS-II, SAS Medical Technology Co., Ltd., Beijing) to measure sperm concentration and assess the percentage of sperm exhibiting PR.

The sperm DNA fragmentation index (DFI) was assessed via flow cytometry (EasyCell, Shenzhen Wellgrow Biotechnology Co., Ltd., Shenzhen) and laser confocal fluorescence microscopy (Leica TCS SP8 X, Leica Microsystems Trading Co., Ltd., Shanghai). Acridine orange staining (Hunan Wellgrow Biotechnology Co., Ltd., Hunan) quantified DFI, while JC-1 fluorescent dye (Hunan Wellgrow Biotechnology Co., Ltd., Hunan) was employed to evaluate mitochondrial membrane potential. Calcium ion carrier A23187, anti-CD46-FITC fluorescent dye staining, and PI fluorescent dye [Celula (China) Medical Technology Co., Ltd., Sichuan] were utilized to assess sperm viability

and acrosome reaction capacity. For detecting hydrogen peroxide levels in sperm plasma membrane and mitochondrial superoxide, DCFH-DA combined with MitoSOX Red fluorescent dye (Shenzhen BRED Life Science Technology Inc., Shenzhen) was used.

Quantification of Indicators in Seminal Plasma AA Metabolic Network

In this study, we had two main groups: the experimental group and the control group as described in Introductory Paragraph. For the quantification of seminal plasma concentrations of PL, sPLA2, AA, COX1, COX2, PGE1, PGF2α, 5-LOX, LTB4, IL-1, and IL-6, we used ELISA assays from Shanghai Duma Biotechnology Co., Ltd., Shanghai.

For the experimental and control groups alike, the following ELISA assay types were applied for each analyte. For the quantification of PL, a competitive ELISA assay was employed. The sPLA2 levels were measured using a direct ELISA assay. AA was quantified with an indirect ELISA assay. COX1 and COX2 concentrations were determined by competitive ELISA assays. PGE1 and PGF2α were assayed using direct ELISA assays. 5-LOX and LTB4 were measured with indirect ELISA assays, while IL-1 and IL-6 levels were quantified using competitive ELISA assays. This approach ensured consistent measurement across both the experimental and control groups, without any sub-division within this quantification process other than the differentiation between these two main study groups.

In vitro Heat Stress Experiment of Human Semen and Allocation

Semen samples from infertile men with normal BMI (sperm concentration $\geq 15\times10^6$ and proportion of sperm with PR $\geq$ 32%) were assigned to two main conditions, cultured in incubators set to 37 °C and 42 °C. Each temperature condition was further subdivided into three groups: a control group, a group with added AA (Merck Co., Ltd., Germany, LOT: 4051772), and a group with combined AA+COX1 (Merck Co., Ltd., Germany, LOT: SLCF6929). Based on variations in the concentrations of AA (10 pg, 1 ng, 100 ng, 10 μg; 10 pg, 100 pg, 500 pg, 1 ng, 5 ng) and COX1 (300 pg), these groups were subsequently divided into specific subgroups for incubation. Each subgroup was treated with 0.5 mL semen and 10 μL of the designated reagent.

Detection of Sperm CatSper1 by Real-Time qPCR

For qPCR analysis, fluorescence-based quantitative PCR was carried out on an ABI 7500 system (Thermo Fisher Scientific, USA), following the instructions provided with the corresponding kit. The total RNA of spermatozoa was extracted by Trizol method at 4°C, and the sample RNA was reverse transcribed into cDNA using PrimeScriptTM RT-PCRKit (RR096A, TaKaRa Co., Ltd., Beijing, China). cDNA was used as a template for detecting the relative expression of CatSper1 gene using real-time fluorescence quantitative PCR. 20 μL of PCR system was used for the detection of *CatSper1* gene, and the cDNA template (50 ng/μL) 2 μL was used for the detection of *CatSper1* gene. The PCR reaction system was 20 μL: cDNA template (50 ng/μL) 2 μL, SYBRPremixExTaqTM II (2×) 10 μL, upstream and downstream primers (10 μmol/L) 0.8 μL each, and ddH_2O 6.4 μL. The PCR reaction conditions were as follows: 95 °C for 50s, 95 °C for 5s, and 60 °C for 30s for a total of 40 cycles; 95 °C for 15s, 60 °C for 1 min, and 95 °C for 1.5s. The β-activator was used as a template. The relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$ method with β-actin as the internal reference gene. The CatSper 1 qRT-PCR primers: F, CGGAACCTGACCCAATC; R, CTCTCCTGCTTCGCTTT.

Detection of Sperm CatSper1 Using Western Blotting

Protein extraction from the selected sperm was conducted using the bicinchoninic acid (BCA) method (Jiangsu Keygen Biotechnology Co., Ltd., Nanjing, China), followed by SDS-PAGE gel separation (Jiangsu Keygen Biotechnology Co., Ltd., Nanjing, China). The CatSper1 primary antibody (ab165120) sourced from Abcam (Shanghai, China) Co., Ltd., was applied, and detection was achieved with an HPR-conjugated Affinipure Goat Anti-Rabbit IgG secondary antibody from Proteintech Group, Inc.

Statistical Analysis

Data were organized in EXCEL and analyzed via SPSS 17.0. For measurement data, nonparametric tests and *t*-tests, contingent on variance homogeneity, determined statistical significance, with results presented as □x ± s or M (25%, 75%). A predictive model was formulated through Logistic regression, assessing sensitivity and specificity by analyzing the area under ROC curve and Youden index. The net reclassification index (NRI) evaluated the comparative performance of relevant indicators.

Results

Correlation of Seminal Plasma AA Metabolic Network Indicators with Sperm Quality

Indicators within the seminal plasma AA metabolic network (PL, SPLA2, AA, COX1, COX2, PGE1, PGF2α, 5-LOX, and LTB4) demonstrated strong associations with sperm quality parameters, including semen volume, sperm concentration, PR percentage, grade A sperm proportion, spontaneous and induced acrosome reactions, mitochondrial membrane potential, superoxide anion presence in both live and dead sperms, and DFI. Among these, a predictive model comprising seminal plasma PL, AA, and COX1 was constructed: Y3 = −0.108×PL + 0.442×AA + 0.025×COX1, achieving a sensitivity of 0.729 and specificity of 0.794. Using the Y3 model's cutoff value as a reference, predictions of spontaneous and induced acrosome reactions, superoxide anions in live and dead sperms, and DFI showed significant improvement compared to sperm motility alone.

Correlation Between Seminal Plasma AA Metabolic Network Indicators and Sperm Quality Parameters

Semen volume correlated positively with PL ($P < 0.05$), while sperm concentration showed an inverse relationship with COX1 and PGF2α ($P < 0.05$). The PR proportion correlated positively with COX1 and IL-1 ($P < 0.05$), and spontaneous acrosome reaction was inversely associated with COX1 and LTB4 ($P < 0.05$). Induced acrosome reaction exhibited a positive correlation with COX1 and PGE1 ($P < 0.05$). Mitochondrial membrane potential showed positive correlations with PL, sPLA2, COX1, and PGE1 ($P < 0.05$). Live sperm superoxide anion levels were positively associated with TNF-α and IL-6 ($P < 0.05$), whereas dead sperm superoxide anion levels were negatively correlated with PL, AA, COX1, COX2, PGE1, 5-LOX, LTB4, and IL-1 ($P < 0.05$). Additionally, DFI displayed a negative correlation with sPLA2, AA, COX1, IL-1, and IL-6 ($P < 0.05$) (Figure 3).

Correlation Analysis of Various Indicators in Seminal Plasma AA Metabolic Network

Significant positive correlations were observed among PL, sPLA2, AA, COX1, COX2, PGE1, PGF2α, 5-LOX, and LTB4, with the exception of PGF2α and LTB4, which showed no significant association ($P > 0.05$). IL-1 displayed a positive correlation with PL, sPLA2, AA, COX1, COX2, PGE1, PGF2α, 5-LOX, and LTB4 ($P < 0.05$). Conversely, IL-6 correlated positively with PL, COX1, COX2, PGF2α, and 5-LOX ($P < 0.05$) but negatively with LTB4 ($P < 0.05$) (Figure 4).

ROC Analysis of Seminal Plasma AA Metabolic Network Indicators

With a significance level of $P < 0.05$, the AUC values for AA and COX1 demonstrated statistical relevance. Sensitivity and specificity values were observed at AA (0.500, 0.794) and COX1 (0.688, 0.618), respectively (Table 1).

Logistic Linear Regression Analysis and Equation Setting of Seminal Plasma AA Metabolic Network Indicators

Logistic linear regression analysis identified indicators with $P < 0.05$ and $P < 0.1$, from which four indicators ($P < 0.1$) were selected and combined into five predictive equations. ROC curve analysis of these equations revealed that equation Y3 = −0.108 × PL + 0.442 × AA + 0.025 × COX1 achieved a sensitivity of 0.729 and specificity of 0.794, both exceeding 0.7, indicating its potential as an effective predictive marker (Table 2 and Table 3).

Comparison of Groups Based on Sperm Motility and Y3 Equation Cutoff Value

Among semen parameters, significant differences ($P < 0.05$) were observed between the two groups (normal vs asthenozoospermia) in dead sperm superoxide anion levels, DFI, and HDS when grouped by sperm motility. Additionally, when grouped by the Y3 equation cutoff value, significant differences ($P < 0.05$) were noted in

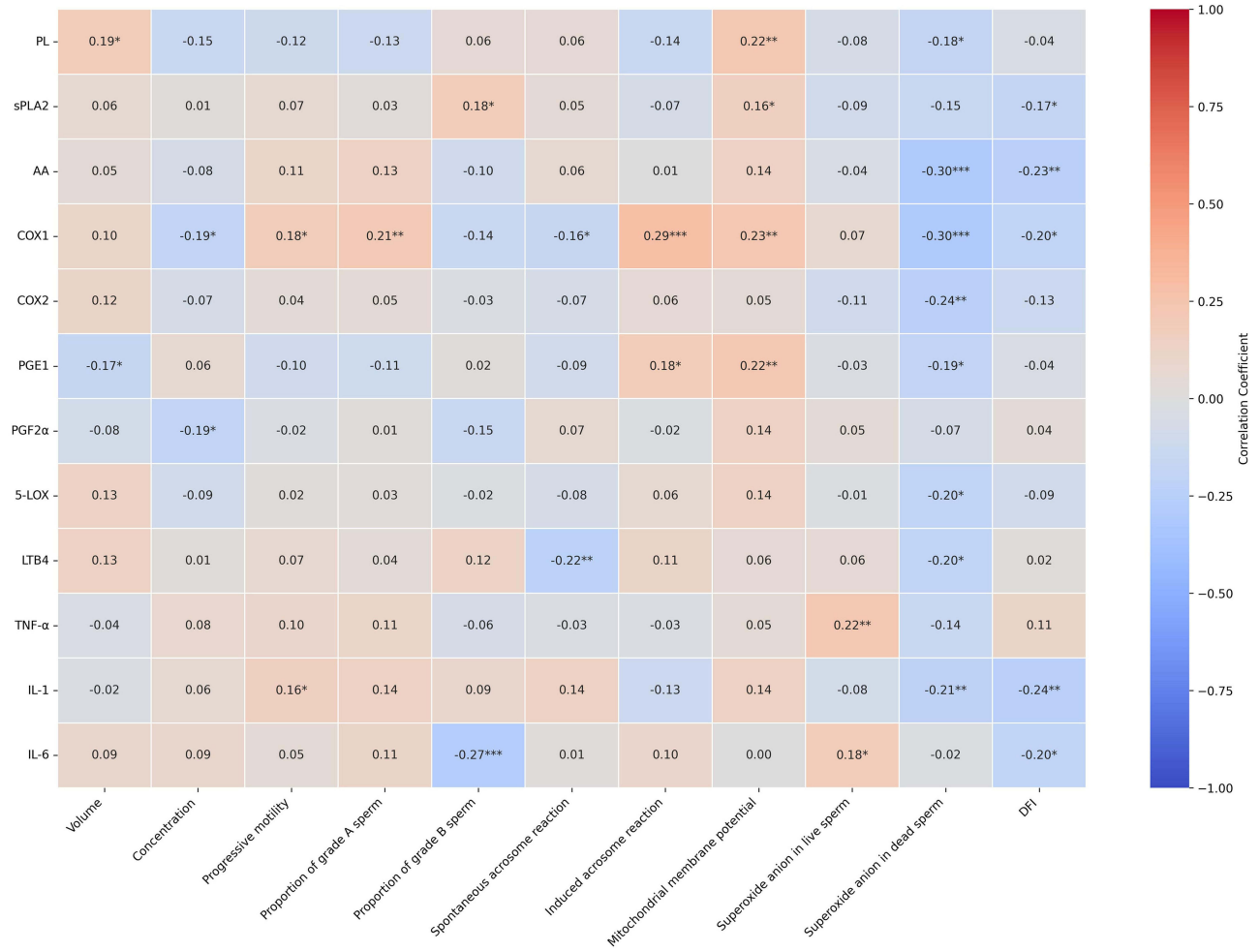

Figure 3 Correlation analysis of seminal plasma AA metabolic network indicators and semen routine indicators. n = 164; ***p<0.001, **p<0.01, *p<0.05.

spontaneous and induced acrosome reactions, superoxide anion in live and dead sperms, DFI, and HDS. No statistically significant differences ($P > 0.05$) were found between the two grouping methods for any parameters examined (Table 4).

In the analysis of seminal plasma AA metabolic network parameters, significant differences in AA and COX1 levels ($P < 0.05$) were observed between groups categorized by sperm motility. When grouped by the Y3 equation Cutoff value, significant differences ($P < 0.05$) emerged across all parameters, excluding PL, specifically in sPLA2, AA, COX1, COX2, PGE1, PGF2α, 5-LOX, LTB4, TNF-α, IL-1, and IL-6. Additionally, in a comparison of grouping methods, COX1 categorized by the Y3 equation Cutoff demonstrated higher discriminatory power than grouping by sperm motility ($P < 0.05$), while other indicators showed no significant differences ($P > 0.05$) (Table 4).

Comparison of Groups Based on Sperm Motility and COX1 Cutoff Value

Significant differences were observed in semen parameters when grouped by the COX1 Cutoff value, specifically in induced acrosome reaction, sperm mitochondrial membrane potential, and superoxide anion levels in both live and dead sperms ($P < 0.05$). No statistically significant differences were identified when comparing the corresponding indicators between the two grouping forms ($P >0.05$) (Table 5).

In the seminal plasma AA metabolic network parameters, grouping by COX1 Cutoff value demonstrated statistically significant differences in all parameters except TNF-α, including PL, sPLA2, AA, COX1, COX2, PGE1, PGF2α, 5-LOX, LTB4, IL-1, and IL-6 ($P < 0.05$). Additionally, in comparing the indicators between grouping forms, COX1 grouping yielded notably higher significance in PL, COX1, PGE1, and 5-LOX values than sperm motility grouping ($P < 0.05$), while no significant differences were found in other indicators ($P >0.05$) (Table 5).

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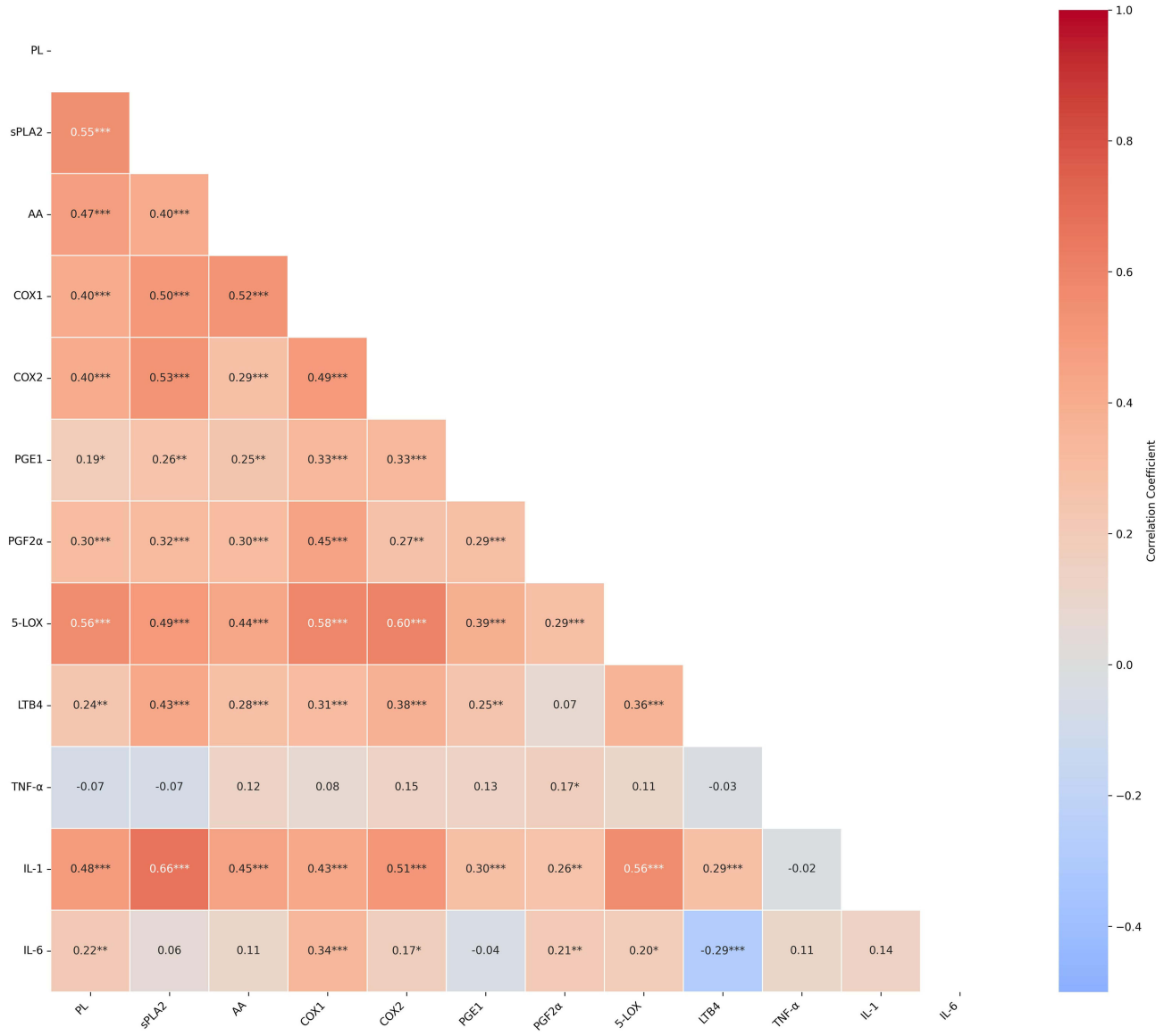

Figure 4 Correlation analysis of various indicators in seminal plasma AA metabolic network. n = 164; ***p<0.001, **p<0.01, *p<0.05.

Application of Induced Acrosome Reaction in Calculating NRI to Assess the Predictive Value of the Y3 Equation and COX1

The NRI for the Y3 equation was 0.309, indicating a 30.9% improvement in predicting induced acrosome reaction over sperm motility alone. Similarly, the NRI for COX1 was 0.302, reflecting a 30.2% enhancement in predictive accuracy compared to sperm motility (Table 6).

Application of DFI to Calculate NRI to Determine the Predictive Value of Y3 Equation and COX1

The NRI calculated using the Y3 equation stood at 0.106, indicating that the DFI predicted by the Y3 equation surpassed the predictive capacity for sperm motility by 10.6%. In comparison, the NRI of COX1 was 0.053, signifying a 5.3% increase in predictive accuracy for DFI over sperm motility (Table 7).

Establishment of Heat Stress-Induced Asthenozoospermia Model

Previous research by Kurhanewicz et al[25] has demonstrated that even a minor elevation in testicular temperature can detrimentally affect sperm quality. Given that the normal human body temperature is approximately 37°C, this value was set

Table 1 ROC Analysis of Seminal Plasma AA Metabolic Network Indicators

	AUC	*P*	Cutoff	Sensibility	Specificity
PL	0.457	0.350	30.705	0.438	0.647
sPLA2	0.521	0.645	7.235	0.208	0.941
AA	0.638	0.003	6.925	0.500	0.794
COX1	0.649	0.001	270.45	0.688	0.618
COX2	0.538	0.404	165.15	0.229	1.000
PGE1	0.466	0.459	422.74	0.313	0.794
PGF2α	0.550	0.274	468.41	0.292	0.882
5-LOX	0.487	0.779	93.28	0.104	0.971
LTB4	0.540	0.382	19.70	0.292	0.912

Abbreviations: PL, phospholipids; sPLA2, Secretory phospholipase A2; AA, arachidonic acid; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; PGE1, Prostaglandin E1; PGF2α, Prostaglandin F2α; 5-LOX, 5-lipoxygenase; LTB4, leukotriene B4.

Table 2 Logistic Linear Regression Analysis of Seminal Plasma AA Metabolic Network Indicators

	B	P	OR (95% CI)
PL	0.108	0.012	1.114 (1.024, 1.213)
sPLA2	0.054	0.883	1.055 (0.517, 2.151)
AA	−0.442	0.094	0.643 (0.383, 1.078)
COX1	−0.025	0.011	0.975 (0.957, 0.994)
COX2	−0.017	0.238	0.983 (0.956, 1.011)
PGE1	0.010	0.051	1.010 (1.000, 1.021)
PGF2α	−0.003	0.499	0.997 (0.989, 1.005)
5-LOX	0.038	0.233	1.039 (0.976, 1.107)
LTB4	−0.078	0.429	0.925 (0.764, 1.121)
Constant	3.648	0.147	38.401

Abbreviations: PL, phospholipids; sPLA2, Secretory phospholipase A2; AA, arachidonic acid; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; PGE1, Prostaglandin E1; PGF2α, Prostaglandin F2α; 5-LOX, 5-lipoxygenase; LTB4, leukotriene B4.

Table 3 ROC Analysis of the Five Equations

	Equation	AUC	*P*	Cutoff	Sensibility	Specificity	OR (95% CI)
P<0.05	Y_1=−0.108×PL+0.025×COX1	0.690	0.000	3.621	0.604	0.794	0.365 (0.218–0.611)
P<0.1	Y_2=−0.108×PL+0.442×AA+0.025×COX1-0.010×PGE1	0.734	0.000	2.493	0.688	0.735	0.362 (0.231–0.567)
N0 PGE1	Y3=−0.108×PL+0.442×AA+0.025×COX1	0.721	0.000	6.529	0.729	0.794	0.402 (0.261–0.620)
No AA	Y_4=−0.108×PL+0.025×COX1-0.010×PGE1	0.689	0.000	0.605	0.750	0.588	0.375 (0.231–0.608)
AA+COX1	Y_5=0.442×AA+0.025×COX1	0.663	0.000	9.863	0.604	0.735	0.579 (0.403–0.833)

Abbreviations: AA, arachidonic acid; COX1, cyclooxygenase 1; PGE1, Prostaglandin E1.

as the baseline for comparison. Temperatures of 39°C, 40°C, and 42°C, which are above normal body temperature, were selected to investigate the impact of heat stress on sperm. In the experiment, normal semen samples, defined as having a concentration of ≥ 15×10^6 and a progressive motility (PR) proportion of ≥ 32%, were cultured at 37°C, 39°C, 40°C, and 42°C. Exposure to 42°C for 1 hour successfully induced an asthenozoospermia model, leading to an average 22.49% reduction in sperm motility. A step-by-step decrease in motility was observed with each incremental increase in temperature. Specifically, sperm motility at 42°C was

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Table 4 Comparison of Metabolic Network-Related Indicators Grouped by Sperm Motility and Grouped by Y3 Equation Cutoff Value and AA Metabolism Network

	Grouped by Vitality				Grouped by Y_3 Equation				Normal VS ≥6.529		Asthenospermia VS <6.529	
	Normal (n=96)	Asthenospermia (n=68)	*Z*	*P*	≥6.529 (n=84)	<6.529 (n=80)	*Z*	*P*	*Z*	*P*	*Z*	*P*
Volume (mL)	3.29 (2.41, 4.67)	3.57 (2.97, 4.10)	0.968	0.333	3.33 (2.20, 4.81)	3.51 (3.01, 3.96)	1.178	0.239	0.252	0.801	0.077	0.939
Concentration (10^6/mL)	57.27 (30.65, 93.73)	52.48 (24.29, 99.46)	0.628	0.530	58.97 (31.00, 94.82)	46.67 (22.87, 106.20)	0.908	0.364	0.178	0.859	0.100	0.920
Progressive motility (%)	49.10 (42.62, 57.05)	23.64 (16.36, 28.99)	10.521	0.000	44.80 (38.80, 54.02)	28.85 (20.60, 43.55)	5.185	0.000	2.254	0.024	3.040	0.002
Portion of grade A sperm (%)	41.25 (34.65, 52.12)	16.24 (8.76, 21.43)	10.895	0.000	38.35 (28.27, 49.55)	21.18 (14.90, 35.16)	5.646	0.000	2.139	0.032	3.087	0.002
Spontaneous acrosome reaction (%)	62.03 (51.26, 70.92)	58.67 (47.56, 66.07)	1.589	0.112	58.67 (47.41, 64.86)	61.05 (53.11, 71.64)	2.290	0.022	1.921	0.055	1.932	0.053
Induced acrosome reaction (%)	19.42 (10.67, 31.67)	18.91 (16.19, 26.67)	0.561	0.575	26.64 (16.19, 37.04)	17.67 (8.51, 22.80)	4.323	0.000	1.876	0.061	1.894	0.058
Mitochondrial membrane potential (%)	29.08 (22.21, 37.81)	29.67 (23.11, 39.51)	0.260	0.795	30.43 (24.09, 37.88)	27.81 (21.48, 39.34)	1.079	0.281	0.556	0.578	0.816	0.415
Superoxide anion in live sperm (%)	5.79 (3.13, 9.29)	5.41 (2.62, 7.00)	1.442	0.155	6.28 (4.28, 9.89)	4.73 (2.10, 6.79)	2.704	0.007	0.763	0.446	0.500	0.617
Superoxide anion in dead sperm (%)	28.18 (18.39, 38.84)	39.83 (29.18, 49.97)	4.460	0.000	28.18 (20.15, 42.46)	36.31 (23.08, 49.91)	2.369	0.018	0.786	0.432	1.316	0.188
Hydrogen peroxide in live sperm (%)	6.68 (2.96, 7.97)	5.03 (3.24, 11.55)	0.247	0.805	6.08 (3.06, 8.04)	5.57 (3.37, 12.11)	0.790	0.430	0.212	0.832	0.354	0.723
Hydrogen peroxide in dead sperm (%)	6.41 (4.76, 8.83)	6.08 (4.56, 7.64)	0.681	0.496	6.31 (4.69, 8.38)	6.10 (4.28, 9.88)	0.303	0.762	0.453	0.650	0.531	0.595
DFI (%)	6.31 (3.83, 11.44)	15.13 (6.96, 21.60)	4.720	0.000	6.72 (3.70, 11.98)	12.45 (5.96, 20.84)	3.695	0.000	0.192	0.848	0.848	0.396
HDS (%)	6.76 (4.07, 8.72)	3.84 (1.48, 6.99)	3.421	0.001	6.34 (3.91, 8.67)	4.62 (2.32, 7.77)	2.161	0.031	0.363	0.717	0.918	0.359
PL (mg/L)	29.05 (25.91, 32.69)	29.18 (26.56, 34.57)	0.935	0.350	29.05 (25.88, 32.24)	29.18 (26.13)	1.171	0.241	0.212	0.832	0.008	0.994
sPLA2 (μg/L)	6.32 (6.04, 6.93)	6.30 (6.08, 6.90)	0.461	0.645	6.47 (6.10, 7.17)	6.20 (5.91, 6.78)	3.080	0.002	1.388	0.165	1.209	0.227
AA (pmol/L)	6.91 (6.42, 7.27)	6.51 (6.11, 6.85)	3.004	0.003	6.99 (6.51, 7.37)	6.41 (5.96, 6.76)	5.995	0.000	1.755	0.079	1.193	0.233
COX1 (ng/L)	277.43 (261.23, 298.08)	264.81 (246.95, 276.67)	3.238	0.001	286.13 (274.79, 301.32)	252.59 (239.54, 270.17)	8.950	0.000	2.822	0.005	2.949	0.003
COX2 (ng/L)	142.60 (135.43, 162.88)	146.04 (133.01, 155.50)	0.835	0.404	149.29 (136.03, 165.97)	139.26 (130.44, 149.75)	3.284	0.001	1.233	0.217	1.224	0.221
PGE1 (ng/L)	394.03 (359.54, 432.27)	396.21 (377.49, 417.43)	0.741	0.459	401.20 (374.37, 435.56)	388.73 (364.39, 412.75)	2.685	0.007	1.658	0.097	1.771	0.077
PGF2α (pg/L)	434.14 (404.01, 472.42)	432.76 (401.97, 457.63)	1.095	0.274	439.64 (407.42, 477.13)	428.98 (397.89, 450.35)	2.395	0.017	0.717	0.473	0.577	0.564
5-LOX (ng/L)	80.78 (75.86, 87.66)	82.12 (77.43, 87.46)	0.280	0.779	82.18 (79.31, 90.00)	81.05 (74.02, 86.20)	2.586	0.010	1.520	0.128	1.309	0.191
LTB4 (ng/L)	18.37 (17.29, 19.98)	18.26 (17.61, 18.97)	0.875	0.382	18.88 (17.97, 20.11)	18.10 (17.22, 18.78)	3.422	0.001	1.205	0.228	1.378	0.168
TNF-α (ng/L)	325.19 (296.71, 356.71)	312.73 (293.21, 329.32)	1.898	0.085	325.19 (296.01, 351.26)	312.73 (293.21, 329.32)	2.114	0.035	0.184	0.854	0.386	0.700
IL-1 (ng/L)	64.13 (61.58, 70.34)	63.61 (60.59, 67.91)	1.379	0.168	64.84 (62.89, 70.40)	63.09 (59.71, 67.61)	3.139	0.002	0.729	0.466	0.439	0.661
IL-6 (ng/L)	5.67 (5.38, 6.03)	5.71 (5.37, 6.08)	0.414	0.697	5.78 (5.44, 6.19)	5.78 (5.29, 6.00)	2.153	0.031	0.884	0.377	0.855	0.393

Abbreviations: DFI, DNA-fragmentation-index; HDS, High DNA Stainability; PL, phospholipids; sPLA2, Secretory phospholipase A2; AA, arachidonic acid; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; PGE1, Prostaglandin E1; PGF2α, Prostaglandin F2α; 5-LOX, 5-lipoxygenase; LTB4, leukotriene B4; TNF-α, tumor necrosis factor-α; IL-1, Interleukin-1; IL-6, Interleukin-6.

Table 5 Comparison of Semen-Related Indicators Grouped by Sperm Motility and Grouped by Cutoff Value of COX1 and AA Metabolic Network

	Grouped by COX1				Normal VS ≥270.45		Asthenospermia VS <270.45	
	≥270.45 (n=90)	<270.45 (n=74)	*Z*	*P*	*Z*	*P*	*Z*	*P*
Volume (mL)	3.58 (2.48, 4.86)	3.20 (2.67, 3.87)	1.533.	0.125	1.014	0.311	1.561	0.119
Concentration (10^6/mL)	54.82 (24.50, 89.08)	83.51 (25.60, 140.55)	1.064	0.287	0.763	0.445	0.939	0.347
Progressive motility (%)	42.59 (31.42, 52.46)	32.29 (21.53, 49.59)	2.327	0.020	3.931	0.000	4.109	0.000
Portion of grade A sperm (%)	35.87 (22.52, 44.38)	22.41 (15.87, 39.40)	2.710	0.007	3.816	0.000	4.265	0.000
Spontaneous acrosome reaction (%)	60.46 (49.93, 66.10)	61.13 (50.19, 71.33)	1.117	0.264	1.319	0.187	1.348	0.178
Induced acrosome reaction (%)	22.44 (15.97, 34.76)	17.89 (7.50, 23.83)	3.596	0.000	1.439	0.150	1.601	0.109
Mitochondrial membrane potential (%)	31.50 (24.02, 40.36)	26.94 (21.23, 35.31)	2.809	0.005	1.363	0.173	1.748	0.080
Superoxide anion in live sperm (%)	5.84 (4.21, 9.46)	4.75 (1.98, 7.31)	2.168	0.030	0.431	0.667	0.294	0.769
Superoxide anion in dead sperm (%)	28.58 (20.34, 40.09)	37.18 (22.75, 52.89)	2.902	0.004	0.861	0.389	0.580	0.562
Hydrogen peroxide in live sperm (%)	6.69 (3.14, 8.20)	5.15 (2.63, 12.92)	0.682	0.530	0.534	0.593	0.278	0.781
Hydrogen peroxide in dead sperm (%)	6.45 (5.15, 8.41)	5.31 (4.08, 8.97)	2.177	0.239	0.213	0.832	0.302	0.762
DFI (%)	7.67 (5.18, 12.45)	9.65 (3.96, 20.59)	1.271	0.204	1.824	0.068	1.511	0.131
HDS (%)	6.06 (3.84, 8.50)	4.97 (2.15, 8.26)	1.459	0.144	0.966	0.334	0.951	0.342
PL (mg/L)	30.78 (27.04, 34.72)	27.92 (25.21, 30.89)	3.285	0.001	2.121	0.034	2.026	0.043
sPLA2 (μg/L)	6.47 (6.14, 7.02)	6.18 (5.89, 6.74)	3.186	0.001	1.374	0.169	1.315	0.188
AA (pmol/L)	6.95 (6.49, 7.29)	6.43 (5.91, 6.78)	5.004	0.000	1.009	0.313	0.997	0.319
COX1 (ng/L)	286.13 (275.07, 298.84)	252.59 (238.98, 262.08)	10.424	0.000	3.146	0.002	4.209	0.000
COX2 (ng/L)	148.98 (136.18, 160.52)	138.86 (129.64, 149.19)	3.391	0.001	1.156	0.248	1.438	0.150
PGE1 (ng/L)	402.45 (373.75, 439.63)	388.10 (362.67, 404.79)	3.120	0.002	1.837	0.066	2.010	0.044
PGEF2α (pg/L)	448.62 (410.32, 473.99)	417.67 (392.46, 446.54)	3.596	0.000	1.167	0.243	1.357	0.175
5-LOX (ng/L)	86.26 (79.68, 91.60)	77.70 (73.52, 83.39)	5.837	0.000	2.912	0.004	3.195	0.001
LTB4 (ng/L)	18.52 (17.94, 19.68)	18.21 (17.09, 18.85)	2.300	0.021	0.578	0.563	0.907	0.364
TNF-α (ng/L)	321.04 (296.01, 346.47)	315.51 (293.21, 341.00)	0.873	0.383	0.807	0.419	1.006	0.314
IL-1 (ng/L)	65.04 (62.12, 70.04)	63.21 (59.98, 67.03)	2.796	0.005	0.436	0.663	0.409	0.683
IL-6 (ng/L)	5.82 (5.47, 6.15)	5.48 (5.24, 5.80)	3.253	0.001	1.369	0.171	1.463	0.143

Abbreviations: DFI, DNA-fragmentation-index; HDS, High DNA Stainability; PL, phospholipids; sPLA2, Secretory phospholipase A2; AA, arachidonic acid; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; PGE1, Prostaglandin E1; PGF2α, Prostaglandin F2α; 5-LOX, 5-lipoxygenase; LTB4, leukotriene B4; TNF-α, tumor necrosis factor-α; IL-1, Interleukin-1; IL-6, Interleukin-6.

Table 6 Calculation of NRI by Evoked Acrosome Reaction to Determine the Application Value of Y3 Equation and COX1

Induced acrosome reaction <15%		Grouped by Y_3 equation		Induced acrosome reaction ≥ 15%		Grouped by Y_3 equation	
		< 6.529	≥ 6.529			< 6.529	≥ 6.529
Vitality	Asthenozoospermia	14	2	Vitality	Asthenospermia	40	12
	Normal	16	16		Normal	10	54
NRI=0.309							
Induced acrosome reaction <15%		**Grouped by COX1**		**Induced acrosome reaction ≥ 15%**		**Grouped by COX1**	
		< 270.45	≥ 270.45			< 270.45	≥ 270.45
Vitality	Asthenozoospermia	14	2	Vitality	Asthenospermia	28	24
	Normal	14	18		Normal	18	46
NRI=0.302							

Abbreviations: NRI, net reclassification index; COX1, cyclooxygenase 1.

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Table 7 Application of DFI to Calculate NRI to Determine the Predictive Value of the Y3 Equation and COX1

DFI >15%		**Grouped by Y_3 equation**		**DFI ≤15%**		**Grouped by Y_3 equation**	
		<6.529	**≥6.529**			**<6.529**	**≥6.529**
Vitality **NRI=0.106**	Asthenospermia Normal	28 12	4 12	Vitality	Asthenospermia Normal	24 16	12 56
DFI >15%		**Grouped by COX1**		**DFI ≤15%**		**Grouped by COX1**	
		<6.529	**≥6.529**			**<6.529**	**≥6.529**
Vitality **NRI=0.053**	Asthenospermia Normal	24 10	6 16	Vitality	Asthenospermia Normal	18 22	20 48

Abbreviations: NRI, net reclassification index; COX1, cyclooxygenase 1.

significantly lower than that at 37°C ($P < 0.05$) (Figure 5). Furthermore, compared to the 37°C group, at 42°C, there were increases in the spontaneous acrosome reaction, superoxide anion levels in both live and dead sperm, and the DNA fragmentation index (DFI). Conversely, the induced acrosome reaction was remarkably reduced ($P < 0.05$) (Figure 5). These results indicate that

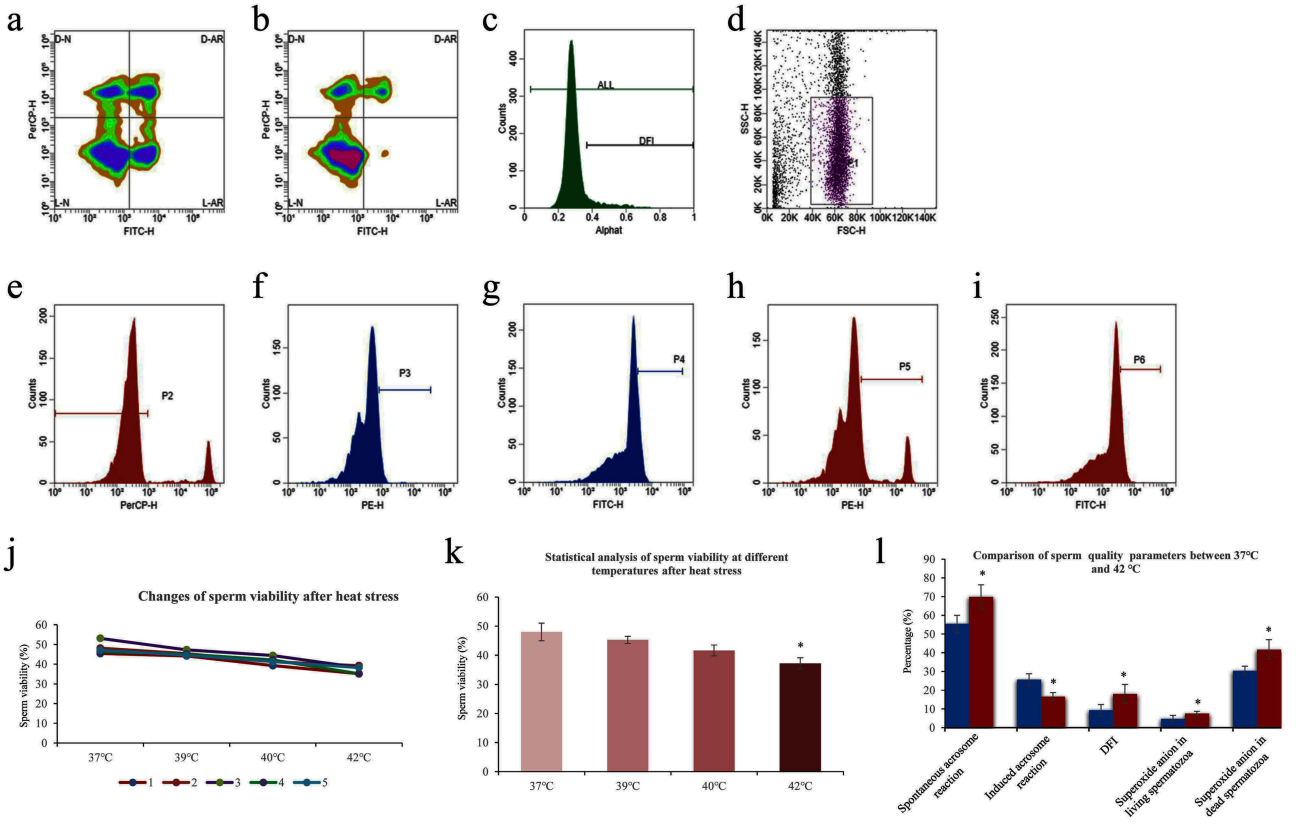

Figure 5 Development of the heat stress-induced asthenozoospermia model. Heat stress was applied to normal semen specimens to assess alterations in sperm quality. Sperm motility was analyzed using CASA, while other metrics were evaluated via flow cytometry. (**a**) represented the induced acrosome reaction; (**b**) showed the spontaneous acrosome reaction, distinguishing D-AR (acrosome reaction in dead sperm) from L-AR (acrosome reaction in live sperm). (**c**) illustrated the DFI; (**d–i**) depicted ROS-related metrics: P3 denoted superoxide anion presence in live sperm, P4 identified hydrogen peroxide in live sperm, P5 showed the total superoxide anion count, and P6 represented the total hydrogen peroxide count. (**j**) detailed the motility changes across four temperature gradients (37°C, 39°C, 40°C, and 42°C) in five samples; (**k**) provided statistical analysis of sperm motility across these gradients; (**l**) compared sperm quality parameters at 37°C and 42°C. *p < 0.05.

heat stress, especially at 42°C, has significant negative effects on sperm quality, as manifested by changes in motility, acrosome reaction, and oxidative stress-related parameters.

Mechanism of in vitro Addition of AA and COX1 to Protect Sperm

To evaluate the impact of AA on sperm, varying concentrations of AA were introduced to normal semen samples (concentration ≥ 15×10^6; PR ≥ 32%) and to a heat stress-induced asthenozoospermia model. Results indicated that AA concentrations exceeding 5 ng significantly reduced sperm motility, with a marked decline observed at 10 μg. Under 42°C heat stress, the addition of 100 pg AA preserved sperm quality, achieving an average motility increase of 38.38% relative to the 42°C-only heat stress group. Furthermore, combining 100 pg AA with 300 pg COX1 under the same conditions enhanced motility by 47.11% on average compared to the 42°C-only group; however, no statistically significant difference was detected between the 100 pg AA+300 pg COX1 and 100 pg AA treatments.

The heat stress asthenozoospermia model confirmed that in vitro addition of AA produced no significant changes in seminal plasma COX1 levels; however, COX2 consumption markedly increased alongside significant elevations in PGE1 and PGF2α production. Conversely, LOX consumption and LTB4 production decreased significantly. Sperm Ca^{2+} concentration increased significantly, while seminal plasma Ca^{2+} concentration showed a marked decline. Additionally, significant reductions were observed in sperm spontaneous acrosome reaction, superoxide anion levels in both live and dead sperms, and DFI, while induced acrosome reaction significantly rose. With the combined addition of AA and COX1 in vitro, compared to AA alone, COX2, LOX consumption and LTB4 production were further reduced, while PGE1 and PGF2α production notably increased. Sperm Ca^{2+} levels were elevated significantly, while seminal plasma Ca^{2+} levels decreased markedly. Compared to the simple heat stress group, sperm spontaneous acrosome reaction, superoxide anion in live and dead sperms, and DFI were significantly lower, with induced acrosome reaction significantly elevated.

Influence of Varying in vitro AA Concentrations on Sperm Motility

Relative to the Control group, no statistically significant differences in sperm motility were observed at 30, 60, and 120 minutes in the 10 pg and 1 ng AA groups ($P > 0.05$). However, the 100 ng and 10 μg AA groups exhibited a significant decline in motility ($P < 0.05$, $P < 0.01$, $P < 0.001$) (Figure 6).

Impact of Varying in vitro AA Concentrations on Sperm Motility Under Heat Stress

Under heat stress conditions at 42°C, sperm motility in the 100 pg group was significantly greater than in the other five groups ($P < 0.05$). When comparing the 100 pg group at 42°C to its counterpart at 37°C, no statistically significant difference was observed ($P > 0.05$). However, sperm motility in all other groups showed a marked decrease at 42°C, with statistically significant differences ($P < 0.05$). Additionally, relative to the 37°C "Control" group, sperm motility at 42°C declined significantly across all groups ($P < 0.05$) (Figure 7).

Effect of in vitro Addition of 100 pg AA on Sperm Quality Under 42 °C Heat Stress

Following heat stress at 42°C, spontaneous acrosome reaction, superoxide anion levels in both live and dead sperms, and DFI showed significant increases, while induced acrosome reaction notably declined ($P < 0.05$). The addition of 100 pg AA at 42°C markedly decreased spontaneous acrosome reaction, superoxide anion levels in live and dead sperms, and DFI, while significantly enhancing the induced acrosome reaction compared to the heat stress-only group ($P < 0.05$). Although all indicators in the AA-supplemented 42°C group differed from those in the 37°C group, these variations lacked statistical significance ($P > 0.05$) (Table 8).

Protective Effects of in vitro Addition of 100 pg AA + 300 pg COX1 on Sperm Quality Under 42 °C Heat Stress

In comparison with the 42°C control group, significantly reduced spontaneous acrosome reaction, superoxide anion levels in both live and dead sperms, and DFI, while markedly increased induced acrosome reaction were observed in remaining groups ($P < 0.05$). Although all indicators in the 100 pg AA + 300 pg COX1 group at 42°C showed lower or higher values than those in the 100 pg AA group alone, these differences lacked statistical significance ($P > 0.05$) (Table 9).

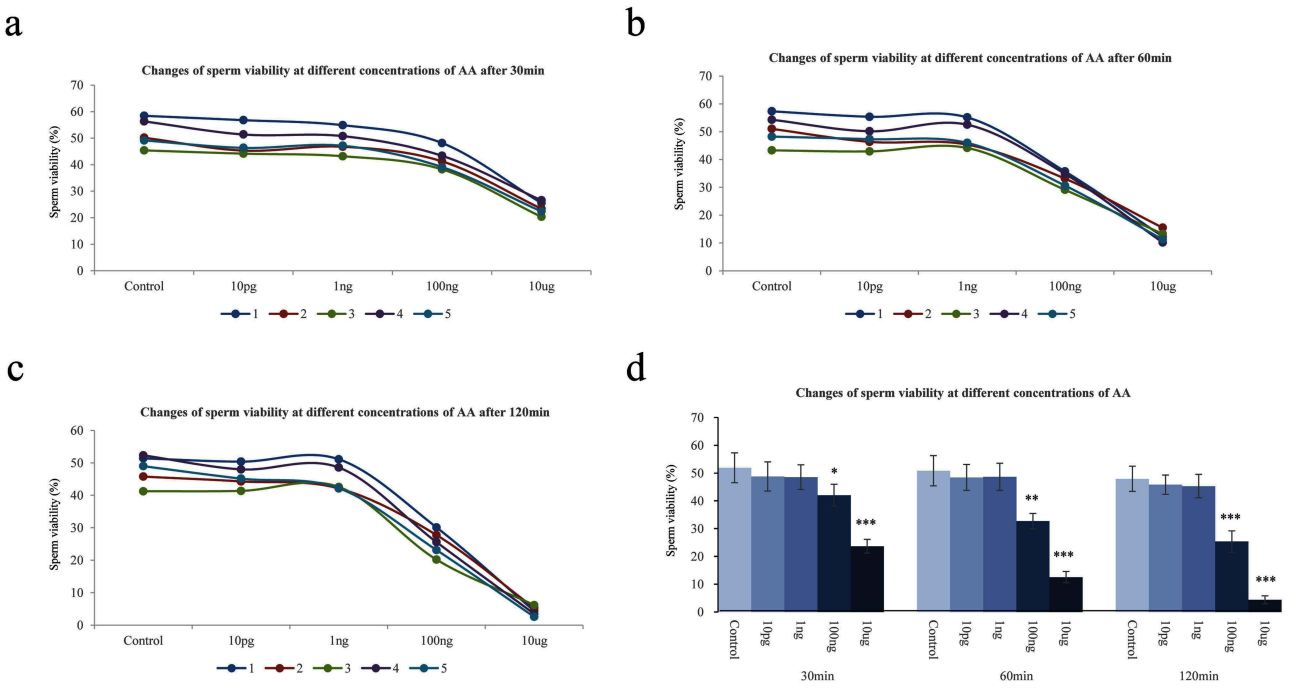

Figure 6 The alterations in sperm motility following the in vitro addition of AA at varying concentrations. Semen samples with normal concentration and vitality were selected, thoroughly mixed, and 0.5 mL from each group was extracted for experimentation. AA was introduced in increasing concentrations of 10 pg, 1 ng, 100 ng, and 10 μg, combined with 10 μL of normal saline. Subfigures (**a**–**c**) depicted motility changes at 30, 60, and 120, respectively, while (**d**) presented a bar chart summarizing motility changes across these time intervals. *p < 0.05, **p < 0.01, ***p < 0.001.

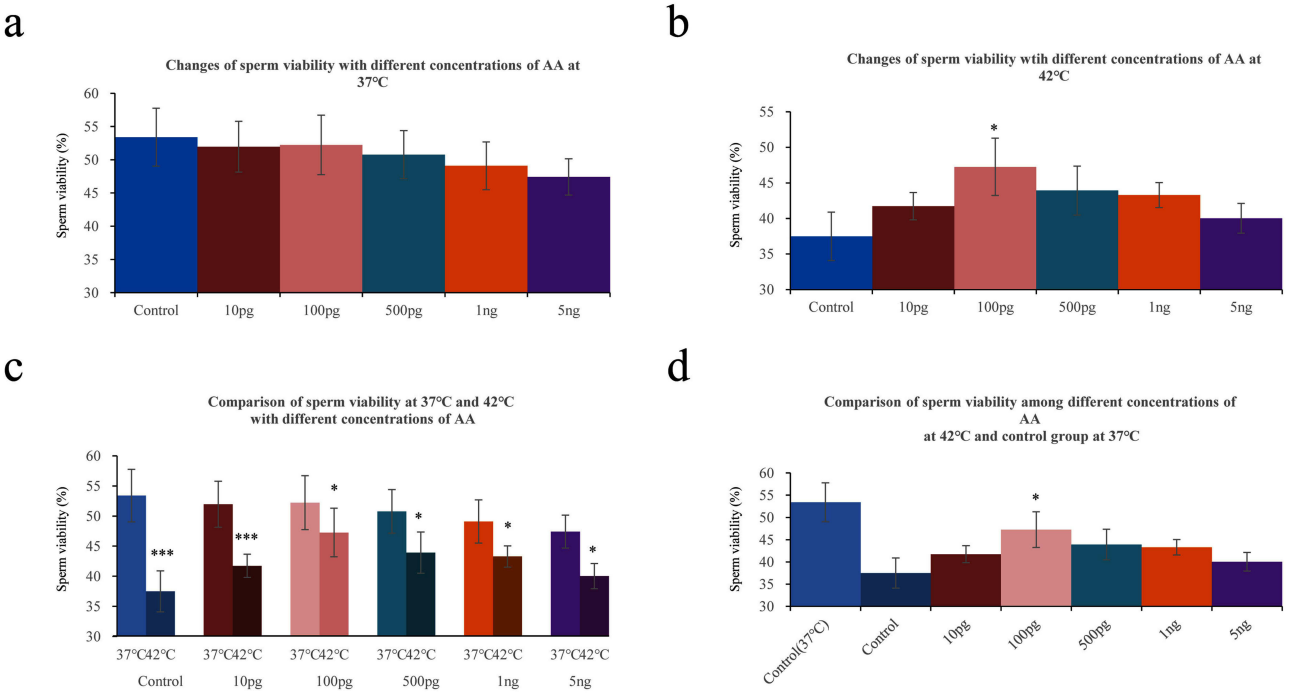

Figure 7 The sperm motility changes under heat stress (42°C) with different AA concentrations added in vitro. Semen samples of normal concentration and vitality were selected, mixed, and 0.5 mL was taken from each group. Samples were divided into two temperature groups (37°C and 42°C) and further subdivided into six concentration groups per temperature. AA was added at 10 pg, 100 pg, 500 pg, 1 ng, and 5 ng, with normal saline used as a control. Subfigures (**a** and **b**) compared motility among groups at 37°C and 42°C, respectively, while (**c**) contrasted motility between 37°C and 42°C groups, and (**d**) compared the 42°C groups with the control at 37°C. *p < 0.05, ***p < 0.001.

Table 8 Protection of Sperm Quality by in vitro Addition of 100 pg AA Under Heat Stress at 42°C

	37°C	42°C	AA 100 pg + 42°C	*F*	*P*
Spontaneous acrosome reaction (%)	43.49 ± 4.25[c]	61.54 ± 4.74[a]	46.41±4.07[b]	24.674	0.000
Induced acrosome reaction (%)	29.71 ± 2.96[c]	14.33 ± 2.21[a]	27.58±2.73[b]	49.287	0.000
Spontaneous acrosome reaction (%)	4.14 ± 1.46[c]	8.45 ± 1.64[a]	5.31±0.76[b]	13.787	0.001
Induced acrosome reaction (%)	27.60 ± 2.67[c]	39.60 ± 3.47[a]	29.43±1.81[b]	27.868	0.000
DFI (%)	9.03 ± 3.16[c]	20.03 ± 3.41[a]	9.95±2.78[b]	19.035	0.000

Notes: Different letters indicate differences between columns. [a]Indicates P<0.01 compared with 37°C; [b]Indicates P<0.01 compared with 42°C; [c]Is used to identify the 37°C group in order to reflect the relationship of differences between columns.
Abbreviations: DFI, DNA-fragmentation-index; AA, arachidonic acid.

Table 9 Protective Effects of in vitro Addition of 100 pg AA + 300 pg COX1 on Sperm Quality Under 42 °C Heat Stress

	37°C			42°C			*F*	*P*
	Control	100 pg AA	100 pg AA + 300pg COX1	Control	100 pg AA	100 pg AA + 300pg COX1		
Progressive motility (%)	51.28 ± 3.87[c]	50.02 ± 3.06[c]	50.25 ± 3.70[c]	32.67 ± 2.58[a]	45.21 ± 2.66[b]	48.06 ± 2.68[b]	24.868	0.000
Spontaneous acrosome reaction (%)	41.58 ± 3.37[c]	42.62 ± 3.14[c]	42.75 ± 3.30[c]	61.12 ± 2.24[a]	43.45 ± 2.56[b]	42.25 ± 3.54[b]	30.931	0.000
Induced acrosome reaction (%)	25.74 ± 3.98[c]	24.02 ± 4.00[c]	24.76 ± 3.57[c]	12.50 ± 1.92[a]	23.48 ± 4.23[b]	25.13 ± 3.64[b]	9.492	0.000
Spontaneous acrosome reaction (%)	3.62 ± 1.41[c]	3.72 ± 1.48[c]	3.69 ± 1.19[c]	9.09 ± 2.08[a]	3.89 ± 1.54[b]	3.70 ± 1.25[b]	8.253	0.000
Induced acrosome reaction (%)	29.51 ± 3.37[c]	30.46 ± 3.14[c]	30.01 ± 2.61[c]	42.87 ± 2.77[a]	30.78 ± 3.03[b]	27.66 ± 4.71[b]	10.415	0.000
DFI (%)	8.88 ± 3.31[c]	9.11 ± 2.86[c]	9.18 ± 2.91[c]	19.60 ± 4.33[a]	9.94 ± 3.49[b]	8.78 ± 2.81[b]	13.502	0.000

Notes: Different letters indicate differences between columns. [a]Indicates P<0.01 compared with 42°C "Control"; [b]Indicates P<0.05 compared with 42°C "100 pg AA"; [c]Is used to identify the 37°C group in order to reflect the relationship of differences between columns.
Abbreviations: DFI, DNA-fragmentation-index; AA, arachidonic acid; COX1, cyclooxygenase 1.

Impact of in vitro Addition of 100 pg AA + 300 pg COX1 on Seminal Plasma AA Metabolic Network Indicators Under 42°C Heat Stress

Following 1-hour incubation, indicators of the AA metabolic network (excluding 5-LOX) exhibited statistically significant alterations ($P < 0.05$) compared with the baseline semen solution. Under 42°C heat stress, all AA metabolic indicators (except PGF2α) showed further significant shifts relative to both the 37°C "Control" group and baseline, with statistical significance ($P < 0.05$). The addition of 100 pg AA under 42°C heat stress induced notable alterations in metabolic indicators compared to the baseline solution and the 37°C "Control" group (excluding PL and sPLA2), and relative to the 42°C heat stress "Control" group (excluding AA and COX1), with all differences reaching statistical significance ($P < 0.05$). Both 100 pg AA + 300 pg COX1 and 100 pg AA alone under 42°C exhibited similar variation trends in AA metabolic network indicators; significant differences were observed between the two groups (except indicators PL, sPLA2, AA, COX1, and seminal plasma Ca^{2+} levels) ($P < 0.05$) (Table 10).

Table 10 Impact of in vitro Addition of 100 pg AA + 300 pg COX1 on Seminal Plasma AA Metabolic Network Indicators Under 42°C Heat Stress

	Original	37°C Control	42°C			*F*	*P*
			Control	100 pg AA	100 pg AA + 300pg COX1		
PL (mg/L)	31.26 ± 1.22[d]	26.83 ± 0.88[a]	24.04 ± 1.44[a,b]	26.49 ± 0.99[a,c]	25.53 ± 1.34[a]	25.616	0.000
sPLA2 (μg/L)	6.45 ± 0.22[d]	5.60 ± 0.29[a]	4.43 ± 0.27[a,b]	5.49 ± 0.28[a,c]	5.54 ± 0.23[a,c]	38.446	0.000
AA (pmol/L)	6.66 ± 0.23[d]	5.47 ± 0.20[a]	4.38 ± 0.29[a,b]	4.59 ± 0.23[a,b]	4.37 ± 0.22[a,b]	85.743	0.000
COX1 (ng/L)	302.43 ± 16.45[d]	260.21 ± 3.80[a]	243.42 ± 6.51[a,b]	244.04 ± 6.10[a,b]	243.82 ± 4.08[a,b]	42.357	0.000
COX2 (ng/L)	156.26 ± 3.99[d]	146.10 ± 5.99[a]	128.47 ± 5.94[a,b]	104.24 ± 6.27[a,b,c]	119.35 ± 5.34[a,b,c,d]	69.566	0.000
PGE1 (ng/L)	372.05 ± 12.62[d]	432.81 ± 8.42[a]	394.17 ± 9.66[a,b]	476.67 ± 6.64[a,b,c]	492.26 ± 4.40[a,b,c,d]	171.879	0.000

(*Continued*)

Table 10 (Continued).

	Original	37°C Control	42°C			*F*	*P*
			Control	100 pg AA	100 pg AA + 300pg COX1		
PGEF2α (pg/L)	431.86 ± 11.01[d]	462.39 ± 7.47[a]	441.95 ± 11.85[b]	490.47 ± 4.92[a,b,c]	518.94 ± 12.77[a,b,c,d]	63.254	0.000
5-LOX (ng/L)	82.34 ± 2.66[d]	80.14 ± 2.08	56.48 ± 4.61[a,b]	62.11 ± 4.88[a,b,c]	67.70 ± 4.24[a,b,c,d]	42.416	0.000
LTB4 (ng/L)	15.32 ± 1.40[d]	18.20 ± 0.79[a]	26.57 ± 1.34[a,b]	23.36 ± 0.93[a,b,c]	20.89 ± 1.04[a,b,c,d]	75.676	0.000
Sperm Ca^{2+} (%)	44.28 ± 2.16[d]	47.48 ± 1.99[a]	33.22 ± 2.71[a,b]	52.81 ± 1.67[a,b,c]	56.33 ± 1.41[a,b,c,d]	95.860	0.000
Seminal plasma Ca^{2+} (mmol/L)	4.43 ± 0.29[d]	4.04 ± 0.21[a]	6.08 ± 0.24[a,b]	3.39 ± 0.20[a,b,c]	3.23 ± 0.16[a,b,c]	129.036	0.000

Notes: Different letters indicate differences between columns, with the significance of the differences, [a]Compared with Original, $P<0.01$; [b]Compared with 37°C "Control", $P<0.01$; [c]Compared with 42°C "Control", $P<0.05$. [d]Is used to identify the "Original" column in order to reflect the relationship of differences between columns.
Abbreviations: PL, phospholipids; sPLA2, Secretory phospholipase A2; AA, arachidonic acid; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; PGE1, Prostaglandin E1; PGF2α, Prostaglandin F2α; 5-LOX, 5-lipoxygenase; LTB4, leukotriene B4.

Effect of in vitro Addition of 100 pg AA and 300 pg COX1 on Sperm Catsper1 Under 42°C Heat Stress

Exposure to 42°C heat stress resulted in a significant reduction in Catsper1 content compared to the original solution and Control groups. The addition of AA or AA+COX1 significantly increased Catsper1 levels, although these levels remained below those of the original solution and Control. No statistically significant difference was observed between the AA and AA+COX1 groups (Figure 8).

Discussion

Semen composition consist of 99% seminal plasma and 1% sperm. Seminal plasma supplies essential energy substrates and plays a critical role in protecting sperm.[26] When sperm is damaged, the buffering and protective functions of seminal plasma are essential, even as the sperm attempts self-repair.[27] Physiologically, maintaining a balanced level of reactive oxygen species (ROS) is essential for sustaining sperm function.[28] However, an excess of ROS impairs sperm integrity and reduces its fertilization ability. During spermatogenesis, there is significant cytoplasmic reduction, leaving sperm with limited resources for repair.[29] Moreover, sperm cell membranes, rich in polyunsaturated fatty acids (PUFAs), are highly vulnerable to external stressors, emphasizing the need for the protective role of seminal plasma.[30]

Arachidonic acid (AA), a type of PUFA, is abundant in various human tissues and participates in essential physiological processes. These include cholesterol esterification, enhancement of vascular elasticity, reduction of blood viscosity, and modulation of blood cell function.[31,32] Its metabolites and related enzymes have attracted considerable research interest,[7,8] and high levels of AA have been detected in seminal plasma.[4] Many studies have established a close relationship between AA, lipid oxidation, and ROS.[33,34] In this study, we identified substantial amounts of AA metabolic network indicators in seminal plasma. We found positive correlations among leukotriene B4 (LTB4). These results suggest three key points: 1) There is a positive feedback mechanism among AA metabolic network indicators. 2) There is a close connection between seminal plasma AA metabolic network indicators and the inflammatory response. 3) There is a negative correlation between LTB4 and IL-6, indicating potential antagonism between the AA metabolic network-related inflammatory pathway and IL-6. Further

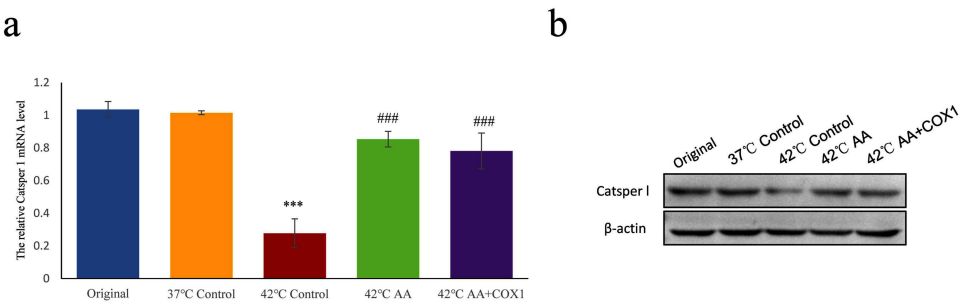

Figure 8 Effect of in vitro addition of AA and COX1 on sperm Catsper1 level under 42°C heat stress. (**a**) The relative Catsper 1 mRNA level was detected by qRT-PCR; (**b**) Western blot was used to detect the Catsper 1 protein level. ***$p < 0.001$ vs. Original group. ###$p < 0.001$ vs.42°C control group.

analysis of the relationship between the AA metabolic network and sperm quality showed distinct positive and negative correlations between AA network indicators and sperm quality metrics. This indicates that the AA metabolic network significantly affects sperm quality.

Our analysis of the AA metabolic network indicators based on the area under the curve (AUC) yielded statistically significant results for AA and cyclooxygenase 1 (COX1). AA had sensitivity and specificity values of 0.500 and 0.794, respectively, while COX1 had values of 0.688 and 0.618. Using logistic regression, we formulated equations based on $P < 0.05$ and $P < 0.1$. By integrating four indicators ($P < 0.1$), we derived five different equations. Among them, the equation $Y3 = -0.108 \times PL + 0.442 \times AA + 0.025 \times COX1$ had sensitivity and specificity values of 0.729 and 0.794, both exceeding 0.7. This highlights its superior predictive value compared to AA or COX1 alone. Validating Y3 as a cutoff criterion showed that it had a better predictive capacity than sperm motility alone in assessing spontaneous and induced acrosome reactions, superoxide anion levels in live and dead sperm, and DNA fragmentation index (DFI). Y3 was particularly effective in evaluating various AA metabolic network parameters, while COX1 had advantages when considered as an individual metric. Additionally, using COX1 as a cutoff showed improved efficacy compared to sperm motility alone in assessing induced acrosome reaction, sperm mitochondrial membrane potential, and superoxide anion levels in both live and dead sperm. When evaluating the AA metabolic network, the indicators phospholipid (PL), COX1, prostaglandin E1 (PGE1), and 5 - lipoxygenase (5-LOX) showed distinct benefits. These results suggest that Y3 and COX1 play complementary roles as predictors of sperm quality, each with its own unique strengths. This enhanced prediction model surpassed basic sperm motility assessments and provided a reliable method for evaluating sperm quality.

We developed an asthenozoospermia model under heat stress conditions. After 1 hour at 42°C, sperm motility decreased by an average of 22.49%. Heat exposure at 42°C led to statistically significant increases in spontaneous acrosome reaction, superoxide anion levels in both live and dead sperm, and DFI compared to 37°C, while the induced acrosome reaction decreased. At 42°C, the addition of 100 pg AA significantly decreased spontaneous acrosome reaction, superoxide anion levels in both live and dead sperm, and DFI, while significantly enhancing the induced acrosome reaction compared to the heat - stress - only group. Notably, 100 pg AA had a protective effect on sperm quality, reducing heat-induced damage. The combination of 100 pg AA and 300 pg COX1 further improved all measured indicators at 42°C compared to the 100 pg AA group alone, although this difference did not reach statistical significance. This suggests a slightly better protective effect on sperm quality with the AA + COX1 combination than with AA alone.

Our study confirmed the feedback regulation within the AA metabolic network in the heat-stress asthenozoospermia model. After heat exposure at 42°C, seminal plasma levels of PL, secretory phospholipase A2 (sPLA2), AA, COX1, COX2, and 5-LOX increased significantly, while the production of PGE1 and prostaglandin F2α (PGF2α) decreased significantly compared to the 37°C control group; LTB4 production also increased significantly. We observed enhanced CatSper1 expressions in sperm, accompanied by significantly reduced sperm Ca^{2+} levels and elevated seminal plasma Ca^{2+}. This suggests the involvement of the seminal plasma AA metabolic network in stress responses. The significant increase in sperm superoxide anions indicated enhanced 5-LOX-mediated lipid oxidation and increased ROS production, exacerbating LTB4-mediated inflammation and thus reducing sperm quality. After heat stress, decreased CatSper1 expression and reduced Ca^{2+} influx in sperm led to lower intracellular Ca^{2+} levels. At the same time, the release of Ca^{2+} from dead sperm increased seminal plasma Ca^{2+}, which was associated with a decrease in induced acrosome reactions and an increase in spontaneous acrosome reactions, thus impairing sperm fertilization potential.

In vitro addition of AA significantly reduced the consumption rates of PL, sPLA2, AA, COX1, and 5-LOX, while significantly increasing COX2 consumption. The production of PGE1 and PGF2α was substantially enhanced, while LTB4 production decreased significantly. This intervention increased sperm CatSper1 expression, significantly increased sperm Ca^{2+} levels, and significantly decreased seminal plasma Ca^{2+}, indicating a protective effect of AA against heat stress in semen. Specifically, AA seemed to slow down LOX-mediated lipid oxidation under heat stress, mitigate LTB4-mediated inflammation, enhance the COX2 stress response, stimulate CatSper1 expression, and promote Ca^{2+} influx in sperm. The enhanced sperm induced acrosome reaction, along with reduced ROS levels, increased CatSper1 expression, elevated Ca^{2+} influx, and increased PGE1 production, collectively suggest improvements in sperm quality and fertilization potential.

After the combined addition of AA and COX1 in vitro, there were no significant differences in the consumption of PL, sPLA2, AA, or COX1 compared to the addition of AA alone. However, COX2 and 5-LOX consumption slowed

down significantly, while the production of PGE1 and PGF2α increased significantly, and LTB4 production decreased significantly. The expression levels of CatSper1, sperm Ca^{2+}, and seminal plasma Ca^{2+} remained stable, indicating that the addition of COX1 had a protective effect against heat stress in semen. These findings suggest a trade-off interaction between COX and 5-LOX as AA-metabolizing enzymes, further supporting the idea that the increased production of PGE1 and PGF2α improves sperm quality. Combining with sperm quality metrics, the dual addition of AA and COX1 was slightly more effective than AA alone.

The novelty of this study were as below: 1. Novel insights into the AA metabolic network in sperm function. Our study is one of the first to comprehensively analyze the entire seminal plasma sPLA2 - COX/5-LOX-Ca2+ pathway in relation to sperm motility. Previous research has mainly focused on individual components of this pathway. For example, while some studies have investigated the role of sPLA2 in sperm motility, they did not explore its connection with the subsequent steps involving 5-LOX and COX enzymes. By evaluating the complete network, we have discovered a previously unreported feedback mechanism. We found that the activation of sPLA2 by Ca2+ initiates a cascade of events that enhance sperm motility, but over time, the decreasing extracellular Ca2+ levels lead to a decline in sPLA2 activity and subsequent sperm motility. This dynamic regulation of sperm motility through the integrated metabolic pathway is a novel finding. 2. Linking dietary-induced fatty acid imbalances to sperm function. In recent years, there has been an increasing awareness of the impact of diet on male reproductive health, but few studies have directly linked the specific dietary-induced changes in polyunsaturated fatty acid (PUFA) profiles, especially imbalances in linoleic acid, linolenic acid, and arachidonic acid (AA), to the molecular mechanisms of sperm motility. Our results show that the altered AA metabolism, which is affected by these dietary-related PUFA imbalances, significantly influences sperm motility. We demonstrated that AA, through its metabolism by 5-LOX and COX, generates compounds like LTB4, PGE, and PGF2α that target the sperm CatSper1 channel to modulate Ca2+ influx and motility. This provides new insights into how dietary factors can affect sperm function at the molecular level. 3. Potential clinical implications. The identification of COX1 as a potential physiological marker for evaluating sperm retrieval status is a novel contribution. To our knowledge, this has not been reported in previous literature. Additionally, the positive correlation we found between PGE, PGF2α, and sperm quality has significant potential for diagnostic and therapeutic applications. These findings could potentially lead to the development of new diagnostic tools and therapeutic strategies for asthenozoospermia and other male infertility conditions, which is a novel aspect of our research in the context of translational medicine. In summary, our results offer new perspectives on the molecular mechanisms of sperm motility regulation, the influence of diet on male reproductive health, and potential clinical applications, all of which contribute to the novelty of our study.

Conclusion

In summary, our research shows the seminal plasma AA metabolic network, via sPLA2-COX/5-LOX-Ca^{2+} mechanisms, impacts sperm motility. The PL-AA-COX1 model may predict sperm quality. Our study provides novel insights into the molecular mechanisms underlying sperm motility, the role of AA and COX1 in modulating sperm quality, and a potential predictive model for sperm quality. These findings contribute significantly to our understanding of male reproductive health and have important implications for both basic research and clinical applications. However, its in-vivo pathways are unclear. Future research should validate it in larger cohorts and study AA/COX1's long-term effects on male fertility.

Highlights

Positive and negative feedback regulatory mechanisms of seminal plasma sPLA2-COX/5-LOX-Ca^{2+} may influence sperm quality.

Data Sharing Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Yinchuan Maternity and Child Health Hospital. Informed consent obtained from the study participants prior to study commencement.

Consent for Publication

All the participants agreed to the manuscript's publication.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interest in this work.

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