DOI: https://doi.org/10.2298/SARH260621074P
UDC: 616.697-02

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

ZIP6 and ZIP14 zinc transporter expression in normozoospermic and asthenozoospermic human spermatozoa – relationship to nuclear chromatin accessibility

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SUMMARY

Introduction/Objective Male infertility accounts for approximately 40% of infertility cases, with asthenozoospermia affecting nearly one in five infertile men. Beyond conventional semen parameters, nuclear DNA organization and chromatin accessibility provide deeper insight into sperm quality. Zinc transporters ZIP6 and ZIP14 have been implicated in chromatin stabilization and zinc homeostasis, potentially essential to sperm maturation and fertilization competence.

Methods Semen samples from normozoospermic and asthenozoospermic men undergoing intrauterine insemination were purified by density gradient. ZIP6 and ZIP14 were detected by immunofluorescence, nuclear DNA visualized by using Sytox™ Orange (Thermo Fisher Scientific, Waltham, MA, USA). Expression levels, nuclear area, and staining intensity were quantified in ImageJ (National Institutes of Health), and analyzed using Kruskal–Wallis test with Dunn's post-hoc correction ($p \leq 0.05$).

Results ZIP6 immunopositivity was significantly reduced in asthenozoospermic compared to normozoospermic samples, while ZIP14 expression remained stable. Nuclear area and overall Sytox™ Orange (Thermo Fisher Scientific) intensity did not differ significantly between transporter-positive and -negative sperm, though ZIP6-positive asthenozoospermic cells showed a modest reduction in nuclear surface area. Normozoospermic ZIP14-negative spermatozoa displayed lower Sytox™ Orange (Thermo Fisher Scientific) fluorescence than ZIP6-negative cells, suggesting focal differences in chromatin accessibility between transporter-defined subpopulations.

Conclusion ZIP6 expression reflects sperm maturation and functional competence, while ZIP14 remains constitutively stable. Both transporters may serve as molecular markers for selecting high-quality sperm in assisted reproduction, particularly in populations exposed to occupational stressors that compromise DNA integrity.

Keywords: ZIP6; ZIP14; spermatozoa; chromatin accessibility; asthenozoospermia; normozoospermia

INTRODUCTION

Male infertility contributes to approximately 40% of infertility cases [1], and while routine semen analysis focuses on concentration, motility, and morphology, increasing attention has been directed toward nuclear integrity as an additional dimension of sperm quality [2].

Spermatozoa represent a highly heterogeneous cell population, both morphologically and molecularly, even within normozoospermic samples. This heterogeneity includes differences in chromatin compaction, oxidative susceptibility, and protein composition, arising during spermatogenesis and epididymal maturation and reflecting variations in nuclear stability, capacitation potential, and protein expression profiles, including zinc transporters [3, 4]. Proper chromatin packaging is essential for sperm functional competence, as the paternal genome must remain transcriptionally silent and structurally protected until fertilization.

Chromatin remodeling and DNA protection mechanisms

During spermatogenesis, histones are sequentially replaced by transition proteins and protamines, resulting in highly compacted toroidal chromatin stabilized by disulfide bonds. This organization renders the sperm genome transcriptionally inactive and provides strong protection against oxidation, elevated temperatures in the female reproductive tract, and other external stressors [5, 6, 7]. However, the degree of chromatin compaction is not uniform across all spermatozoa, and incomplete protamination or structural irregularities may compromise nuclear integrity and DNA stability. Such imperfections, arising from defective histone-to-protamine transition, impaired recombination or repair, or abortive apoptosis, result in a baseline level of DNA damage detectable in testicular, epididymal, and ejaculated spermatozoa. Importantly, inadequate chromatin condensation may leave portions of the genome in a transcriptionally accessible state, which is at odds with the biological

Received • Примљено: June 21, 2026
Accepted • Прихваћено: September 21, 2026
Online first: September 24, 2026

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requirement for transcriptional silencing of the paternal genome prior to fertilization [3, 5].

Factors contributing to sperm DNA damage

Beyond intrinsic spermatogenic imperfections, sperm DNA damage has been associated with multiple lifestyle, environmental, and health-related factors, including smoking, environmental pollutants, and other conditions that promote oxidative stress [8]. Although damaged germ cells are typically eliminated via apoptosis, some may escape this process and still participate in fertilization, with potential consequences for embryo development and pregnancy outcomes [6, 9, 10].

Zinc (Zn^{2+}) in male reproductive physiology

Zinc plays a crucial role in chromatin stabilization during spermatogenesis. It contributes to DNA condensation by stabilizing protamine–DNA interactions and supporting the formation of disulfide bonds that reinforce nuclear structure [11, 12]. Through these mechanisms, zinc plays an important role in maintaining chromatin stability and protecting sperm DNA from fragmentation [10–13]. Beyond its structural role, zinc may also function as an additional safeguard upon ejaculation, when exogenous Zn^{2+} is incorporated into the sperm nucleus and helps stabilize chromatin, inhibit premature metabolic activity, preserve membrane integrity, and exert antioxidant effects [11]. In this way, zinc contributes both to DNA integrity and to the acrosome reaction, with membrane-associated transporters such as ZIP6 and ZIP14 influencing intracellular zinc availability [12, 14, 15]. This raises the possibility that differences in zinc transporter expression among individual spermatozoa could underlie part of the functional heterogeneity observed within the ejaculate.

Zinc transporters as determinants of sperm subpopulations

Zinc homeostasis in spermatozoa is regulated through the coordinated activity of ZIP importers, which increase cytosolic zinc, and ZnT exporters, which reduce it. In our previous study, we characterized the localization and differential expression of ZIP6 and ZIP14 in normozoospermic and asthenozoospermic spermatozoa, demonstrating the existence of distinct ZIP-positive and ZIP-negative subpopulations within the ejaculate [16]. These findings suggest that individual sperm cells differ in their capacity for zinc uptake, providing a molecular basis for functional diversity. Whether this variability in zinc transporter expression is associated with differences in nuclear chromatin organization and DNA integrity, however, remains to be established.

Although zinc transporters of the ZIP family define distinct subpopulations of ZIP-positive and ZIP-negative spermatozoa within the ejaculate, it remains unknown whether these subpopulations differ in nuclear chromatin organization or DNA integrity. Therefore, the aim of this study was to examine the association between ZIP6 and ZIP14 expression and nuclear characteristics of human spermatozoa, specifically, nuclear surface area and chromatin accessibility as assessed by Sytox™ Orange (Thermo Fisher Scientific, Waltham, MA, USA) fluorescence intensity in both normozoospermic and asthenozoospermic samples.

METHODS

Sample collection

Semen samples were obtained from male patients undergoing intrauterine insemination at the University Clinical Center of Serbia, Višegradska Clinic for Gynecology and Obstetrics, Department for Medically Assisted Reproduction, Belgrade, Serbia, and written informed consent was obtained from all participants prior to sample collection. The sperm samples used in this study were drawn from the same cohort previously analyzed for compartmental and subcompartmental ZIP6 and ZIP14 localization [16]; the present analysis extends that work by examining the relationship between transporter expression and nuclear characteristics.

Sample preparation and classification

A total of 50 individual semen samples were collected in sterile containers following 3–5 days of sexual abstinence and allowed to liquefy at room temperature. Semen quality was assessed according to 2010 World Health Organization laboratory guidelines [17], and samples were classified as normozoospermic or asthenozoospermic. Men with known risk factors for male infertility or relevant medical history were excluded. For in-depth cellular analysis, four samples were selected, two normozoospermic and two asthenozoospermic, from donors aged 31–36 years, without children, and with a mean body mass index of 26.83.

Spermatozoa were purified using a two-layer density gradient to remove leukocytes, non-spermatic cells, and immotile spermatozoa. SpermGrad (Vitrolife®, Göteborg, Sweden) solutions at 90% and 45% were layered and centrifuged at 300 g for 20 minutes. The resulting pellet was washed twice in 5 mL of 0.9% NaCl at 300 g for 15 minutes each, then resuspended in 0.5 mL of 0.9% NaCl [16]. Sperm smears were prepared from the final suspension for immunofluorescence analysis.

Immunofluorescence

Smears were fixed in methanol for 15 minutes, air-dried, then post-fixed and permeabilized in acetone for five minutes, followed by incubation in 0.3% Triton X-100 in Tris-buffered saline (TBS) for 10 minutes. Non-specific binding was blocked for 30 minutes using a solution of 0.1%

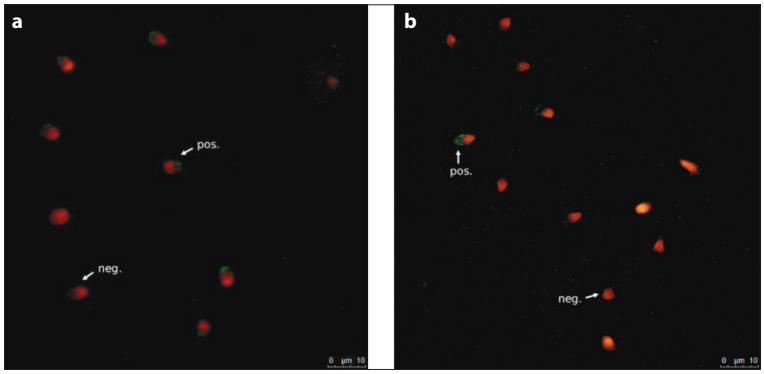


Figure 1. Sytox™ Orange (Thermo Fisher Scientific, Waltham, MA, USA) reaction intensity in the ZIP6 positive (+, pos.) and negative (-, neg.) reactions in normozoospermic (a) and asthenozoospermic (b) semen samples using confocal laser scanning microscopy, scale bar = 10 µm

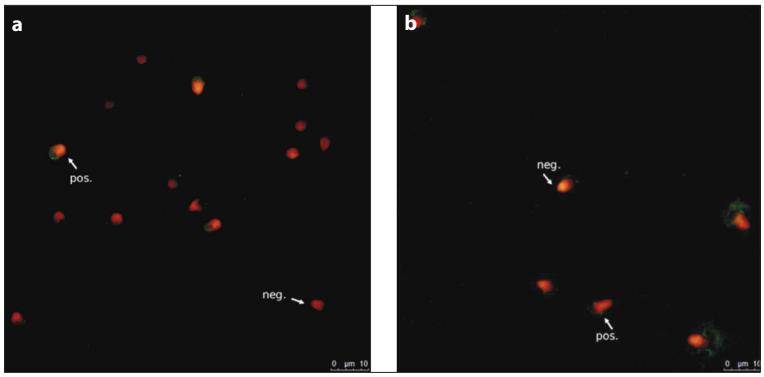


Figure 2. Sytox™ Orange (Thermo Fisher Scientific, Waltham, MA, USA) reaction intensity in the ZIP14 positive (+, pos.) and negative (-, neg.) reactions in normozoospermic (a) and asthenozoospermic (b) semen samples using confocal laser scanning microscopy, scale bar = 10 µm

bovine serum albumin, 3% normal goat serum, 0.2% Triton, and 0.05% Tween 20 in TBS. Smears were then incubated overnight at 4°C with primary antibodies against ZIP6 (1:50, PA5-21071, Invitrogen, Thermo Fisher Scientific) and ZIP14 (1:50, PA5-21077, Invitrogen, Thermo Fisher Scientific) diluted in blocking solution. After three washes in TBS with Tween 20, smears were incubated for one hour at room temperature with Alexa Fluor 488-conjugated secondary antibody (1:400, A-11029, Invitrogen, Thermo Fisher Scientific). Nuclei were counterstained with Sytox™ Orange (Thermo Fisher Scientific) (1:1000, S11368, Life Technologies, Thermo Fisher Scientific) for five minutes, after which slides were rinsed in TBS and mounted in Mowiol (Sigma-Aldrich, St. Louis, MO, USA) [18]. Specificity was confirmed by parallel negative controls in which primary antibodies were omitted.

Images were acquired using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany) operated in sequential mode to prevent channel crosstalk. ZIP immunofluorescence was excited at 488 nm (argon laser) and Sytox™ Orange (Thermo Fisher Scientific) at 543 nm (helium–neon laser). Signals were captured in the green channel (ZIP) and the orange-red channel [Sytox™ Orange (Thermo Fisher Scientific)].

Quantification of immunopositivity

Immunofluorescence images were analyzed using ImageJ (National Institutes of Health) version 1.52p. Spermatozoa displaying any level of ZIP6 or ZIP14 immunoreactivity were classified as ZIP-positive, irrespective of signal intensity or subcellular distribution, while cells showing no detectable signal were classified as ZIP-negative. For each sample, nuclear surface area and Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity were measured in a minimum of 100 spermatozoa per group.

Statistics

Data was analyzed using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA), version 8.4.3. Differences in nuclear area and Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity between ZIP-positive and ZIP-negative spermatozoa were assessed using the Kruskal–Wallis test with Dunn's post-hoc correction for multiple comparisons. Statistical significance was set at $p \leq 0.05$.

RESULTS

As previously reported, ZIP6 immunopositivity was significantly higher in normozoospermic than in asthenozoospermic spermatozoa, while ZIP14 immunopositivity did not differ significantly between groups [16]. Building on those findings, the present study examined whether this differential transporter expression is associated with differences in nuclear characteristics, specifically, sperm head nuclear surface area and Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity as a measure of chromatin accessibility, comparing ZIP-positive and ZIP-negative spermatozoa within both normozoospermic and asthenozoospermic samples (Figures 1 and 2).

Sperm head nuclear surface area

Sperm head nuclear surface area did not differ significantly between ZIP-positive and ZIP-negative spermatozoa in normozoospermic samples for either ZIP6 or ZIP14 (Figure 3a). In asthenozoospermic samples, ZIP6-positive spermatozoa had a slightly but significantly smaller nuclear surface area compared to ZIP6-negative cells, while no such difference was observed for ZIP14 (Figure 3b). Overall, nuclear surface area remained within a comparable range across all groups, suggesting that sperm head morphology is largely consistent regardless of ZIP transporter expression status.

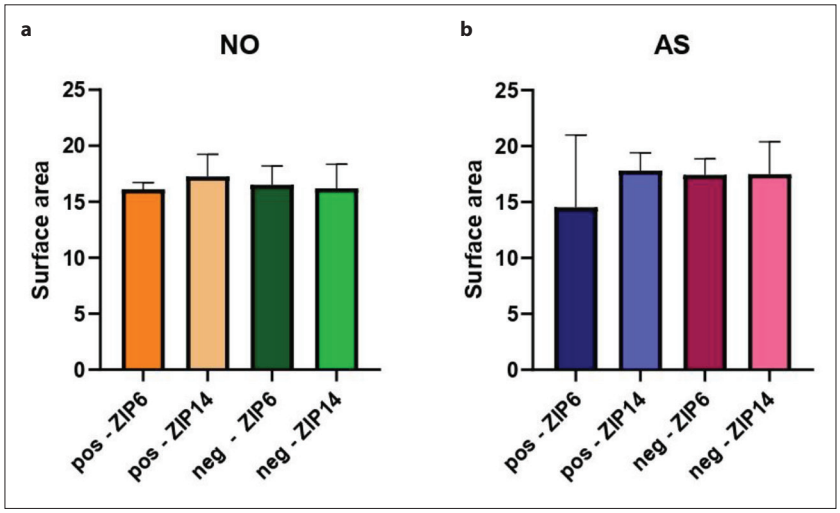


Figure 3. Sperm head nuclear surface area; sample types: a.) NO (normozoospermia); b.) AS (asthenozoospermia); pos-ZIP6, pos-ZIP14 – positive reactions for ZIP; neg-ZIP6, neg-ZIP14 – negative reactions for ZIP

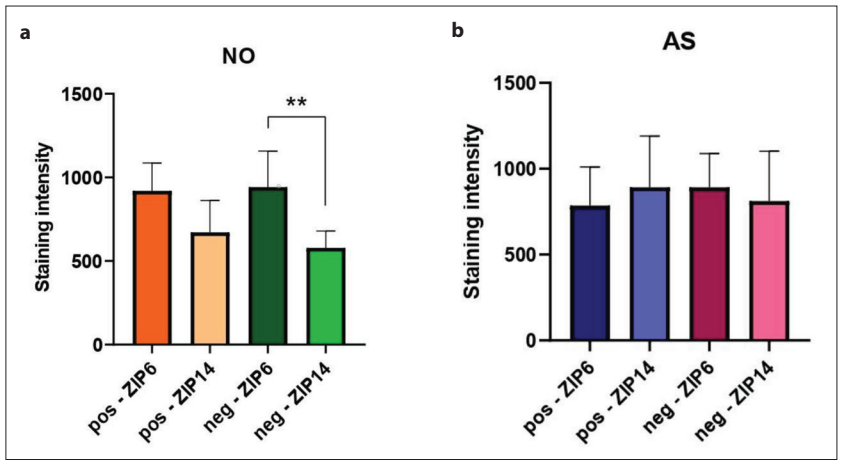


Figure 4. DNA staining intensity with Sytox™ Orange (Thermo Fisher Scientific, Waltham, MA, USA): a.) NO (normozoospermia); b.) AS (asthenozoospermia); pos-ZIP6, pos-ZIP14 – positive reactions for ZIP; neg-ZIP6, neg-ZIP14 – negative reactions for ZIP;
**$p \leq 0.01$

Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity

In normozoospermic samples, Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity did not differ significantly between ZIP6-positive and ZIP14-positive spermatozoa, nor between ZIP6-positive and ZIP6-negative cells (Figure 2a). However, a statistically significant difference was observed between ZIP6-negative and ZIP14-negative spermatozoa, with ZIP14-negative cells displaying lower fluorescence intensity than ZIP6-negative cells ($p \leq 0.01$; Figure 4a). In asthenozoospermic samples, no statistically significant differences in Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity were observed between ZIP-positive and ZIP-negative spermatozoa for either ZIP6 or ZIP14 (Figure 4b).

DISCUSSION

The present study examined whether ZIP6 and ZIP14 zinc transporter expressions are associated with differences in nuclear characteristics among human spermatozoa, using Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity and nuclear surface area as indicators of chromatin accessibility and nuclear morphology. The analysis was performed in ZIP-positive and ZIP-negative subpopulations within normozoospermic and asthenozoospermic samples, building on previously reported compartmental and subcompartmental localization data for these transporters [16].

Consistent with earlier observations, ZIP14 immunoreactivity was relatively stable between normozoospermic and asthenozoospermic groups, whereas ZIP6 expression was reduced in asthenozoospermia [16]. This differential pattern suggests that ZIP6 and ZIP14 reflect distinct aspects of sperm cellular biology rather than equivalent functional roles. The reduction in ZIP6 immunopositivity in asthenozoospermic spermatozoa supports the notion that ZIP6 expression may possibly be linked to sperm maturation status and functional competence, consistent with the recognized role of zinc transporter dynamics in spermatogenesis and maintenance of sperm cellular homeostasis [12, 19]. Whether this reduction reflects impaired zinc uptake capacity, altered transporter trafficking, or simply a marker of a less mature sperm subpopulation remains to be determined. By contrast, the relative stability of ZIP14 across groups suggests that its expression may be maintained independently of motility status, possibly reflecting a more constitutive role in zinc homeostasis.

The central question of this study was whether transporter-defined sperm subpopulations differ in nuclear characteristics. No major differences in sperm head nuclear surface area or Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity were observed between ZIP-positive and ZIP-negative spermatozoa in either group, indicating preserved global nuclear morphology and overall chromatin organization regardless of ZIP expression status. As Sytox™ Orange (Thermo Fisher Scientific) fluorescence reflects DNA accessibility rather than DNA fragmentation, these findings suggest comparable bulk chromatin compaction while not excluding subtle or localized DNA damage detectable only by more sensitive assays [20]. Importantly, the preservation of global nuclear features does not preclude functional heterogeneity. Differential immunoreactivity of ZIP6 and ZIP14 suggests that these transporters may represent distinct aspects of sperm cellular

diversity rather than equivalent functional roles [4]. The immunofluorescence pattern suggests that ZIP6 may be associated with sperm functional competence, supported by its reduced presence in asthenozoospermia and previous links between zinc transporter expression and sperm maturation [12, 15, 19]. Differences between ZIP6 and ZIP14 may reflect transporter-specific regulation during spermatogenesis or define sperm subpopulations with altered chromatin remodeling dynamics despite preserved global nuclear structure.

Beyond its fundamental role in zinc homeostasis, intracellular zinc availability has been increasingly associated with sperm chromatin organization and nuclear heterogeneity [12, 13]. During spermatogenesis, chromatin condensation and the histone-to-protamine transition are tightly regulated processes influenced by histone acetylation and epigenetic remodeling [5, 21]. Recent studies have shown that histone retention, protamine replacement, and post-translational histone modifications contribute to the establishment of localized regions of accessible chromatin within the highly condensed sperm nucleus [3, 22], supporting the concept that spermatozoa within a single ejaculate represent structurally heterogeneous subpopulations which may affect future offspring [23].

Consistent with this concept of nuclear heterogeneity, one finding warrants specific attention. In normozoospermic samples, a statistically significant difference in Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity was observed between ZIP6-negative and ZIP14-negative spermatozoa, with ZIP14-negative cells displaying lower intensity. This may suggest that, among spermatozoa lacking transporter immunoreactivity, the absence of ZIP14 may be associated with reduced DNA accessibility, potentially reflecting differences in chromatin organization compared to cells lacking ZIP6 [22, 23]. This distinction may possibly indicate distinct regulatory roles of the two transporters during spermatogenesis [15, 16]. Zinc is known to contribute to chromatin stabilization through protamine–DNA interactions and disulfide bond formation [11, 14], raising the possibility that ZIP14-dependent zinc availability could influence local chromatin condensation in ways not captured by ZIP6 expression alone. Although this finding is subtle and based on a limited sample, it highlights a biologically meaningful observation that warrants a follow-up with larger cohorts and complementary chromatin assays.

The sperm nuclear surface area findings further support the overall picture of structural consistency across subpopulations. The slightly smaller nuclear area observed in ZIP6-positive asthenozoospermic spermatozoa may reflect a subpopulation with subtly altered chromatin compaction or incomplete nuclear maturation [3, 5, 22], though this observation must be interpreted cautiously given the known sensitivity of nuclear area measurements to fixation and staining conditions [24], and requires confirmation in larger, independent cohorts. This differential pattern suggests that ZIP6 and ZIP14 head dimensions in human spermatozoa are generally tightly constrained by the final stages of spermiogenesis, and it is plausible that major morphological divergence between ZIP-positive and ZIP-negative cells would be unlikely in purified, motility-selected samples [5, 25].

It is important to note that Sytox™ Orange (Thermo Fisher Scientific) intensity, while informative as an indicator of chromatin accessibility, is not equivalent to established measures of DNA integrity such as the DNA fragmentation index obtained by sperm chromatin structure assay (SCSA), the Comet assay, or terminal deoxynucleotidyl transferase dUTP nick end labeling staining [9, 20, 26]. The interpretation of intensity differences reflecting chromatin organization rather than DNA damage is therefore appropriate, but limits the conclusions that can be drawn regarding fertilization competence. Future studies should combine zinc transporter immunoprofiling with validated DNA fragmentation assays in larger, well-characterized cohorts to determine whether ZIP6 or ZIP14 expression predicts sperm DNA integrity in a clinically meaningful sense [27].

Taken together, the present findings indicate that ZIP6 and ZIP14 define distinct sperm subpopulations that do not differ substantially in global nuclear morphology or bulk chromatin staining. The heterogeneity in Sytox™ Orange (Thermo Fisher Scientific) intensity observed among transporter-negative cells in normozoospermia suggests that subtle differences in chromatin organization may exist between ZIP6- and ZIP14-dependent zinc handling pathways, a distinction that Sytox™ Orange (Thermo Fisher Scientific) alone cannot fully resolve. Future research should, therefore, extend to validated assays of sperm DNA fragmentation and chromatin integrity to better elucidate the fertilization potential of these subpopulations and their impact on male reproductive health.

CONCLUSION

This study demonstrates that ZIP6 and ZIP14 identify molecularly distinct subpopulations of human spermatozoa in both normozoospermic and asthenozoospermic samples. The reduced ZIP6 immunopositivity observed in asthenozoospermia, in contrast to the stable expression of ZIP14, suggests a possible association between ZIP6 and sperm maturation status and functional competence.

The absence of pronounced differences in sperm head nuclear surface area and Sytox™ Orange (Thermo Fisher Scientific) fluorescence intensity between ZIP-positive and ZIP-negative spermatozoa indicates that transporter-defined subpopulations do not differ substantially in global nuclear morphology or bulk chromatin organization. The subtle intensity differences observed among transporter-negative cells in normozoospermic samples may reflect focal variations in chromatin accessibility rather than generalized DNA fragmentation, consistent with the known structural heterogeneity of ejaculated sperm populations.

Collectively, these findings suggest that ZIP6 and ZIP14 represent potential molecular markers of sperm heterogeneity whose relevance extends beyond zinc homeostasis to broader questions of sperm nuclear quality. The present results indicate that transporter-defined heterogeneity is

primarily molecular rather than associated with major nuclear structural alterations, highlighting that subtle molecular differences may exist despite preserved global morphology. Given the potential relevance of molecular differences among individual spermatozoa to fertilization potential, future studies integrating zinc transporter immunoprofiling with validated DNA fragmentation assays, such as SCSA, terminal deoxynucleotidyl transferase dUTP nick end labeling, or the Comet assay, in larger and more diverse cohorts, including those exposed to environmental or occupational stressors, may help determine whether differential ZIP expression is associated with DNA damage and ultimately influences fertilization potential and assisted reproductive outcomes.

Ethics: The study was approved by the Ethics Committee of the University Clinical Center of Serbia, Belgrade, Serbia (No.: 1730/12), and written informed consent was obtained from all participants prior to sample collection.

Conflict of interest: None declared.

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Експресија транспортера цинка *ZIP6* и *ZIP14* у хуманим сперматозоидима из нормозооспермичних и астенозооспермичних узорака – повезаност са приступачношћу нуклеарног хроматина

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САЖЕТАК

Увод/Циљ Мушки инфертилитет чини приближно 40% свих случајева неплодности, при чему астенозооспермија погађа скоро сваког петог инфертилног мушкарца. Поред конвенционалних параметара анализе семена, организација нуклеарне ДНК и приступачност хроматина пружају дубљи увид у квалитет сперматозоида. Транспортери цинка *ZIP6* и *ZIP14* доводе се у везу са стабилизацијом хроматина и хомеостазом цинка, процесима који могу бити од суштинског значаја за сазревање сперматозоида и њихову фертилизациону способност.

Методе Узорци семена нормозооспермичних и астенозооспермичних мушкараца укључених у програм интраутерине инсеминације пречишћени су путем градијента густине. *ZIP6* и *ZIP14* детектовани су имунофлуоресценцијом, док је нуклеарна ДНК визуализована применом боје *Sytox™ Orange (Thermo Fisher Scientific,* Валтам, МА, САД). Ниво експресије, површина нуклеуса и интензитет бојења квантификовани су у програму *ImageJ* (Национални здравствени институт), а разлике између група анализиране су Крускал–Валисовим тестом уз Данову *post hoc* корекцију ($p \leq 0,05$).

Резултати Имунопозитивност *ZIP6* била је значајно смањена у астенозооспермичним у односу на нормозооспермичне узорке, док је експресија *ZIP14* остала стабилна. Површина једра и укупни интензитет флуоресценције боје *Sytox™ Orange (Thermo Fisher Scientific)* нису се значајно разликовали између сперматозоида позитивних и негативних на транспортере, иако су *ZIP6*-позитивне астенозооспермичне ћелије показале умерено смањење површине нуклеуса. У нормозооспермичним узорцима, *ZIP14*-негативни сперматозоиди показали су нижи интензитет флуоресценције боје *Sytox™ Orange (Thermo Fisher Scientific)* у односу на *ZIP6*-негативне ћелије, што указује на локализоване разлике у приступачности хроматина између субпопулација дефинисаних експресијом транспортера.

Закључак Експресија *ZIP6* одражава степен сазревања и функционалну компетентност сперматозоида, док *ZIP14* показује конститутивно стабилан образац експресије. Оба транспортера могу представљати молекуларне маркере за селекцију сперматозоида високог квалитета у асистираним репродуктивним технологијама, нарочито у популацијама изложеним професионалним стресорима који нарушавају интегритет ДНК.

Кључне речи: *ZIP6*; *ZIP14*; сперматозоиди; доступност хроматина; астенозооспермија; нормозооспермија