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ORIGINAL ARTICLE

Differential sperm histone retention in normozoospermic ejaculates of infertile men negatively affects sperm functional competence and embryo quality

Riddhi Kirit Pandya[1] | Ameya Jijo[1] | Aswathi Cheredath[1] | Shubhashree Uppangala[2] | Sujith Raj Salian[1] | Vani R Lakshmi[3] | Pratap Kumar[4] | Guruprasad Kalthur[5] | Sanjay Gupta[6] | Satish Kumar Adiga[1]

[1]Centre of Excellence in Clinical Embryology, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[2]Division of Reproductive Genetics, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[3]Department of Data Science, Prasanna School of Public Health, Manipal Academy of Higher Education, Manipal, India

[4]Department of Reproductive Medicine and Surgery, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[5]Division of Reproductive Biology, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India

[6]KS313, Epigenetics and Chromatin Biology Group, Gupta Lab, Cancer Research Institute, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai, India

Correspondence
Satish Kumar Adiga, Centre of Excellence in Clinical Embryology, Department of Reproductive Science, Kasturba Medical College, Manipal Academy of Higher Education, Manipal 576104, India.
Email: satish.adiga@manipal.edu

Sanjay Gupta, KS313, Epigenetics and Chromatin Biology Group, Gupta Lab, Cancer Research Institute, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai, India.
Email: sgupta@actrec.gov.in

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Abstract

Background: The unique epigenetic architecture that sperm cells acquire during spermiogenesis by retaining <15% of either canonical or variant histone proteins in their genome is essential for normal embryogenesis. Whilst heterogeneous levels of retained histones are found in morphologically normal spermatozoa, their effect on reproductive outcomes is not fully understood.

Methods: Processed spermatozoa ($n = 62$) were tested for DNA integrity by sperm chromatin dispersion assay, and retained histones were extracted and subjected to dot-blot analysis. The impact of retained histone modifications in normozoospermic patients on sperm functional characteristics, embryo quality, metabolic signature in embryo spent culture medium and pregnancy outcome was studied.

Results: Dot-blot analysis showed heterogeneous levels of retained histones in the genome of normozoospermic ejaculates. Post-wash sperm yield was affected by an increase in H3K27Me3 and H4K20Me3 levels in the sperm chromatin ($p < 0.05$). Also, spermatozoa with higher histone H3 retention had increased DNA damage ($p < 0.05$). Spermatozoa from these cohorts, when injected into donor oocytes, correlated to a significant decrease in the fertilisation rate with an increase in sperm histone H3 ($p < 0.05$) and H3K27Me3 ($p < 0.01$). An increase in histone H3 negatively affected embryo quality ($p < 0.01$) and clinical pregnancy outcome post-embryo transfer ($p < 0.05$). On the other hand, spent culture medium metabolites assessed by high-resolution (800 MHz)

nuclear magnetic resonance showed an increased intensity of the amino acid methionine in the non-pregnant group than in the pregnant group ($p < 0.05$) and a negative correlation with sperm histone H3 in the pregnant group ($p < 0.05$).

Discussion and conclusion: Histone retention in spermatozoa can be one of the factors behind the development of idiopathic male infertility. Such spermatozoa may influence embryonic behaviour and thereby affect the success rate of assisted reproductive technology procedures. These results, although descriptive in nature, warrant further research to address the underlying mechanisms behind these clinically important observations.

KEYWORDS
assisted reproductive technology, infertility, metabolomics, pregnancy outcome, retained histone post-translational modifications, sperm epigenetics

1 | INTRODUCTION

Classically, the contribution of spermatozoa to embryogenesis was limited to the transfer of paternal DNA[1,2] and centrioles[3] and as a signal to start the metabolic activity of the oocytes.[4] However, recent literature involving high-throughput technologies indicates that their contribution is much more central than previously thought.[5–7] Several reports have demonstrated that the male gamete imparts various epigenetic marks, RNA and protein molecules to the resultant zygotes.[7–9] These factors might have a vital role in embryo development and future health of the progeny.[5,10–12]

During spermiogenesis, human spermatozoa attain a unique epigenetic architecture by replacing nucleosome-bound DNA with protamines and retaining <15% of either canonical or variant histones in the genome.[13–15] The retained nucleosomes in mature spermatozoa exhibit specific post-translational modifications (PTMs), such as acetylation and methylation majorly on H3 and H4 histones. These heritable marks are highly enriched in the imprinted gene regions (microRNA and HOX gene clusters), promoters and regulatory regions of various developmentally important genes.[8,16–18] The epigenetic memory delivered by spermatozoa during fertilisation may aid in the epigenetic reprogramming of zygotes and the development of early embryos by regulating their gene expression patterns.[19–21]

Earlier reports have demonstrated that an abnormal histone-to-protamine ratio in infertile men leads to altered semen parameters, fertilisation failure and low pregnancy outcomes.[22–24] In addition, the differential profiles of sperm DNA methylation were detected on imprinted and developmental genes located at retained histones in oligozoospermic patients.[25,26] Furthermore, patients with astheno and teratozoospermic conditions had differential epigenetic signatures.[27] A recent report also demonstrated that normozoospermic patients have differential epigenetic signatures making a sub-cohort of unexplained infertility,[28] where changes in the sperm histone retained epigenome were associated with fertilisation failure, poor blastocyst development and development of imprinted disorders in the progeny.[28–31]

Heterogeneous levels of histone PTMs in mature normal human spermatozoa have been reported.[15,18,32,33] The enrichment of gene activation marks (H3K4Me3 and H4K12Ac) and repression marks (H3K9Me3, H3K27Me3 and H4K20Me3) were observed at the developmental promoter region and certain imprinted genes in the spermatozoa.[8,26,33] Also, these histone modifications are transmitted to embryos during fertilisation.[15,18,21] However, the influence of altered retained histone PTMs of the paternal genome on embryogenesis has not been elucidated. Importantly, concerns associated with increased imprinting disorders among those born through assisted reproductive technology (ART)[34,35] raise the need to study the sperm epigenetic signature and its impact on embryos. Therefore, the current study looked at the retained histones and their PTMs in the processed sperm fraction of men undergoing ART treatment and their impact on sperm functional characteristics and embryo development.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Samples obtained from patients ($n = 62$) visiting the university infertility clinic for their treatment were included in the study after obtaining ethical approval from the Institutional Ethics Committee (IEC 464/2017 and 568/2020) and written informed consent from all the patients. Ejaculates were evaluated according to World Health Organization criteria.[36] Based on semen analysis reports, only normozoospermic subjects were included. These patients did not have varicocele, urogenital infections, hypogonadotropic hypogonadism, obstruction or agenesis of the seminal ducts. The patients' sperm characteristics are described in Table 1. The study outline is depicted in Figure 1. Briefly, processed sperm samples ($n = 62$) were used to

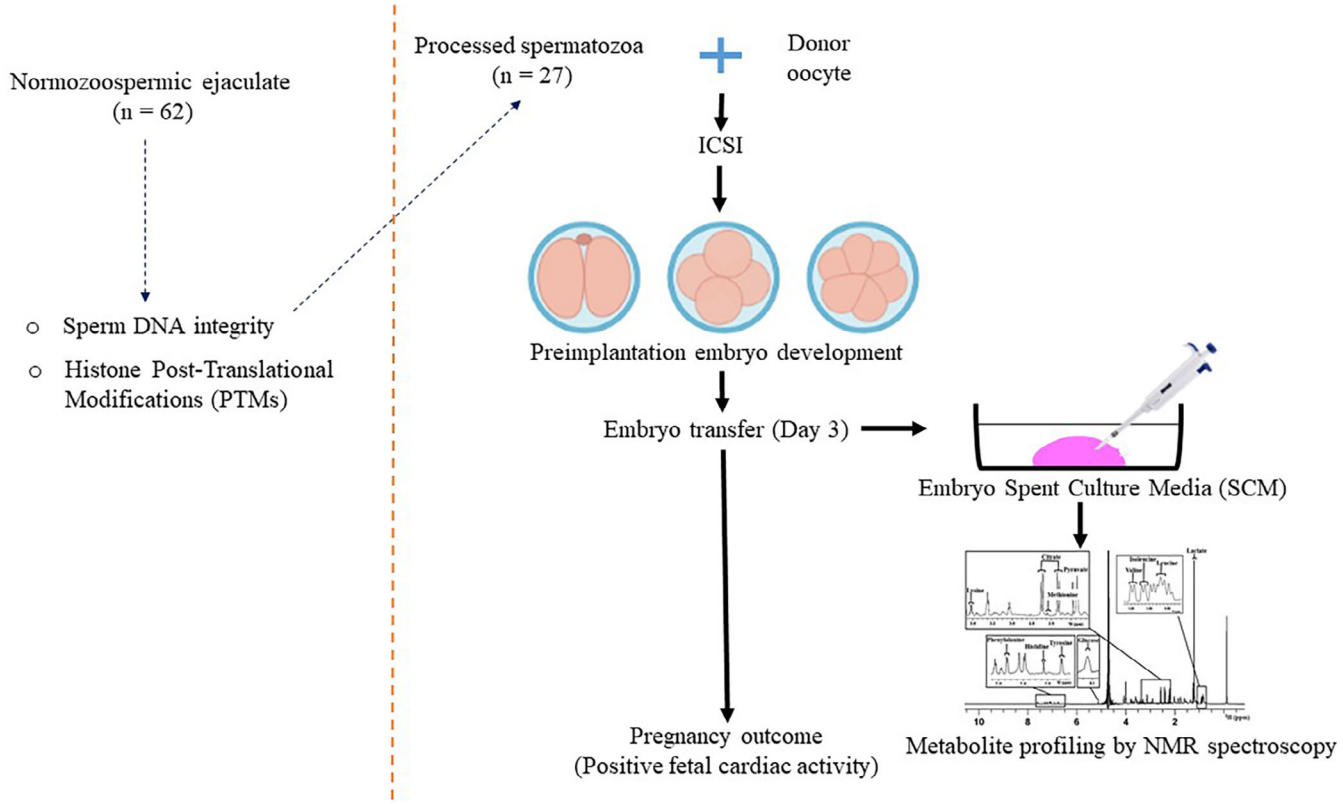

FIGURE 1 Schematic outline of the study: experimental outline to determine the influence of retained sperm epigenetic signature on pre-implantation embryos. NMR, nuclear magnetic resonance.

TABLE 1 Sperm characteristics in neat ejaculate and processed fraction.

	Median (Q_1 –Q_3)
Neat ejaculate ($n = 62$)	
Male age (years)	36.00 (34.00–39.75)
Semen volume (mL)	2.50 (1.87–3.63)
Sperm concentration (10^6/mL)	62.50 (46.50–80.75)
Total sperm number (10^6/ejaculate)	157.00 (95.25–204.75)
Total motility (%)	70.00 (59.25–76.75)
Processed fraction ($n = 62$)	
Sperm concentration (10^6/mL)	27.66 (20.51–44.13)
DNA damage (%)	9.00 (2.00–23.25)

extract retained histones using the acid extraction method followed by dot-blot analysis. Furthermore, part of the sample was also subjected to a sperm chromatin dispersion (SCD) test to understand its sperm chromatin integrity. To understand the association between histone PTMs and sperm characteristics, a correlation analysis was performed. Out of 62 patients, 27 underwent Intracytoplasmic Sperm Injection (ICSI) cycles using donor oocytes. All the recipient females ($n = 27$; partner of the male patients) had a history of poor ovarian reserve as evidenced by low Anti-Mullerian Hormone <0.5 ng/mL in addition to previous in vitro fertilisation failure because of poor ovarian response ($n = 8$) and advanced maternal age ($n = 2$). The processed sperm samples of these patients were used to understand the impact of sperm histone PTMs on embryo developmental competence and reproductive outcomes in pregnant ($n = 17$) and non-pregnant groups ($n = 10$). Post-embryo transfer spent culture medium was collected from both the groups and analysed for the levels of metabolites using nuclear magnetic resonance (NMR) spectroscopy and associated with sperm histone PTMs to determine the relationship between them.

2.2 | Semen sample and sperm preparation

After semen analysis, ejaculates were subjected to density gradient separation as described earlier.[37] Briefly, samples were overlaid on the gradient solutions (80% and 40%) (^{V}GRAD, Cat. No. V-GRD83_100P, Vitromed GmbH) and centrifuged at 350 *g* for 20 min. The pellet was resuspended in pre-warmed V-HEPES plus media (Cat. No. V-HTF-P1, Vitromed GmbH) and washed by centrifuging at 300 *g* for 8 min. The pellet was resuspended in pre-warmed V-ONESTEP (Cat. No. V-OSM-20, Vitromed GmbH) and washed again by centrifuging at 200 *g* for 8 min. After wash, the sperm concentration was calculated and 0.1 million spermatozoa were used to determine sperm chromatin integrity. The remaining fraction was stored in phosphate buffered saline (PBS) at −80°C for histone PTM analysis.

2.3 | Sperm chromatin integrity assay

On processed sperm samples, an SCD assay was carried out as described by Fernández et al.[38] with a few minor modifications. A mixture of 0.1 million spermatozoa and 1% low melting agarose kept at 37°C was layered on slides pre-coated with 0.65% normal melting agarose and allowed to solidify. The slides were immersed in denaturation solution (0.08 N HCl), lysis solution 1 (0.4 m Tris, 20 mM Dithiothreitol (DTT), 1% SDS, 50mM Ethylenediamine tetraacetic acid) and lysis solution 2 (0.4 M Tris, 2 M NaCl). The slides were then air-dried after being neutralised in Tris buffer (0.4 M Tris) and serially dehydrated in alcohol. Sperm chromatin integrity was assessed under a fluorescent microscope (Imager-A1; Zeiss) after staining the cells with ethidium bromide (7 μg/mL). The large halo spermatozoa (without any DNA damage or normal) produce halos with a thickness equal to or greater than the length of the minor diameter of the core, whereas small halo spermatozoa produce halos with a thickness equal to or smaller than one-third diameter of the minor diameter of the core.[38,39] The percentage of spermatozoa with damaged DNA was calculated by counting spermatozoa with a small + no halo, and the percentage of spermatozoa with severely damaged DNA was calculated by counting spermatozoa with no halo.

2.4 | Histone extraction

Acid extraction of sperm histone was carried out as described previously with minor modifications.[33,40] Approximately 2 million spermatozoa were washed twice with PBS at 4000 *g* for 5 min at 4°C. Pellet was resuspended in hypotonic lysis buffer (10 mM Tris–Cl pH 8.0, 0.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM DTT) along with a protease inhibitor and vortexed thoroughly. After incubation for 1 h at 4°C, their nuclei were pelleted by centrifuging at 10,000 *g* for 10 min at 4°C, and 0.4 N of H_2SO_4 solution was added. The tubes were vortexed with intermittent incubation on ice for 2 h. The solution was centrifuged at 16,000 *g* for 10 min at 4°C and the supernatant was transferred to a fresh tube. The proteins were allowed to precipitate by adding 4 volumes of chilled acetone and stored at −20°C overnight. The next day, they were pelleted down and washed once with chilled acidified acetone (0.05 M HCl in 100% acetone) and twice with chilled 100% acetone at 16,000 *g* for 10 min at 4°C. The pellet was vacuum dried and dissolved in 0.1% β-mercaptoethanol and stored at −20°C.

2.5 | Dot-blot analysis

Total protein extracted from 2 million spermatozoa was slotted on a polyvinylidene difluoride membrane using a vacuum and proteins were visualised. The membrane was incubated in 5% Bovine serum albumin in Tris-buffered saline with 0.1% Tween (TBST) for 1 h and probed with histone H3, histone H4 and histone PTMs such as H3K4Me3, H4K12Ac, H3K9Me3, H3K27Me3 and H4K20Me3 antibodies overnight at 4°C. The membrane was washed three times in TBST and incubated with a secondary antibody at room temperature for 1 h. Blots were washed using three washes and developed using Clarity Max western ECL substrate (Cat. No. 1705062, Bio-Rad). Antibodies used and dilutions are given in Table S1. The intensity of the signal for all the modifications for each patient was calculated by densitometric analysis using ImageJ software. The relative expression of each modification was expressed in fold change by normalising the signal intensity to the total protein spotted on the membrane (Figure S1).

2.6 | Fertilisation and embryo grading

Controlled ovarian hyperstimulation of the donors was performed by a standard antagonist protocol. Follicular aspiration was performed and retrieved oocyte cumulus complexes were washed and then incubated in V-ONESTEP medium media (Cat. No. V-OSM-20, Vitromed GmbH) at 37°C in 6% CO_2 and 5% O_2 in an incubator. An average of 10-11 donor MII oocytes per couple (n = 27) were inseminated with the spermatozoa by ICSI and cultured individually in V-ONESTEP culture media. After 16–18 h of insemination, oocytes were assessed for fertilisation. On day 3, fertilised embryos were graded as per the ESHRE consensus,[41] and per patient (n = 2), embryos were transferred to the recipient females (partner of the male patients). Grade I and II embryos were considered good- and average-quality embryos, respectively, whereas Grade III embryos were considered poor-quality embryos.

2.7 | Biochemical and chemical pregnancy outcomes

Fourteen days after embryo transfer, a blood test was performed to determine the level of human chorionic gonadotropin (β-hCG). β-hCG levels above 100 mIU/mL were considered positive for biochemical pregnancy. After 4 weeks, ultrasonography was performed to determine the presence of a gestational sac or foetal cardiac activity to confirm clinical pregnancy.

2.8 | Collection of embryo spent culture media, NMR sample preparation and data analysis

The spent culture medium samples and media controls were collected post-embryo transfer, snap-frozen and stored at −80°C for the NMR analysis. The samples were prepared as described in Cheredath et al.[42] Briefly, 25 μL of embryo spent culture media samples were thawed for 10 min at room temperature. The samples were prepared by dilution with sodium salt of 2,2,3,3 tetradeutero 3-(trimethyl silyl) propionate (TSP) as a standard reference compound and transferred to 1.7 mm

NMR tubes for profiling the spectra. All spectra were acquired on a Bruker AVANCE NMR spectrometer operating at a ^{1}H resonance frequency of 800 MHz equipped with a 1.7 mm cryo-probe at 298 K. The NMR experiments were recorded using Carr–Purcell–Meiboom–Gill sequence 'cpmgpr1d' pulse program available in the Bruker library. Data were analysed using the Bruker TOPSPIN 3.6.2 software. The peak integrals were measured with respect to the corresponding integral of the TSP signal.

2.9 | Statistical analysis

All the data are presented as the mean and SD, median or interquartile range (Q_1–Q_3) after checking the normality assumption with the help of the Shapiro–Wilk test. Subsequently, an age-adjusted Pearson's correlation analysis was performed using Jamovi (version 2.2.5; RRID:SCR_016142).[43] The strength of the correlation was represented by the value of the correlation coefficient (r value). To compare male age, semen parameters, DNA damage, female and donor age, the number of retrieved oocytes, endometrial thickness and the number of embryos transferred between pregnant and non-pregnant groups, two samples independent t-tests or Mann–Whitney U-test were performed after appropriate assumption checks. Female age, male age and sperm DNA damage-adjusted analysis of covariance (ANCOVA) were performed between the levels of sperm histone PTMs and clinical pregnancy. To determine the levels of methionine in media control and spent culture medium (SCM) of the pregnant and non-pregnant groups, the Kruskal–Wallis test was performed. All graphs were plotted using GraphPad Prism 8 (GraphPad Prism software). The level of significance was set at 5% for the entire study.

3 | RESULTS

3.1 | Sperm histone PTMs and post-wash sperm characteristics

Sperm characteristics analysed in neat and processed samples are summarised in Table 1. To determine the association between processed sperm characteristics, retained core histones and histone PTM levels, age-adjusted Pearson's correlation was performed as our data demonstrated age-related changes in sperm characteristics such as total motility ($r = -0.305$, $p < 0.05$), progressive motility ($r = -0.313$, $p < 0.01$) and tail defects ($r = 0.341$, $p < 0.01$) in the neat ejaculate (Table S2). Correlation analysis demonstrated a weak negative correlation between sperm concentration and H3K27Me3 ($r = -0.27$, $p < 0.05$) and H4K20Me3 ($r = -0.285$, $p < 0.05$). Although the percentage of spermatozoa with DNA damage (% small halo + % no halo) did not show any correlation with core histones and PTMs, the percentage of spermatozoa with severe damage (% no halo) had a weak positive correlation with H3 histone ($r = 0.26$, $p < 0.05$) (Table 2).

3.2 | Core histone (H3) and its modification (H3K27Me3) demonstrated a relationship between fertilisation and embryo quality

Sperm histone modifications play an important role during fertilisation and embryo development. Therefore, the age-adjusted correlation analysis was carried out between retained sperm histone levels, their PTM levels, and embryological outcomes such as fertilisation rate and embryo quality on day 3 of the development. Table 3 represents the embryological outcomes. Among the histones and modifications considered in this study, histone H3 ($r = -0.397$, $p < 0.05$) and H3K27Me3 ($r = -0.503$, $p < 0.01$) showed a moderate negative correlation with the fertilisation rate (Table 4). However, no correlation was obtained between sperm histone PTMs and cleavage rate. Interestingly, histone H3 showed a moderate negative correlation with the percentage of good-quality embryos on day 3 of development ($r = -0.568$, $p < 0.01$).

3.3 | Pregnancy outcome is associated with core histone H3 in spermatozoa

As we found a negative association of retained histone PTMs present in spermatozoa with fertilisation rate and embryo quality, the association between sperm histone PTMs and pregnancy outcome was assessed. Out of 27 infertile couples from the donor oocyte program, 17 patients conceived successfully, resulting in a 62.9% clinical pregnancy rate. Here, the patients were categorised into two groups. Patients who had positive foetal cardiac activity post-embryo transfer were considered pregnant ($n = 17$) and those with negative foetal cardiac activity were considered non-pregnant ($n = 10$). The factors influencing pregnancy outcomes such as the age of the male patients, semen characteristics, sperm DNA damage, donor age, recipient age, oocytes used, embryos transferred and endometrial thickness, were comparable between the groups, as presented in Table S3. The mean levels of sperm histone and their PTMs were compared between the pregnant and non-pregnant groups by applying ANCOVA adjusting for the influence of the female recipients' age, male age and sperm DNA damage. It was found that the pregnant group had a significantly low level of histone H3 in sperm fraction used in ICSI than the non-pregnant group (Figure 2). However, other histone modification levels did not show any significant difference between the groups (Table S4).

3.4 | Sperm histone H3 is associated with altered metabolite levels in SCM of preimplantation embryos

As we found that the high histone H3 levels might influence pregnancy outcomes, we explored whether sperm histones and their modifications have any role in the embryo metabolomic signature. Hence, SCM was profiled using high-sensitivity enhanced 800 MHz NMR spectroscopy. A total of 12 metabolites consisting of eight amino acids and four carbohydrates were identified by 1D ^{1}H NMR spectroscopy in

TABLE 2 Correlation analysis between sperm histone post-translational modifications and processed sperm parameters ($n = 62$).

	Histone 3	H3K4Me3	H3K9Me3	H3K27Me3	Histone 4	H4K12Ac	H4K20Me3
Sperm concentration (10^6/mL)	0.058	−0.095	−0.125	−0.270*	0.154	−0.153	−0.285*
DNA damage (%)	0.075	0.060	0.075	0.139	0.052	−0.168	0.120
Severe DNA damage (%)	0.260*	0.021	0.143	−0.007	0.086	−0.249	0.058

Note: Pearson's correlation coefficient (r value) is presented in the table (adjusted for men's age). DNA damage includes small halo + no halo (%). Severe DNA damage includes no halo (%).
*Significant at alpha = 5%.

TABLE 3 Pre-implantation embryo developmental outcomes ($n = 27$).

	Mean ± SD
Fertilisation rate (%)	73.94 ± 16.08
Cleavage rate (%)	96.50 ± 6.50
Good-quality embryos (%) (day 3) (Grade I embryos)	38.66 ± 28.74

the medium control (blank) and SCM of embryos in the pregnant and non-pregnant categories (Table S5). The methionine intensity in SCM of the embryos that resulted in pregnancy was significantly reduced when compared to embryos that did not lead to pregnancy. ($p < 0.05$, Figure 3A). Further correlation analysis demonstrated that levels of methionine in SCM of the pregnant group showed a moderate negative correlation with sperm histone H3 levels ($r = -0.648$, $p < 0.05$) (Figure 3B and Table S6).

4 | DISCUSSION

In this study, heterogeneous levels of retained histones in spermatozoa demonstrated a significant association with sperm DNA fragmentation, fertilisation, embryo quality, and pregnancy outcome suggesting that histone retention in the spermatozoa from normozoospermic ejaculate might influence embryonic behaviour and pregnancy outcome.

Heterogeneous levels of histone PTMs in the chromatin of normal human spermatozoa have been reported.[15,32,44,45] Sperm chromatin enriched with histone PTMs such as H3K4Me2, H3K36Me3 and H4S1ph is indicative of poor sperm quality.[46–48] In line with this, our findings demonstrated the heterogenous levels of retained histones and their PTMs in processed sperm fractions of normozoospermic ejaculates. Also, a negative correlation was observed between post-wash sperm concentration and heterochromatin marks, H3K27Me3 and H4K20Me3, indicating that increased histone retention in chromatin could affect the post-wash sperm yield. Histone methylation marks such as H3K9Me3, H3K27Me3 and H4K20Me3 are present in the heterochromatin region and are crucial for the progression of normal spermatogenesis.[49,50] Any alterations during this process can lead to poor sperm competence and decreased fertility.[15,51]

It has been shown that an altered histone-to-protamine ratio, or more specifically, increased histone retention in sperm chromatin affects male fertility by decreasing sperm concentration, and motility, increasing DNA damage and poor embryo outcome.[22,23,26,32,52] We observed a positive association between histone H3 and spermatozoa with high DNA damage. Nuclear compaction by the incorporation of protamines protects the genetic and epigenetic integrity of sperm chromatin from external stress.[53,54] Therefore, sperm chromatin with increased histone retention reduces the chromatin structure stability, thereby rendering it more vulnerable to DNA fragmentation.[22,55] Our findings and reports mentioned above imply that alteration in the histone-to-protamine ratio can hinder the epigenetic stability of sperm chromatin, thereby impairing sperm competence.

Genes important for embryonic development are marked by specific histone PTMs in spermatozoa.[8,56] It has been shown that retained histone marks, H3K9Me3 and H4K20Me3 of the spermatozoa are transmitted to the embryos and demarcate the heterochromatin region in the zygote; furthermore, they act as a template for copying epigenetic information through maternal machinery.[57] Therefore, to understand the influence of normozoospermic ejaculates with heterogenous paternal histone PTMs on fertilisation and preimplantation embryo developmental competence, we selected sperm samples used for the donor oocyte ICSI program from the above normozoospermic cohort. The use of donor oocytes likely minimised the influence of female factors and thus enabled the study to assess the contribution of paternal epigenome to embryogenesis. Sperm histone H3

TABLE 4 Correlation analysis between sperm histone post-translational modifications and embryo developmental competence ($n = 27$).

	Histone H3	H3K4Me3	H3K9Me3	H3K27Me3	Histone H4	H4K12Ac	H4K20Me3
Fertilisation rate (%)	−0.397*	−0.316	−0.305	−0.503**	0.111	0.033	−0.337
Cleavage rate (%)	−0.136	−0.138	−0.332	−0.275	−0.025	0.126	0.255
Good-quality embryos (%) (day 3)	−0.568**	0.072	−0.067	−0.114	0.014	−0.218	0.119
Poor-quality embryos (%) (day 3)	0.365	−0.115	−0.038	0.085	−0.039	0.103	−0.039

Note: Pearson's correlation coefficient (r value) is presented in the table (adjusted for men's age).
*Significant at alpha = 5%; * Significant at alpha = 1%.

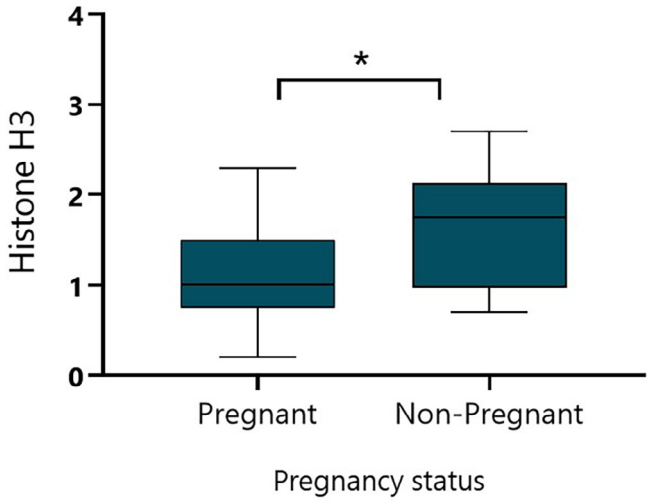

FIGURE 2 Box-and-whisker plot showing the level of histone H3 in the spermatozoa of male partners in pregnant ($n = 17$) and non-pregnant groups ($n = 10$). The data were analysed by unpaired student's *t*-test and represented as mean ± SD. Asterisk (*) represents significance at $p < 0.05$ (adjusted for the female recipient's age, male age and sperm DNA damage).

and H3K27Me3 showed a negative association with the fertilisation rate. The enrichment of H4K12Ac in the sperm nucleus and the zygote following fertilisation has been demonstrated and is associated with genes expressed at the four-cell stage to blastocyst.[20] Whereas experimentally induced abnormal histone retention during spermiogenesis showed differential expression of genes in the two-cell stage to blastocyst,[58] suggesting abnormal retention of histones during spermiogenesis leading to altered gene expression in early embryos.[49,59,60] These studies support our findings that altered levels of paternally retained histones and their PTMs in normozoospermic individuals may have an impact on the fertilisation process. Interestingly, a negative correlation between histone H3 and good-quality embryos was observed, indicating that an increase in histone retention can affect the development of human preimplantation embryos. Earlier studies have demonstrated that the cleavage rate and quality of the embryos are adversely affected because of increase in histone retention in sperm chromatin.[61] However, embryo production was not affected.[62] Furthermore, poor-quality blastocysts were observed in normozoospermic spermatozoa with abnormal histone CpG island methylation profiles, suggesting that alteration in the epigenetic signature on the paternal genome is associated with unexplained male infertility and poor blastocyst development.[29]

We noticed unsuccessful pregnancies in couples with a significantly increased level of histone H3 in spermatozoa. When normozoospermic spermatozoa were subjected to dot-blot analysis, the non-pregnant group had higher levels of histones than the pregnant group, demonstrating the impact on pregnancy outcome.[10] The clinical pregnancy rate was lower in spermatozoa with a lower protamine 1:protamine 2 ratio in the chromatin.[23,24] Together, these findings support the fact that levels of retained histones in spermatozoa and their enrichment at developmental genes are essential for fertilisation and early embryo development. Since the establishment of a successful pregnancy is multifactorial, our argument suggesting the association between retained histones and clinical pregnancy may be overstated. However, there could be a link between histone levels in spermatozoa and pregnancy outcome.

Previous studies have shown that the uptake of metabolites from culture media can be a predictive tool for embryo viability and implantation potential.[62–65] Data from our laboratory have shown that sperm DNA integrity negatively affects embryo metabolism.[65,66] In the current study, we observed that the intensity of methionine was significantly lower in the SCM of embryos in successful pregnancy groups than in those of failed pregnancy. Methionine is a key element in the 1-C metabolism cycle that provides methyl moieties to S-adenosyl methionine, which is required for DNA and histone methylation.[67,68] Also, methionine is a precursor to the methyl donors required for protein synthesis during embryonic development.[69] Methionine in embryo culture media is essential because the methylation levels on embryo genomes are highly maintained after fertilisation and start to decline after the four-cell stage.[70,71] Abnormal methionine metabolism

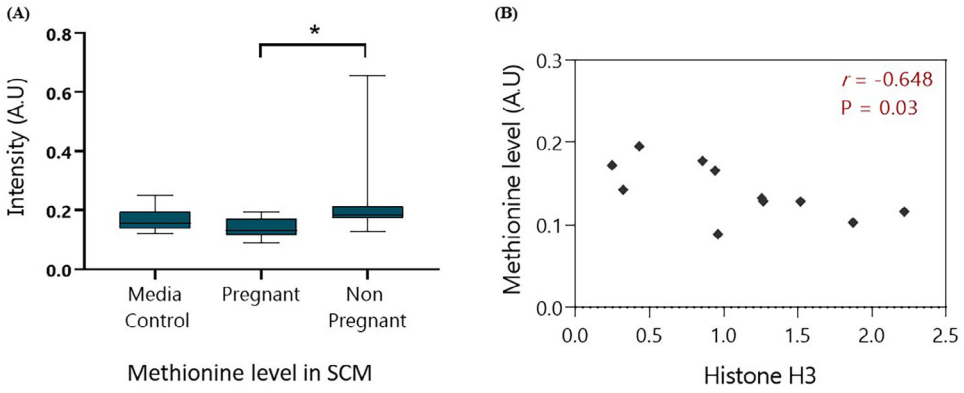

FIGURE 3 (A) Levels of methionine in spent embryo culture media obtained from media control ($n = 13$), pregnant ($n = 11$) and non-pregnant ($n = 7$) groups are represented by a box-and-whisker plot. The data were analysed by the Kruskal–Wallis test. Asterisk (*) represents significance at $p < 0.05$. (B) Scatterplot depicting the association between levels of sperm histone H3 and methionine obtained from spent embryo culture media of the pregnant group ($n = 11$). '*r*' represents Pearson's correlation coefficient. Data are significant at $p < 0.05$. SCM, spent culture medium.

affects the transition of the morula to the blastocyst because of hypomethylation of DNA, which changes the expression of developmental genes, indicating a critical role for methionine metabolism during pre-implantation embryo development.[72,73] However, at this juncture, it is not possible to claim whether a similar phenomenon exists in our study as we did not use SCM beyond day 3 of development. On the other hand, we noticed that the level of methionine in SCM of the successful pregnancy group was found to be negatively correlated with the sperm histone H3 levels. According to earlier reports, differential levels of amino acids in spent embryo media were observed in pregnant and non-pregnant groups.[74–76] Our results are in line with previous studies, where methionine levels in the SCM of embryos might regulate embryo development, which is also associated with sperm histone H3. A molecular understanding of the significance of methionine metabolism in preserving H3K4Me3 levels in the chromatin during early embryogenesis has been provided by expression experiments in human embryonic stem cells.[77,78] However, we could not identify the molecular or biochemical mechanisms to link methionine metabolism and sperm histone H3 levels in the current study.

Various intrinsic and extrinsic factors, such as altered lifestyle, dietary exposure, nutritional status (malnutrition or obesity) or environmental toxicants (smoking, alcoholism and other environmental toxins) can affect the sperm epigenome.[79–82] Our findings provide support for existing research that morphologically normal spermatozoa may contain altered epigenetic signatures that could potentially be a contributing factor to idiopathic/unexplained infertility.[15,18,32,33] By minimising the influence of the female factor using donor oocytes, the current study demonstrates that the heterogeneous amounts of retained sperm histones in normozoospermic individuals can affect fertilisation and embryo quality, thereby impacting the pregnancy outcome. Importantly, altered epigenetic signatures in spermatozoa may be passed on to the next generation, potentially impacting the health of the offspring.[81,83–85] Patients with abnormal semen characteristics were not included because the sperm numbers obtained after the ICSI procedure were not adequate to perform epigenetic analysis. Due to restrictions on using human embryos for research, we were unable to determine the underlying molecular mechanism responsible for the retained histone H3 and H3K27Me3-mediated alterations during fertilisation, development of the embryos, and changes in the levels of methionine in SCM.

5 | CONCLUSION

Heterogenous levels of retained histones in spermatozoa can be one of the factors underlying the aetiology of idiopathic male infertility. Such spermatozoa may influence fertilisation, the quality of the embryos, and embryo metabolism, thereby affecting the assisted reproductive technology outcome. These results, although descriptive in nature, warrant further research to address the underlying mechanisms behind these clinically important observations.

AUTHOR CONTRIBUTIONS

Satish Kumar Adiga conceived and designed the experiments. Riddhi Kirit Pandya, Ameya Jijo, Aswathi Cheredath and Sujith Raj Salian performed the experiments and was involved in the acquisition of data. Riddhi Kirit Pandya, Vani Lakshmi R. and Shubhashree Uppangala analysed and interpreted the data. Satish Kumar Adiga, Riddhi Kirit Pandya and Sanjay Gupta wrote the manuscript. Guruprasad Kalthur, Sanjay Gupta and Pratap Kumar revised the manuscript critically for important intellectual content. Riddhi Kirit Pandya is the guarantor of this work and as such, has full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have given final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data related to this article are available in both the article and the online supporting information.

ORCID

Guruprasad Kalthur https://orcid.org/0000-0002-4554-2917
Satish Kumar Adiga https://orcid.org/0000-0002-2897-4697

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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