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Sperm DNA fragmentation affects epigenetic feature in human male pronucleus

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Summary

To evaluate whether the sperm DNA fragmentation affects male pronucleus epigenetic factors, semen analysis was performed and DNA fragmentation was assessed by the method of sperm chromatin structure assay (SCSA). Human-mouse interspecies fertilisation was used to create human male pronucleus. Male pronucleus DNA methylation and H4K12 acetylation were evaluated by immunostaining. Results showed a significant positive correlation between the level of sperm DNA fragmentation and DNA methylation in male pronuclei. In other words, an increase in DNA damage caused an upsurge in DNA methylation. In the case of H4K12 acetylation, no correlation was detected between DNA damage and the level of histone acetylation in the normal group, but results for the group in which male pronuclei were derived from sperm cells with DNA fragmentation, increased DNA damage led to a decreased acetylation level. Sperm DNA fragmentation interferes with the active demethylation process and disrupts the insertion of histones into the male chromatin in the male pronucleus, following fertilisation.

KEYWORDS
DNA fragmentation, epigenetic, male pronucleus, sperm

1 | INTRODUCTION

Spermatozoon is a highly specialised cell that functions to transfer the paternal genome to the next generation (Filipescu, Szenker, & Almouzni, 2013). This specialised cell lacks a chromatin repair system, and DNA is protected by the condensing of this structure (Björndahl & Kvist, 2014; Lee, Chen, Leung, Chen, & Hsu, 2016). Nevertheless, sperm chromatin is vulnerable to damage both within and outside testis (Gavriliouk & Aitken, 2015). The main causes of sperm DNA fragmentation are from environmental toxins, reactive oxygen species (ROS), caspase and endogenous endonucleases (Aitken, Bronson, Smith, & De Iuliis, 2013; Gavriliouk & Aitken, 2015; Sakkas & Alvarez, 2010).

Sperm cells with DNA fragmentation can fertilise the oocyte, but the embryos derived from these sperm cells are less likely to reach the blastocyst stage (Haghpanah et al., 2015). In many cases, chromatin-damaged spermatozoon has resulted in male infertility (Evenson, 2016; Simoni et al., 2016; Sivanarayana et al., 2014; Xue et al., 2016). Sometimes, in assisted reproductive techniques (ART) such as intracytoplasmic sperm injection (ICSI), it is inevitable that low-quality and potentially chromatin-damaged spermatozoon is used (Evenson & Wixon, 2006b). Base on literature study, approximately 50% of spermatozoa with normal morphology are DNA-fragmented in subfertile individuals and these spermatozoa find the opportunity being inseminated during ICSI (Avendaño, Franchi, Duran, & Oehninger, 2010). Chromatin damage can involve DNA strand fracture; it can also produce epigenetic abnormalities (Fernández-Gonzalez et al., 2008; Mohammad et al., 2005; Palermo & Neri, 2015).

Recently, considerable importance has been given to the impact of epigenetic sperm abnormalities on successful fertilisation and potential health of a future baby (Pliushch et al., 2015; Rajabi, Mohseni-Kouchesfehani, Mohammadi-Sangcheshmeh, Farifteh-Nobijari, & Salehi, 2016; Tavalaee, Razavi, & Nasr-Esfahani, 2009). For example, a relationship has been determined between global sperm DNA

methylation and male infertility (Bahreinian et al., 2015; Tavalaee, Bahreinian, Barekat, Abbasi, & Nasr-Esfahani, 2014). In recent years, there has been concern about the transfer of epigenetic abnormalities through ICSI (Carrell, Nyboe Andersen, & Lamb, 2015; Hiura et al., 2014; Lazaraviciute, Kauser, Bhattacharya, Haggarty, & Bhattacharya, 2014). It is now known that there is a link between sperm DNA fragmentation and genetic and epigenetic abnormalities in mouse embryo (Ramos-Ibeas et al., 2014). In addition, a relationship has been determined between abnormal sperm imprinting and spermatogenesis (Boissonnas et al., 2010). Previous studies have shown that it is likely that the structure of sperm chromatin effects the creation and maintenance of epigenetic patterns during spermatogenesis (Brunner, Nanni, & Mansuy, 2014; Carrell, 2012). Thus, epigenetic evaluation of sperm cell is essential to consider likely epigenetic changes on future embryos. Sperm injection into mouse oocyte and assessment of pronuclei is a frequently performed test to estimate the ability of a semen sample to activate oocyte. This test is known as the mouse oocyte activation test (MOAT; Meerschaut, Nikiforaki, Heindryckx, & De Sutter, 2014). MOAT functions only to assess the ability of spermatozoa to activate the oocyte, but makes no evaluation of other sperm parameters. The purpose of this study was to evaluate the effects of DNA fragmentation on the epigenetic factors of DNA methylation and H4K12 acetylation in human male pronuclei following MOAT and to suggest a method for more complete sperm assessment.

2 | MATERIAL AND METHODS

Materials were purchased from Sigma Chemical Corporation (St. Louis, MO) except where the specific manufacturer is mentioned. Animal experiments were performed with the guiding principles accepted by the Shahid Beheshti University of Medical Sciences committee on animal research. Semen samples obtained from patients were referred to Shahid Taleghani Infertility Center following written consent.

2.1 | Sperm preparation

Semen samples were obtained (n = 25) after 2–3 days of sexual abstinence and transferred to a laboratory. Count and motility of semen samples were analysed according to WHO (World Health Organization) criteria (World Health Organization, 2010), and sperm morphology was evaluated according to strict criteria following Diff-Quik staining (World Health Organization, 2010). Then, a fraction of each sample was washed with Ham's F10 medium containing 10% human serum albumin (HSA) and was used for ICSI.

2.2 | Sperm chromatin structure assay (SCSA)

To evaluate DNA fragmentation, the SCSA technique was used (Evenson & Wixon, 2006a; Ribas-Maynou et al., 2013). First, a volume of semen, which contained 1–2 million sperm cells, was mixed with TNE buffer (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, 1 mmol/L EDTA, pH 7.4). This suspension was treated with acid solution (0.1% Triton X-100, 0.15 mol/L NaCl, 0.08 N HCl, pH 1.2) for 30 s and then was stained with 6 μg/ml acridine orange in phosphate-citrate buffer (pH = 6.0). Stained sperm cells were studied by flow cytometry. The level of DNA fragmentation was determined as the DNA fragmentation index (DFI). The sample was defined as normal, when its DFI was lower than 30%, but when DFI was more than 30%, the sample was considered DNA fragmented (Ribas-Maynou et al., 2013).

2.3 | Oocyte preparation

BDF1 female mice (6–8 week old; Pasteur Institute, Tehran, Iran) were super ovulated by intraperitoneal injection with 10 IU pregnant mare serum gonadotropin (PMSG), followed by 10 IU human chorionic gonadotropin (HCG) 48 hr later. Cumulus oocyte complexes (COCs) were collected from oviduct ampullae 13–14 hr after injection of the HCG in FHM medium, and oocytes separated from cumulus cells by transferring the COCs to FHM medium containing hyaluronidase (Dehghani-Mohammadabadi et al., 2014; Rajabi et al., 2016). Oocytes were selected following washing in FHM. Criteria for oocyte selection were meiosis II with normal morphology. Good-quality oocytes were collected in a KSOM until ICSI.

2.4 | Intracytoplasmic sperm injection (ICSI)

Washed human spermatozoa from two groups were injected into mouse oocytes with a PMM-150FU Piezo-actuated micromanipulator (Prime Tech Ltd, Tsuchiura, Japan; Fulka, Barnetova, Mosko, & Fulka, 2008). The injected oocytes were cultured in a KSOM in an incubator at 37°C with 5% CO_2 for 6 hr without artificial oocyte activation. The zygote formation was evaluated by creation of two pronuclei and considered as successful fertilisation. Zygote was used for immunostaining (Rajabi et al., 2016).

2.5 | Immunostaining for Acetyl H4K12 and 5-methylcytosine

The process of immunostaining was described previously. Zygotes with two, male and female, pronuclei were fixed by paraformaldehyde 4% for 30 min and then permeabilised with PBS containing 0.2% Triton X-100 for 30 min (Fulka et al., 2008; Tavalaee et al., 2014). Zygotes derived from each semen sample were divided into two groups: acetyl H4K12 (H4K12ac) and 5-methylcytosine (5-mC). For evaluation of H4K12ac, samples were blocked in PBS/5% BSA for 45 min and incubated with a primary antibody of anti-H4K12ac (Abcam, Cambridge, UK; 1:200 dilution) overnight at 4°C. Then, after being washed several times with PBS/1% BSA, the samples were incubated with a secondary antibody conjugated with FITC for 1 hr at 37°C. The samples were washed several times with PBS/1% BSA and stained with Hoechst H33342 (Fulka et al., 2008).

For evaluation of methylation level of DNA, samples were treated with HCl (2 mol/L) for 30 min, followed by Tris-HCl (pH = 8) for 15 min. After being washed several times with PBS/1% BSA/0.1% Tween-20, the samples were blocked in PBS/2% BSA overnight at 4°C

and incubated with anti-5-mC (Epigentek, NY, USA; 1:100 dilution). After extensive washing, they were incubated with secondary antibody conjugated with propidium iodide (PI) at 37°C for 1 hr. Finally, the samples were washed and stained with Hoechst H33342 (Fulka et al., 2008).

To evaluate the level of DNA methylation and H4K12 acetylation, the photographs were analysed with ImageJ version 1.45 s; National Institutes of Health, USA) software.

2.6 | Statistical analysis

Statistical analyses were performed using SPSS Ver.20 (SPSS, Chicago, IL, USA). The fluorescent intensities were compared by a nonparametric analysis test (Mann–Whitney). Correlation between studied parameters was performed using a correlation coefficient (Pearson correlation). $p < .05$ was considered statistically significant.

3 | RESULTS

To evaluate the impact of sperm DNA fragmentation on male pronuclei epigenetic changes, sperm parameters, count, motility and percentage of normal morphology were analysed. Analysis was performed on 25 semen samples from men who had been referred to an infertility centre, and DFI was evaluated for each sample.

The number of injected oocytes and means of fluorescent intensity in male pronuclei are shown in Table 1. In this experiment, 380 oocytes survived after ICSI and were cultured for 6 hr. Formation of the male pronucleus was considered as a mark of successful fertilisation (Figure 1). The larger pronucleus originated from the decondensed sperm head, and the smaller pronucleus, which was located near the secondary polar body, originated from oocyte (Fulka et al., 2008). Among the 222 zygotes with both male and female pronuclei, 112 cases were derived from sperm cells with DFI of less than 30% (the group of normal sperm cells) and 110 cases were derived from sperm cells with of DFI of more than 30% (the group of sperm cells with DNA fragmentation; Table 1).

3.1 | Comparison of sperm parameters in samples with DFI less and more than 30%

The comparison between the mean of sperm counts in samples with DFI less than 30% and DFI more than 30% indicated significant difference (Table 2). The percentage of normal morphology demonstrated significant difference between the two groups (Table 2). Likewise, a significant difference was detected between the successful fertilisation rate in normal and DNA-fragmented groups (Table 2).

Correlation analysis of DNA fragmentation with sperm parameters, in total population of the samples, revealed a relationship between the DNA damage and sperm parameters including, motility, morphology and fertilisation rate (Table 3). However, there was no correlation between the DNA fragmentation and count (Table 3).

Correlation analysis of DNA damage and sperm parameters in each groups indicated no relationship between DNA fragmentation and

TABLE 1 The number of injected oocytes and means of fluorescent intensity in male pronuclei

Groups	No. of injected oocyte	No. of survived oocyte	No. of 2PN	No. of zygotes assessed for 5-mC	No. of zygotes assessed for H4K12ac	Fluorescence intensity 5-mC	Fluorescence intensity H4K12ac
DNA Fragmentation Index <30%	223	169 (75.78%)	112 (66.27%)*	55	57	22.48 ± 0.6*	30.57 ± 3
DNA Fragmentation Index >30%	293	211 (72.01%)	110 (52.13%)*	54	56	31.78 ± 1.4*	35.66 ± 2.4
Total	516	380 (73.64%)	222 (58.42%)	109	113	—	—

5-mC, 5-methylcytosine; H4K12ac, acetylation of lysine 12 histone 4.
*$p < .05$.

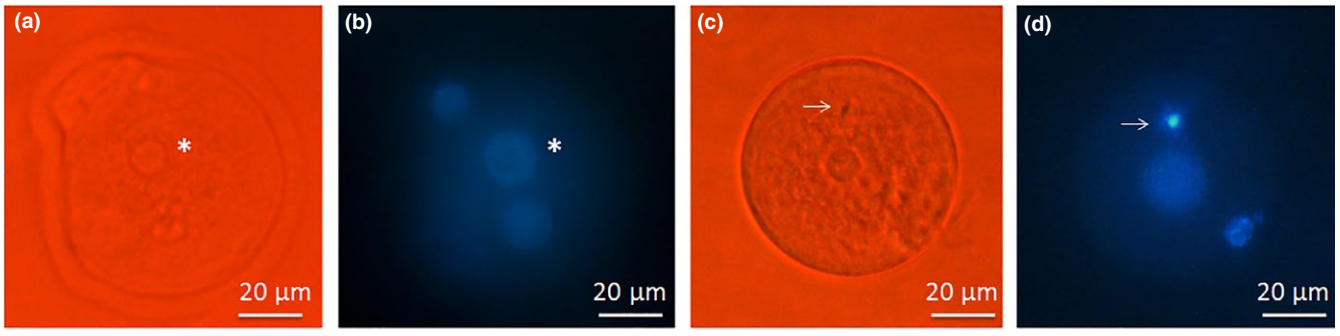

FIGURE 1 Light and fluorescent micrograph of successful and unsuccessful fertilisation, (a and b) light micrograph and Hoechst staining of a successfully fertilised oocyte, (c and d) light micrograph and Hoechst staining of an unsuccessfully fertilised oocyte. Asterisks show male pronuclei, and arrows demonstrate sperm cells chromatin, which are not decondensed

TABLE 2 Comparison of sperm parameters in normal and DNA-fragmented semen samples

Groups	Average sperm			
	Count	Motility%	Morphology%	Successful fertilisation%
DNA Fragmentation Index <30%	70.62 ± 2.3*	27.44 ± 0.9	5.58 ± 0.1*	66.27 ± 1*
DNA Fragmentation Index >30%	65.26 ± 4.1*	26.46 ± 1.5	2.55 ± 0.2*	52.13 ± 1.4*

*$p < .05$.

TABLE 3 Correlation of epigenetic factors and sperm parameters with DNA fragmentation in total population of samples

Groups	5-mC (*p* value)	H4K12ac (*p* value)	Count (*p* value)	Motility (*p* value)	Morphology (*p* value)	Successful fertilisation (*p* value)
DFI	0.673 (.001)*	−0.006 (.963)	0.073 (.517)	−0.3 (.007)*	−0.632 (.001)*	0.615 (.001)*

DFI, DNA Fragmentation Index; 5-mC, 5-methylcytosine; H4K12ac, acetylation of lysine 12 histone 4.
*$p < .05$.

count in normal sample as well as the samples with DFI more than 30% ($p > .05$; Table 4). No correlation was observed between morphology and level of DNA fragmentation in normal semen samples ($p > .05$). In contrast, there was a relationship between morphology and level of DNA damage in semen samples with DNA fragmentation of more than 30% ($p < .05$; Table 4). Similarly, a relationship was observed between motility and the level of DNA fragmentation in DNA damaged semen samples ($p < .05$; Table 4). In the case of successful fertilisation, there were significant negative correlations between fertilisation in normal ($p < .05$) and DNA-damaged ($p < .05$) semen samples (Table 4).

3.2 | Comparison of epigenetic factors in male pronuclei derived from normal sperm cells and sperm cells with DNA fragmentation

Epigenetic factors were assessed in both groups, pronuclei derived from normal and DNA-fragmented sperm cells (Figure 2). The fluorescence intensity of 5-mC in samples with DFI less than 30% and more than 30% indicated significant difference ($p < .05$). The fluorescence intensity of H4K12ac in samples with of DFI less than 30% and in samples with more than 30% indicated no significant difference (Table 1). Results of the correlation test are shown in Tables 3 and 4.

3.2.1 | H4K12ac

Correlation analysis between DFI and H4K12ac in total population of samples revealed no significant relationship (Table 3). However, correlation analysis of DNA fragmentation with the acetylation level of H4K12, in the group with DFI, was more than 30%, demonstrating a significant negative correlation ($p < .05$; Table 4).

Correlation analysis of sperm parameters with the level of H4K12ac demonstrated a relationship between the acetylation level of H4K12 with count and motility in total population of the samples (Table 5). However, no correlation was detected between the H4K12ac with semen parameters in the group with the normal range of DFI ($p > .05$; Table 6). In the group with DFI more than 30%, a negative correlation was determined between H4K12ac level and sperm parameters including motility, morphology and fertilisation rate ($p < .05$; Table 6). This means that a decreased quality of spermatozoa resulted in a decreased level of H4K12ac in male pronuclei.

3.2.2 | 5-mC

Correlation analysis between DFI and 5-mC in total population of samples indicated a significant relationship between the DNA fragmentation and the level of this epigenetic factor (Table 3). Furthermore, a comparative study on 5-mC level between the normal group and that with DNA fragmentation more than 30% showed that in both groups, there was a significant correlation between DFI and 5-mC level ($p < .05$; Table 4).

The results of correlation analysis between 5-mC level and sperm parameters revealed no relationship in total population of samples ($p > .05$; Table 5) as well as in the normal group with DFI less than 30% ($p > .05$; Table 6). In contrast, a negative correlation was determined between 5-mC level and the sperm parameters: motility, morphology and successful fertilisation rate in the DNA-fragmented group ($p < .05$; Table 6).

TABLE 4 Correlation of epigenetic factors and sperm parameters with DNA fragmentation in two groups

Groups	5-mC (*p* value)	H4K12ac (*p* value)	Count (*p* value)	Motility (*p* value)	Morphology (*p* value)	Successful fertilisation (*p* value)
DNA fragmentation <30%	0.345 (.032)*	−0.109 (.503)	−0.265 (.164)	−0.109 (.503)	−0.185 (.180)	−0.498 (.001)*
DNA fragmentation >30%	0.615 (.005)*	−0.544 (.004)*	−0.258 (.065)	−0.868 (.001)*	−0.902 (.003)*	−0.597 (.001)*

5-mC, 5-methylcytosine; H4K12ac, acetylation of lysine 12 histone 4.
*$p < .05$.

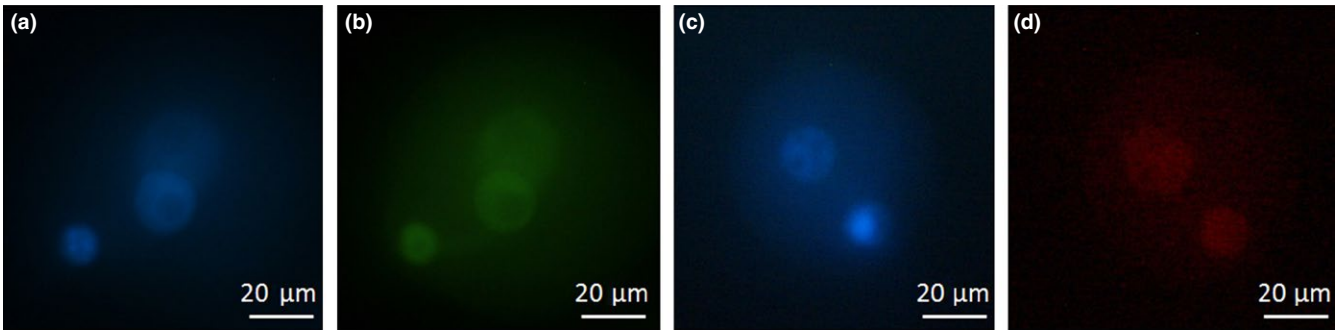

FIGURE 2 Fluorescent micrograph of zygotes for DNA fragmented samples, labelled with (a) Hoechst H33342, (b) same zygote by antibody acH4K12, (c) Hoechst H33342 and (d) same zygote by antibody 5-mC

TABLE 5 Correlation of epigenetic factors and sperm parameters in total population of samples

Groups	Count (*p* value)	Motility (*p* value)	Morphology (*p* value)	Successful fertilisation (*p* value)
H4K12ac	0.607 (.001)*	−0.249 (.044)*	−0.045 (.718)	−0.062 (.619)
5-mC	−0.097 (.468)	0.264 (.045)*	−0.531 (.001)*	−0.432 (.001)*

5-mC, 5-methylcytosine; H4K12ac, acetylation of lysine 12 histone 4.
*$p < .05$.

TABLE 6 Correlation of epigenetic factors and sperm parameters in two groups

Groups	Epigenetic factors	Count (*p* value)	Motility (*p* value)	Morphology (*p* value)	Successful fertilisation (*p* value)
DNA fragmentation <30%	H4K12ac	0.258 (.065)	−0.284 (.076)	−0.145 (.380)	0.056 (.733)
	5-mC	−0.127 (.440)	−0.178 (.272)	0.126 (.315)	−0.106 (.523)
DNA fragmentation >30%	H4K12ac	0.265 (.164)	0.544 (.004)*	0.65 (.001)*	0.571 (.002)*
	5-mC	−0.374 (.060)	−0.615 (.005)*	−0.633 (.004)*	−0.612 (.005)*

5-mC, 5-methylcytosine; H4K12ac, acetylation of lysine 12 histone 4.
*$p < .05$.

4 | DISCUSSION

Epigenetic signals are important for proper transcription of the paternal genome at the later stage of fertilisation (Aitken et al., 2013; Beaujean, 2015; Osman, Alsomait, Seshadri, El-Toukhy, & Khalaf, 2014). It is shown that there is a relationship between male fertility and epigenetic marks (Bahreinian et al., 2015; Tavalaee et al., 2009). According to importance of sperm epigenetic structure in successful fertilisation and gene expression in future embryos, comparison was made of the sperm parameters: count, motility and morphology in normal semen samples and those having sperm DNA fragmentation. Sperm cells were then injected into mouse oocytes to assess success fertilisation rates of sperm samples. DNA methylation and H4K12 acetylation in male pronuclei were evaluated following the formation of pronuclei.

Results showed that sperm count had no significant correlation with DNA damage, demonstrating that the process of DNA damage was independent of sperm concentration in the seminal fluid. In this case, findings were consistent with those of previous studies (Tavalaee et al., 2009). Previous studies demonstrate a significant correlation between the extent of DNA damage and sperm morphology (Choucair et al., 2016; Kim, Jee, Lee, Suh, & Kim, 2014; Tavalaee et al., 2009). In terms of morphology, we observed a relationship between DFI and sperm parameters in total population of samples, but no correlation was detected between morphology and DNA fragmentation level in which DFI was less than 30%. In contrast, those groups with more DNA damage than normal demonstrated that normal sperm morphology was less in the more damaged sperm cells. This means that as long as the amount of DNA damage is low, then there is no effect on normal sperm morphology. The relationship between sperm motility and amount of DNA damage findings showed that there is a correlation in total population. This is consist with previous studies (Choucair et al., 2016). We found no correlation between the percentage of motile sperm cells and DNA fragmentation, in the normal group. However, motility was less in DNA-fragmented samples and with an increased amount of DNA damage, resulted in decreased motility of spermatozoa, showing that these findings were consistent with those of Tavalaee et al. (2009). In this study, a significant association was found between the decreased fertilisation rate and sperm DNA fragmentation. As in previous studies, one can conclude that sperm DNA fragmentation is correlated with unsuccessful fertilisation (Cissen et al., 2016; Mohammad et al., 2005; Simon, Zini, Dyachenko, Ciampi, & Carrell, 2016).

The findings in the study of DNA methylation showed that methylation of male pronucleus and sperm DNA fragmentation had significant correlation. With an increasing amount of DNA damage, male pronucleus methylation increased. Tavalaee et al. (2014) report on level of DNA damage and sperm methylation and mention low methylation in spermatozoa with DNA damage and suggest that fragmented DNA has lower tendency to attach DNA methyl transferase (DNMT). The same is probably true for demethylation of DNA in pronucleus. Pronuclei from sperm cells with DNA fragmentation have a high level of methylation that is probably due to low tendency for demethylation in cases of damaged DNA.

The assessment of the relationship between DNA methylation and sperm parameters in the normal group showed than there is no relationship between DNA methylation and sperm parameters including sperm count, motility and percentage of normal morphology, but in DNA-fragmented group, the level of DNA methylation in the male pronucleus had a relationship with motility and percentage of normal morphology. That means that if the injected spermatozoon was high quality, then there was no interference with the demethylation process. In the group with damaged DNA, an inverse relationship was observed between DNA damage and fertilisation rate, and these results were consistent with those of previous findings (Tavalaee et al., 2009).

It has been shown that impaired spermatogenesis is associated with H4 acetylation disorders (Sonnack, Failing, Bergmann, & Steger, 2002). Comparison of H4K12 acetylation in normal and DNA-fragmented sperm cells showed no relationship between level of acetylation and percentage of DNA fragmentation (Kim et al., 2014).

Observations in this study revealed no correlation between level of H4K12ac and DNA fragmentation in total population of male pronuclei. In addition, no relationship was observed between the H4K12 acetylation level and DFI in male pronuclei derived from sperm cells with DNA fragmentation less than 30%, but in the second group, in which DNA fragmentation was more than 30%, there was an inverse relationship between DNA fragmentation and level of H4K12ac in male pronuclei. Kim et al. (2014) reports no correlation between sperm DNA fragmentation and sperm chromatin H4K12ac (Kim et al., 2014). Most of the acetylated histones, which have been replaced in male pronucleus chromatin, originated from the oocyte (McLay & Clarke, 2003; Yang, Wu, & Macfarlan, 2015). DNA strand breaks are likely to cause inappropriate localisation of acetylated histones in male chromatin. Thus, suitable replacement of protamines with histones needs an intact DNA structure.

Investigation was made on the correlation between H4K12ac level and sperm parameters. In total population, a relationship was detected between this epigenetic factor with sperm count and sperm motility. In normal sperm samples, in which DNA fragmentation was less than 30%, no relationship was observed between these two characteristics. In contrast, in the group with more damage than normal, a significant direct correlation was determined between H4K12ac level and the sperm parameters of motility, percentage of normal morphology and fertilisation rate. This result demonstrates that high-quality sperm cells had a healthier DNA structure and localisation of the acetylated histones in the chromatin performed well.

5 | CONCLUSION

In this study, comparison was made of epigenetic factors in pronuclei derived from normal and DNA-fragmented sperm cells. Male pronucleus methylation level was higher in pronuclei with DNA fragmentation. In the case of H4K12ac, in the group with DNA fragmentation of more than 30%, there was a negative significant relationship between DNA damage and level of histone acetylation. This result demonstrates that increased level of damage results in a decreased level of acetylation. We believe that there was a high 5-mC level in pronuclei with damaged DNA, because of disorder in the demethylation process and that the reason for the decrease in the H4K12ac level in pronuclei with damaged DNA was disruption in the process of insertion of histones into the male chromatin.

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