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Full Title:	DNA methylation profile at a satellite DNA region is associated with aberrant placentation in cloned calves
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Abstract:	Background: Cloning via somatic cell nuclear transfer (SCNT) has been associated with a variety of pathologic alterations, mainly in the placenta of cattle, and these alterations could be associated with an aberrant epigenetic reprogramming of the donor cell genome. Thus, in this study, we tested the hypothesis that DNA methylation patterns are not appropriately established after the nuclear transfer and that those altered patterns are associated with specific aberrant phenotypes. Results: Here, we compared global and specific placental DNA methylation patterns between aberrant and healthy SCNT-produced calves. Foetal cotyledon samples of ten SCNT pregnancies were collected. Global DNA methylation and hydroxymethylation levels were measured using ELISA-based assay and Satellite I, and α-Satellite repeat elements were measured using bisulfite sequencing. Our analysis revealed that the SCNT-produced calves that showed aberrant phenotypes exhibited a methylation pattern of the satellite I region that was less methylated than that of healthy calves. In contrast, global methylation and hydroxymethylation analyses showed higher levels for both cytosine modifications in SCNT-produced female calves with aberrant phenotypes. Concerning perinatal viability, the satellite I region showed most of the sequences to be hypermethylated in live cloned calves compared with the dead calves. Conclusions: This study is the first to characterize global methylation and hydroxymethylation in cloned Nellore (Bos taurus indicus) cattle. Our results suggest that the satellite I region could be used as an epigenetic-based biomarker for predicting offspring viability, which supports the development and adaptation of new protocols for cloning. Studies that evaluate DNA methylation patterns of this satellite region by assessing the donor cell nucleus or embryo biopsies could shed light on how to improve the efficiency of cloning.

DNA methylation profile at a satellite DNA region is associated with aberrant placentation in cloned calves

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Abstract

Background: Cloning via somatic cell nuclear transfer (SCNT) has been associated with a variety of pathologic alterations, mainly in the placenta of cattle, and these alterations could be associated with an aberrant epigenetic reprogramming of the donor cell genome. Thus, in this study, we tested the hypothesis that DNA methylation patterns are not appropriately established after the nuclear transfer and that those altered patterns are associated with specific aberrant phenotypes.

Results: Here, we compared global and specific placental DNA methylation patterns between aberrant and healthy SCNT-produced calves. Foetal cotyledon samples of ten SCNT pregnancies were collected. Global DNA methylation and hydroxymethylation levels were measured using ELISA-based assay and Satellite I, and α-Satellite repeat elements were measured using bisulfite sequencing. Our analysis revealed that the SCNT-produced calves that showed aberrant phenotypes exhibited a methylation pattern of the satellite I region that was less methylated than that of healthy calves. In contrast, global methylation and hydroxymethylation analyses showed higher levels for both cytosine modifications in SCNT-produced female calves with aberrant phenotypes. Concerning perinatal viability, the satellite I region showed most of the sequences to be hypermethylated in live cloned calves compared with the dead calves.

Conclusions: This study is the first to characterize global methylation and hydroxymethylation in cloned Nellore (*Bos taurus indicus*) cattle. Our results suggest that the satellite I region could be used as an epigenetic-based biomarker for predicting offspring viability, which supports the development and adaptation of new protocols for cloning. Studies that evaluate DNA methylation patterns of this satellite region by

assessing the donor cell nucleus or embryo biopsies could shed light on how to improve the efficiency of cloning.

Keywords: SCNT, Nuclear Reprogramming, Epigenetics, Nellore cattle

Background

Cloning via somatic cell nuclear transfer (SCNT) is an assisted reproductive technique that has many potential applications, such as multiplication of high genetic value animals and animals of endangered species [1, 2], transgenic research [3], and stem cell research [4], as well as a model of studies for human diseases [5, 6], etc. Although it is already a technique that is in use, its efficiency is still very low, because SCNT could be associated with a variety of pathologic changes in the placental and foetal phenotypes. In cattle, placentation problems in SCNT pregnancies is the main cause of low survival rates, with only approximately 5 to 10% of transferred embryos producing viable offspring [7]. During the first trimester of gestation, more than 50% of the pregnancies are lost [8], and impaired placental angiogenesis appears to be the main cause of foetal mortality [9]. In the later stages of gestation, the placenta exhibits thicker and oedematous membranes, and placentomes are reduced in number and enlarged [10]. These alterations are thought to be caused by aberrant epigenetic reprogramming in early embryogenesis [11, 12], especially in trophoblast cells [13]. These cells frequently show atypical hypermethylation [13], which could result in placental pathologies and the onset of developmental abnormalities [14-16].

DNA methylation is the major epigenetic modification of the mammalian genome [17], and it has a critical role in gene expression regulation [18], genomic imprinting [19], X-chromosome inactivation [20], and suppression of repetitive elements [21]; it is also involved in many diseases [22-25], and it is essential for normal embryo development [26, 27]. DNA methylation is widely reprogrammed after fertilization [26] and during the process of SCNT [12]. In early embryogenesis, the nuclear reprogramming mechanism

involves two steps: the first step refers to the erasure of epigenetic patterns through global demethylation, which especially occurs abnormally in SCNT within a shorter period of time, before zygotic genome activation [28]. The second step refers to genome-wide *de novo* methylation in early embryos, from a totipotent status to various differentiated states for tissue generation or organogenesis during post-implantation development [7]. The complex patterns of methylation in SCNT embryogenesis highlight the significance of profiling DNA methylation to answer biological questions [23, 29, 30]. An epigenetic biomarker through DNA methylation could represent an important contribution to enhancing the effectiveness of SCNT-based cloning.

Satellite DNA sequences act as epigenetic signals that are required for the organization of pericentromeric heterochromatin during embryogenesis and that are necessary for developmental progression [31]. Satellite DNA are highly repetitive DNA sequences that constitute a considerable part of the eukaryotic genomes [32]. Considering that the satellite DNA are widespread sequences in the genome and that they are normally methylated, they could reflect specific methylation patterns in the genome [31].

Taking all of this information together, in this study, we tested the hypothesis that DNA methylation patterns are not appropriately established after nuclear transfer in SCNT and that they are associated with specific aberrant phenotypes in cloned cattle. To test this hypothesis, we evaluate global methylation and hydroxymethylation in the genome as well as methylation in two specific satellite DNA in the placenta of cloned calves with different phenotypes.

Methods

Placenta sampling

Ten SCNT pregnancies and two artificial insemination (AI) pregnancies (control) were used. Placenta samples (cotyledon) were collected during caesarean sections at a private animal reproduction laboratory (GENEAL Genetics and Animal Biotechnology, Brazil). Placenta samples that showed different phenotypes (Fig. 1) of ten Nellore (*Bos taurus indicus*) cloned calves (n= 6 male and n= 4 female) and placenta samples of two Nellore calves produced by AI (control) were collected, snap frozen on dry ice, and stored at −80°C until DNA isolation. SCNT embryo production and embryo transfer were according to the routinely used protocol at the GENEAL laboratory.

Calves without placental abnormalities were considered to be healthy. In contrast, calves that presented at least one placental abnormality, such as enlarged placentomes (≥ 6 cm, according to a previous description [14]), placental edema, enlarged umbilical cord (≥ 5 cm, as based on the ref. [33]), large offspring syndrome (LOS) (≥ 59.5 kg, according to previously described [34]), and meconium-stained amniotic fluid, were considered to be aberrant (Fig. 1).

Calves that died during the first week of life, in the perinatal period, were considered to be dead offspring, and live offspring were the calves that survived beyond that period.

Genomic DNA isolation

Genomic DNA was isolated from placenta biopsies using a salting out protocol as described in Biase et al [35]. Briefly, biopsies were ground up in liquid nitrogen to a powder to ensure homogeneity for DNA sampling, and they were incubated overnight

at 55°C in 600 µL of extraction buffer (50 mM Tris-HCL, pH 7.8; 25 mM EDTA, 400 mM NaCl and 1% SDS), 0.5 mg/ml of proteinase K and 25 µg/ml RNAse. After incubation, protein and cellular debris were separated by adding 600 µL phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated by adding precipitation solution [glycogen (20 µg/µL); NH4OAc (7.5 M); 100% ethanol)]. Next, pellets were washed with 70% ethanol, and the DNA was resuspended in 150µL TE buffer (Tris-HCl 25 mM; EDTA 10 mM pH 8). DNA quality was evaluated by agarose gel electrophoresis, and the concentration and purity were assessed by using the Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA samples were stored at -20°C.

Primers for DNA methylation analysis

Primers were designed using the MethPrimer software [36] to flank and amplify the CpG islands in the repetitive DNA sequences *Bos taurus* bovine testis satellite I (Satellite I) and *Bos taurus* alpha satellite I DNA (α-Satellite) (Table 1).

Table 1 Primers for methylation analysis of repetitive DNA sequences

Genomic region	Primer Sequence (5'- 3')	*GenBank* accession number	Number of CpG sites	Amplicon length (bp)
Satellite I	F: TGTAGATTGGGGATAGGAGAGTTAG R: CCCCTACTTTATCTAAAAAAAATTACCTT	AH001157.2	23	347
α-Satellite	F: TTTTTTTTGATTTGGATAGGAGG R: TATATTTAAAACCAAAAATTTTTCC	AJ293510.1	18	277

F (forward); R (reverse); bp (base pair).

Sodium bisulfite treatment, PCR amplification, cloning and bisulfite sequencing

DNA samples were treated with sodium bisulfite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer`s instructions. After the sodium bisulfite treatment, the samples were stored at -80°C until PCR amplification. Sodium bisulfite-treated DNA samples were subjected to PCR amplification. The primer sequences, *GenBank* accession number, CpG number and amplicon size are listed in Table 1.

PCRs were performed in a total volume of 20 µL using 1x Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1U Platinum™ Taq polymerase (Invitrogen, CA, USA), 10 µM of each primer (forward and reverse) and 3 µL of bisulfite-treated DNA. The temperature and time conditions for each PCR used an initial denaturing step at 94°C for 3 min followed by 40 cycles at 94°C for 40 s, 45°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 15 min.

After PCR, amplicons were purified from an agarose gel using the Wizard SV Genomic DNA Purification System (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Then, the purified amplicons were cloned into the TOPO TA Cloning vector (pCR™II-TOPO® vector system, Invitrogen, Carlsbad, CA, USA) and transferred into DH5α cells using a heat shock protocol. Plasmidial DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, CA, USA), and individual clones were sequenced using the dideoxy methodology.

The sequencing quality was analysed using Chromas®, and the methylation pattern was analysed using the BiQ Analyser program [37] (MPI for Informatics, Saarland, Germany). The DNA sequences were compared with the sequences from *GenBank* (accession numbers in Table 1). Only sequences that originated from clones with ≥ 95% of homology and cytosine conversion were used.

The DNA methylation profiles of 0% to 20% methylation were considered to be low DNA methylation levels, profiles of 21% to 50% were considered to be moderate DNA methylation levels, and profiles of 51% to 100% were considered to be high DNA methylation levels in the bisulfite sequence analysis. This classification was based on a method previously described by Zhang et al [38].

Global DNA methylation and hydroxymethylation analysis

Global DNA methylation and hydroxymethylation analysis were performed using genomic DNA from placenta samples (n= 12) using the Methylflash Global DNA Methylation kit and Methylflash Global DNA Hydroxymethylation kit, respectively (Epigentek, Farmingdale, NY, USA), according to the manufacturer's instructions, with minor modifications. Briefly, each DNA sample (approximately 100 ng), in quadruplicate, is bound to strip-wells that are specifically treated to have high DNA affinity. For global DNA methylation analysis, the methylated fraction of DNA, 5-methylcytosine (5-mC), is detected using capture and detection antibodies. For global DNA hydroxymethylation analysis, the hydroxymethylated fraction of DNA is detected using a 5-hydroxymethylcytosine (5-hmC) monoclonal antibody-based detection complex. Both were quantified colourimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer (Bio-Rad Microplate Reader, Bio-Rad Laboratories, Redmond, WA, USA). The percentage of methylated or hydroxymethylated DNA was proportional to the optical density (OD) intensity measured. Relative quantification was used to calculate the percentage of 5-mC and 5-hmC in the total cytosine content of the bovine genome [39].

Statistical Analysis

The methylation of satellite regions and global hydroxymethylation data were compared among experimental groups using the Kruskal-Wallis test or the Mann-Whitney test. The data on global methylation were compared by using a one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test or the independent samples t-test. The data collected were analysed using the Graph Pad Prism software (https://www.graphpad.com/scientific-software/prism/). The results are presented as the mean ± standard error of the mean. *P*-value ≤ 0.05 denoted a statistically significant difference.

Results

DNA methylation profile at the satellite regions

With regard to the phenotypes (Fig. 1), the animals that were evaluated in this study showed a higher incidence of enlarged umbilical cords followed by enlarged placentomes and large offspring syndrome (LOS).

The bisulfite sequencing results are shown in Figures 1 to 5. Satellite I and α-Satellite were chosen as the repeat elements to be investigated for the DNA methylation status in foetal cotyledons of SCNT calves. Satellite I of SCNT-produced calves that showed abnormalities were less methylated compared to the controls in both sexes (p val<0.05) (Fig. 2 and Fig. 4c, g). Furthermore, males presented differences in their methylation status between aberrant and healthy cloned calves (Fig. 4g). Healthy clones showed a higher methylated pattern than aberrant clones (p val= 0.0003). However, on the α-Satellite region, all of the groups of animals showed most of the sequences hypermethylated (p val>0.05) (Fig. 3 and Fig. 4d, h), except for animal IIIB, which presented most of the sequences as less methylated than the male control (p val<0.05) (Fig. 3).

SCNT-produced calves showed less methylation on the Satellite I region compared to the AI controls (p val<0.05) (Fig. 2c, d), and no differences were found for the α-Satellite region (p val>0.05) (Fig. 3c, d). When comparing the methylation status between live and dead SCNT-produced calves, bisulfite sequencing analysis demonstrated that the placenta of the animals that survived have higher methylation compared to placenta from dead animals for the Satellite I region (p val<0.05) (Fig. 5c, d). In contrast, there

were no significant differences in the methylation status on an α-Satellite region between live and dead calves (*p* val>0.05). (Fig. 5 e, f).

Global methylation and hydroxymethylation

The global methylation and hydroxymethylation results are shown in Figure 1 and Figures 4 and 5. With regard to the global methylation and hydroxymethylation status, hydroxymethylation showed much lower levels compared to the methylation levels (0.06% vs 8.08%, respectively) (Fig. 1).

In female calves, the methylation and hydroxymethylation levels were significantly different among the three groups of animals (*p* val<0.05) (Fig. 4 a, b); the control calves exhibited lower levels, the healthy calves showed intermediate levels, and aberrant calves showed higher levels of methylation and hydroxymethylation. In contrast, no differences were found for the male calves (*p* val>0.05) (Fig. 4 e, f).

Concerning viability, no differences were found in both evaluations between the animals that survived and the animals that died during the perinatal period (*p* val>0.05) (Fig. 5 a, b).

Discussion

Two decades after Dolly's birth [40], cloning by nuclear transfer remains a riddle, despite advances in the biotechnology of reproduction. The bottleneck most likely is the inefficient epigenetic reprogramming of the somatic cell genome [13], which can entail problems with placental development and function [8]. Consequently, the SCNT procedure has low efficiency [41], which represents an obstacle to advances in promising technologies, such as transgenic livestock production [42], autologous therapeutic cells [12], etc. A biomarker by DNA methylation that is the most stable epigenetic mark in the genome could be useful as a measurable indicator of the offspring's biological conditions. This approach could represent an important contribution to enhancing the effectiveness of SCNT-based cloning.

Initially, we determined the most frequent abnormalities that were observed at the moment of parturition of SCNT-produced calves. We found that an enlarged umbilical cord followed by enlarged placentomes were recurrent abnormalities in cloned calves (Fig. 1), which is consistent with several studies [33, 43, 44]. Previous studies have suggested that an enlarged umbilical cord is associated with an increase in the allantoic duct size and with the thickness of both the duct and blood vessel walls, which is caused by both excessive tissue growth and edema [14]. Thus, the high occurrence of alterations in the umbilical cord in cloned animals suggests that a failure in the foeto-maternal exchange of nutrients, which results from a default in the umbilical cord tissue, could be an important cause of the low efficiency of the SCNT procedure [45].

Enlarged placentomes were associated with placental edema in most cases (Fig. 1). A previous study has found that edema around placentomes is a relatively thin, jelly-like

layer, and the increase in the placentome weight is mainly due to the enlarged placentome tissue [46]. Placentome, which is the point of materno-foetal interface, consists of the foetal cotyledon contributed by the chorion and a maternal cotyledon, which originates from the caruncular regions of the uterus [47, 48]. Compensatory overgrowth of individual placentomes has been reported in SCNT pregnancies [10, 14, 44], where the number of placentomes was reduced, which is in agreement with our data. This reduced number of placentomes might not necessarily be harmful to the foetal viability if the total surface area for the nutrient exchange remains within normal limits [49, 50], despite it being a frequent phenotype that is related to abnormal SCNT pregnancies.

Another altered phenotype that we found was the large offspring syndrome (LOS), which is a foetal overgrowth condition, similar to the Beckwith–Wiedemann syndrome in humans [51]. The most striking feature of the syndrome is a large size at birth [52]. In this study, overgrowth showed a high incidence (Fig. 1), which reflects similar findings previously reported [46, 49]. Previous studies have suggested that assisted reproductive technologies (ART) are related to the LOS pathogenesis due to misregulation of imprinted genes [53-57]. In almost all deliveries, overgrowth was accompanied by meconium-stained amniotic fluid (Fig. 1), which is related to hypoxic stress and foetal maturation level [58] and represents a high-risk status for the cloned neonates [44, 59]. All of the aberrant phenotypes of the SCNT-produced calves reported here could be due to problems in the epigenetic reprogramming of the donor nucleus after nuclear transfer. This epigenetic reprogramming involves the erasure of the post-translational histone modifications and the DNA methylation patterns of the somatic genome followed by the establishment of a new epigenetic pattern in the genome of the embryo

cells [12, 38]. During embryogenesis, the inner cell mass (ICM) and trophoblast are first distinguished at the blastocyst stage. The ICM will give rise to the body of the foetus, and the trophoblastic cells will give rise to the foetal component of the placenta [47]. Reprogramming of the genome of cells from the ICM appears to be efficient, but in the trophoblast, it is especially vulnerable to problems in the context of SCNT protocols, which contributes to defects in the placental function and development [13]. Therefore, the altered patterns of methylation that can be established in the SCNT embryos highlight the importance of profiling DNA methylation to understand the pathophysiological conditions frequently observed in cloned calves.

With regard to the DNA methylation analysis, the data presented here showed the genomic methylation status in extraembryonic tissue, which corresponds to the placental unit of trophoblastic origin called foetal cotyledon [47]. It is important to highlight that for each nucleus donor animal, there was a healthy cloned calf produced, and it was used as a control for the other aberrant clones of the same donor animal (Fig. 4). Thus, we eliminated any genetic effect that could interfere in the DNA methylation profile since they were genetically identical copies. Therefore, we tested the hypothesis that they were epigenetically different.

DNA methylation is the major epigenetic modification of the mammalian genome [17], occurring by the covalent modification of a cytosine to 5-methylcytosine (5 mC), by the DNA methyltransferase (DNMTs) enzymes [18]. The newly discovered ten-eleven translocation (TET) family of enzymes facilitated the oxidation of 5 mC to 5-hydroxymethylcytosine (5 hmC) and other oxidation derivatives as part of the active genomic demethylation process [60]. The hydroxymethylation level is a predictive

indicator for a variety of diseases [61], such as cancers [62, 63] and neurological abnormalities [64, 65], but the biological functions of the 5 hmC are still controversial.

In this study, we observed lower levels of 5 hmC compared to 5 mC (Fig. 1). This result corroborates with the literature, which demonstrates that 5 hmC modification is relatively rare, with levels that vary from < 0.1% to 0.7%, depending on the tissue [66-69]. Previous studies in human tissues that used the same methodology that we used here showed that the percentage of 5 hmC was very low in the placenta (0.05-0.06%) [70], and this result was similar to our findings. Importantly, analysing global methylation and hydroxymethylation in the foetal cotyledon, we showed higher levels for both cytosine modifications in SCNT-produced female calves with aberrant phenotypes (Fig. 4 a, b), which is in accordance with a previous study that showed that trophoblast exhibited abnormal hypermethylation in cloned embryos [13].

Based on our knowledge, this study is the first to characterizing global methylation and hydroxymethylation in cloned Nellore (*Bos taurus indicus*) cattle using an ELISA-based assay. We believe that this information could help us to better understand some epigenetic causes of specific human syndromes and mainly SCNT embryogenesis, thus contributing to improving the efficiency of SCNT.

Inefficient epigenetic reprogramming of the satellite regions was found in cloned bovine embryos [38, 71-74] and foetuses [75-78]. However, DNA methylation patterns in cloned cattle at calving is poorly understood. Satellite DNA are highly repetitive DNA sequences that constitute a considerable portion of eukaryotic genomes. They are typically located at pericentromeric regions of all mammalian chromosomes and are frequently methylated [79-81]. Considering that satellite DNA are very widespread in the genome [82], they could reflect specific methylation patterns of the genome [31]. Thus, two

satellite regions, *Bos taurus* testis satellite I (Satellite I) and *Bos taurus* alpha satellite I DNA (α-Satellite), were chosen to be analysed in this study.

For satellite I region, placenta from healthy cloned calves were more methylated compared to aberrant clones (Fig. 4 c, g). These high levels of methylation observed in healthy clones as well as in the AI controls in the satellite region can be explained by the necessity of transcriptional repression by methylation marks in repetitive DNA, which is in accordance with the literature [21, 32, 79]. This transcriptional repression is necessary because satellite DNAs are the major component of constitutive heterochromatin, playing an essential role in the development of the compact structure of the heterochromatin. Heterochromatin plays an essential role in the preservation of epigenetic information and proper chromosome segregation [31], therefore ensuring genomic stability [81].

However, in the same satellite region, placenta from animals that exhibit aberrant phenotypes showed hypomethylation in both sexes (Fig. 4 c, g). In pathological conditions, such as diseases and cancer, misregulation of pericentromeric satellites, together with decondensation and demethylation of pericentromeric DNA, have been reported [81, 83]. This demethylation causes a transcriptional activation of the pericentromeric satellite DNA that appears to be part of a general stress response programme that is activated by environmental stimuli [31], and it could be extrapolated to the microenvironment of the *in vitro* maturation and *in vitro* culture to which the cloned embryos are submitted [84, 85].

With regard to the methylation status of the α-Satellite region, all of the animals showed this region to be hypermethylated, except for animal III B, which was different from the controls (Fig. 3). The alpha satellite repeats are located at the centromeres [81], and this

region is the locus at which each chromosome maintains sister chromatid cohesion and regulates accurate chromosome segregation during cell division [32]; therefore, it must be appropriately methylated [75]. However, the satellite I repeats are located in the pericentromeric region [81], and this difference of location within the chromosome between these two repetitive regions that we analysed can explain why there were differences in the methylation profile on satellite I and not for the α-satellite. Satellite I is not located in an essential region that critical for cell division, such as the centromeric position of α-satellite. We suggest that loci located at the centromeric regions could be evolutionarily more protected from deleterious environmental effects. Thus, we did not find important variations in the methylation status of the α-satellite region.

When cloned calves, regardless of phenotypes or sex, were compared to the AI controls, those cloned animals showed the Satellite I region to be less methylated (Figure 2 c, d). These data, along with a high mortality rate (Fig. 1), indicated that many of those animals were viable offspring that survived to term but died in the perinatal period due to a variety of anomalies. We suggest that the altered methylation patterns found here in this study could be involved, which is in agreement with many published studies [7, 12, 13, 34, 86].

Evaluating the viability of neonates in the perinatal period, we showed that the satellite I region was hypermethylated in live cloned calves (Fig. 5c), which is in contrast to dead calves which exhibited hypomethylation (Fig. 5d). This finding reveals that cloned calves that survived in the perinatal period probably had an appropriate epigenetic reprogramming in this satellite region, since high levels of methylation were expected in the satellite DNA [31].

Conclusions

In summary, these results suggest that satellite I could be used as an epigenetic-based biomarker for predicting offspring viability in the context of SCNT, which would support the development and adaptation of new SCNT protocols in a direct, timely and specific manner. From this perspective, studies that evaluate DNA methylation patterns of this satellite region by assessing the donor cell nucleus or embryo biopsies could shed light on how to improve the efficiency of cloning.

Declarations

Ethics approval

The Ethics Committee on Animal Use (CEUA-protocol no. 078/16) of the Federal University of Uberlândia, Brazil approved all procedures.

Availability of data and materials

All of the data generated or analysed during this study are included in this published article.

Competing interests

No potential conflicts of interest were disclosed.

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Authors' contributions

HB, RR, MMS and NAB participated in the collection of materials for analysis. MMF, MMS, AdaSM, and LNV performed the genomic analyses. MMS and MMF designed the experiment, interpreted the results and wrote the manuscript.

All authors read and approved the final manuscript.

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Consent for publication

Not applicable

Consent to participate

Not applicable

Figure legends

Fig. 1 Different phenotypes and percentages of global methylation and hydroxymethylation and specific methylation statuses of two satellite regions in the foetal placenta (cotyledon) of Nellore (*Bos taurus indicus*) cloned calves at birth. Each phenotypic trait is represented by a different colour. Each line represents a calf. I, II, III, IV represent different cell lines (nucleus donor animals). A, B and C represent different cloned animals. Controls are calves produced by artificial insemination (AI). **%** met sat I (percentage of methylation on satellite I region); % met α-sat (percentage of methylation on α-satellite region); % 5 mC global (percentage of global methylation); % 5 hmC global (percentage of global hydroxymethylation); ♀ (female); ♂ (male).

Fig. 2 DNA methylation of the Satellite I region in the foetal placenta (cotyledon) of SCNT-produced calves according to sex and phenotypes and according to the type of assisted reproduction technique. (**A**) Female calves: IA, IB and IIA, IIB represent SCNT-produced calves. I and II are different cell lines (nucleus donor animals). Controls are calves produced by artificial insemination (AI). (**B**) Male calves: IIIA, IIIB, IIIC and IVA, IVB, IVC represent SCNT-produced calves. III and IV are different cell lines (nucleus donor animals). (**C**) Representation of all sequenced alleles of satellite I of all cloned calves by somatic cell nuclear transfer (SCNT). (**D**) Representation of all sequenced alleles of satellite I of all controls by artificial insemination (AI). Brackets indicate the groups that belong to each phenotype (aberrant and healthy). Each line represents one individual DNA clone, and each circle represents one CpG dinucleotide (23 CpGs). The white circles represent the unmethylated CpGs, the filled black circles represent the methylated

CpGs, and the grey circles represent a CpG that could not be analysed. The numbers to the right of each line indicate the number of times that the allele was sequenced, and the numbers to the bottom of each group represent the DNA methylation means ± standard errors for each group. (*, #, ■, ♦, ▲, ♠) represents significantly different means of methylation between each SCNT-produced calf compared to its respective control (p val ≤ 0.05). ♀ (female); ♂ (male).

Fig. 3 DNA methylation of the α-Satellite region in foetal placenta (cotyledon) of SCNT-produced calves according to the sex and phenotype, and according to the type of assisted reproduction technique. (**A**) Female calves: IA, IB and IIA, IIB represent SCNT-produced calves. I and II are different cell lines (nucleus donor animals). Controls are calves produced by artificial insemination (AI). (**B**) Male calves: IIIA, IIIB, IIIC and IVA, IVB, IVC represent SCNT-produced calves. III and IV are different cell lines (nucleus donor animals). (**C**) Representation of all sequenced alleles of the α-satellite of all cloned calves by somatic cell nuclear transfer (SCNT). (**D**) Representation of all sequenced alleles of the α-satellite of all controls by artificial insemination (AI). Brackets indicate the groups that belong to each phenotype (aberrant and healthy). Each line represents one individual DNA clone, and each circle represents one CpG dinucleotide (18 CpGs). The white circles represent unmethylated CpGs, the filled black circles represent methylated CpGs, and the grey circles represent a CpG that could not be analysed. The numbers to the right of each line indicate the number of times that the allele was sequenced, and the numbers to the bottom of each group represent the DNA methylation means ± standard errors for each group. (*) represents significantly different means of

methylation between each SCNT-produced calf compared to its respective control (*p* val ≤ 0.05). ♀ (female); ♂ (male).

Fig. 4 Comparison of the global methylation and hydroxymethylation levels and specific methylation statuses of two satellite regions in foetal placenta cotyledons of male and female SCNT-produced calves classified as healthy and aberrant. (**A**) Percentage of global methylation in females according to phenotype. (**B**) Percentage of global hydroxymethylation in females according to phenotype. (**C**) Percentage of methylation on the satellite I region in females according to phenotype. (**D**) Percentage of methylation on the α-satellite region in females according to phenotype. (**E**) Percentage of global methylation in males according to phenotype. (**F**) Percentage of global hydroxymethylation in males according to phenotype. (**G**) Percentage of methylation on the satellite I region in males according to phenotype. (**H**) Percentage of methylation on the α-satellite region in males according to phenotype. *P-value* represents significantly different means between phenotypes (*p* val≤ 0.05). ♀ (female); ♂ (male).

Fig. 5 Comparison of global methylation and hydroxymethylation levels and specific methylation statuses of two satellite regions in foetal placenta cotyledons between live and dead SCNT-produced calves. (**A**) Percentage of global methylation according to viability. (**B**) Percentage of global hydroxymethylation according to viability. (**C**) Percentage of methylation on satellite I region according to viability. (**D**) Percentage of methylation on α-satellite region according to viability. (*) represents significantly different means (*p* val ≤ 0.05).

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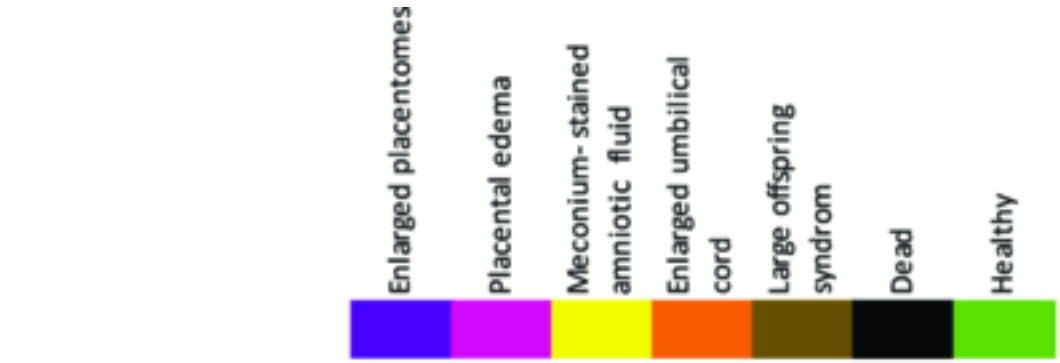

Sex	Nucleus donor	Clones	%met sat I	%met α-sat	%5mC global	%5hmC global
♀	I	A	34.78	80.00	12.73	0.10
		B	43.14	68.38	8.69	0.08
♀	II	A	28.85	77.08	10.99	0.12
		B	46.58	59.72	8.77	0.05
♂	III	A	30.80	77.35	9.73	0.07
		B	29.77	42.42	6.94	0.02
		C	68.77	74.54	7.48	0.07
♂	IV	A	30.14	53.70	8.96	0.11
		B	19.40	71.53	5.39	0.01
		C	54.35	53.33	3.85	0.07
♀	Control		59.94	83.33	4.98	0.01
♂	Control		64.64	79.63	8.46	0.08

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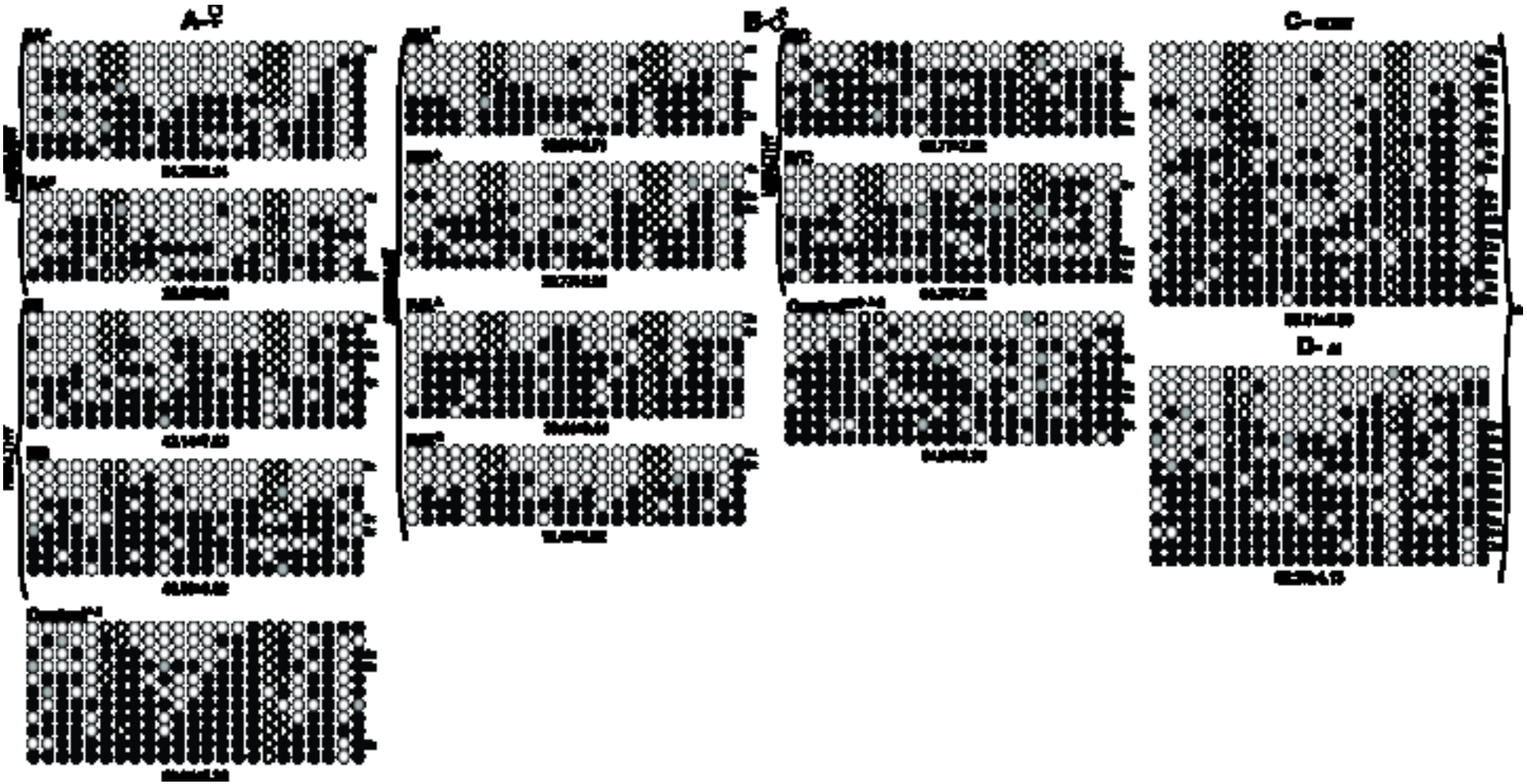

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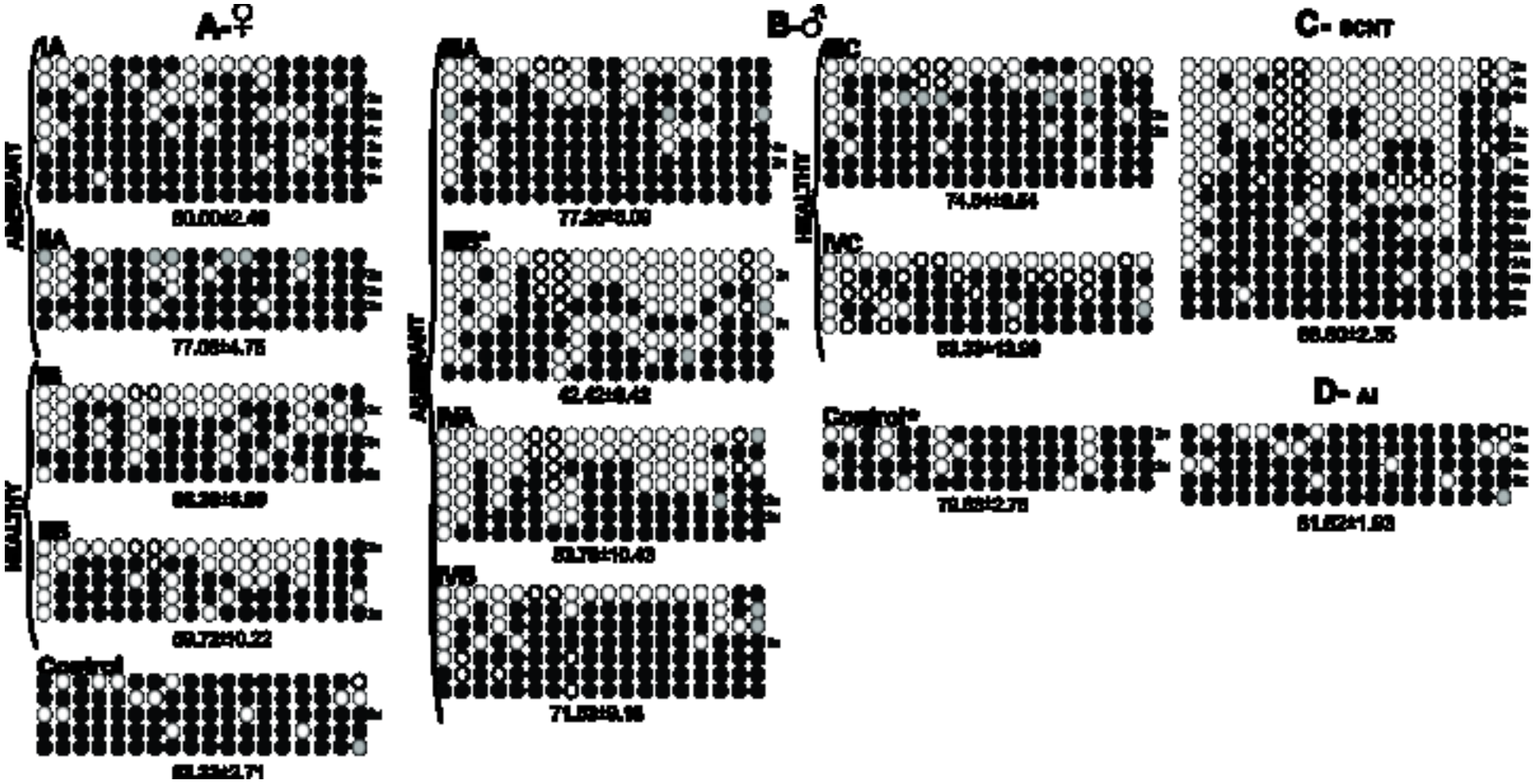

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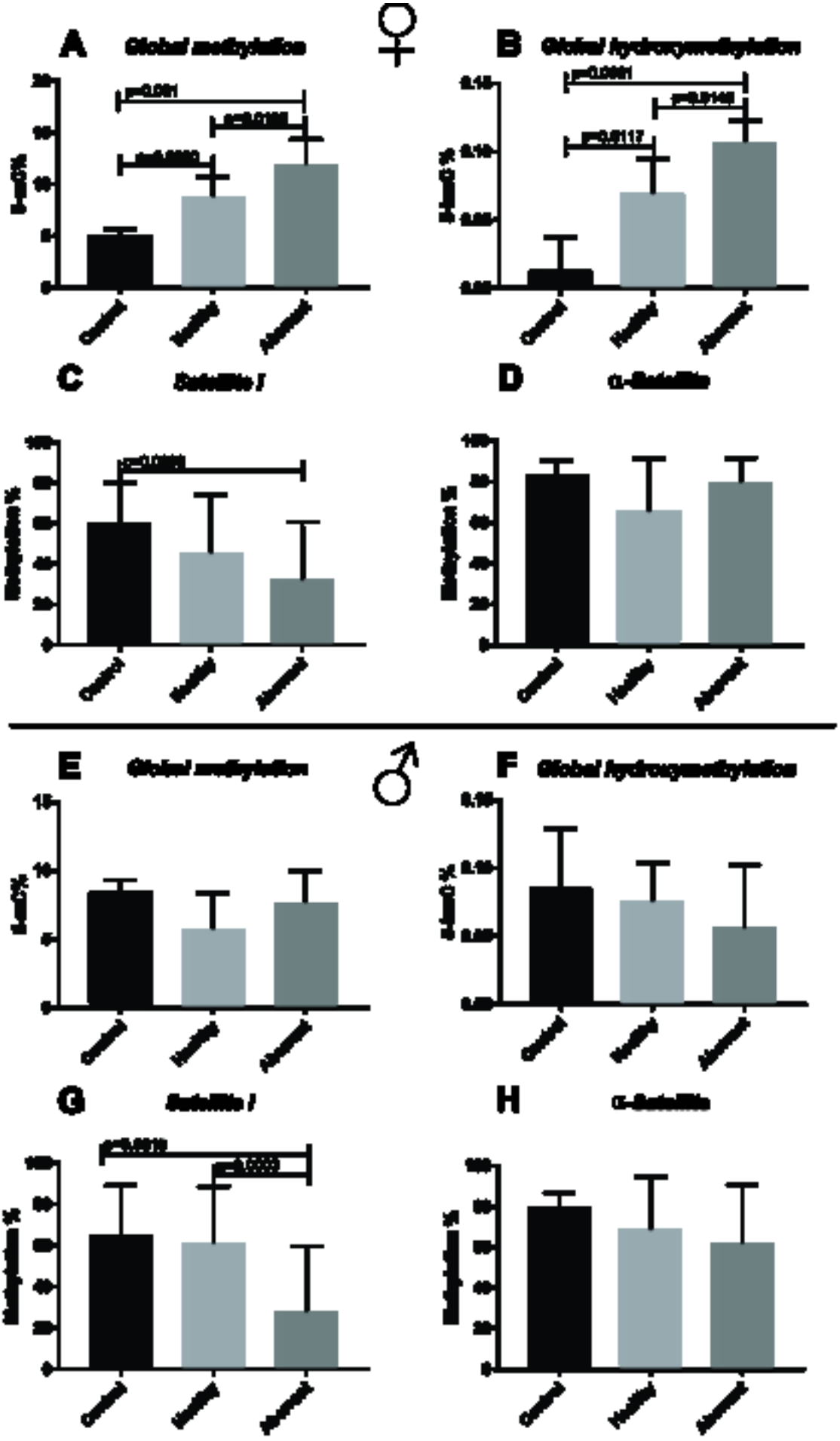

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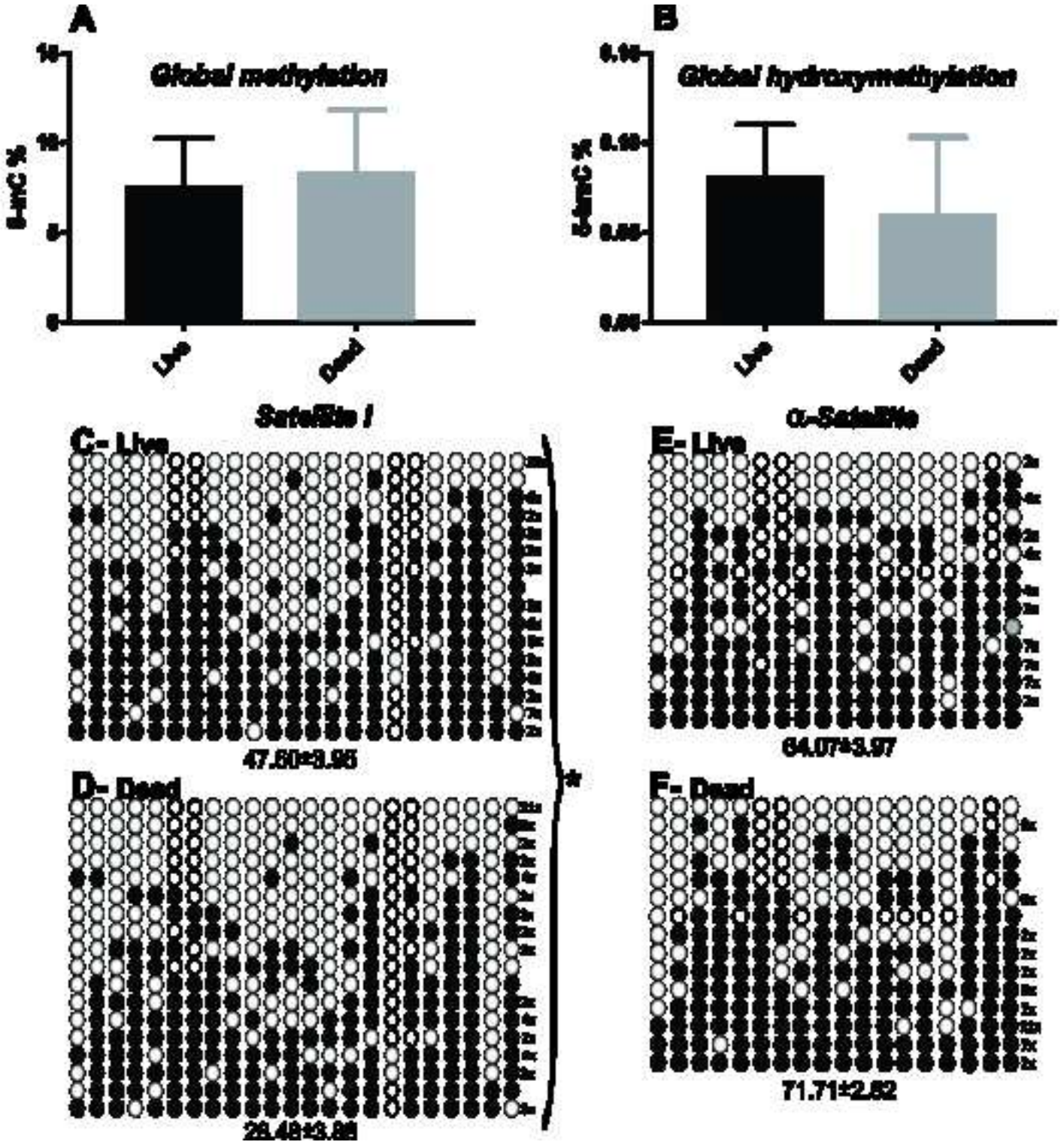

DNA methylation profile at a satellite DNA region is associated with aberrant placentation in cloned calves

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