1 **Procaine and S-adenosyl-L-homocysteine (SAH) affect the expression of genes**

2 **related to the epigenetic machinery and change the DNA methylation status of *in***

3 ***vitro* cultured bovine skin fibroblasts**

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24 # Abstract

25 Cloning using somatic cell nuclear transfer (SCNT) has many potential applications such

26 as in transgenic and genomic-edited animal production. Abnormal epigenetic

27 reprogramming of somatic cell nuclei is probably the major cause of the low efficiency

28 associated with SCNT. Strategies to alter DNA reprogramming in donor cell nuclei may

29 help improve the cloning efficiency. In the present study, we aimed to characterise the

30 effects of procaine and S-adenosyl-L-homocysteine (SAH) as demethylating agents

31 during the cell culture of bovine skin fibroblasts. We characterised the effects of

32 procaine and SAH on the expression of genes related to the epigenetic machinery,

33 including the DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, and OCT4 genes, and on

34 DNA methylation levels of bovine skin fibroblasts. We found that DNA methylation levels

35 of satellite I were reduced by SAH (P=0.0495) and by the combination of SAH and

36 procaine (P=0.0479) compared with that in the control group. Global DNA methylation

37 levels were lower in cells that were cultivated with both compounds than in control cells

38 [procaine (P=0.0116), SAH (P=0.0408), and both (P=0.0163)]. Regarding the

39 transcriptional profile, there was a decrease in total DNMT transcript levels in cells

40 cultivated with SAH and procaine. There was a higher level of TET3 transcripts in treated

41 cells than in the controls. Our results showed that the use of procaine and SAH during

42 bovine cell culture was able to alter the epigenetic profile of the cells. This approach

43 may be a useful alternative strategy to improve the efficiency of reprogramming the

44 somatic nuclei after fusion, which in turn will improve the SCNT efficiency.

45

46

Introduction

48   Although two decades have passed since the first cloned mammal was born from an

49   adult animal (1) and despite the somatic cell nuclear transfer (SCNT) technique having

50   become a commercially available technique, its efficiency is still extremely low (2, 3).

51   SCNT routinely involves the use of differentiated somatic cells, which display changed

52   totipotency states through mechanisms dependent on epigenetic modifications (1, 4).

53   Despite the successful cloning of several species, the use of differentiated somatic cells

54   as donor nuclei is associated with a range of concerns, such as increased abortion rates,

55   high embryonic lethality, and severe abnormalities in cloned foetuses and placentas in

56   ruminants (5). Abnormal DNA methylation patterns represent one possible reason for

57   the frequent developmental and metabolic anomalies and the very low survival rates of

58   cloned calves (5-7).

59   Correct epigenetic reprogramming involves, among other modifications, the

60   methylation of genomic DNA and post-translational modifications to histone proteins,

61   which are involved in chromatin organisation (5, 8, 9). Epigenetic reprogramming during

62   initial development is a dynamic process that involves two waves of methylation and

63   demethylation and starts at the beginning of gametogenesis, continuing until the

64   formation of all foetal tissues (9, 10). Some studies have reported that cloned embryos

65   only partially demethylate their genomes and begin the *de novo* methylation process

66   earlier than their counterparts (5, 7). Epigenetic processes such as DNA methylation and

67   demethylation are regulated by different groups of enzymes. DNA methylation is

68   performed by the DNA methyltransferase enzymes (DNMTs) (11). In fertilised embryos,

69   through early development, methylation is reduced on or removed from numerous

70   sequences, and from the 8-16 cell stage, a new embryonic methylation pattern

71   generated by the *de novo* methyltransferases DNA methyltransferase 3 alpha (Dnmt3a)

72   and DNA methyltransferase 3 beta (Dnmt3b) is established (5, 7). New patterns of DNA

73   methylation on the genome are maintained by DNA methyltransferase 1 (DNMT1) and

74   determine gene expression in embryo development (8). The mechanism of DNA

75   demethylation involving the oxidisation of 5-methylcytosine (5mC) in 5-

76   hydroxymethylcytosine (5hmC) is triggered by the ten-eleven translocation enzymes

77   (TETs) (12). The TET family contains the TET1, TET2, and TET3 dioxygenases, which

78   enable the conversion of 5-methylcytosine (5mC) to 5-hydroxylmethylcytosine (5-hmC)

79   and 5-formylcytosine and 5-carboxylcytosine, ultimately leading to the replacement of

80   the 5mC with a cytosine (13-15). TET proteins are also involved in histone modification,

81   binding to metabolic enzymes and other proteins, influencing gene transcription (16).

82   The difficulty in achieving efficient methylation reprogramming in SCNT embryos may

83   be a consequence of the resistance of tissue-specific epigenetic patterns to be

84   reprogrammed by the oocyte (17). Studies have reported that embryo development can

85   be improved by reducing DNMT expression using epigenetic modifier agents and siRNA

86   technology (18). It has also been found that SCNT embryo development can be

87   significantly improved by strategies that stimulate changes in DNA methylation and

88   histone modifications, such as the use of 5-Aza-2-deoxycytidine (zdC)(19-21), procaine

89   (para-amino-benzoyl-diethylamino-ethanol) (22, 23), S-adenosyl-L-homocysteine (SAH)

90   (24, 25), and Scriptaid (25), during cell culture. Procaine and SAH are demethylating

91   compounds that are not analogous to nucleosides. Thus, they do not incorporate into

92   the DNA molecule and therefore do not have cytotoxic effects, distinguishing them from

4

93　　most previously tested compounds (23, 26, 27). Procaine inhibits human cancer cell

94　　growth (27) through interactions with CpG-rich genomic regions and prevents the action

95　　of DNMT enzymes (23). Gao et al. (2009) showed that procaine can reverse the

96　　abnormal methylation of the Wif-1 gene and be used as a demethylating drug (28). SAH

97　　is a physiological by-product of the transmethylation reaction in cells involving S-

98　　adenosyl-L-methionine (SAM) and is found in the nucleus, cytoplasm, and extracellular

99　　environment (29). Increasing SAH concentrations promote its binding to the DNMT

100　　active sites, which in turn decreases DNA methylation (30). Jeon et al. (2008)

101　　demonstrated that SAH treatment of fibroblasts induces global DNA demethylation and

102　　increases the developmental potential for SCNT embryos with them also exhibiting

103　　higher telomerase activity levels, which is indicative of enhanced nuclear

104　　reprogramming (24).

105　　Skin fibroblasts are the most commonly used donor cells used for nuclear transfer (NT)

106　　in cattle production. Thus, it is important to gain a better understanding of the dynamics

107　　of DNA methylation patterns in these cells in *in vitro* culture to improve SCNT protocols.

108　　Moreover, it is important to evaluate the dynamics of cells cultured with demethylating

109　　agents, as these culture conditions may offer promising prospects to verify the

110　　pluripotency status of treated cells and analyse the hypothesis that demethylating

111　　compounds could drive cells towards a dedifferentiated status.

112　　Taken together, we aimed to evaluate the effects of procaine and/or SAH during the *in*

113　　*vitro* culture of bovine skin fibroblasts on the global and specific DNA methylation

114　　patterns and on the mRNA levels of the genes encoding the epigenetic machinery, such

115　　as DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, and the pluripotency gene OCT4.

Material and Methods

Ethics statement

Experiments were performed in accordance with the Brazilian Law for Animal Protection and the Institutional Guidelines for Animal Care and Experimentation. The project was approved by the Ethics Committee in Animal Use at EMBRAPA Genetic Resources and Biotechnology (CEUA/CENARGEN) at a special meeting held on July 2, 2015.

Experimental design

Adult skin fibroblasts were obtained from a biopsy taken from a male Nellore (*Bos taurus indicus*) bull. Procaine and SAH were used alone or in combination for 2 weeks during bovine skin fibroblast *in vitro* culture in four experimental treatments. Four biological replicates were performed for each treatment: (1) control, (2) cells cultured with 1.0 mM procaine, (3) cells cultured with 1.0 mM SAH, and (4) cells cultured with 1.0 mM SAH and 1.0 mM procaine. We specifically focused on evaluating the effects of procaine and SAH on cell growth, specific and global DNA methylation, and the transcript levels of genes encoding the epigenetic machinery, including DNMT1, DNMT3A, DNMT3B, TET1, TET2, and TET3. In addition, to verify the pluripotency status of the treated cells, we analysed the expression of the OCT4 gene. The experimental design is presented in Fig. 1.

Fig. 1. Scheme of the experimental design. Skin biopsy was obtained from a male Nellore (*Bos taurus indicus*) bull. From this biopsy, an *in vitro* culture of skin fibroblasts was established. Cells from the control group were cultured only with DMEM. The other groups were supplemented with procaine, S-adenosyl-L-homocysteine (SAH), or a

6

139   combination of both substances for 2 weeks. After 2 weeks of *in vitro* culture, genomic

140   DNA and total RNA extractions were performed. Genomic DNA was used for global (5-

141   mC DNA ELISA Kit -Zymo Research) and specific (satellite I region – BS-PCR) methylation

142   analyses. Total RNA was used for analysis of gene expression by RT-qPCR. Genes

143   involved in the epigenetic machinery (DNMT1, DNMT3A, DNMT3B, TET1, TET2, and

144   TET3) and pluripotency status (OCT4) were analysed. The β-actin and GAPDH genes were

145   used as endogenous controls.

146

Cell culture establishment

148   The skin biopsy was cut into small pieces (2–3 mm^2), and the explants were cultured in

149   Dulbecco's modified Eagle Medium (DMEM-Gibco® Life Technologies, Carlsbad, CA,

150   USA) supplemented with 3.7 g/L sodium bicarbonate, 110 mg/L pyruvate, 10% foetal

151   bovine serum (FCS), and antibiotics in 25 cm^2 bottles at 39°C in a humidified atmosphere

152   with 5% CO_2 until confluence. After establishing the cell culture, cells were frozen

153   (second passage) in DMEM containing 10% dimethyl sulphoxide (DMSO, Gibco,

154   Carlsbad, CA, USA). Cell pallets were stored at -80°C for 24 h and subsequently

155   transferred to liquid nitrogen, where they were stored until use.

Cell culture using procaine and/or SAH

157   Cells were *in vitro* cultured using procaine or SAH according to a previously established

158   protocol in the Laboratory of Animal Reproduction (Embrapa Genetic Resources and

159   Biotechnology, Brazil). Briefly, cells were thawed, subcultured, and grown until they

160   reached confluence in DMEM supplemented with 3.7 g/L sodium bicarbonate, 110 mg/L

161   pyruvate, 10% foetal bovine serum (FBS), and antibiotics for 2 weeks. The culture media

162   also contained 1.0 mM procaine, 1.0 mM SAH, or a combination of the two compounds,

163   as described in the experimental design (Fig. 1). Cells were evaluated after reaching

164   confluence using an inverted Axiovert 135M microscope at 10X magnification (ph1) with

165   an attached CFI60 Nikon optical system and an ECO-LED lighting system.

DNA isolation

167   *In vitro* cultured cells were used for genomic DNA isolation for use in methylation

168   analyses. Genomic DNA was isolated using the DNeasy® Blood & Tissue Kit (Qiagen,

169   Hilden, Germany) according to manufacturer's protocol. DNA samples were diluted in

170   20 µL buffer AT and stored at -20°C after measuring the DNA concentration.

Sodium bisulphite treatment

172   Genomic DNA was treated with sodium bisulphite using the EZ DNA Methylation-

173   Lightning™ Kit (Zymo Research, Orange, CA, USA) according to manufacturer's protocol.

174   After bisulphite treatment, the DNA samples were stored at -80°C until PCR

175   amplification or global methylation analyses.

PCR of bisulphite-converted DNA

177   Bisulphite-converted DNA samples were submitted to PCR amplification. The primers

178   were designed with MethPrimer software to flank and amplify a CpG island in the

179   repetitive DNA sequence of the *Bos taurus* bovine testis satellite I (satellite I)(31).

180   PCR was performed in a total volume of 20 µL using 1x Taq buffer, 1.5 mM $MgCl_2$, 0.4

181   mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), 0.5 µM each primer

182   (forward and reverse), and 2 µL of bisulphite-treated DNA. The following temperature

183   and time conditions were used: (1) an initial denaturing step at 94°C for 3 min, (2) 40

184   cycles at 94°C for 40 s, 45°C for 1 min, and 72°C for 1 min, and (3) a final extension at

185   72°C for 15 min.

Cloning and bisulphite sequencing

187   After PCR, the amplicons were purified from an agarose gel using the Wizard SV Genomic

188   DNA Purification System (Promega Corp., Madison, WI, USA) according to the

189   manufacturer's protocol. Then, the purified amplicons were inserted into the TOPO TA

190   Cloning vector (pCR™II-TOPO® vector system, Invitrogen, Carlsbad, CA, USA) and

191   transferred into DH5α cells using a heat shock protocol. Then, plasmid DNA was isolated

192   using the QIAprep Spin Miniprep Kit (Qiagen, CA, USA), and individual clones were

193   sequenced using BigDye® cycle sequencing chemistry.

194   The sequencing quality was analysed using Chromas®, and the methylation patterns

195   were analysed using the BiQ Analyser program (32) (MPI for Informatics, Saarland,

196   Germany). The DNA sequences were compared with a reference sequence from

197   GenBank, accession number AH001157.2. Only sequences that originated from clones

198   with ≥ 95% homology and cytosine conversion were used.

Global DNA methylation analysis

200   Genomic DNA samples were also used for global DNA methylation analysis using the 5-

201   mC DNA ELISA Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's

202   protocol. Briefly, each DNA sample (~100 ng), in triplicate, was bound to strip-wells that

203   were specifically treated to have high DNA affinity. Global DNA methylation was

204   detected using specific antibodies and was quantified colorimetrically by reading the

205   absorbance at 450 nm in a microplate spectrophotometer (Bio-Rad Microplate Reader,

206   Bio-Rad Laboratories, Redmond, WA, USA). The percentage of methylated DNA was

207 proportional to the measured optical density (OD) intensity. Relative quantification was

208 used to calculate the percentage of 5-mC in the total cytosine content in the bovine

209 genome.

Total RNA isolation and cDNA synthesis

211 Total RNA isolation was performed in biological quadruplicates of exponentially growing

212 fibroblasts using the PureLink^{TM} RNA Mini kit (Invitrogen, USA) following the

213 manufacturer's protocol. Total RNA was eluted in 20 μL of DEPC-treated water.

214 Immediately before use for cDNA synthesis, total RNA was treated with 2 U of RQ1

215 RNase-Free DNase (Promega, USA) at 37°C for 30 min, and the DNase was inactivated

216 by incubation at 65°C for 10 min. cDNA was synthesised using 1 μg of total RNA in a 20

217 μL final volume using the GoScript Reverse Transcription System (Promega, USA)

218 according to the manufacturer's protocol. cDNA synthesis was performed in a Veriti 96-

219 Well Thermal Cycler Gradient (Applied Biosystems) using the following conditions: 5 min

220 at 25°C, 60 min at 42°C, 15 min at 70°C, and holding at 4°C. cDNA samples were stored

221 at -20°C until use.

Real Time PCR

223 RT-qPCR reactions were performed in triplicate in a final volume of 25 μL containing 1

224 μL of cDNA, 12.5 μL of GoTaq® qPCR Master Mix (Promega, USA), and 0.2 μM each

225 primer in a 7500 Fast Real-Time PCR System (Applied Biosystems, USA). RT-qPCR

226 conditions were as follows: 95°C for 20 s and 40 cycles of a denaturation step at 95°C for

227 3 s and an annealing/extension step at 60°C for 30 s. Reactions were optimised to

228 provide a maximum amplification efficiency for each gene using a relative standard

229 curve, and the efficiencies of each pair of primers are shown in Table 1. Each sample was

230   analysed in triplicate, and the specificity of each PCR product was determined by

231   analysing the melting curve and the size of amplicons in an agarose gel. The

232   glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (BACT) genes were

233   used for data normalisation using the geometric means of their Ct (cycle threshold) and

234   their efficiencies of amplification. The control group (cells cultivated without procaine

235   and SAH) was used as the reference sample. The relative abundance of mRNA for each

236   gene was calculated and compared among the experimental groups using the ΔΔCt

237   method, with efficiency correction using the Pfaffl method (33).

238   Six genes related to DNA methylation reprogramming were selected for analysis,

239   DNMT1, DNMT3A, DNMT3B, TET1, TET2 and TET3. In addition, OCT4, related to

240   pluripotency status, was also analysed. Details on the genes and primers are presented

241   in Table 1.

242

243   Table 1: Primers for gene expression analysis

Gene	Primer sequence	Amplicon (bp)	*GenBank accession number*	Primer efficiency
DNMT1	F: TTG GCT TTA GCA CCT CAT TTG CCG	82	NM_182651.2	91.244 %
	R: TCC TGC ATC ACG CTG AAT AGT GGT			
DNMT3A	F: TTT CCA ATG TGC CAT GAC AGC GAC	82	NM_001206502.1	114.726%
	R: GGG CCC ACT CGA TCA TTT GTT TGT			
DNMT3B	F: CAA CAA GCA ACC AGA GAA TAA G	161	NM_181813.2	112.048%
	R: CAA CAT CCG AAG CCA TTT G			
TET1	F: GTA TGC TCC AGC TGC TTA TC	167	XM_015469834.1	108.166%
	R: CCA CTG TGC TCC CAT TAT TC			
TET2	F: GTA GGG ACA TTT CCT CCT TAT TC	157	XM_010828077.2	105.302%
	R: CAG CTG CAC TGT AGT TAT GG			
TET3	F: GTA ACC CAG GTG ATT CTG ATA C	200	XM_015465317.1	101.853%
	R: CAG CAG CCT ATC TGC TAA TC			

OCT4	F: TTCAGCCAAACGACTATCTGCCGT	112	AY490804.1	90.941%
	R: TCTCGTTGTTGTCAGCTTCCTCCA			
GAPDH	F: GGC GTG AAC CAC GAG AAG TAT AA	119	NM_001034034.2	101.988%
	R: CCC TCC ACG ATG CCA AAG T			
B-ACT	F: GGC ACC CAG CAC AAT GAA GAT CAA	134	NM_173979.3	109.929%
	R: ATC GTA CTC CTG CTT GCT GAT CCA			

244   F: forward primer; R: reverse primer; bp: base pair

Statistical analyses

246   All analyses were performed using GraphPad Prism software (Version 6.0). Data were

247   compared among experimental groups using ANOVA and Tukey's test or the Kruskal-

248   Wallis and Mann-Whitney tests for data showing or not showing normality, respectively.

249   The normality of the data was analysed using the Shapiro-Wilk test. The results are

250   presented as the mean ± the standard deviation.

12

Results

Cell culture and morphology

253   After *in vitro* culture, the cells from all treatments showed normal cell growth and

254   reached confluence. No morphological changes were observed during culture (Fig. 2).

255

256   Fig. 2. Photomicrographs of bovine skin fibroblast cell lines in *in vitro* culture. A - Control

257   group. B - Culture supplemented with 1.0 mM procaine. C – Culture supplemented with

258   1.0 mM S-adenosyl L-homocysteine (SAH). D - Culture supplemented with 1.0 mM

259   procaine and 1.0 mM SAH. 100x magnification. Bar: 0.20 mm.

260

Total cell counting

262   The cells were separately counted after treatment with procaine, SAH, or procaine and

263   SAH. The total cell number observed for each treatment, in quadruplicate, is shown in

264   Table 2 and Fig. 3. No differences were found among the treatments (P=0.0899).

265

266   Table 2. Total number of cells from the *in vitro* culture of bovine skin fibroblasts cultured

267   with procaine, SAH, or procaine and SAH counted in a Neubauer chamber.

	CONTROL	PROCAINE	SAH	PROCAINE + SAH
	62×10^4	65×10^4	55×10^4	54×10^4
	59×10^4	60×10^4	60×10^4	56×10^4
	66×10^4	51×10^4	54×10^4	66×10^4
	48×10^4	52×10^4	53×10^4	58×10^4
Mean ($\pm$SD)	$58{,}75 \times 10^4 (\pm10{,}2)$	$57 \times 10^4 (\pm9{,}5)$	$55{,}5 \times 10^4 (\pm5{,}4)$	$58{,}5 \times 10^4 (\pm8{,}9)$

268

13

269   Fig. 3. Mean ± standard deviation (four biological replicates) of the number of cells for

270   each treatment (procaine, S-adenosyl L-homocysteine (SAH), procaine and SAH, and

271   control groups). The cells were counted in a Neubauer chamber.

272   ## DNA methylation profile of satellite I

273   The bisulphite sequencing results are shown in Fig. 4 and Fig. 5. The DNA methylation

274   status of satellite I, a DNA repeat element, was investigated. We found that the genomic

275   DNA from cells cultured for 2 weeks with SAH and with procaine + SAH was less

276   methylated compared to that from cells in the control group (P=0.0495 and P=0.0479,

277   respectively; Fig. 4 and Fig. 5). However, no differences were found for cells cultured

278   with procaine alone.

279

280   Fig. 4. DNA methylation pattern of the satellite I region in bovine skin fibroblasts. A -

281   Control cells. B - Cells cultured with 1.0 mM procaine. C - Cells cultured with 1.0 mM S-

282   adenosyl L-homocysteine (SAH). D - Cells cultured with 1.0 mM procaine and SAH. Each

283   line represents one individual clone, and each circle represents one CpG dinucleotide (a

284   total of 23 CpGs were analysed). White circles represent unmethylated CpGs, filled black

285   circles represent methylated CpGs, and grey circles represent CpGs that could not be

286   analysed. Arrows indicate that for all treatments, the cytosines in position 17 were

287   always demethylated.

288

289   Fig. 5. Mean ± standard deviation of DNA methylation levels of the satellite I region in

290   bovine skin fibroblasts. Control cells and cells cultured with procaine, S-adenosyl L-

291   homocysteine (SAH), or procaine and SAH. An asterisk represents a significant

292   difference.

14

293

Global methylation analysis

295 We found that global methylation levels were lower in all treated cell groups, procaine

296 (P=0.0116), SAH (P=0.0408), and procaine and SAH (P=0.0163), than in the control group

297 (Fig. 6).

298

299 Fig. 6. Mean ± standard deviation of global DNA methylation levels in bovine skin

300 fibroblasts cultured with procaine, S-adenosyl L-homocysteine (SAH), or procaine and

301 SAH. Biological quadruplicates and technical triplicates were performed for each

302 measurement.

303

Quantification of relative mRNA abundance

305 We quantified the mRNA levels of genes involved in the epigenetic methylation process,

306 DNMT1, DNMT3A, DNMT3B, TET1, TET2, and TET3, and pluripotency status, OCT4.

307 The mRNA levels of all genes that were evaluated were detected in bovine skin

308 fibroblasts. Specific amplicon sizes determined in agarose gels and specific melting

309 temperatures for each gene studied confirmed the high specificity of the primers that

310 were used in this study (Fig. 7 and Fig. 8). When analysing each DNMT individually, per

311 each treatment, no differences were found among the groups, but when considering

312 the expression of each DNMT individually, regardless of the treatment, the mRNA levels

313 of DNMT1, DNMT3A, and DNMT3B were lower in cells cultivated with both substances

314 (Fig. 9). Moreover, when considering the expression of the group of three DNMTs and

315 analysing per treatment individually, procaine, SAH, and procaine in combination with

316   SAH decreased the mRNA levels of DNMTs (Fig. 10). Regarding the TET genes, the only

317   significant difference that was verified was for TET3, for which there were higher levels

318   of transcripts in cells cultivated with both substances, regardless of the treatment, when

319   compared to the control (Fig. 11). Figure 12 shows the log-fold change for all analysed

320   genes related to DNA methylation reprogramming, presenting the downregulated or

321   upregulated profile of each gene compared to that of the control (Fig. 12). For the OCT4

322   gene, we verified that there was no significant difference in treated cells in relation to

323   the control group (Fig. 13 and Fig. 14).

324

325   Fig. 7. Agarose gel (1.5%) showing amplicons relative to the genes evaluated. Lane 1 -

326   DNMT1 (82 bp); lane 2 - DNMT3A (82 bp); lane 3 - DNMT3B (161pb); lane 4 - TET1 (167

327   bp); lane 5 - TET2 (157 bp); lane 6 - TET3 (200 bp); lane 7 - GAPDH (119 bp); and lane 8 -

328   β-ACTIN (134 bp). M: 100 bp DNA ladder. Lanes 9 to 16 represent the negative control

329   PCR for each gene, respectively. bp – base pair.

330

331   Fig. 8. Melting curves for primers used in RT-qPCR.

332

333   Fig. 9. mRNA levels of DNMT1, DNMT3A, and DNMT3B determined by RT-qPCR in bovine

334   skin fibroblasts treated with procaine and/or S-adenosyl L-homocysteine (SAH).

335   Differences were considered significant when $p < 0.05$.

336

337   Fig. 10. mRNA levels of the group of DNMTs (DNMT1, DNMT3A, DNMT3B) determined

338   by RT-qPCR in bovine skin fibroblasts treated with procaine and/or S-adenosyl L-

339   homocysteine (SAH). Differences were considered significant when $p < 0.05$.

16

340

341 Fig. 11. mRNA levels for the TET3 gene determined by RT-qPCR in bovine skin fibroblasts

342 treated with procaine and/or S-adenosyl L-homocysteine (SAH). Differences were

343 considered significant when $p < 0.05$.

344

345 Fig. 12. Fold change values for the DNMT1, DNMT3A, DNMT3B, TET1, TET2, and TET3

346 genes in skin fibroblasts cultured *in vitro* for 14 d with procaine, S-adenosyl L-

347 homocysteine (SAH), or both substances in relation to the control group.

348

349 Fig. 13. mRNA levels for the OCT4 gene determined by RT-qPCR in bovine skin fibroblasts

350 treated with procaine and/or S-adenosyl L-homocysteine (SAH). Differences were

351 considered significant when $p < 0.05$.

352 Fig. 14. mRNA levels for the OCT4 determined by RT-qPCR in bovine skin fibroblasts

353 treated with procaine and/or S-adenosyl L-homocysteine (SAH) regardless the

354 treatments. Differences were considered significant when $p < 0.05$.

355

356

Discussion

358 Widespread epigenetic reprogramming, including changes in DNA methylation and post-

359 translational histone modifications, occurs during mammalian gametogenesis and initial

360 embryogenesis (9, 10). Most evidence suggests that the developmental failures and

361 abnormalities in SCNT embryos and foetuses may be due to the incomplete epigenetic

362 reprogramming of the somatic genome from the donor cell (7). Similar to the somatic

363 genome of the donor cells, the genomes of SCNT embryos are hypermethylated

364 compared to those of embryos produced *in vitro* or *in vivo* (5, 7). Thus, a strategy that

365 decreases DNA methylation levels in somatic cells in culture could be useful for

366 increasing SCNT efficiency.

367 Our results showed that the addition of procaine, SAH, or both compounds in

368 combination to cell culture media did not impair cell growth or morphology (Fig. 2).

369 Chromatin modulating agents that are analogous to nucleosides normally have high

370 toxicity and cause apoptosis (23). Procaine and SAH are non-cytotoxic compounds that

371 are able to inhibit DNMT enzymes (23, 34). Procaine was shown to reduce tumour

372 volumes by 42.2%, not increase apoptosis rates, and have no adverse effects when used

373 in the culture of the HLE hepatoblastoma cell line (35). Jeon et al. reported that SAH can

374 reversibly inhibit DNMTs in somatic cells (24). Jafari et al. also reported that SAH induced

375 global DNA demethylation (36). Taken together, these results support the potential use

376 of procaine and SAH as demethylating agents in cell culture for different applications.

377   Studies have shown that DNA methylation is an important epigenetic mark that plays a

378   role in repressing gene activity, especially because it is associated with the chromatin

379   state (37-39). Satellite DNA, such as satellite I, LINE-1, and α-satellite DNA, are normally

380   methylated (40). Here, we showed that the DNA methylation of satellite I was lower in

381   cells cultured with SAH (62.8%) and with SAH + procaine (64.2%) compared to that in

382   control cells (72.5%) (Fig. 5). Thus, we believe that SAH may work well as a global DNA

383   demethylating agent, especially considering that satellite I is widespread in the genome.

384   We also found that SAH had a noticeable effect, at least in this repetitive region

385   evaluated here, as it caused a significant reduction in methylation levels compared to

386   those in the control, different from procaine, which resulted in levels similar to those in

387   the control (Fig. 5). This higher level of methylation observed in the control group in the

388   satellite region may be explained by the necessity for transcriptional repression by the

389   methylation marks on the repetitive DNA, which is in agreement with the literature (41).

390   Interestingly, the CpG site at position 17 was totally demethylated in all sequenced

391   clones in all treatment groups, suggesting that this pattern may have some biological

392   role that was not investigated in this study (Fig. 4).

393   The global methylation analysis revealed that cells from all treatments were less

394   methylated than the control cells (Fig. 6). Cells from the control group showed 2.9%

395   global methylation. A very relevant reduction was observed in the cells treated with

396   procaine (1.3%), SAH (1.6%), and both compounds in combination (1.5%),

397   demonstrating the significant potential demethylating action of both substances on

398   bovine fibroblasts. We also found no differences among the groups that were treated

399   with procaine, SAH, or both compounds.

400   The presence of tissue-specific epigenetic marks in somatic/differentiated genomes

401   renders essential genes related to embryo development transcriptionally inactive,

402   leading to abnormal global gene expression patterns in embryos (7, 37). Moreover, the

403   incomplete DNA methylation reprogramming normally observed in SCNT could be

404   caused by repressive factors present in donor cells (17, 42). Therefore, our proposal to

405   reduce the methylation levels of the donor cell genome could improve reprogramming

406   to more effectively erase cellular memory and promote the better activation of

407   developmental genes.

408   Studies evaluating the expression profiles of genes encoding the enzymes related to the

409   DNA methylation process in bovine fibroblasts are scarce. However, knowledge of the

410   gene expression profile in somatic cells commonly used for the donor nucleus in SCNT

411   may be important for efforts to improve the SCNT efficiency. The epigenomic state of

412   early-developing human embryos is defined and governed by a group of genes that

413   encode the enzymes involved in chromatin remodelling (41). Min et al. (2015) observed

414   that the expression of genes involved in initial development were more altered in

415   blastocysts produced using fibroblasts for the donor nucleus than in embryos obtained

416   from cumulus cells (43). In addition, they also found that cloned embryos showed an

417   altered gene expression profile compared to embryos produced *in vitro* (43).

418   In this study, we also evaluated the expression profile of genes related to DNA

419   methylation reprogramming. The log-fold change analysis revealed that the overall

420   profile of the genes encoding the DNMTs was downregulated in cells treated with SAH

421   or a combination of procaine and SAH (Fig. 12). In addition, when we compared the

422   mRNA levels of the group of three DNMT enzymes or compared the levels of transcripts

423   between treated cells and control cells, we observed that the treatments decreased the

424   DNMT mRNA levels (Fig. 9 and Fig. 10). At the cellular level, the enzymatic concentration

425   may influence its own transcription rate. Molecules that are able to interact with

426   enzymes in their active form may influence their activity (44, 45). Thus, this may lead to

427   the initial accumulation of these enzymes, inducing the cell to then reduce the

428   transcription rate (45). This mechanism could explain our results, in which we found a

429   reduction in mRNA levels for DNMTs in the presence of procaine and SAH.

430   In addition to DNMTs, we also evaluated the levels of gene transcripts encoding TET

431   enzymes; these act in the DNA demethylation process, and their correct expression is

432   essential for embryo development (14, 46). Although it has been discussed in the

433   literature in different contexts (47-50), reports on the expression profile of TET genes in

434   cattle are scarce, especially those focusing on cloning using NT. TET enzymes oxidise 5-

435   mC to 5-hmC, and in general, 5-hmC is transient in the epigenome, as its frequency is

436   less than that of 5-mC (51). However, some cells have high levels of TETs, such as

437   embryonic stem cells (52) and neurons (53). Here, we found higher levels of TET3

438   transcripts in cells cultivated with both substances, individually or together, compared

439   to those in the control (Fig. 11). Studies with bovine embryos have shown that the TET1

440   enzyme does not participate in the DNA demethylation process in early development,

441   with only TET2 and TET3 being detected and TET3 being present at higher levels in

442   embryos (54) and oocytes (55). Our results demonstrated that TET3 expression

443   increased in cells treated with SAH and procaine. Pagage-Lariviere and Sirard (2014)

444   showed that TET3 is present in high abundance in embryos (54). In this context, the

445   increased levels of TET3 found here may be favourable for the development of cloned

446   embryos when using these cells as a nucleus donor.

447 Interestingly, we found that fibroblasts from all treatments expressed OCT4, even

448 fibroblasts from the control group, even though there were not expected to express

449 genes related to pluripotency because of their highly differentiated status. This result

450 may be explained by the results of Pan et al. (2015) from their evaluation of skin

451 fibroblast samples showing the presence of a heterogeneous population of fibroblasts

452 containing multipotent stem cells (56). Moreover, we found no significant differences in

453 OCT4 transcript levels among the treatments, suggesting that the molecular changes

454 induced by procaine and SAH were not sufficient to alter the expression of pluripotency

455 genes such OCT4. Because the control of gene expression is a mechanism involving many

456 factors such as DNA methylation, histone modifications, and microRNAs, just the DNA

457 demethylation induced by procaine and SAH might not have been sufficient to alter

458 OCT4 expression.

459 In general, our data suggest that the use of procaine, SAH, or both compounds in

460 combination is a promising strategy for SCNT protocols because their use in cell culture

461 did not affect cell growth and morphology and because they reduced specific and global

462 DNA methylation levels. However, it is important to highlight that the reduction in global

463 methylation was higher than that for the methylation of the satellite region. This result

464 may be explained by the fact that these repetitive regions are more resistant to being

465 demethylated (57). A recent study demonstrated that Dnmt1s, a specific isoform in

466 somatic cells, is a barrier for zygotic genome activation and genomic methylation

467 reprogramming, leading to a developmental stop in SCNT embryos (18). Moreover, its

468 removal allows for an efficient activation of genes essential for embryonic development,

469 thus increasing SCNT efficiency (18). Based on this information, the use of procaine and

470 SAH can be a useful strategy to improve SCNT efficiency considering that our results

471   showed that these compounds decreased DNMT transcript levels (Fig. 9). Taken

472   together, the use of these DNA demethylating agents in cell culture decreased DNMT

473   expression and DNA methylation levels, suggesting that these agents have the potential

474   to induce cells into a less differentiated state, which may improve SCNT protocols.

475

476   Authors' contributions

477   NAB participated in the collection of materials for analysis. NAB, AdosSM, MMS, LNV,

478   LOL, and RVS performed the cellular and molecular analyses. MMF designed the study.

479   NAB and MMF interpreted the results and wrote the manuscript. All authors read and

480   approved the final manuscript.

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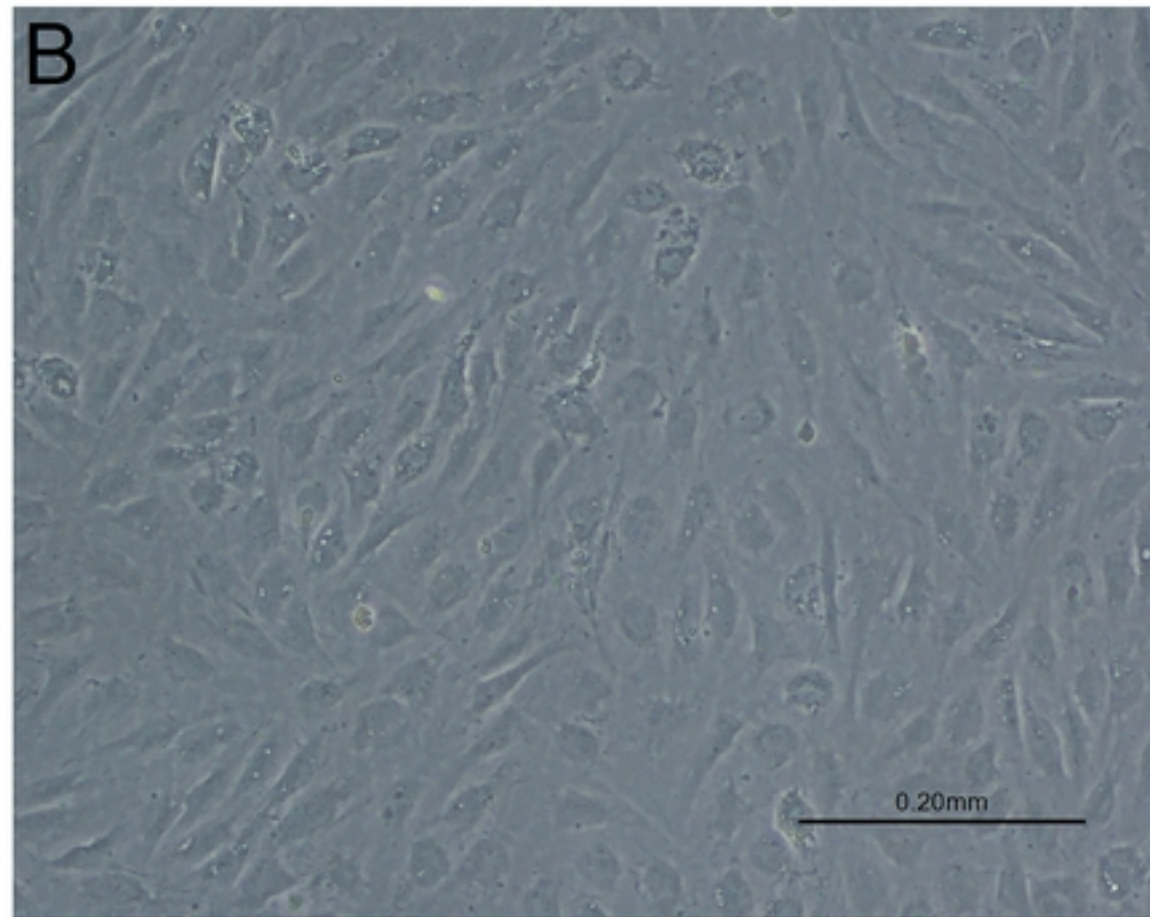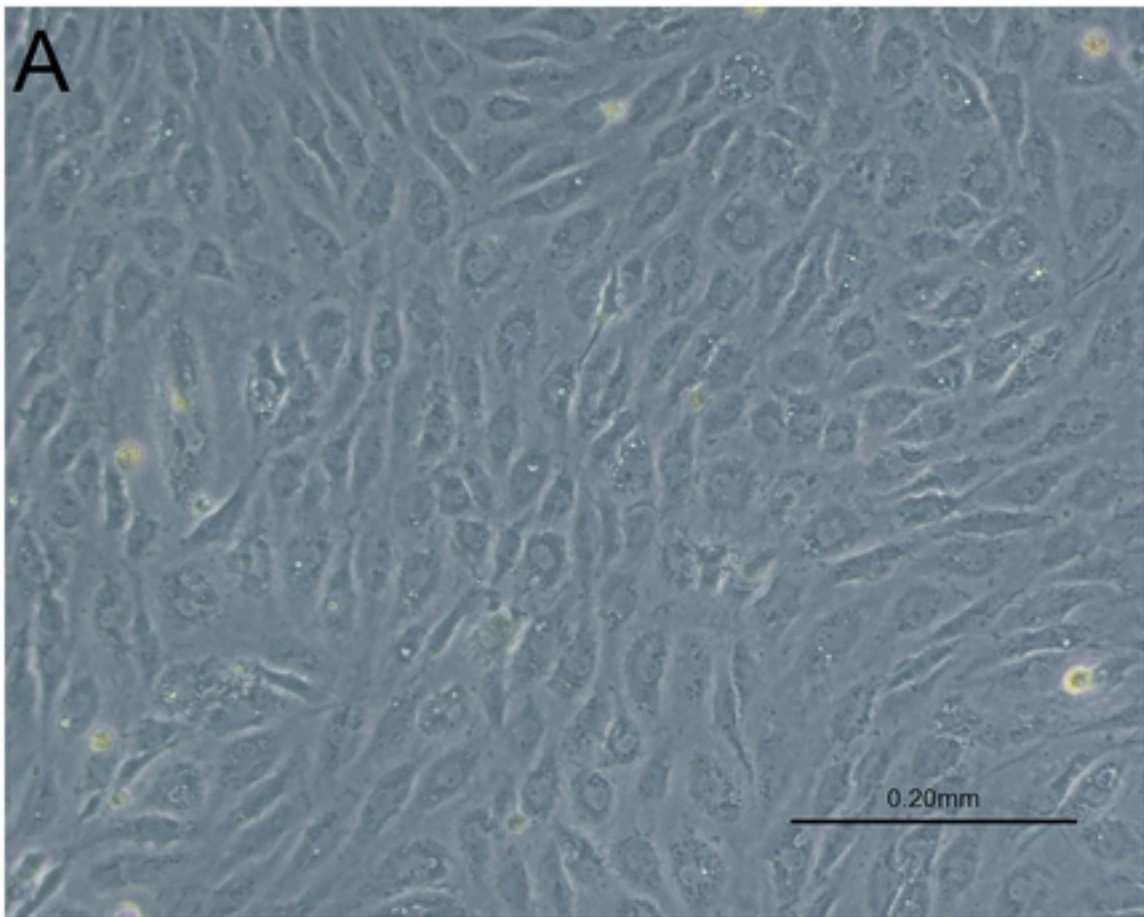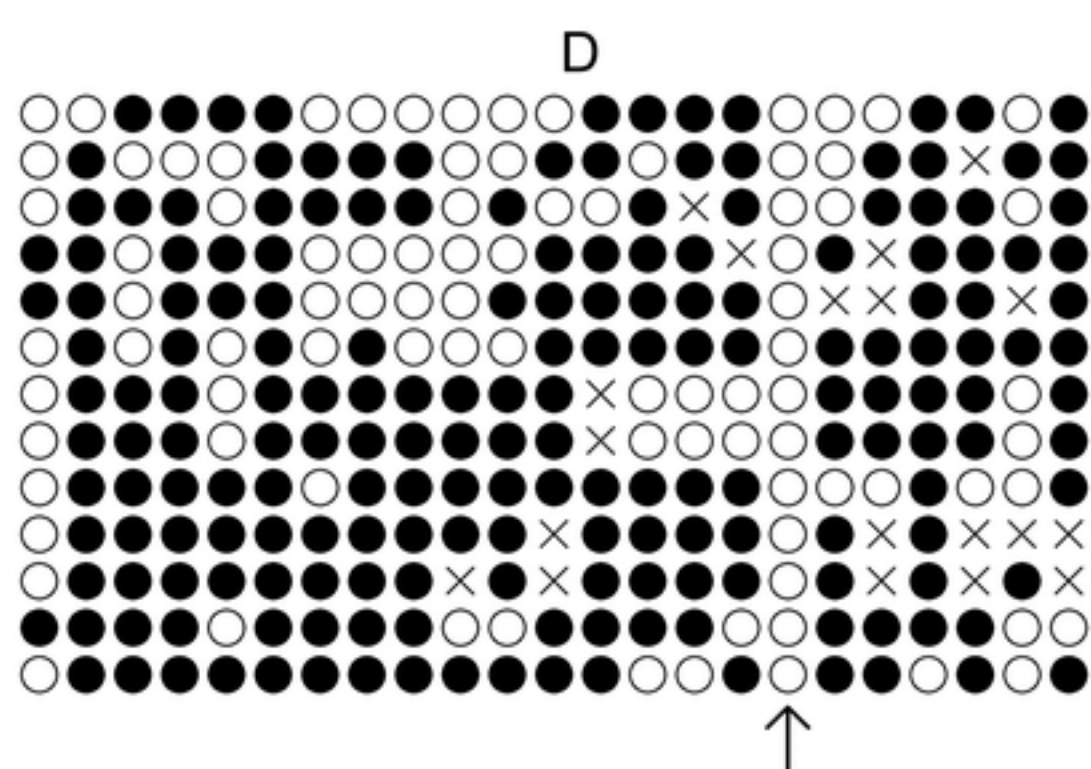643   genome regulation. Genome Dyn 7: 153-69, 2012.
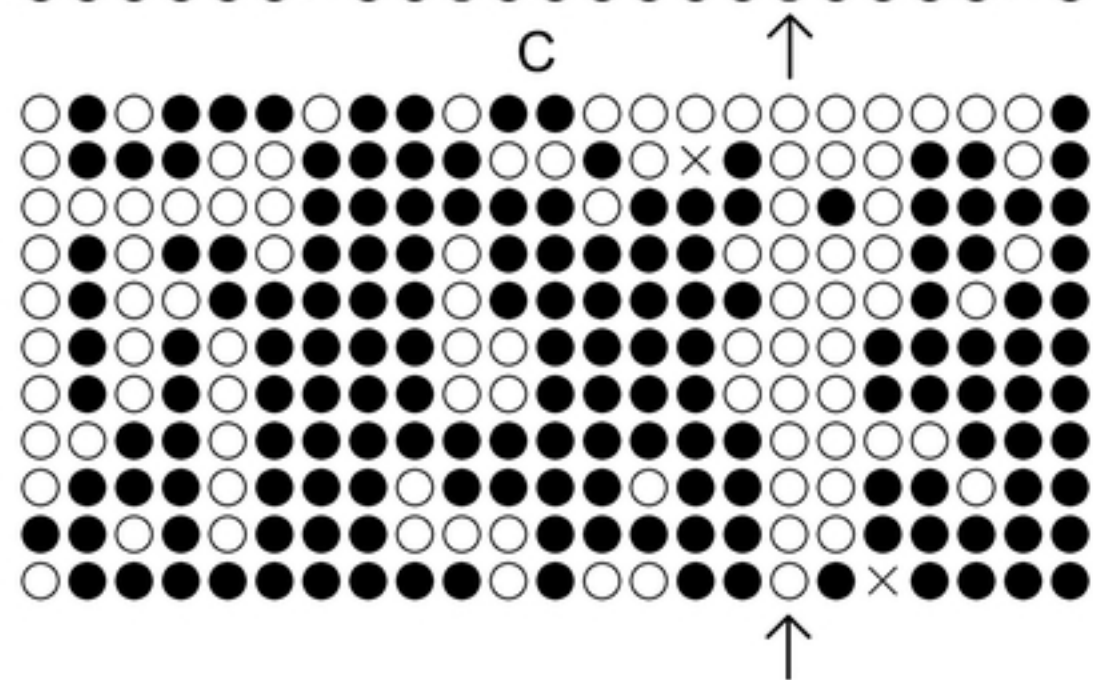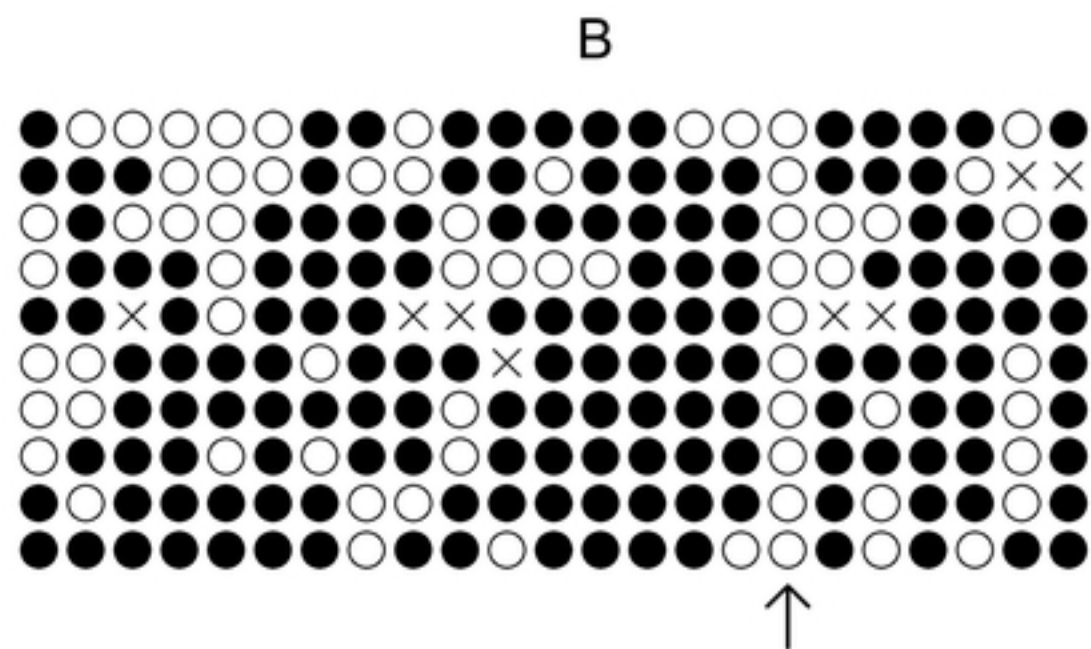
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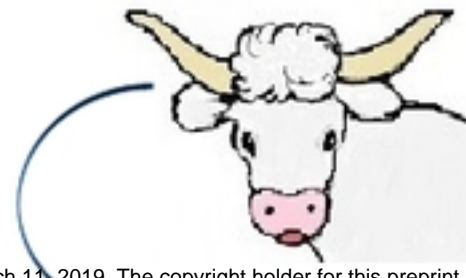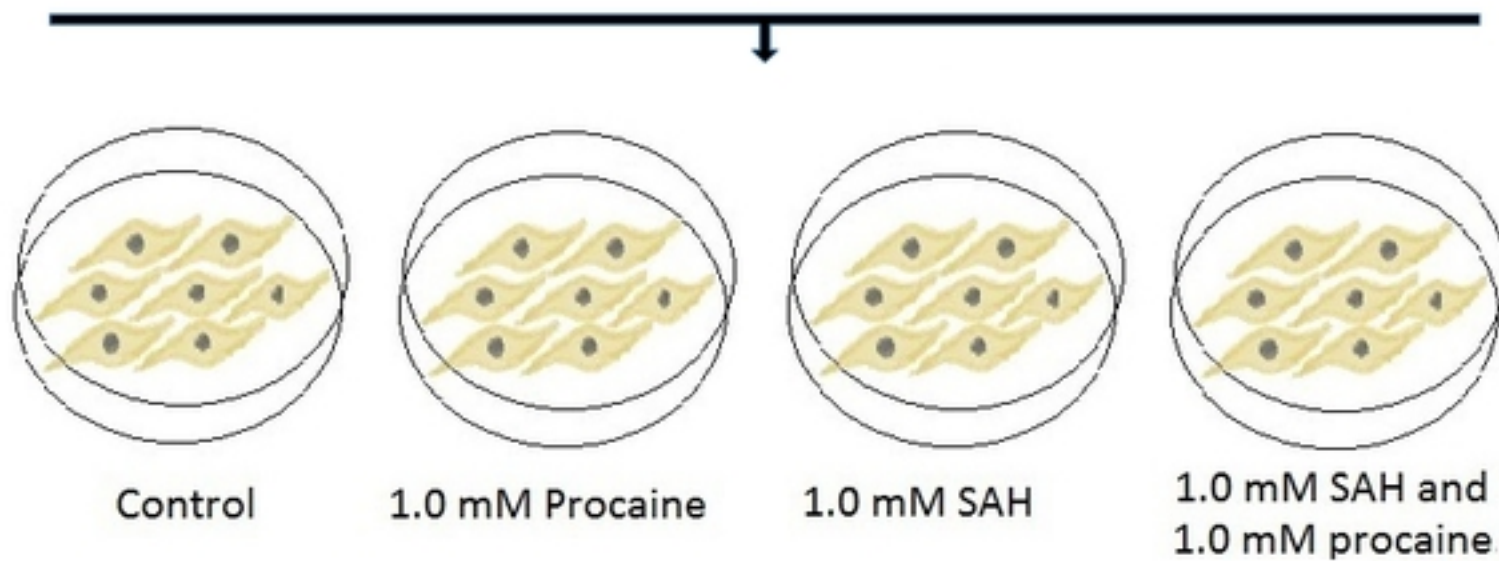
Fig1
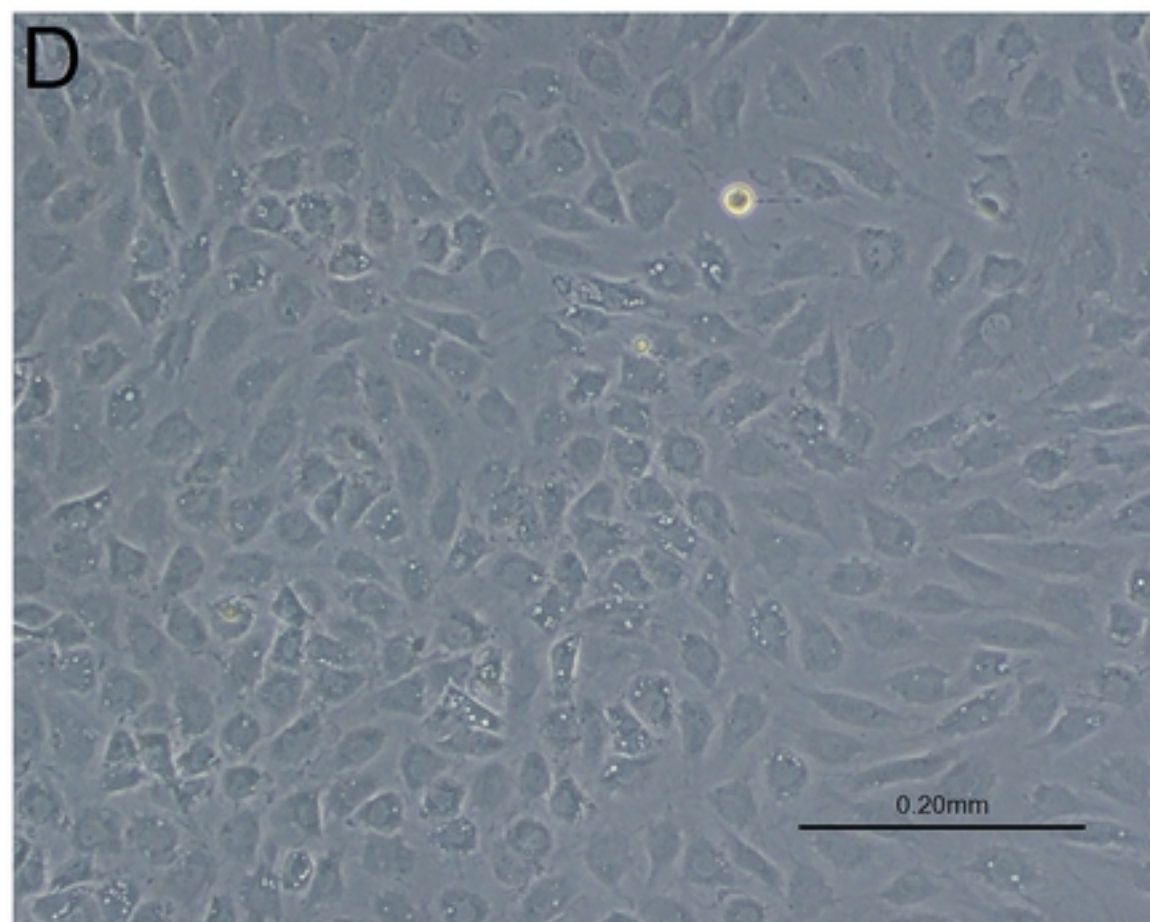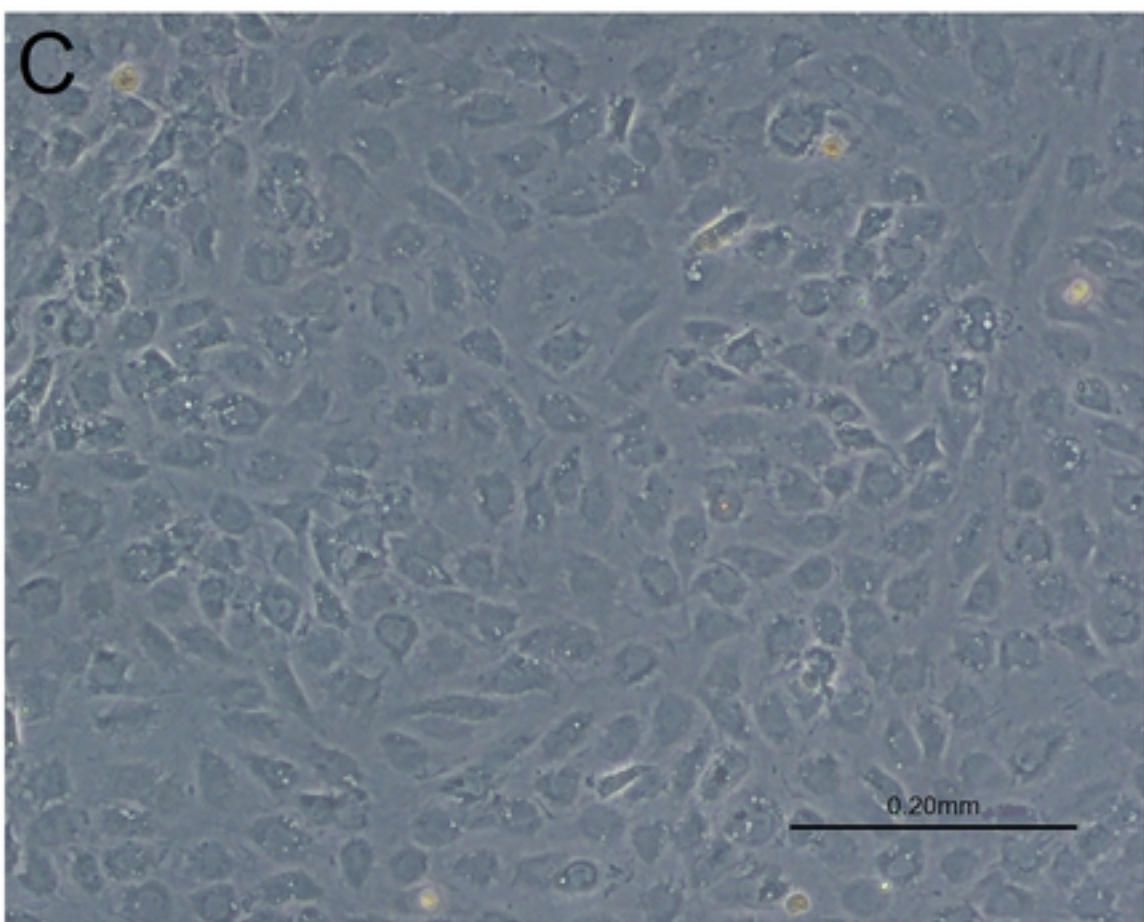
Fig2

Number of cells
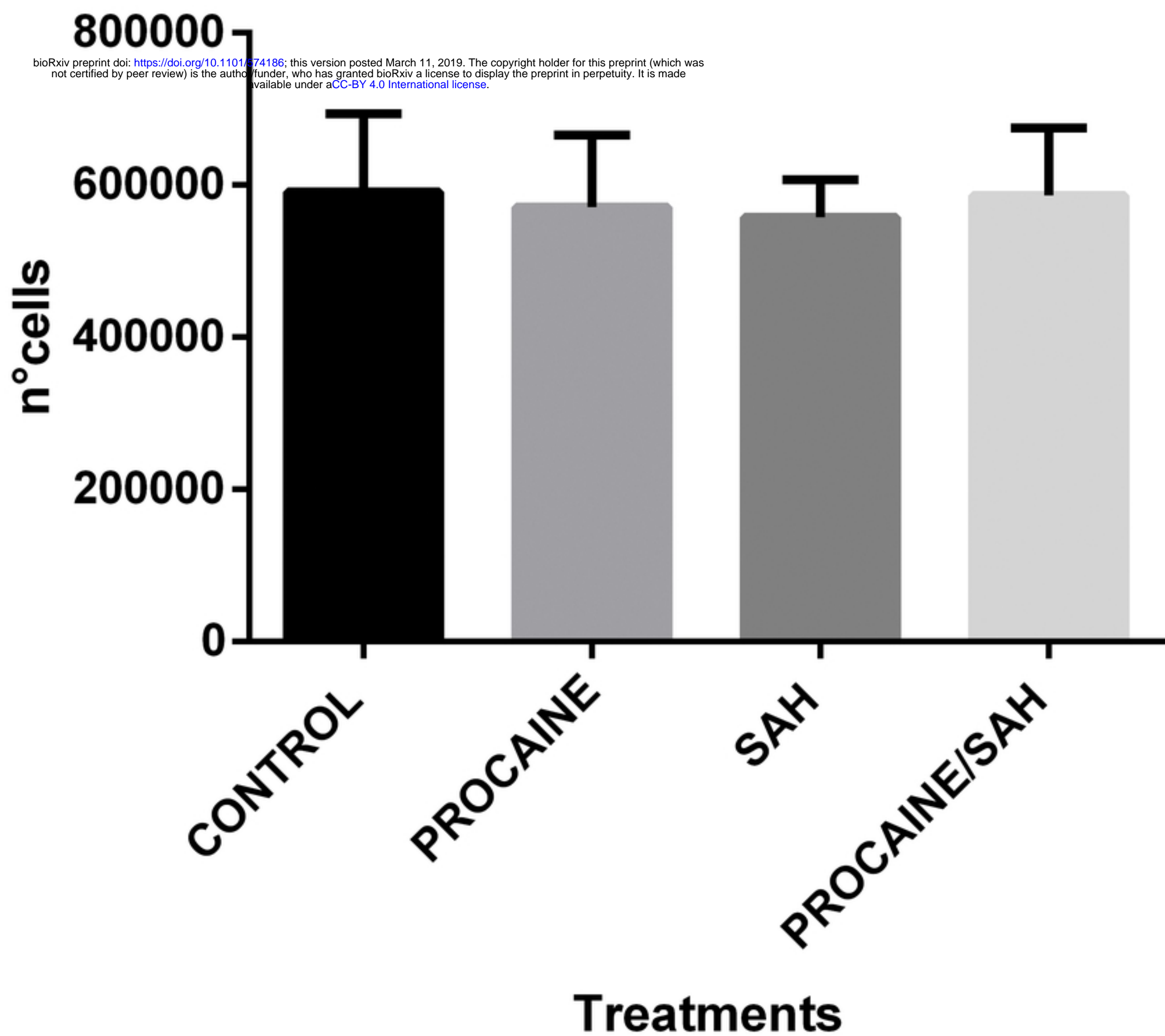
Fig3
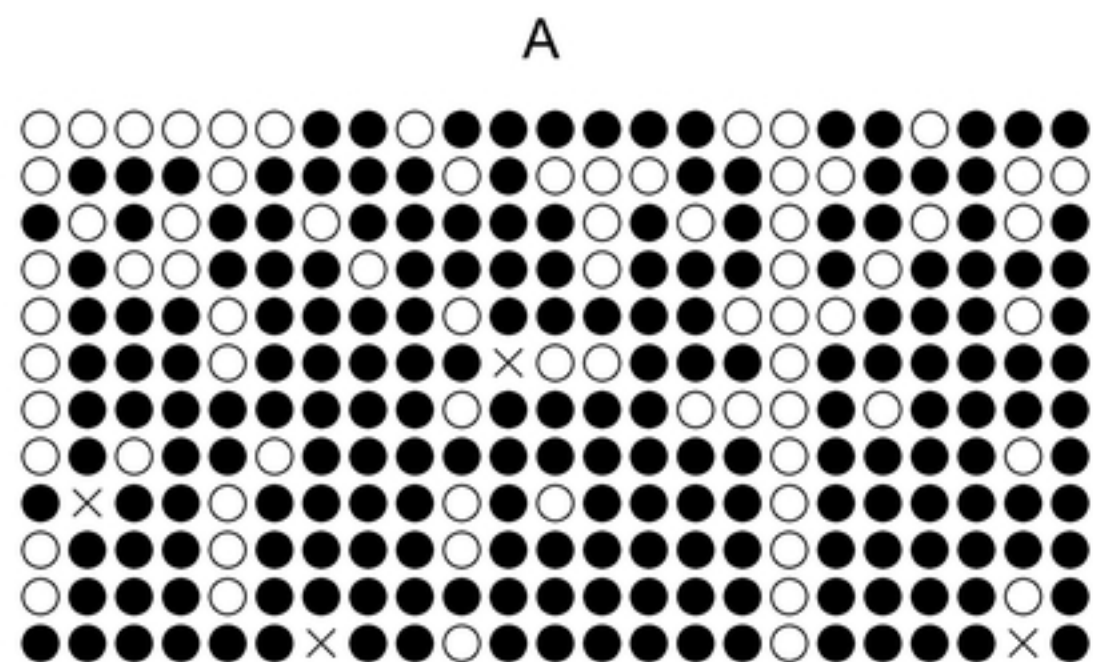
Fig4

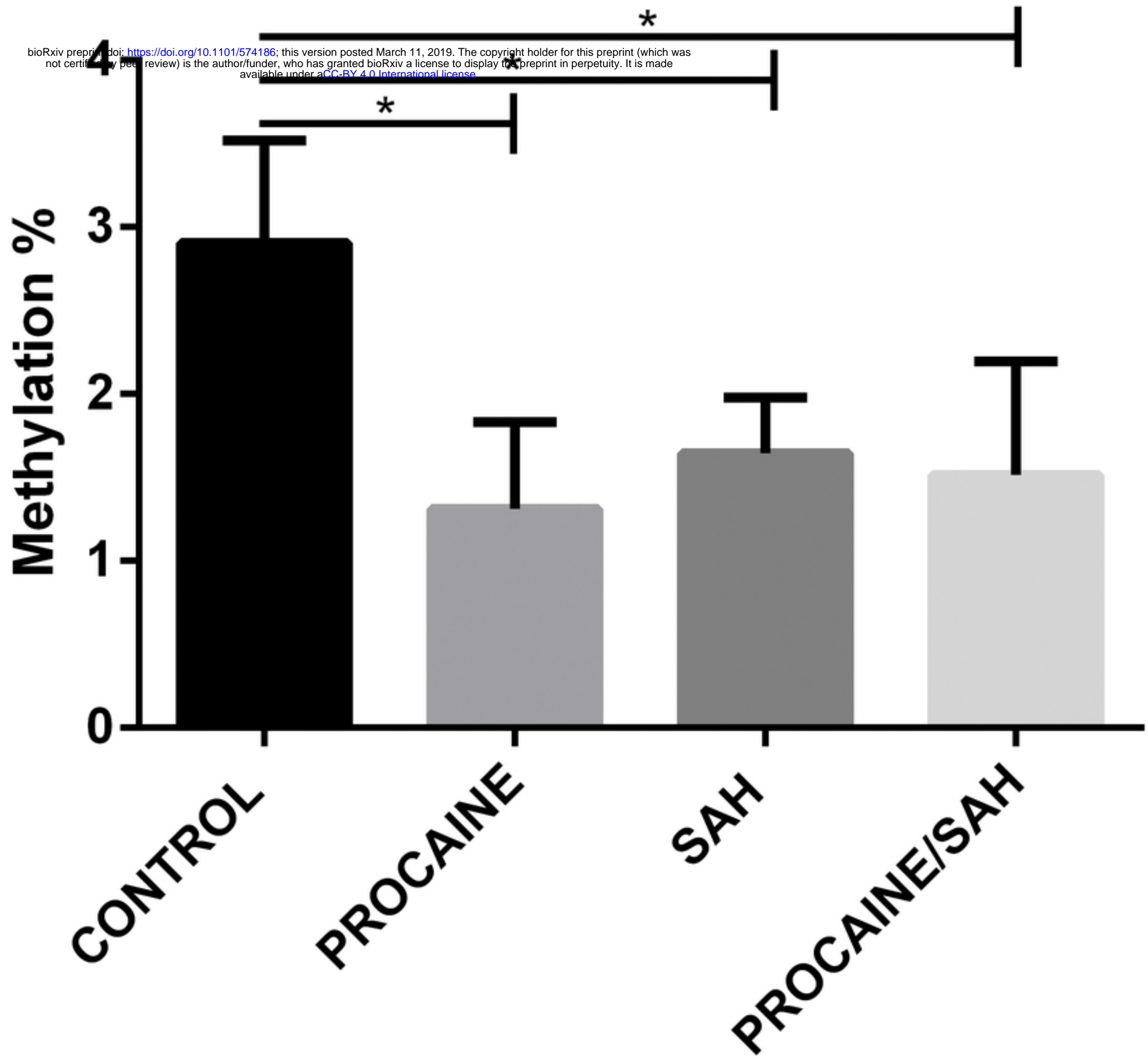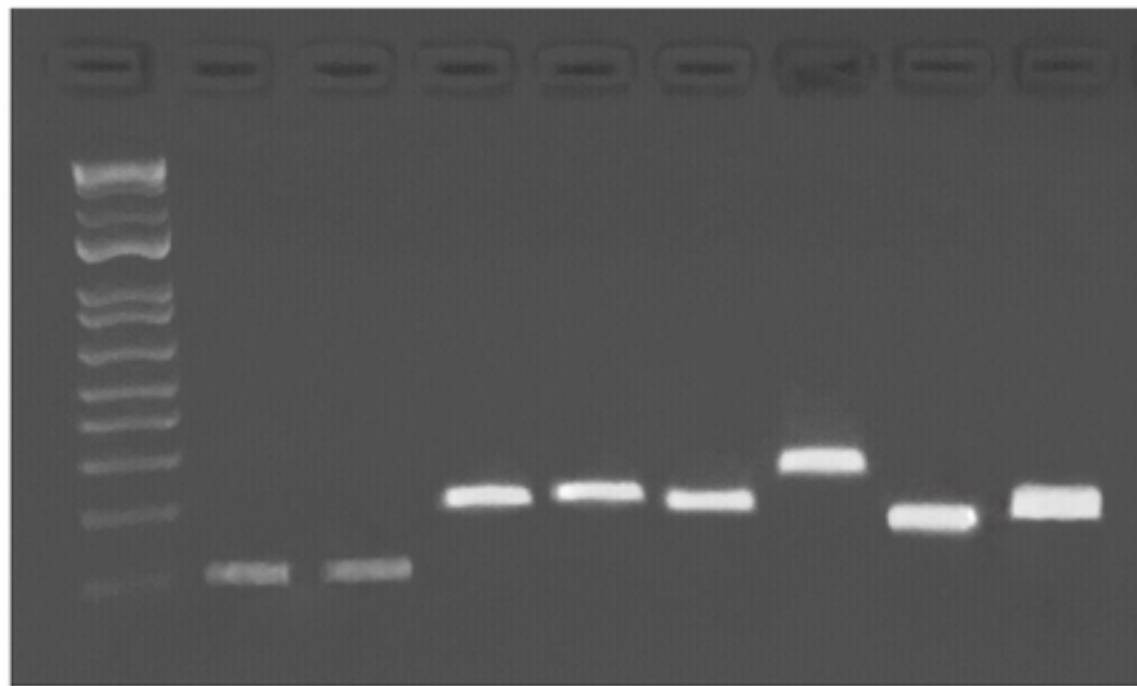# *Satellite I* Methylation Level
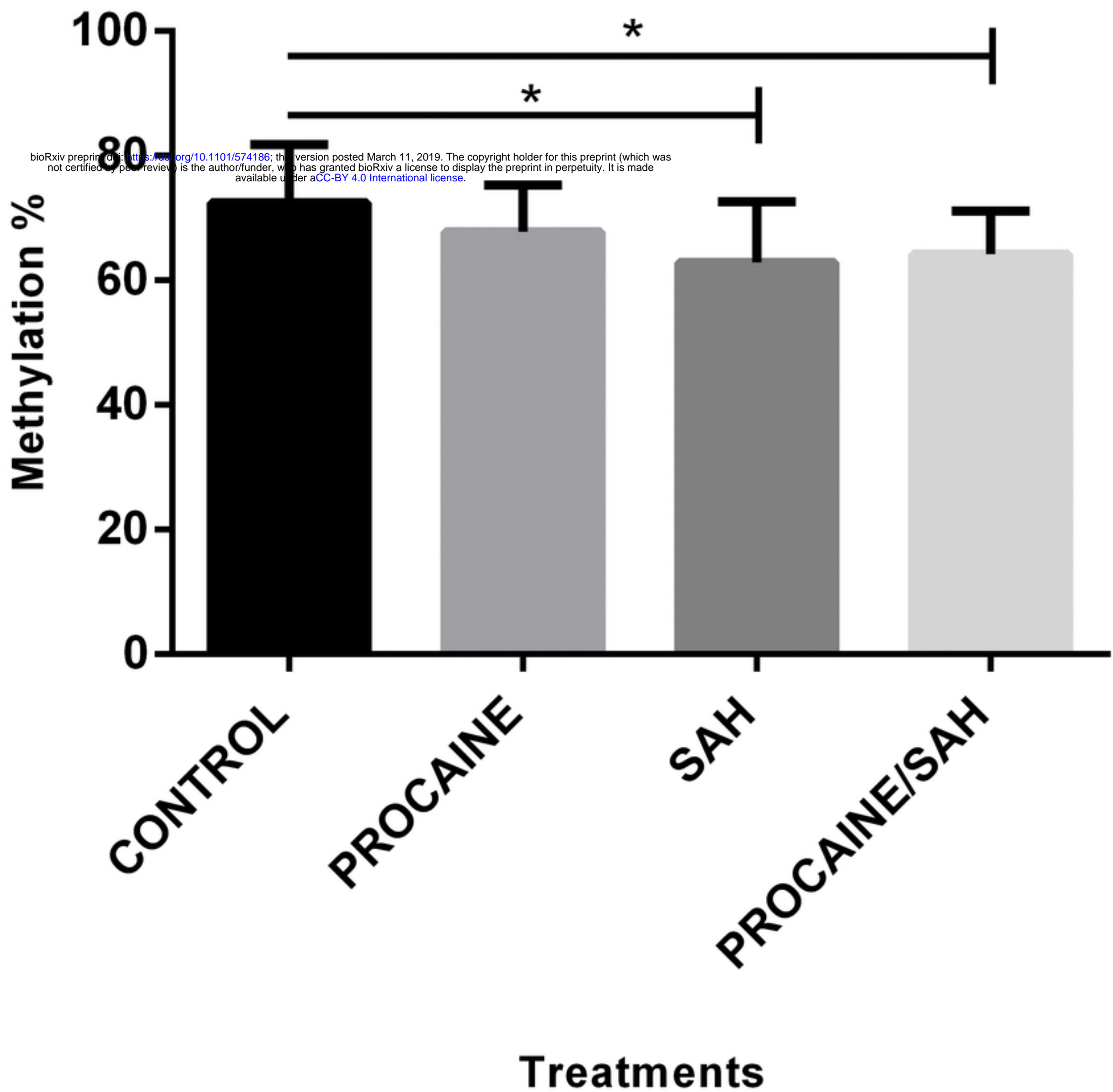
Fig5

Global Methylation Level

Fig6
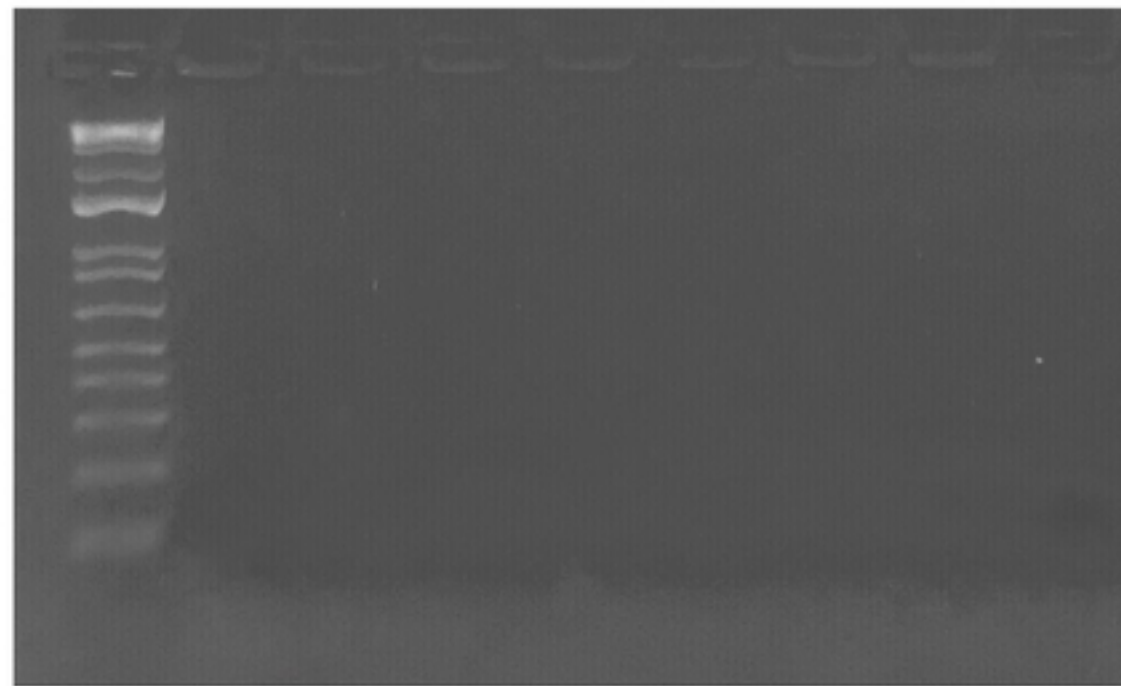
Fig7

DNMT1 – 80,42 °C
DNMT3A – 83,92 °C
DNMT3B – 85,67 °C

TET1 – 83,82 °C
TET2 – 81,25 °C
TET3 – 81,24 °C

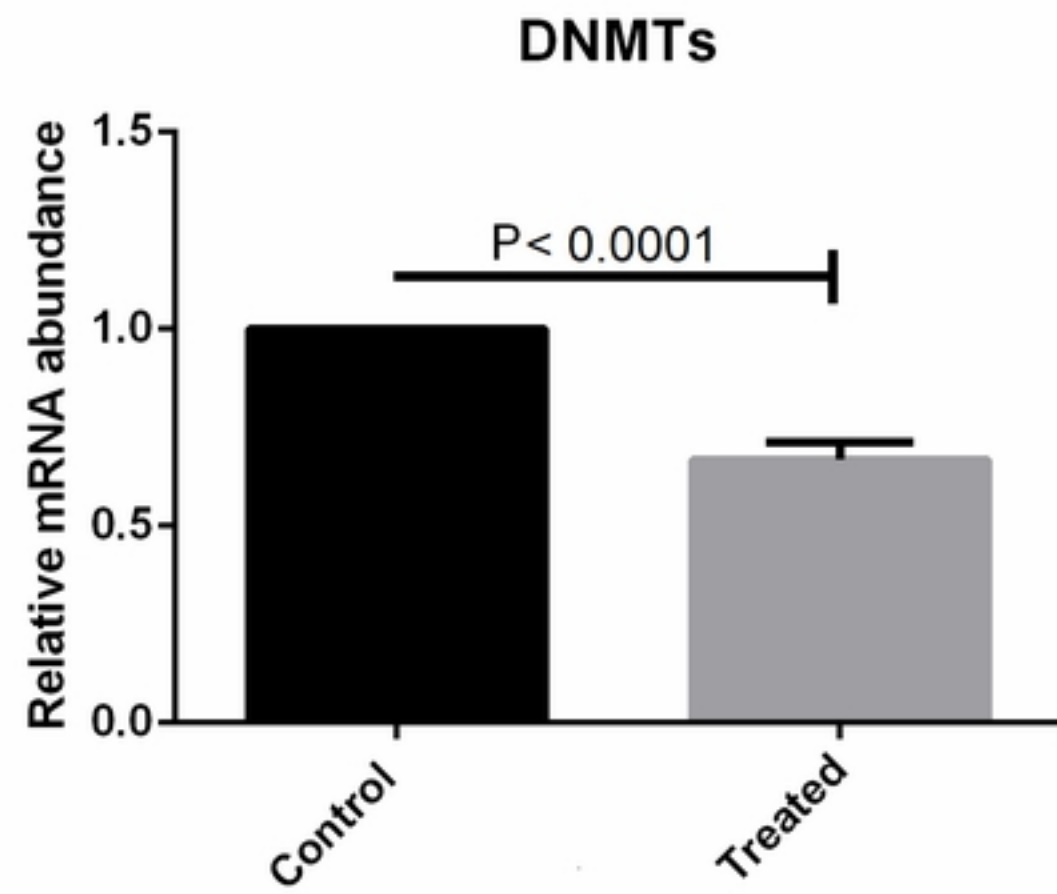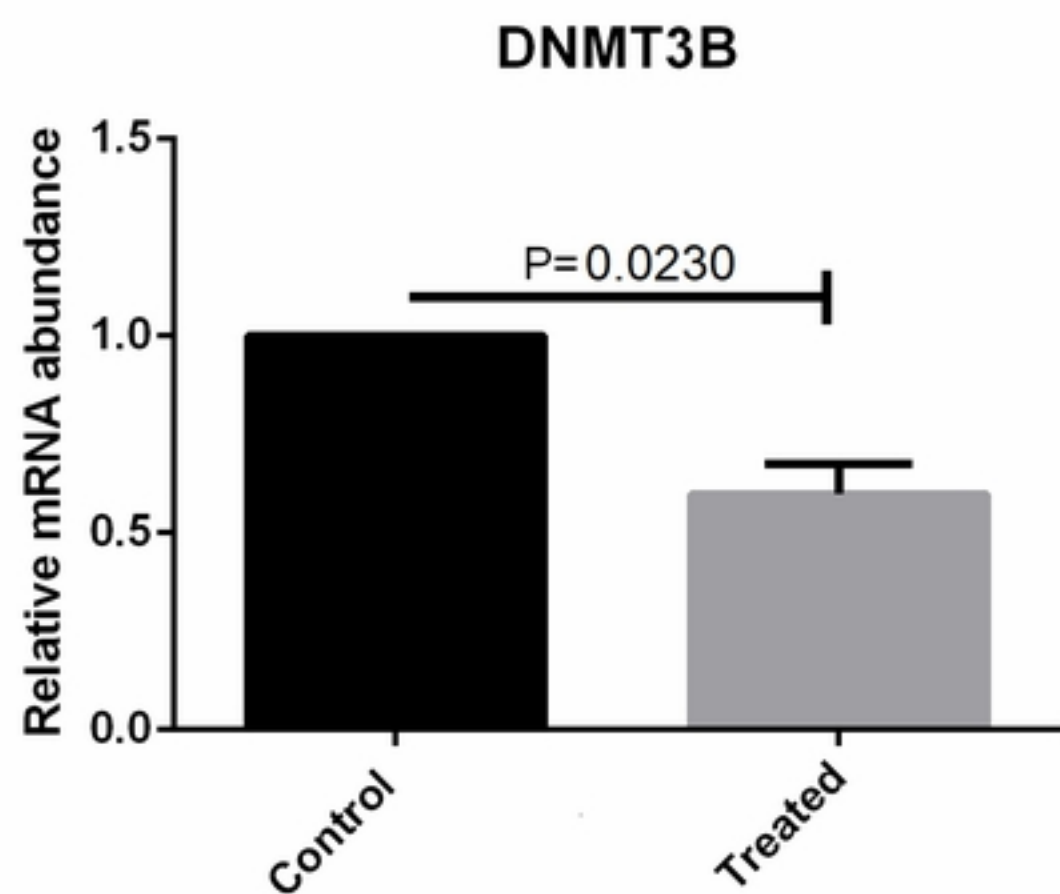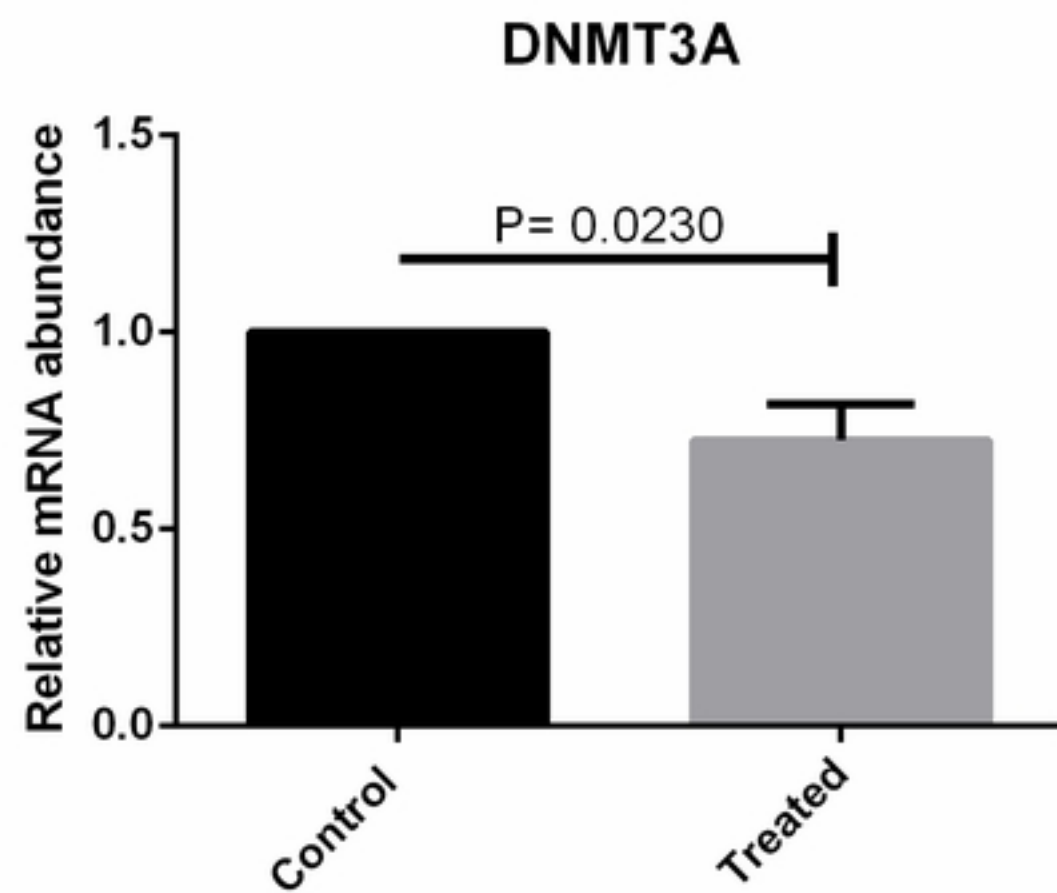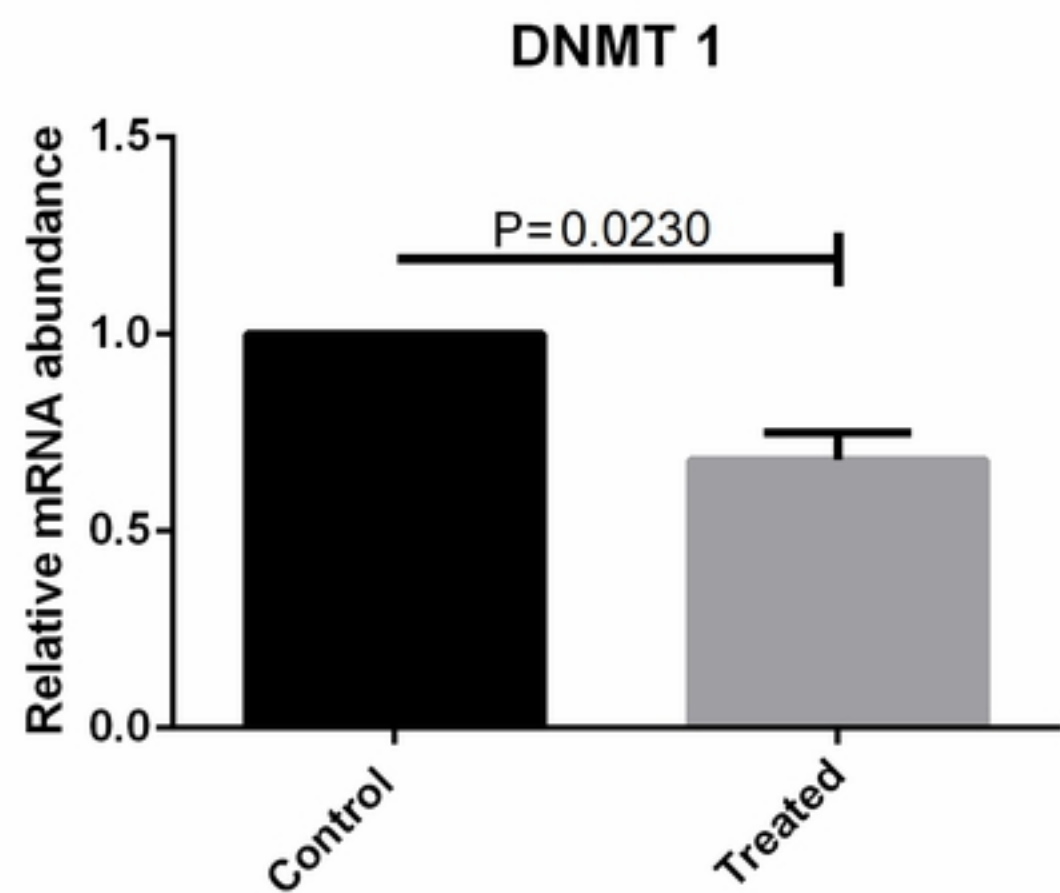OCT4 – 83,22 °C
GAPDH – 84,24 °C
B-ACTIN – 85,67 °C

Fig8

Fig9

Fig10

Fig11

Fig12

OCT4

$P = 0.4527$

Fig13

OCT4

Fig14