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Estimation of Global Content of 5-methylcytosine in DNA during Allantoic and Pulmonary Respiration in the Chicken Embryo*

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DNA methylation is an epigenetic modification that plays an important role in the proper development and functioning of an organism. The DNA methylation level is species-, tissue- and organelle-specific, and the methylation pattern is determined during embryogenesis. A correlation between methylation and age is also observed. Epigenetic phenomena are an enormously interesting research subject, not only from the perspective of pure science, but also due to their possible applications in medicine. The aim of this study was to determine the global DNA methylation level in relation to the developmental stage of the embryo. The global level of 5-methylcytosine in the DNA during pulmonary respiration was found to be higher than during allantoic respiration. The analysis shows a clear dependence between the stage of individual development and the global DNA level of 5-methylcytosine. In the future, methylation level may be a determinant of age and perhaps even a tool for predicting life expectancy. Abnormalities in the methylation process result in premature ageing at the cellular and individual level.

Key words: DNA methylation, DNA methylation level, epigenetics, *Gallus gallus domesticus*.

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In a normal cell, continuous transcriptional activity occurs only in certain genes. These are the genes that determine basic cellular functions, and are referred to as housekeeping genes. The expression of most genes, however, is subject to regulation, and this depends on nucleosomal chromatin organisation (BALLESTAR & ESTELLER 2002).

Silencing of gene expression is associated with two different mechanisms. The first mechanism requires the presence of methyl-sensitive transcription factors, capable of binding to unmethylated gene promoters. This is because DNA methylation affects protein-DNA interactions, leading to changes in chromatin structure and in the accessibility of DNA to the transcriptional machinery. The presence of methylated CpG dinucleotides in a given gene promoter directly prevents binding of transcription factors (HENDRICH & BIRD 2000). The second mechanism requires the presence of MBP proteins (methyl-CpG-binding proteins – proteins that bind to methylated CpGs) that are able to bind to specific methylated DNA sequences. The silencing of gene expression is associated with a decrease in histone acetylation mediated by MBP proteins (JONES & TAKAI 2001).

Embryonic development is a highly organised and complex process which begins with a totipotent zygote stage and leads to the emergence of many types of specialised cells that build tissues and organs forming a structural and functional whole. During embryogenesis no genes are lost and thus the cause of the differences between cells is a diversely operating gene expression pattern which is strictly defined for each cell (SZPECHT-POTOCKA 2004). After fertilisation, the zygote and the developing embryo have a pattern of epigenetic modifications inherited from both parents. DNA methylation is maintained in somatic cells

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and in primordial germ cells during migration to developing gonads. Epigenetic modifications in the genome of the embryo comprise two main stages, the first of which takes place in primordial germ cells and the second in the preimplantation stage in the embryo (BIRD 2002).

During the first divisions of the embryo, DNA is completely demethylated. Prior to the fusion of the pronuclei, the paternal genome is immediately and completely demethylated, while in the maternal genome demethylation occurs gradually during the first cell divisions. Hypomethylation of the genomes attains an equal level at the stage of the 16-cell embryo. Then the embryonic genome is methylated *de novo* after implantation during the blastocyst stage (ALLEGRUCCI *et al.* 2005; GÓRSKA *et al*. 2006). A hypothesis was put forth that the global DNA methylation pattern changes at critical periods of embryonic development in the chicken.

The embryonic development of a chicken (*Gallus gallus domesticus*) lasts 21 days. Within three weeks a non-organised cluster of cells turns into a chick that will hatch independently. On the 6th day of incubation the allantois takes on respiratory functions, while on the 18th day the embryo begins to breathe with lungs. The embryo mortality rate is highest during these two periods of embryonic development, and for this reason they are referred to as critical periods of incubation (PIJARSKA & MALEC 2007).

The use of *Gallus domesticus* in this study was not accidental. *Gallus domesticus* is a model organism used in biomedical and evolutionary research, as well as in comparative genomics and epigenetic studies (ANDRASZEK *et al.* 2012; GRYZINSKA *et al.* 2013b). Polbar is a native Polish hen breed, belonging to a genetic resource flock created by crossing Green-legged Partridgenous hens and barred Plymouth Rock cocks (GRYZINSKA *et al.* 2013c; WÓJCIK *et al*. 2012).

The aim of this study was to evaluate the content of 5-methylcytosine in the DNA on the sixth day of incubation during allantoic respiration and on the eighteenth day of incubation during pulmonary respiration in chicken embryos of the Polbar breed.

Material and Methods

Subjects

Embryos collected on the sixth and eighteenth day of embryonic development were the experimental material (25 embryos for each age group). The protocol was approved by the 2nd Lublin Local Ethical Commission for Animal Experiments (Permit Number 8/11 of 15/03/2011).

DNA extraction and estimation of global DNA methylation level

DNA was isolated from the embryo using the DNeasy Tissue Kit (Qiagen) according to the enclosed protocol. The total DNA methylation was determined using the Imprint Methylated DNA Quantification Kit from Sigma according to the enclosed protocol. The procedure consisted of three stages: DNA binding, methylated DNA capture and detection. Prior to determining the relative DNA methylation level, DNA concentration was calculated so that the final DNA concentration after dilution in the appropriate buffer volume was 150 ng/μl for all the tested samples.

Statistical analysis

The results obtained were analysed statistically using Student's *t*-test for the significance of difference between means. Differences between means at $P \leq 0.05$ were considered significant.

Results

The concentration of the DNA obtained from 6-day-old embryos ranged from 19 ng/μl to 65 ng/μl, compared to 24-48 ng/μl on the 18th day (Fig. 1).

The absorbance at a wavelength of 450 nm was measured twice for the same amount of DNA originating from the same individual. The measurements were also repeated for the methylated control sample and the blank sample. The average of the two measurements and the total DNA methylation level were calculated. The results showed that the overall DNA methylation level increased with the age of the Polbar chicken embryos. The total DNA methylation level on the 6th day of incubation, as a relative methylation level in the samples compared to the methylated control, was 5.8%. The total DNA methylation level on the 18th day of incubation, as a relative methylation level in the samples compared to the methylated control, was 17.98. The difference between the means was statistically significant ($P<0.05$).

The global level of 5-methylcytosine in the DNA during pulmonary respiration was higher than during allantoic respiration. The analysis shows a clear effect of age (during embryonic development) on the global DNA level of 5-methylcytosine in Polbar chickens.

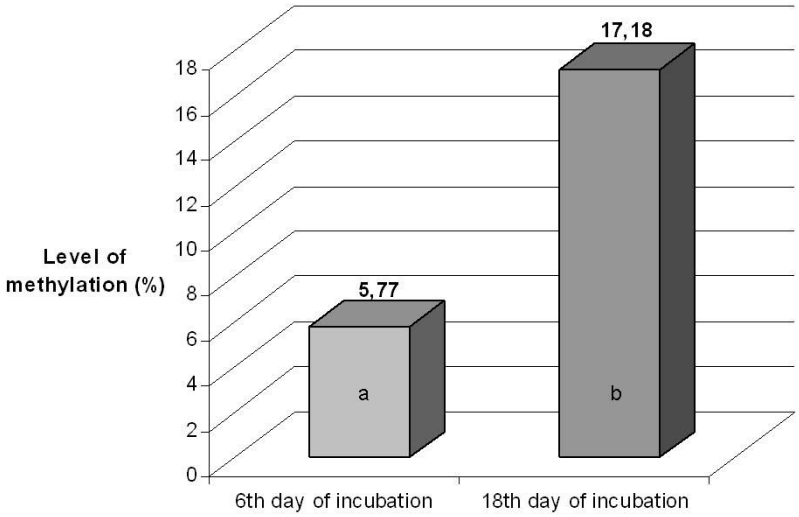

Fig. 1. Total level of DNA methylation at 6 and 18 days of incubation; a, b – means with different letters differ significantly ($P<0.05$).

Discussion

The present study analysed results obtained during critical periods of incubation. The results confirmed the hypothesis that the global DNA methylation pattern changes at critical periods of embryonic development in the chicken. The results show a clear dependence between the stage of individual development and the global DNA level of 5-methylcytosine. Methylation and demethylation reactions are highly specific and associated with a particular developmental stage in a given organism. Methylated cytosine is an important epigenetic factor regulating the activity of genes responsible for the differentiation process. Most results presented by other authors concern the postnatal period, for which a decrease in the methylation level with age has been noted in salmon (BERDYSHEV *et al.* 1967), rats (VANYUSHIN *et al.* 1973), mice, and humans (WILSON *et al.* 1987; BJORNSSON *et al.* 2008). While many authors have presented studies demonstrating that the DNA methylation level increases with age (FUKE *et al.* 2004; KWABI-ADDO *et al.* 2007; BOCKLANDT *et al.* 2011), there are few publications available concerning the global DNA methylation level during the prenatal period (GRYZINSKA *et al.* 2013b). The results obtained in the present study were confronted only with these studies. In recent years considerable attention has been paid to the role of epigenetic mechanisms in the formation of a new organism. During the development of multicellular organisms, cells and tissues acquire different gene expression programs depending on the functions they perform (BIRD 2002). In mammals, methylation plays an important role in the regulation of gene expression during embryogenesis, and later during cell differentiation (AZHIKINA & SVERDLOV 2005). The significance of the DNA methylation process for the organism's development is evidenced by the fact that mice lacking the DNA-methyltransferase gene die within eight days after fertilisation (SULEWSKA *et al.* 2007). Methylation is a very dynamic process. epigenetic modifications in the embryonic genome comprise two main stages the first taking place in primordial germ cells, and the second in the preimplantation stage in the embryo (BIRD 2002). prior to fusion of the pronuclei, the paternal genome undergoes global and instant demethylation, while the maternal genome is gradually demethylated during the first cellular divisions. Immediately after fertilisation the zygote undergoes substantial changes, including DNA demethylation and histone acetylation. Global demethylation takes place in the early embryo stage, followed by *de novo* methylation of the majority of CpGs after implantation (SULEWSKA *et al.* 2007). During gastrulation, tissue-specific genes are demethylated and expressed in their respective tissues, but it should be emphasised that most of the genome is still methylated. The next stage of the *de novo* methylation is sex-dependent and occurs during gametogenesis. A slight decrease in methylation has been observed in the post-embryonic stage as well as in senescent cells *in vitro* (SALOZHIN *et al.* 2005).

The DNA methylation map of the entire genome has already been described in many species, in-

cluding humans, Arabidopsis, silkworms and rice, but the DNA methylation pattern in birds is still rarely studied. research carried out under the direction of Qinghe Li showed that the DNA methylation pattern in the chicken genome was analogous to the methylation pattern in mammals and plants. Regions within a gene (located between the transcription start site and the transcription termination site), as well as repeated sequences, are hypermethylated, while the regions containing the transcription start site and the transcription termination site are hypomethylated. Most of the chicken genome, however, remains unmethylated (LI *et al.* 2011). Studies by XU *et al*. (2007, 2011) demonstrated differences in DNA methylation patterns in different organs and tissues in Leghorn, Plymouth Rock and their F1 hybrids.

Methylation is not only species-, tissue- and organelle-specific, but dependence of methylation on age, both in mammals and plants, is observed as well. Two phenomena may be distinguished during the ageing of an organism: global hypomethylation of 5-methylcytosine and hypermethylation of CpG islands within promoters (GRYZIŃSKA *et al.* 2013a). Increased methylation, i.e. hypermethylation, of gene promoter sequences is associated with the loss of gene function, as in the case of ordinary mutations. Hypermethylation of the genome has an enormous impact on cellular function. The inactivation of key genes and biological pathways affects such diversified processes as ageing and heart disease, contributing to the occurrence of developmental disorders, especially tumours (LI 2002). Epigenetic changes occurring during ageing may directly contribute to the initiation of a neoplastic transformation (JAENISCH & BIRD 2003).

The DNA methylation profile is also influenced by diet, single nucleotide polymorphisms in specific genes, and environmental factors. deficiencies in nutrients such as folic acid, methionine or selenium may cause DNA hypomethylation, contributing to improper gene expression and genetic instability (FENECH *et al.* 2005). In the early stage of embryogenesis, the mother's diet and the environment can have a significant impact on the methylation profile, and disturbances in this process may lead to the consolidation of an abnormal DNA methylation profile in the embryo (MOSS & WALLRATH 2007). An aberrant profile of DNA and histone modifications can be the cause of many diseases, including tumours, metabolic diseases and neurodegenerative diseases. The interest in epigenetic regulation of transcription is not only academic, but also results from the search for new types of therapy.

DNA methylation is an ideal parameter for comprehensive diagnostics of many diseases. Methylation patterns are an invaluable source of information providing insight into the current state of gene activity and potential means of activating or inhibiting genes, which could be stimulated with medications (OLEK & WALTER 1997; PLASS & SOLOWAY 2002).

Discovering and understanding epigenetic mechanisms which are involved in mammalian development and modify gene expression is of key importance in medicine. This knowledge is used in research on therapeutic cloning, in the treatment of tumours and genetically determined diseases, in clinical application of stem cells, and in molecular diagnostics. Epigenetic technologies as commercial products for diagnostics and therapy have great potential whose realization is awaited by patients and physicians (SZPECHT-POTOCKA 2004).

In conclusion, DNA methylation is an age-dependent process. In the future, methylation level may be a determinant of age and perhaps even a tool for predicting life expectancy. Abnormalities in the methylation process result in premature ageing at the cellular and individual level.

References

ALLEGRUCCI A., THURSTON A., LUCAS E. 2005. Epigenetics and the germline. Reproduct. **129**: 137-149.

ANDRASZEK K., GRYZIŃSKA M., KNAGA S., WÓJCIK E., SMALEC E. 2012. Number and Size of Nucleoli in the Spermatocytes of Chicken and Japanese Quail. Folia Biol. (Kraków) **60**: 121-127.

AZHIKINA T.L., SVERDLOV E.D. 2005. Study of Tissue Specific CpG Methylation of DNA in Extended Genomic Loci. Biochemistry **70**: 596-603.

BALLESTAR E., ESTELLER M. 2002. The impact of chromatin in human cancer: linking DNA methylation to gene silencing. Carcinogenesis **23**: 1103-1109.

BERDYSHEV G.D., KOROTAEV G.K., BOIARSKIKH G.V., VANIUSHIN B.F. 1967. Nucleotide composition of DNA and RNA from somatic tissues of humpback salmon and its changes during spawning. Biokhimiia **32**: 988-993.

BIRD A. 2002. DNA methylation patterns and epigenetic memory. Gen. Dev. **6**: 6-21.

BJORNSSON H.T., SIGURDSSON M.I., FALLIN M.D., IRIZARRY R.A., ASPELUND T., CUI H., YU W., RONGIONE M.A., EKSTROM T.J., HARRIS T.B., LAUNER L.J., EIRIKSDOTTIR G., LEPPERT M.F., SAPIENZA C., GUDNASON V., FEINBERG A.P. 2008. Intra-individual change over time in DNA methylation with familial clustering. JAMA. **299**: 2877-2883.

BOCKLANDT S., LIN W., SEHL M.E., SANCHEZ F.J., SINSHEIMER J.S., HORVATH S., VILAIN E. 2011. Epigenetic predictor of age. PLOS One **6**: 1-6.

FENECH M., BAGHURST P., LUDERER W., TURNER J., RECORD S., CEPPI M., BONASSI S. 2005. Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability – results from a dietary intake and micronucleus index survey in South Australia. Carcinogenesis **26**: 991-999.

FUKE C., SHIMABUKURO M., PETRONIS A., SUGIMOTO J., ODA T., MIURA K., MIYAZAKI T., OGURA C., OKAZAKI Y., JINNO Y. 2004. Age related chan ges in 5-methylocytosine

content in human peripheral leukocytes and placentas: and HPLC-based study. Ann. Hum. Genet. **68**: 196-204.

GÓRSKA I., KEMPISTY B., JAGODZIŃSKI P. 2006. Epigenetic modifications in the germ cells. Pract. Gynaecol. **3**: 2-5.

GRYZIŃSKA M., ANDRASZEK K., JOCEK G. 2013a. DNA methylation analysis of the gene CDKN2B in *Gallus gallus* (Chicken). Folia Biol. (Kraków) **61**: 165-171.

GRYZINSKA M., BLASZCZAK E., STRACHECKA A., JEZEWSKA-WITKOWSKA G. 2013b. Analysis of Age-Related Global DNA Methylation in Chicken. Biochem. Genet. **51**: 554-563.

GRYZINSKA M., KRAUZE M., KLEBANIUK R., STRACHECKA A. 2013c. Influence of gender and age on haematological indicators of Polbar's breed chickens. Acta Vet. Beograd **63**: 601-608.

HENDRICH B., BIRD A. 2000. Mammalian methylotransferases and methyl-CpG-binding domains: proteins involved in DNA methylation. Curr. Top. Microbiol. Immunol. **249**: 55-74.

JAENISCH R., BIRD A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. **33**: 245-254.

JONES P.A., TAKAI D. 2001. The role of DNA methylation in mammalian epigenetics. Science **293**: 1068-1070.

KWABI-ADDO B., CHUNG W., SHEN L., ITTAMANN M., WHEELER T., JELINEK J., ISSA J.P. 2007. Age-related DNA methylation changes in normal prostate tissues. Clin. Cancer Res. **13**: 3796-3802.

LI E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. Nat. Rev. Gen. **3**: 662-673.

LI Q.L., HU X., LI J., DU Z. 2011. Genome-Wide Mapping of DNA Methylation in Chicken. PLoS ONE **6**, e19428.

MOSS T.J., WALLRATH L.L. 2007. Connections between epigenetic gene silencing and human disease. Mutat. Res. **618**: 163-174.

OLEK A., WALTER J. 1997. The pre - implantation ontogeny of the H19 methylation imprint. Nat. Genet. **17**: 275-276.

PIJARSKA I., MALEC H. 2007. Mileage embryogenesis in chickens. Polskie Drobiarstwo **4**: 31-35. (In Polish).

PLASS B., SOLOWAY P.D. 2002. DNA methylation, imprinting and cancer. Eur. J. Hum. Genet. **10**: 6-16.

SALOZHIN S.V., PROKHORCHUK E.B., GEORGIEV G.P. 2005. Methylation of DNA – One of the Major Epigenetic Markers. Biochemistry **70**: 525-532.

SULEWSKA A., NIKLINSKA W., KOZŁOWSKI M., MINAROWSKI L., NAUMNIK W., NIKLIŃSKI J., DĄBROWSKA K., CHYCZEWSKI L. 2007. DNA methylation in states of cell physiology and pathology. Folia Histochem. Cytobiol. **45**: 149-158.

SZPECHT-POTOCKA A. 2004. Healthy and sick DNA "corset", or medical aspects of epigenetics. Kosmos **53**: 281-293.

VANYUSHIN B.F., NEMIROVSKI L.E., KLIMENKO V.V., VASILIEV V.K., BELOZERSKY A.N. 1973. *The 5-methylcytosine in DNA of rats.* Gerontologia **19**: 138-152.

WILSON V.L., SMITH R.A., MA S., CUTLER R.G. 1987. Genomic 5-methyldeoxycytidine decreases with age. J. Biol. Chem. **262**: 9948-9951.

WÓJCIK E., ANDRASZEK K., GRYZIŃSKA M., WITKOWSKI A., PAŁYSZKA M., SMALEC E. 2012. Sister chromatid exchange in Greenleg Partridge and Polbar hens covered by the genepool protection program for farm animals in Poland. Poult. Sci. **91**: 2424-2430.

XU Q., ZHANG Y., SUN D., WANG Y., YU Y. 2007. Analysis on DNA methylation of various tissues in chicken. Anim. Biotechnol. **18**: 231-241.

XU Q., ZHANG Y., SUN D.X., WANG Y.C., TANG S.Q., ZHAO M. 2011. Analysis of DNA methylation in different chicken tissues with MSA. Yi Chuan **33**: 620-626.